PROTEIN PHOSPHORYLATION AND TROPOMYOSIN IN CHROMAFFIN CELL FUNCTIONS

by

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ABSTRACT

Protein phosphorylation is a major general mechanism by which intracellular events respond to external physiological stimuli. It has also been postulated that protein phosphorylation may be involved in secretion.

Stimulation of bovine chromaffin cells in culture by either acetylcholine or a depolarizing concentration of K^+ enhanced the phosphorylation of several proteins as well as increased the dephosphorylation of a 20.4kD polypeptide. The degree of phosphorylation of some polypeptides was greater in chromaffin cells stimulated by acetylcholine than those depolarized by K^+. The state of phosphorylation of some of these polypeptides may be regulated by Ca^{2+}-dependent systems since Ca^{2+} deprivation inhibited their phosphorylation. Trifluoperazine, a calmodulin antagonist, significantly inhibited the acetylcholine-induced phosphorylation of several polypeptides, thus suggesting that calmodulin might regulate some phosphorylation events in chromaffin cells. The time course of phosphorylation of several proteins appears to be temporally associated with catecholamine release in chromaffin cells. Among the chromaffin cell phosphoproteins which responded to stimulation, a 38kD polypeptide possessed molecular characteristics similar to tropomyosin. Consequently, the presence of tropomyosin in chromaffin cells was

investigated. Cultured chromaffin cells were found to contain two forms of tropomyosin. These tropomyosin polypeptides possess apparent molecular weights of 32,000 and 38,000 and appear to correspond to the major forms of tropomyosin isolated from the adrenal medulla. In addition, the 38kD adrenal medullary tropomyosin was shown to interact strongly with F-actin.

These findings support the hypothesis that protein phosphorylation may be a major process involved in several cellular events in chromaffin cells, some of which may be related to the secretory cycle of this cell system.

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La Phosphorylation des Protéines et la Tropomyosine
au Niveau des Fonctions des Cellules Chromaffines

RESUME

La phosphorylation des protéines est un mécanisme général majeur par lequel des événements cellulaires répondent aux stimuli physiologiques du milieu extérieur. Il a aussi été postulé que la phosphorylation des protéines peut être impliquée dans la sécrétion.

La stimulation des cellules chromaffines de boeuf en culture, soit par l'acétylcholine ou une concentration dépolarisante d'ions K^+, augmenta la phosphorylation de plusieurs protéines ainsi que la déphosphorylation d'un polypeptide de 20.4kD. Le degré de phosphorylation de certains polypeptides était plus élevé dans le cas des cellules chromaffines stimulées par l'acétylcholine que celles dépolarisées par les ions K^+. La phosphorylation de certains de ces polypeptides peut être contrôlée par des systèmes dépendants en ions Ca^{2+} puisqu'une baisse en ions Ca^{2+} dans le milieu extérieur inhiba leur phosphorylation. La trifluopérazine, un antagoniste de la calmoduline, inhiba de façon significative la phosphorylation de plusieurs polypeptides provoquée par l'acétylcholine, suggérant ainsi que la calmoduline pourrait contrôler certains événements reliés à la phosphorylation dans les cellules chromaffines. La cinétique de phosphorylation de plusieurs protéines semble être associée temporellement à la libération des catécholamines dans les

cellules chromaffines. Parmi les phosphoprotéines des cellules chromaffines qui répondèrent à la stimulation, un polypeptide de 38kD possédait des caractéristiques moléculaires similaires à la tropomyosine. Par conséquent, la présence de la tropomyosine dans les cellules chromaffines fut examinée. Il a été déterminé que les cellules chromaffines en culture contiennent deux formes de tropomyosine. Ces polypeptides de tropomyosine possèdent des poids moléculaires apparents de 32,000 et 38,000 et semblent correspondre aux formes prédominantes de tropomyosine isolées de la médullo-surrénale. De plus, il fut démontré que la tropomyosine de 38kD de la médullo-surrénale interagit fortement avec la F-actine.

Ces résultats favorisent l'hypothèse voulant que la phosphorylation des protéines soit un processus majeur impliqué dans plusieurs événements cellulaires au niveau des cellules chromaffines, dont certains peuvent être reliés au cycle sécrétoire de ce système cellulaire.

Dédiée à mes parents,

Claudette et Roger

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ABBREVIATIONS USED IN THIS THESIS

ACh	acetylcholine
ACTH	adrenocorticotropic hormone
Ad	adrenaline
APUD	amine, precursor uptake and decarboxylation
ATPase	adenosine 5'-triphosphatase
BSA	bovine serum albumin
C	catalytic subunit (kinase)
CaM-PK	calcium/calmodulin-dependent protein kinase
cAMP-PK	cyclic AMP-dependent protein kinase
cGMP-PK	cyclic GMP-dependent protein kinase
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio] 1-propane-sulfonate
DA	dopamine
DAG	1,2-diacylglycerol
DβH	dopamine β-hydroxylase
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid
GABA	γ-amino butyric acid
GFAP	glial fibrillary acid protein
GIP	gastric inhibitory peptide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMM	heavy meromyosin
kD	kilodalton
LMM	light meromyosin
MAP	microtubule-associated protein
MLCK	myosin light chain kinase
MSH	melanocyte-stimulating hormone

MW	molecular weight
NA	noradrenaline
NGF	nerve growth factor
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDE	phosphodiesterase
pI	isoelectric point
PI	phosphatidylinositol
PK	protein kinase
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PPase	protein phosphatase
R	regulatory subunit (kinase)
RC	rate coefficient
SDS	sodium dodecyl sulfate
SP	substance P
TCA	trichloroacetic acid
TEA	tetraethylammonium
TFP	trifluoperazine
TH	tyrosine hydroxylase
TRH	thyrotropin-releasing hormone
Tris	tris(hydroxymethyl)-aminomethane
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide

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CHAPTER 1

INTRODUCTION

A. THE ADRENAL MEDULLA:

ANATOMICAL, PHYSIOLOGICAL AND HISTOCHEMICAL FEATURES

The adrenal gland in humans was first described in 1563 by Eustachius, but functional studies concerning this organ became available only in 1855 from Addison's work.

The adrenal chromaffin tissue as well as the interrenal cortical tissue is present in all mammals and more generally in all vertebrates. The terms chromophil or chromaffin for the medullary homologue of the adrenal gland were first introduced by Stilling in 1898 and Kohn in 1902 respectively, whereas the term interrenal for the cortical homologue was first mentioned by Balfour in 1878. The relative position of the medullary and cortical tissue entities varies greatly depending on the vertebrate classes. In certain species, the medulla and cortex are well separated while in others they are in close juxtaposition or intermingled. True or morphologically separated medulla and cortex exist in the mammals. However, there exists interdigitation as well between these two tissue entities at the frontier between the two morphological zones. In some invertebrate species, the chromaffin tissue is present, although the interrenal counterpart has not been identified yet.

In humans, the adrenal glands are triangular, cap-like organs sitting on the upper poles of the kidneys, enclosed in a tough connective tissue capsule and embedded in perirenal adipose tissue. Each adrenal gland measures approximately 5 cm by 2.5 cm in diameter, weighs approximately 5 grams (4-14 g.) and is yellowish in color. The adrenal gland is essentially a double organ composed of an outer cortical tissue and inner medullary tissue with

the latter constituting about 10% of the total adrenal weight. The two adrenal tissues are unrelated and derived from different embryological entities. The cortex arises in the embryo from a region of the mesoderm, which also gives rise to the gonads, whereas the medulla originates from the ectoderm which also gives rise to the sympathetic nervous system. The adrenal glands are highly vascularized organs which receive their main blood supply from multiple superior, middle and inferior suprarenal arteries arising from the phrenic, aortic and renal arteries, respectively. The medullary and cortical tissues are supplied by distinct arterial blood vessels which are connected with one another through capillary anastomoses in the zona reticularis (most inner cortical zone). The adrenal medulla possesses a complex venous drainage system where the right adrenal suprarenal vein drains into the inferior vena cava and the left one empties into the renal vein (for review see Coupland, 1975). The adrenal glands are mainly innervated by secretomotor sympathetic fibers running through the ipsilateral greater splanchnic nerve branches. These nerve branches form the adrenal plexus which derives mainly from the celiac plexus but is connected to the renal and phrenic plexuses.

The cholinergic nature of the preganglionic sympathetic innervation to the adrenal medulla was demonstrated in the classical experiments of Feldberg and collaborators (Feldberg et al., 1934). It was also shown that acetylcholine (ACh) was the humoral transmitter of splanchnic stimulation to the medullary tissue. Splanchnic stimulation causes the release of the chromaffin cell hormones, which consist mainly of adrenaline (Ad) and noradrenaline (NA). In addition, small but measurable amounts of dopamine (DA) have been detected in adrenal gland effluents of certain species following nico-

tinic stimulation.

The content of the most abundant adrenal medullary catecholamines Ad and NA varies greatly in different mammalian species, ranging from 120 to 2230 µg per gram of medullary tissue (Holzbauer & Sharman, 1972). In healthy humans, their content is about 600 µg per gram of adrenal medulla, which is constituted of 83% of Ad and 17% of NA (Goodall, 1951). During human development, the total catecholamine content of the adrenal medulla changes as well as the ratio of Ad to NA. In foetuses and neonates, NA is the predominant species though the proportion of Ad compared to NA increases with age (West et al., 1951). In vertebrates, it was shown that a change in the Ad to NA ratio was coupled with a change in the topographical association of cortical and chromaffin tissues (Wright & Jones, 1955). Furthermore, it was demonstrated that adrenocorticotrophic hormone (ACTH) and glucocorticoids stimulate phenylethanolamine N-methyltransferase activity and consequently influence the synthesis of Ad in the adrenal medulla (Wurtman & Axelrod, 1966).

The adrenal medullary hormones Ad and NA appear to possess independent functional roles. Different physiological conditions produce a selective release of the two hormones. Several studies in the literature support this hypothesis; for example, hyperglycemia or carotid occlusion decreases the amount of Ad secreted (Burn et al., 1950; Euler & Folkow, 1953). On the other hand, an increased release of Ad was observed under sciatic or branchial plexus stimulation, asphyxia or ACh stimulation (Euler & Folkow, 1953; Redgate & Gellhorn, 1953; Outschoorn, 1952). Recently, it was reported that DA might modulate catecholamine release triggered by stimulation of the

nicotinic cholinoceptor since dopaminergic receptors were shown to be present in cat adrenal medullary chromaffin cell membranes and that dopaminergic receptor antagonists inhibited the effect produced by DA and apomorphine (Artalejo et al., 1985).

The hormonal secretion of the adrenal medulla is controlled by hypothalamic structures and the medulla oblongata (Magoun et al., 1937). Stimulation of discrete areas of the hypothalamus was shown to influence the ratio of Ad to NA released from the gland (Redgate & Gellhorn, 1953; Folkow & Euler, 1954). In addition, Kennard (1945) suggested that autonomic areas of the cerebral cortex may influence the hypothalamic components involved in adrenal medulla secretion, although this was only clearly demonstrated later by Folkow and collaborators (Ferguson et al., 1957; Euler & Folkow, 1958).

The adrenal medullary hormones Ad and NA were recognized as early as the beginning of this century as activators of several physiological mechanisms in emergency situations (Cannon & de la Paz, 1911; Cannon et al., 1924, 1926). Their stimulating effect on the heart, vasodilation of skeletal muscle vessels, venoconstriction, decrease in gut motility, bronchiolar muscle relaxation, pupillary dilation and mobilization of liver glycogen and free fatty acids all contribute to increase the organism's efficiency under emergency conditions. In man, emotional or mental stress, such as anxiety and irritation, or also physical stress produces an increased secretion of catecholamines. Furthermore, several other stimuli such as cold, heat, blood pH (acidosis or alkalosis), asphyxia, anoxia, hypertension and hypoglycemia result in an increased catecholamine secretion (for review see Lewis, 1975).

The existence of two types of cells in the adrenal medulla was first

suggested by Eränkö, although it was only clearly demonstrated by Hillarp and Hökfelt that this tissue possesses distinct Ad and NA containing cells (Eränkö, 1951, 1952; Hillarp & Hökfelt, 1953). Following these studies, Eränkö was able to show that a good correlation existed between these two specific cell types and the contents of the two hormones in adrenal medullary tissue (Eränkö, 1955a, 1955b, 1955c). Electron microscopy studies revealed that the two types of chromaffin cells are different in terms of vesicular ultrastructures. NA forms an electron dense polymeric structure while Ad does not (Coupland & Hopwood, 1966; Benedeczky & Smith, 1972; Grynszpan-Winograd, 1975). In the rat, the mean intramembranous diameter of Ad-containing granules measures 190 nm whereas the mean diameter of the NA-storing granules measures 250 nm. However, the diameter of the dense core of NA-containing types is smaller than of Ad-containing granules (D'Anzi, 1969; Benchimol & Cantin, 1977).

The general ultrastructural features of the two chromaffin cell types are very similar except for their vesicular ultrastructural differences. The cells measure approximately 15 μm in diameter with nuclei which are fairly big and centrally located. However, the nucleus is poor in chromatin and, in addition to marginated-chromatin, some associated-chromatin is detected near the nucleolus. The cell contains stacks of rough endoplasmic reticulum, relatively abundant, elongated mitochondria, a few small lysosomes, multivesicular bodies and numerous residual bodies often showing lipid droplets. The Golgi apparatus located near the nucleus is prominent, and often, coated vesicles can be found scattered between elements of the Golgi complex (Winkler, 1977).

The adrenal medulla also contains a third type of chromaffin cell, the so-called small granule-containing cell (SGC). They have been described in adrenal medulla of rodents, avians, reptiles, dogs and also in human foetal adrenal medulla (Diner, 1965; Kobayashi et al., 1978; Unsicker et al., 1978; Unsicker 1973, 1976a, 1976b; Kajihara et al., 1978; Hernoven, 1971). The SGC cells of the adrenal medulla are also referred to as small granule chromaffin cells since they display a positive chromaffinity staining under the light microscope (Kobayashi, 1977; Kobayashi et al., 1978). They constitute only 1-5% of the total chromaffin cell population with secretory granules measuring around 100-120 nm and overall resemble very much the small intensely fluorescent (SIF) cells of the sympathetic ganglia (Coupland et al., 1977; Kobayashi et al., 1978). The nature and functional significance of the SGC cells of the adrenal medulla are not clear although several possibilities have been suggested. They may be dopamine-containing cells or correspond to an immature type of chromaffin cells or finally, belong to the transitional forms between nerve cells and purely endocrine chromaffin cells (Unsicker, 1973; Kobayashi, 1977; Kobayashi & Coupland, 1977; Kajihara et al., 1978).

The adrenal medullary chromaffin cells are derived embryologically from the neural crest, sharing a common origin with the sympathetic neurons (Euler, 1972). Developmental biology experiments performed in the embryonic neural axis of quail have shown that adrenal medullary cells arise from the 18th to 24th somite level of the neural crest, whereas the sympathetic ganglia originate from a region in the proximity of the 5th somite level. Furthermore, some cells from other somite levels are able to differentiate into

true chromaffin cells if the microenvironment is appropriate at the term of their migration (Le Douarin & Teillet, 1974; Le Douarin et al., 1975).

In 1969, Pearse proposed that a group of apparently unrelated endocrine cells, some in endocrine glands, others in nonendocrine tissues, sharing a number of cytochemical and ultrastructural characteristics would be designated as the APUD (Amine, Precursor Uptake and Decarboxylation) series. The APUD series included the adrenal medullary chromaffin cells as well as other polypeptide-secreting endocrine cells (Pearse, 1969). At the beginning, the hypothesis was that these cells originated from the neural crest only, but later on, as new members were included in the APUD series, it appeared that these cells were derived not only from the neural crest itself but from the neuroectoderm in its fullest sense (Pearse, 1976). As the APUD concept evolved, a new concept appeared which included morphological and physiological tinges to characterize these cells. The secretory cells which exhibited such characteristics were named "paraneurons" (Fujita, 1976, 1977; Fujita & Kobayashi, 1979). Therefore, the adrenal medullary chromaffin cell was recognized as a typical paraneurone (Kobayashi, 1977).

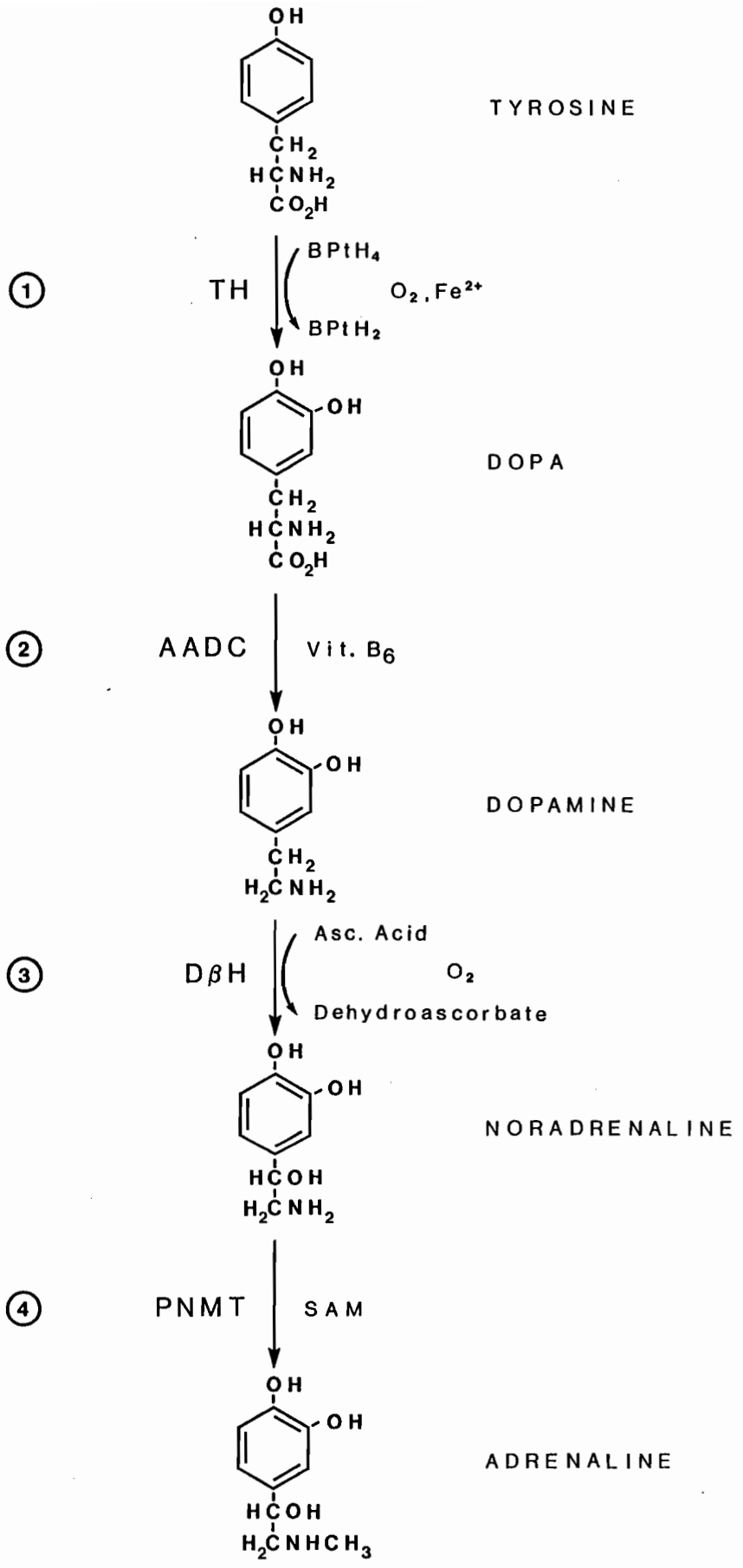
B. CATECHOLAMINE BIOSYNTHESIS

The catecholamines are a group of structurally related substances composed of a catechol nucleus (benzenic ring with two adjacent hydroxyl groups) and an amine-containing side chain. The catecholamines known to occur in man are Ad, NA and DA. Catecholamines are synthesized in brain, sympathetic nerves and ganglia, adrenal medullary chromaffin cells, chromaffin cell tumors (pheochromocytomas) and early in life in the organ of Zuckerkandl. The biosynthesis of catecholamines occurs *in vivo* from their amino acid precursors, tyrosine, obtained from dietary sources, and phenylalanine, via conversion by the enzyme phenylalanine hydroxylase. The biosynthesis pathway of catecholamines was first postulated by Blaschko (1939) and finally confirmed in 1964 by Nagatsu and colleagues. (See Fig. 1 for biosynthesis pathway).

The first step in catecholamine biosynthesis is the conversion of tyrosine to DOPA which involves the metahydroxylation of the former via the catalytic action of the enzyme tyrosine hydroxylase (TH) (E.C. 1.14.16.2) or tyrosine 3-monooxygenase. This enzyme is stereospecific and requires molecular O_2, Fe^{2+} and L-erythro-5,6,7,8-tetrahydrobiopterin as cofactor. The hydroxylation of tyrosine to DOPA is slower than the subsequent decarboxylation and parahydroxylation, and therefore tyrosine hydroxylase is the rate-limiting enzyme (Spector *et al.*, 1963; Levitt *et al.*, 1965). TH exists in two kinetically distinct forms with different affinities for the pterin cofactor (Wilson *et al.*, 1981; Weiner *et al.*, 1982). One of these forms most probably corresponds to the phosphorylated enzyme described in rat striatum

Fig. 1 Catecholamine biosynthesis pathway of the adrenal medulla.

TH, tyrosine hydroxylase; BPt, biopterin; AADC, aromatic L-amino acid decarboxylase; Vit. B_6, vitamin B_6; DβH, dopamine β-hydroxylase; Asc. Acid, ascorbic acid; PNMT, phenylethanolamine N-methyltransferase; SAM, S-adenosylmethionine.


TYROSINE
① TH
BPtH4
O2, Fe2+
BPtH2
DOPA
② AADC
Vit. B6
DOPAMINE
③ DβH
Asc. Acid
O2
Dehydroascorbate
NORADRENALINE
④ PNMT
SAM
ADRENALINE

(Lovenberg et al., 1975) and more recently identified in adrenal medulla (Haycock et al., 1982a; Meligeni et al., 1982; Treiman et al., 1983). The phosphorylation of TH decreases its K_m and appears essential for its activation (Haycock et al., 1982b; Vigny & Henry, 1982). In addition, the level of pterin cofactor which is regulated by the induction of the enzyme GTP-cyclohydroxylase (E.C. 3.5.4.16) seems to be an important factor in the regulation of tyrosine hydroxylase due to the presence of a low cofactor concentration in tissues (Viveros et al., 1981a, 1981b; Abou-Donia & Viveros, 1981). The transsynaptic induction of TH is initiated under physiological conditions by ACh (Thoenen et al., 1969). In addition, the biosynthesis of the enzyme can be induced by long-term as well as short-term regulation by numerous physiological stimuli or exposure to some pharmacological agents (Gordon et al., 1966; Chuang & Costa, 1974; Zigmond et al., 1974; Lerner et al., 1977; Mitchell & Vulliet, 1985). Tyrosine hydroxylase is directly controlled by end-product feedback inhibition as well as by phospholipids and anions (Ikeda et al., 1966; Lloyd & Kaufman, 1974; Lloyd, 1979; Kuczenski & Mandell, 1972). Very recently, an endogenous tyrosine hydroxylase activity was detected in developing chick heart, and it was suggested that it might represent a source of extraneuronal catecholamines (Stewart & Kirby, 1985).

In the next step of catecholamine biosynthesis, DOPA is converted to DA through decarboxylation. This biotransformation is catalyzed by the enzyme DOPA decarboxylase (E.C. 4.1.1.28) also referred to as aromatic-L-amino acid decarboxylase. This enzyme is very active and requires pyridoxal phosphate (vitamin B_6) as a cofactor. The maximal rate of decarboxylation in pheochromocytoma cells was found to be at least an order of magnitude greater than

the rate of DOPA synthesis in these cells, indicating that the decarboxylation was not a rate-limiting step. It was also found that treatments which increased the activity of tyrosine hydroxylase did not alter the activity of the decarboxylase, which indicates that these two enzymes are not regulated coordinately (Wang & Perlman, 1981). The active enzyme, isolated and characterized in bovine adrenal, is a protein of 56kD which is derived from a 78kD polypeptide through proteolytic degradation during posttranslational modification (Albert et al., 1982).

The third step of the pathway consists in the synthesis of NA from DA via the action of the enzyme dopamine beta-hydroxylase (DβH) (E.C. 1.14.17.1) also known as dopamine beta-monooxygenase. This enzyme was first isolated from adrenal medulla extracts and found to be a copper-containing protein (Levin et al., 1960). The copper atoms are believed to bind to the active site of the enzyme although the exact number of atoms bound per subunit is still controversial (Skotland & Ljones, 1983). DβH is a mixed function oxidase which requires molecular oxygen and utilizes ascorbic acid as a cofactor (Diliberto, 1981). There is uncertainty as to how the semidehydroascorbate, a species produced during the enzymatic reaction, is recycled back to ascorbate in vivo. The primary subcellular localization of semidehydroascorbate reductase, a key enzyme in the regeneration of ascorbate, was found to be in the mitochondrial outer membrane (Diliberto et al., 1982). Since the hydroxylation of DA occurs within the chromaffin vesicles and the reductase is located on another organelle in the cytoplasm, this process appears to be rather complex. Consequently, it was proposed that cytochrome b561, the second most abundant protein in the chromaffin granule membrane,

is probably involved in this important function of electron transfer (Wakefield et al., 1982; Njus et al., 1983). DβH exists as two enzymatic forms, a so-called free form localized in the soluble content of the chromaffin granules and a membrane-bound form attached to the inner surface of the chromaffin granule (König et al., 1976; Aunis et al., 1977; Huber et al., 1979). The two forms of DβH are similar but not identical since their carbohydrate composition is identical but their amino acid content is different (Blakeborough et al., 1981; Slater et al., 1981; Fischer-Colbrie et al., 1982). In terms of molecular weight, the two forms are different, the molecular weight of the soluble native form being 300,000 and that of the membrane-bound form being greater than 1,000,000. In electrophoretic studies, it was demonstrated that the membrane-bound form was composed of two nonidentical subunits with molecular weights of 75,000 and 70,000. It was also found that the membrane-bound DβH was about one third as active as the soluble form (Fleming & Saxena, 1982; Saxena & Fleming, 1983). The synthesis, activation and degradation of DβH are regulated by various neuronal and humoral factors (for review see Carmichael, 1984).

The final step in the catecholamine biosynthesis corresponds to the conversion of NA to Ad which is catalyzed by the enzyme phenylethanolamine N-methyltransferase (PNMT) (E.C. 2.1.1.28). The enzyme is located solely in Ad-storing cells. This enzyme is mainly restricted in mammals to the adrenal medulla, although low levels of activity have been detected in certain brain areas as well as in heart and lung cells (Pendleton et al., 1978). PNMT utilizes S-adenosylmethionine as a cofactor in the enzymatic transmethylation. The cofactor is regulated by its synthesizing enzyme methionine adeno-

syltransferase, which in turn appears to be influenced by glucocorticoids (Wong & Ciaranello, 1982; Wong et al., 1982). PNMT may be regulated by an acute response to substrate and end-product inhibition, or another regulatory mechanism may entail the transformation of one form of the enzyme to more active forms (Burke et al., 1982). Although PNMT was considered to be solely a cytosolic enzyme, it may also be directly associated with Ad-containing vesicle membranes (Van Orden et al., 1977; Winkler & Carmichael, 1982).

Recently, it has been postulated that TH, DβH and PNMT, enzymes involved in catecholamine biosynthesis, are co-regulated by their biosynthesis. These three enzymes have been referred to as the "catecholamine enzyme gene family" since they apparently share a common gene sequence and have evolved from a common ancestral precursor (Joh et al., 1983, 1984).

C. THE CHROMAFFIN GRANULE: A CATECHOLAMINE-STORING ORGANELLE

Adrenal medullary catecholamines are stored in membrane-bound organelles, the secretory granules, and are released into the blood stream via exocytosis (Trifaró, 1977).

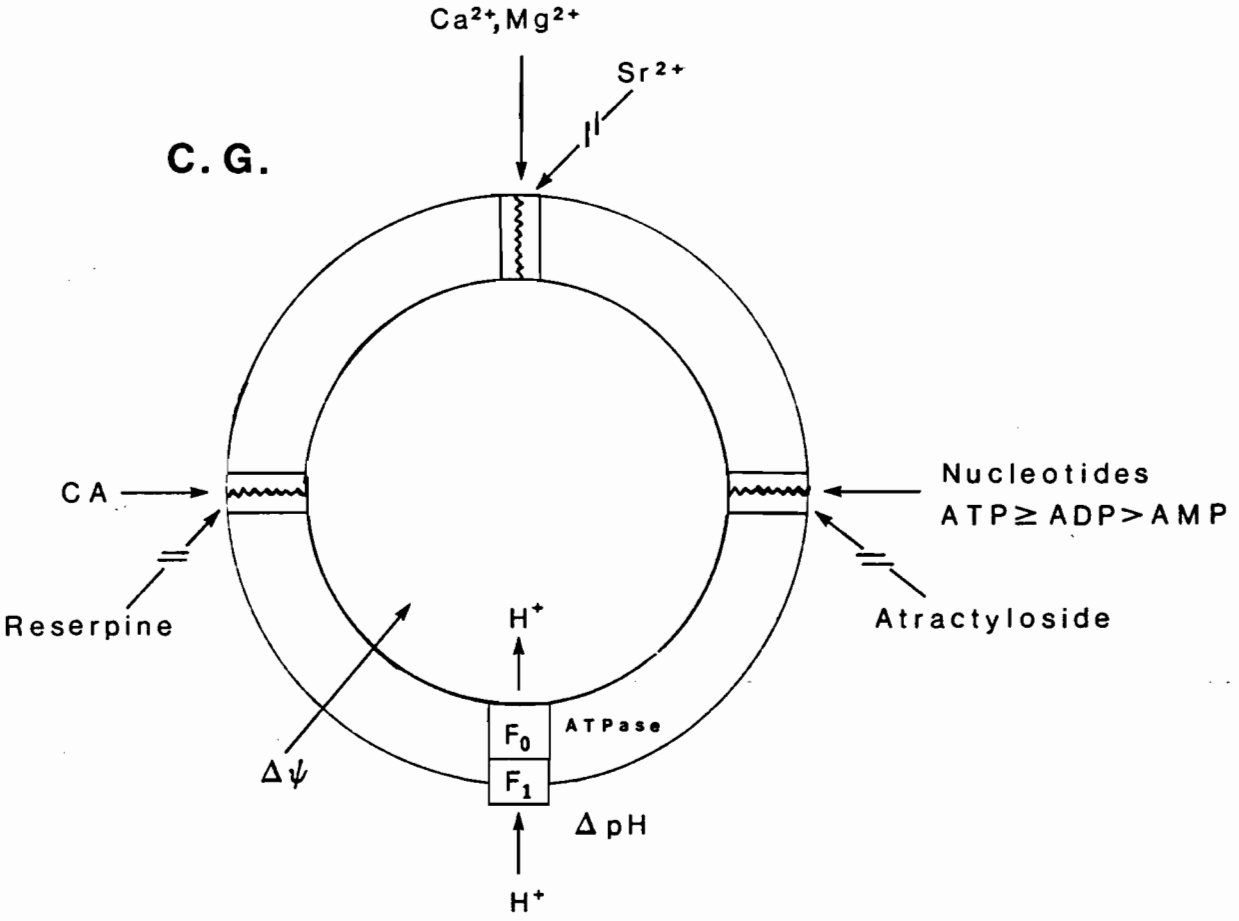
The presence of hormone-storing organelles (chromaffin granules) in the adrenal medulla was first reported in 1953 when it was observed that centrifugation of adrenal medulla homogenates led to the sedimentation of the major portion of the hormones (catecholamines) of this tissue (Blaschko & Welch, 1953; Hillarp et al., 1953). The chromaffin granules are multifunctional organelles involved in the uptake, storage, biosynthesis and secretion of catecholamines (Smith, 1968; Winkler & Smith, 1975). Since techniques are available to isolate the chromaffin granules in large amounts and high purity, their composition, organization and function have been studied extensively (Winkler & Smith, 1975; Winkler, 1976, 1977; Winkler & Westhead, 1980; Winkler & Carmichael, 1982; Carmichael, 1983; Hogue-Angeletti et al., 1985). Consequently, they have been utilized as a model system to study the molecular organization of neurotransmitter-storing vesicles (Winkler et al., 1981; Njus et al., 1985).

The chromaffin granules contain very high concentrations of catecholamines and ATP. ATP is the most abundant nucleotide making up 70% of the total nucleotide content with ADP, AMP, GTP, GDP, UTP and UDP making up the rest (Van Dyke et al., 1977). The catecholamines are stored bound to ATP in a ratio of about 4.5:1 with granular concentration of catecholamines and ATP

of approximately 600 mM and 135 mM, respectively (Phillips & Apps, 1979; Sen et al., 1979). However, it should be pointed out that these values may differ with size and age of the granules. The intragranular pH is 5.6 and therefore it has been calculated that only 0.1% of the catecholamines are present in the unprotonated form (Johnson & Scarpa, 1976; Casey et al., 1977). The concentration of catecholamines and ATP inside the granules is maintained by a carrier-mediated process. The Mg^{2+}-ATPase of chromaffin granules acts as an electrogenic proton pump which is driven by a transmembrane pH gradient (ΔpH) and a membrane potential ($\Delta\psi$) (Bashford et al., 1976; Scherman & Henry, 1979; Apps et al., 1980a; Kanner et al., 1980). The uptake of nucleotides takes place via the action of a separate carrier which is driven by an electrical potential ($\Delta\psi$) (Weber & Winkler, 1981) (see also Fig. 2). The two carriers are selectively inhibited by different agents (Winkler & Carmichael, 1982). Membranes of chromaffin granules have a low permeability for cations; however, it has been demonstrated that calcium and magnesium can be taken up into the granules (Kostron et al., 1977; Daniels et al., 1979).

The coexistence of peptides in neuroendocrine systems has been detected and by now, extensively documented (for review see Sundler et al., 1985). In adrenal chromaffin cells, it was found that in addition to catecholamines, certain opioid peptides (enkephalins and dynorphin), serotonin, γ-amino butyric acid (GABA) as well as neuropeptides such as substance P (SP), neurotensin, vasoactive intestinal polypeptide (VIP), somatostatin and neuropeptide Y are stored within and secreted from chromaffin granules (Viveros et al., 1979; Stine et al., 1980; Livett et al., 1981; Lemaire et al., 1982,

Fig. 2 Uptake of nucleotides, ions and catecholamines into the chromaffin granule. The three uptake mechanisms in chromaffin granules (C.G.) are schematically represented. The proton-translocating ATPase of chromaffin granules acts as an electrogenic proton pump which is driven by a transmembrane pH gradient (ΔpH) and a membrane potential ($\Delta\psi$). The Mg^{2+}-ATPase of chromaffin granules is composed of an ATP-splitting part (F_1) and a proton-translocating part (F_0). Two specific carriers appear to be responsible for the translocation of catecholamines (CA) and nucleotides. These two carriers are selectively inhibited by different agents (e.g. reserpine, atractyloside).


C. G.
Ca2+,Mg2+
Sr2+
CA
Reserpine
Nucleotides
ATP≥ ADP>AMP
Atractyloside
H+
F0
ATPase
F1
Δψ
Δ pH
H+

1984; Verhofstad & Jonsson, 1983; Kataoka et al., 1984, 1985; Role et al., 1981; Goedert et al., 1983; Eiden et al., 1983).

The chromaffin granule membrane is a basic bimolecular lipid leaflet of about 8 nm. The lipids of this membrane consist of cholesterol, phospholipids (with 17% lysolecithin) and gangliosides (for review see Winkler et al., 1981). The topographical arrangement of the lipids within the chromaffin granule membranes has been reviewed extensively (Winkler & Westhead, 1980). By electrophoretic techniques, it was shown that this membrane contains at least 46 proteins (Trifaró et al., 1982). However, it should be pointed out that some of these polypeptides might be present due to contamination during membrane isolation (Huber et al., 1979; Abbs & Phillips, 1980; Corcoran et al., 1982). The major chromaffin granule membrane protein, DβH, represents about 20% of the total membrane protein content and is localized on the inner surface of the granule membrane (Hörtnagl et al., 1972; Redick et al., 1974; König et al., 1976). Cytochrome b561 (also known as chromomembrin B) is the second major protein component and is located on the outer surface of the granule membrane (König et al., 1976; Abbs & Phillips, 1980). The other protein components of the chromaffin granule membrane include adenosine 5'-triphosphatase (ATPase) which consists of an ATP-splitting part (F_1) located on the outer surface of the granule membrane and a proton translocating part (F_0) embedded within the membrane (Njus & Radda, 1978; Apps et al., 1980b); phosphatidylinositol kinase, with its active center located on the outer surface of the membrane (Phillips, 1973; Trifaró, 1973; Trifaró & Dworkind, 1975); NADH:oxidoreductase (Terland & Flatmark, 1980; Zaremba & Hogue-Angeletti, 1982); synaptin, which is localized on the outer surface of the

membrane (Bock & Helle, 1977); actin (Burridge & Phillips, 1975; Phillips & Slater, 1975; Meyer & Burger, 1979; Aunis et al., 1980; Hesketh et al., 1981; Lee & Trifaró, 1981); α-actinin (Jockush et al.,1977; Aunis et al., 1980; Trifaró et al., 1982; Bader & Aunis, 1983) and a spectrin-like protein (Aunis & Perrin, 1984; Perrin & Aunis, 1985).

The chromaffin granules contain a large number of soluble proteins which represent 75-80% of the total granule protein content. These proteins, which have been collectively named chromogranins (Blaschko et al., 1967), are secreted from the chromaffin cell by exocytosis (Smith & Winkler, 1972; Viveros, 1975). However, this term now is mainly restricted to a family of acidic glycoproteins (Winkler et al., 1984). The major secretory protein, chromogranin A, comprises about 40% of the total soluble proteins (Winkler, 1976). It is a polypeptide of 80kD which possesses an O-glycosidically-linked carbohydrate chain (Kiang et al., 1982; Banerjee & Margolis, 1982; Fischer-Colbrie et al., 1982; Somogyi et al., 1984).. It was shown recently, using antibodies against chromogranin A, that this protein belongs to a family of related polypeptides which have the same isoelectric point but different molecular weights, the so-called "chromogranins A" (O'Connor & Frigon, 1984; Kilpatrick et al., 1984). Therefore, it was suggested that chromogranin A is broken down by endogenous proteases within the granules. A second family of acidic polypeptides of identical isoelectric points and molecular weights ranging from 100,000 to 24,000 was named chromogranins B (Fischer-Colbrie & Frischenschlager, 1985). It was demonstrated that these two families of chromogranins derive from different genetic precursors (Falkensammer et al., 1985). The chromaffin granules also contain other

soluble proteins which include the enzyme DβH, glycoprotein III (Fischer-Colbrie et al., 1982, 1984), enkephalin precursors (Udenfriend & Kilpatrick, 1983) and proteoglycans (Kiang et al., 1982; Banerjee & Margolis, 1982).

D. CATECHOLAMINE SECRETION

D.1 Involvement of Different Types of Receptors in Catecholamine Secretion

The adrenal medullary chromaffin cells receive direct innervation from the preganglionic sympathetic fibers of the splanchnic nerve. Splanchnic stimulation causes the release of ACh which then interacts with the cholinergic receptors at the level of the chromaffin cell plasma membrane and finally results in the secretion of catecholamines from the cells. Both nicotinic and muscarinic receptors have been found in the adrenal medulla of several species. However, some species possess only one type of receptor; for example, nicotinic only in hamsters and muscarinic only in chicks (Liang & Perlman, 1979; Ledbetter & Kirshner, 1975). The relative involvement of the nicotinic and muscarinic receptors in catecholamine secretion depends upon the concentration of ACh to which the receptors are exposed. Recent studies have shown that other receptor types are also involved in the secretory process of these cells.

D.1.1 Nicotinic and Muscarinic Receptors

The bovine adrenal medulla possesses both nicotinic and muscarinic receptors, but only the nicotinic receptors are involved in ACh-stimulated secretion of catecholamines (Mizobe et al., 1979; Trifaró & Lee, 1980). In addition, it has been demonstrated that α-bungarotoxin binds with high affinity to chromaffin cells but does not inhibit nicotinic receptor-induced

catecholamine secretion (Kumakura et al., 1980b; Quik & Trifaró, 1982). The muscarinic receptors are activated by low ACh concentration ($<10^{-7}$M) producing an intracellular elevation of cGMP which consequently inhibits the nicotinic receptor-induced secretion of catecholamines (Schneider et al., 1979; Yanagihara et al., 1979; Derome et al., 1981; Fisher et al., 1981). In addition, the muscarinic receptors are also known to enhance phospholipid labelling with ^{32}Pi and mediate a rise in cytosolic Ca^{2+}, which may suggest a possible involvement of protein kinase C in their physiological action (Fisher et al., 1981; Kao & Schneider, 1985). Therefore, under basal conditions which correspond to low ACh concentration released by splanchnic stimulation, the chromaffin cells are probably under inhibitory control. On the other hand, at higher ACh concentrations which may result from intense splanchnic stimulation due to stress conditions, the activation of nicotinic receptors may overcome the muscarinic inhibitory activity. It has been proposed that the dual control of catecholamine secretion via the nicotinic and muscarinic receptors may have a physiological function in maintaining a low basal secretion while allowing increased secretion upon intense stimulation of the splanchnic nerve (Derome et al., 1981).

D.1.2. Opioid and Neuropeptide Receptors

Recently, the presence of opioid peptides and several other neuropeptides was demonstrated in the adrenal medulla and, for some of them, also in the terminals of the splanchnic nerve. These peptides can interact with the chromaffin cells via different receptor species. Therefore, these findings

suggest that these different endogenous neuropeptides may play a role in the regulation or modulation of catecholamine secretion from the adrenal medulla (for review see Livett et al., 1983).

The opioid peptide family has been implicated in modulating catecholamine secretion from the adrenal medulla (Kumakura et al., 1980a). The presence of enkephalin-like immunoreactivity was shown in chromaffin cells as well as in the splanchnic nerve terminals innervating these cells (Schultzberg et al., 1978; Pelto-Huikko et al., 1985). There exists a controversy concerning the co-localization of enkephalins and catecholamines, which are stored in either Ad- or NA-containing cells, or maybe in both, in different species (Lundberg et al., 1979; Linnoila et al., 1980; Hernoven et al., 1980; Pelto-Huikko et al., 1982, 1985; Varndell et al., 1982; Livett et al., 1982; Kobayashi et al., 1983; Kondo & Yui, 1984). However, in bovine adrenal medulla, both Leu- and Met-enkephalins appear to be localized exclusively in Ad-containing chromaffin cells (Livett et al., 1982; Roisin et al., 1983; Bloch et al., 1984). In isolated bovine adrenal chromaffin cells, dynorphin (1-13) appears to be mainly localized in NA-containing chromaffin cells (Dumont et al., 1983). The opioid peptides seem to have a complex mode of action in modulating catecholamine secretion. Enkephalins, β-endorphin, dynorphin (1-13) and opiates (including morphine) were found to inhibit nicotine-evoked secretion of catecholamines without affecting high K^+-evoked release. However, this inhibition occurred only at very high concentrations of enkephalins and morphine ($>10^{-5}M$), lacked stereospecificity and was not reversible by naloxone and naltrexone. Therefore, it is unlikely that this inhibition of catecholamine release by opiates and opioid peptides is medi-

ated via specific opiate receptors (Lemaire et al., 1981; Dean et al., 1982). It should be pointed out though, that a controversy still exists concerning the selectivity issue since other groups maintain that this inhibition takes place via high-affinity stereospecific opiate receptors (Costa et al., 1980; Saiani & Guidotti, 1980, 1982). In contrast, morphine and opioid peptides were found to enhance basal release of catecholamines at concentrations ranging from 10^{-6} to 10^{-3}M. This enhancement by morphine was found to be calcium-dependent, stereospecific and reversed by naloxone and naltrexone (Dean & Livett, 1981). The physiological significance of the inhibition seen by β-endorphin in bovine adrenal medullary catecholamine secretion is still obscure although recently, it was demonstrated that this peptide was present in human adrenal medulla but not detected in other mammalian species (Evans et al., 1983).

Substance P has been mainly localized within the splanchnic nerve terminals that make contact with the chromaffin cells, whereas somatostatin (14 and 28) appears to be contained within the chromaffin cells (Livett, 1984). SP and somatostatin-14 were found to inhibit the ACh- and nicotine-mediated release of catecholamines from isolated chromaffin cells though without affecting basal release or K^+-evoked secretion (Livett et al., 1979; Mizobe et al., 1979). Furthermore, their interaction with the chromaffin cells appears to be specific for the nicotinic receptor-ionophore complex, in which case, SP may possibly interact with the Na^+ transport through the complex (Mizobe et al., 1979; Dean & Livett, 1980). Electrophysiological studies using patch-clamp techniques also support the involvement of SP in nicotinic receptor regulation (Clapham & Neher, 1984a). In addition to its inhibitory

action on nicotine-mediated release of catecholamines, SP also protects catecholamine secretion against nicotinic desensitization (ACh and nicotine at high concentrations) (Boksa & Livett, 1984). Recently, it has been shown that the SP receptors modulating adrenal catecholamine release are neither of the "SP-P" nor of the "SP-E" receptor subtype but rather, belong to a third subtype of SP receptors which shares several characteristics with the receptors mediating histamine release from mast cells (Boksa & Livett, 1985). In addition, recent in vivo studies in neonatal capsaicin-treated rats have provided further evidence for the neuromodulatory role of sensory nerves containing SP in the secretion of catecholamines from the adrenal medulla (Khalil et al., 1984).

The fact that the adrenal medulla and the splanchnic nerve contain high levels of enkephalins, that enkephalins and other opioid peptides enhance basal catecholamine release, and that SP is inhibitory on ACh-mediated release, together suggest that opioid peptides and SP might control the homeostatic status of the adrenal medulla by opposing actions.

D.1.3. Adrenergic and Dopaminergic Receptors

The adrenal medullary chromaffin cells, in response to stimulation, secrete catecholamines directly into the circulation. Presynaptic inhibition of neurotransmitter release via the action of inhibitory presynaptic receptors is thought to be a general phenomenon in secretory cells and is considered to be a mechanism for fine regulation of the release process (Langer, 1976, 1977). At the moment, the presence and functional role of the

adrenergic receptors in the adrenal chromaffin cells are still the subject of a great controversy. Collett and Story (1982) found no evidence for a modulating role for the α-adrenoceptors of the chromaffin cells. In contrast, Wada and collaborators have shown that α-adrenergic receptor activation inhibited catecholamine release from chromaffin cells and also that inhibition is probably mediated via adrenergic receptors of type α_2 (Wada et al., 1982; Sakurai et al., 1983). Finally, Greenberg and Zinder (1982) have demonstrated in isolated bovine chromaffin cells that the α-agonist clonidine inhibited catecholamine release whereas the β-agonist isoproterenol enhanced release from this system. Therefore, it was suggested that the adrenergic receptors of the chromaffin cells are involved in the fine regulation of catecholamine secretion from the adrenal medulla.

Recently, it was demonstrated that a dopaminergic receptor modulates catecholamine release from the cat adrenal gland (Artalejo et al., 1985). DA (1 μM) and apomorphine (1-10 μM) were shown to markedly inhibit catecholamine release evoked by nicotine. The dopamine receptor antagonists, haloperidol, sulpiride and picobenzide, completely reversed the inhibition of catecholamine release produced by dopamine and dopamine receptor agonists. Therefore, it was suggested that dopaminergic antagonists removed a negative feedback mechanism that inhibits nicotine-evoked catecholamine release (Artalejo et al., 1985).

D.2 Catecholamine Secretion: An Exocytotic Event

De Robertis and collaborators were the first to propose that the release of the contents of the adrenal medullary chromaffin granules into the extracellular space occurred via exocytosis (reverse pinocytosis). Exocytosis is a mechanism whereby the membrane of the secretory granule fuses with the plasma membrane of the chromaffin cell (De Robertis & Vaz Ferreira, 1957; De Robertis & Sabatini, 1960). The functionality of the proposed mechanism has been substantiated by several morphological studies (Diner 1967; Malamed et al., 1968; Grynszpan-Winograd, 1971; Smith et al., 1973) and also by ultrastructural studies which showed omega-shaped invaginations associated with extracellular dense cores (Benedeczky & Smith, 1972; Grynszpan-Winograd, 1975). In addition, biochemical evidence supports release by exocytosis rather than by intracytoplasmic release (Smith, 1973; Viveros, 1975).

Upon stimulation of the adrenal medulla or isolated chromaffin cells, catecholamines are released as well as other soluble granular components such as ATP (Douglas et al., 1965; Lastowecka & Trifaró, 1974), chromogranin A (Banks & Helle, 1965), DβH (Viveros et al., 1968; Fenwick et al., 1978), and opiate peptides (Viveros et al., 1979; Lemaire et al., 1982). Although large polypeptides such as DβH and chromogranin A are released during exocytosis, no cytoplasmic marker enzymes such as lactate dehydrogenase or PNMT are detected in the effluents secreted from the chromaffin cells (Kirshner et al., 1966; Schneider et al., 1967; Lastowecka & Trifaró, 1974; Fenwick et al., 1978). Therefore, exocytosis appears to be the mechanism of secretion in the adrenal medulla and other endocrine glands which store hormones in

subcellular granules (for review see Trifaró, 1977).

In recent studies, several approaches have been used in attempts to gain access to the intracellular sites of exocytosis in order to examine the molecular nature of the events involved. Such approaches include the use of "leaky" cells, which are chromaffin cells permeabilized by exposure to intense electric fields (Baker & Knight, 1980, 1981; Knight & Baker, 1982), detergent permeabilized chromaffin cells using saponin or digitonin as detergents (Brooks & Treml, 1983a; Dunn & Holz, 1983; Wilson & Kirshner, 1983) and, more recently, microinjection of macromolecules in chromaffin cells using the erythrocyte ghost technique (Trifaró & Kenigsberg, 1983; Kenigsberg & Trifaró, 1985a, 1985b) (see also reviews Burgoyne, 1984; Creutz, 1984; Holz, 1985; Pollard et al., 1985).

D.3 Ion Channels in Secretion and Electrophysiology of Adrenal Chromaffin Cells

Houssay and Molinelli (1928) were the first to suggest the importance of Ca^{2+} in hormone secretion from the adrenal medulla. However, only many years later, Douglas and Rubin (1961) demonstrated that Ca^{2+} ions were indispensable in the secretory process of the adrenal medulla. Douglas also proposed the concept of "stimulus-secretion coupling", a concept which intended to include all the events involved in the secretory process and which possessed great similarity to the excitation-contraction coupling in muscle (Douglas, 1968). Since Ca^{2+} is an essential requirement in the secretory process, omission of Ca^{2+} from the extracellular environment blocks the release of

hormone in response to different secretagogues. On the other hand, reintroduction of Ca^{2+} into a Ca^{2+}-free medium produces a sharp increase in catecholamine secretion (Douglas & Rubin, 1961). Other divalent cations, such as Mg^{2+} and Ba^{2+}, were found to produce different effects on the secretory process. It was shown that increasing the extracellular Mg^{2+} concentration produced an inhibition of catecholamine release (Douglas & Rubin, 1963). It was also demonstrated that the replacement of extracellular Ca^{2+} by either Sr^{2+} or Ba^{2+} was effective in maintaining the stimulation-induced release of catecholamine, and furthermore, that Ba^{2+} itself, in absence of extracellular Ca^{2+}, was a potent secretagogue (Douglas & Rubin, 1964).

The physiological stimulus for adrenal medullary catecholamine secretion is ACh liberated from splanchnic nerve terminals (Feldberg et al., 1934). It has been demonstrated that in perfused adrenal medulla and in chromaffin cell tissue cultures, ACh produces catecholamine secretion (Douglas & Rubin, 1961; Douglas et al., 1967a, 1967b). In addition, catecholamine output was observed when K^+ concentration was increased in the extracellular environment. However, in both types of stimulation, catecholamine secretion was dependent upon the presence of external Ca^{2+} ions (Douglas & Rubin, 1963; Sorimachi, 1968).

The action of ACh on the adrenal chromaffin cells is associated with the depolarization of the cells which results from an increased membrane permeability and an inward movement of Na^+ and Ca^{2+} ions. Na^+ inward current is the predominant component in the depolarization phenomenon although Ca^{2+} inward current seems to play a crucial role in catecholamine secretion. In early experiments, it was shown that ACh produced catecholamine release and

caused a slight depolarization of the cells even if adrenal medulla perfusion was performed in Na^+-free media (Douglas & Rubin, 1961). These observations were later confirmed by other investigators in cultured chromaffin cells (Douglas et al., 1967b; Brandt et al., 1976; Kidokoro et al., 1982). In addition, it was demonstrated that tetrodotoxin (TTX) blocked catecholamine release but only by 30-50% (Trifaró & Lee, 1980; Amy & Kirshner, 1982). These last findings indicate that activation of the voltage-sensitive Na^+ channel is not essential for secretion. Therefore, these observations suggest that ACh does not induce catecholamine release through depolarization of the cell but rather by directly enhancing Ca^{2+} entry.

Although Na^+ ions may not be essential for catecholamine secretion, some studies suggest that these ions influence Ca^{2+} movements into chromaffin cells (Douglas & Rubin, 1961; Lastowecka & Trifaró, 1974). In addition, perfusion of adrenal medulla in presence of ouabain, a cardiac glycoside which inhibits Na^+-K^+-dependent ATPase, resulted in a cellular accumulation of Na^+ and was found to enhance catecholamine release in a Ca^{2+}-dependent manner (Banks, 1967). There are some doubts about the existence of a direct link between the inhibition of the Na^+ pump and the enhanced release of catecholamine, since several investigators have shown different results concerning this issue (Gutman & Boonyaviroj, 1977; Pocock, 1980; García et al., 1981; Aunis & García, 1981). In recent studies conducted by Pocock (1983a, 1983b), it was demonstrated that ouabain increased the basal rate of secretion which was dependent on extracellular Na^+ but maintained in absence of calcium. Therefore, it was suggested that the stimulatory effect observed with ouabain resulted from a marked decrease in the rate of active calcium extrusion

from the cells. Furthermore, Ca^{2+} movements in chromaffin cells may involve both Na^{+}:Ca^{2+} and Ca^{2+}:Ca^{2+} exchange processes which can be blocked by Gd^{3+} ions (Aguirre et al., 1977; Rink, 1977; Bourne & Trifaró, 1982).

The role of ACh in triggering exocytosis in chromaffin cells by increasing Ca^{2+} entry is quite clear now although its role in altering membrane permeability is still obscure. Recent electrophysiological studies have brought about some insights concerning this issue. It was demonstrated that electrical depolarization or elevated ACh concentrations increased the frequency of spontaneous action potentials in cultured chromaffin cells (Brandt et al., 1976). In gerbil and human chromaffin cells, it was shown that upon the application of depolarizing currents or ACh, the action potentials detected were blocked by TTX but were not affected by Ca^{2+} deprivation or $CoCl_2$. Therefore, it was concluded that these action potentials were mediated predominantly by Na^{+} inward current with little or no Ca^{2+} component (Biales et al., 1976). These last findings were later substantiated by studies conducted in perfused adrenal glands in the presence of ACh (Ishikawa et al., 1977; Ishikawa & Kanno, 1978; Kidokoro & Ritchie, 1980). Recent investigations which involved the use of noise analysis and patch-clamp techniques have demonstrated that action potentials in chromaffin cells are mediated by Na^{+} and Ca^{2+} inward current (Kidokoro et al., 1982; Fenwick et al., 1982a, 1982b). In addition, membrane depolarization is thought to activate voltage-sensitive Ca^{2+} channels, leading to a rise in cytosolic free Ca^{2+}, which in turn evokes catecholamine secretion (Knight & Kesteven, 1983; Kao & Schneider, 1984, 1985; Friedman et al., 1985). Furthermore, ACh response can be blocked by hexamethonium, a ganglionic nicotinic-blocker,

and D600 (methoxyverapamil), a Ca^{2+}-channel antagonist, the latter being also effective at blocking K^+-evoked and Ca^{2+}-dependent catecholamine release (Pinto & Trifaró, 1976; Aguirre et al., 1977, 1979; Knight & Baker, 1983). Thus, these studies suggested the presence of a separate D600-sensitive channel in chromaffin cells.

The pharmacological, physiological and electrophysiological findings mentioned above and reviewed elsewhere (Kidokoro, 1984, 1985) suggest that the chromaffin cells possess at least two kinds of Na^+ channels and three different classes of Ca^{2+} channels which are as follows: a) Na^+ and Ca^{2+} channels associated with the nicotinic receptor-ionophore complex which are insensitive to TTX, Mn^{2+} and Co^{2+} and through which Na^+ and Ca^{2+} appear to compete; b) separate voltage-sensitive Na^+ and Ca^{2+} channels for which the Na^+-sensitive channel is partially inhibited by TTX whereas the Ca^{2+}-sensitive channel is inhibited by Co^{2+}; c) a distinct D600-sensitive Ca^{2+} channel which is activated by Na^+ and Ca^{2+} entry through the voltage-sensitive channels.

In addition, it was recently demonstrated that chromaffin cells possess a big unitary conductance Ca^{2+}-dependent K^+ channel which is activated by depolarization at low intracellular Ca^{2+} levels and blocked by relatively low tetraethylammonium (TEA) concentrations (Marty, 1981). Very recently, quinine, an antiarrhythmic drug, was found to produce a "flickery" block when the Ca^{2+}-dependent K^+ channel was examined by patch-clamp technique in chromaffin cells. It was also determined that this agent does not appear to affect the resting release of NA significantly but does affect high K^+-induced NA release in a dose-dependent manner (Glavinovic et al., 1985).

However, the functional significance of these channels in chromaffin cells still awaits to be characterized further.

In conclusion, as proposed many years ago (Douglas & Rubin, 1961) and confirmed by new electrophysiological findings, Ca^{2+} ions definitely play a crucial role in the secretory process in adrenal chromaffin cells.

D.4 Endocytosis and Membrane Retrieval in Chromaffin Cells

Secretion from the adrenal medulla occurs by exocytosis, a process by which the secretory granule membrane fuses with the plasma membrane and portions of the membrane of the secretory granule are inserted into the plasma membrane. Under normal conditions, the surface area of the plasma membrane remains constant; therefore, some mechanism of membrane retrieval must exist because otherwise, the continuous addition of membrane of secretion organelles to the plasma membrane would lead to an enlargement of the cell.

The first morphological evidence concerning a mechanism of membrane retrieval in the adrenal medulla was provided by Diner (1967). The presence of such a mechanism was substantiated later by electron microscopy studies using exogenous tracers such as horseradish peroxidase and thorium dioxide, which indicated that membrane patches formed by the granule membrane are retrieved by endocytosis (Nagasawa & Douglas, 1972; Abrahams & Holzman, 1973). The endocytotic membrane retrieval process in neurons, in neurosecretory cells of the neurohypophysis and in chromaffin cells has been related to coated pits which invaginate to form coated vesicles (Nagasawa et al., 1971; Grynszpan-Winograd, 1971; Heuser & Reese, 1973). These coated vesicles

possess a "cage"-like or lattice-like network which covers an inner vesicular structure (for review see Keen, 1985). Furthermore, since secretory granule and plasma membranes are different in terms of function and composition, consequently a selective membrane retrieval mechanism must exist to maintain adequate and specific cell functions. Several studies conducted in the adrenal medulla and other secretory tissues suggest that a selective membrane retrieval mechanism does in fact exist (for review see Silverstein et al., 1977; Winkler, 1977). In adrenal chromaffin cells, a selective retrieval process was demonstrated with the use of antibodies against specific granule components such as DβH and glycoprotein III (Lingg et al., 1983; Phillips et al., 1983; Patzak et al., 1984). Therefore, the above-mentioned studies and others substantiate the fact that specific and complete removal of membrane of secretory granules from the plasma membrane does occur during the endocytotic membrane retrieval process.

The nature and properties of the "cage"-like component of the coated vesicles, which are implicated in the membrane retrieval process during endocytosis, have been investigated extensively (for review see Keen, 1985). The "cage"-like structure or "coat" consists of clathrin triskelion subunits which are present in pentagonal or hexagonal arrangements of the complex coat structure (Kirchhausen & Harrison, 1981; Crowther & Pearse, 1981; Ungewickell & Branton, 1981). The clathrin triskelion is composed of a clathrin heavy chain of molecular weight of 180,000 and two light chains of molecular weights ranging between 30,000-36,000 (Pearse, 1978). Clathrin has been isolated from several secretory tissues including the adrenal medulla (Pearse B.M.F. 1975, 1976, 1982; Keen et al., 1979; Mello et al., 1980; Daiss &

Roth, 1983; Brodsky & Parham, 1983). Furthermore, the presence of clathrin has been demonstrated by immunofluorescence in cultured chromaffin cells (Trifaró et al., 1982, 1984b). In addition to clathrin heavy and light chains, several other polypeptides have been isolated from the "coat" and have been referred to as assembly polypeptides since they seem to play a crucial role in the "coat" assembly (Keen et al., 1979; Irace et al., 1982; Zaremba & Keen, 1983). One of these proteins, a 50kD polypeptide, has been studied in detail since it was identified as a substrate of the coated vesicle kinase (Pauloin et al., 1982; Kadota et al., 1982; Usami et al., 1984, 1985). Clathrin was found capable of binding to α-actinin and actin (Schook et al., 1979) and has also been found associated with the microtubule network through specific binding to tubulin (α and β), tau factor and the microtubule-associated protein MAP2 (Kelly et al., 1983; Pfeffer et al., 1983; Pfeffer & Kelly, 1983) and finally associated with calmodulin (Salisbury et al., 1980; Linden et al., 1981; Linden, 1982; Moskowitz et al., 1982a, 1982b).

Therefore, the association of clathrin with the contractile proteins, the microtubules and calmodulin as well as the phosphorylation of some assembly polypeptides may suggest a role for these components in the process of membrane retrieval.

E. THE CHROMAFFIN CELL IN MONOLAYER CULTURE: A GOOD MODEL FOR THE STUDY OF CELLULAR FUNCTIONS

The perfused adrenal gland maintained *in vitro* has been used extensively to study several functional aspects of this tissue. Although studies using this model system have provided considerable information concerning the mechanism of storage, synthesis and secretion of catecholamines, several limitations have been encountered while using this system. Consequently, the isolation and maintenance in culture of chromaffin cells has been proven to be a substantial development in methodology. The chromaffin cell in culture has been shown to be a useful system for studying various aspects of cellular functions and particularly useful in the study of stimulus-secretion coupling. In addition, the possibility of long-term maintenance of these cells in culture allows several types of long-term experiments to be performed.

Adult bovine adrenal medullary chromaffin cells are used most often for culturing since the starting material is readily available, the chromaffin cells can be easily isolated and a high yield of cells is obtained from each gland. Bovine adrenal chromaffin cells are purified from enzyme-dissociated adrenal medullary cells by centrifugation on self-generating Percoll density gradients (see Livett *et al.*, 1978; Trifaró *et al.*, 1978). The purified chromaffin cells are plated on collagen-coated plastic petri dishes and maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum and several other components (see Trifaró & Lee, 1980). The adrenal medullary chromaffin cell in monolayer culture has

been well characterized (Mizobe et al., 1979; Kilpatrick et al., 1980; Trifaró & Lee, 1980; Unsicker et al., 1980; Livett, 1984). It has been demonstrated in these studies that the secretory characteristics and also other functional properties of these cultured cells are well maintained in culture and similar to those described for the cells in the intact gland. The integrity of catecholamine stores of isolated chromaffin cells has been shown to be well maintained even after relatively long periods of time (2 to 4 weeks) in culture (Trifaró & Lee, 1980).

At first, chromaffin cells in culture are round with a diameter of about 20 μm. With time in culture, these cells progressively extend processes or neurite-like structures, and growth cone-like structures are often observed at their extremities. These processes display varicosity-like structures which contain high levels of catecholamines and enkephalins (Livett & Dean, 1980; Trifaró & Lee, 1980). In contrast to dissociated sympathetic neurones and rat PC12 cells, the spontaneous outgrowth of processes from adult bovine chromaffin cells is independent of nerve growth factor (NGF) and is not affected by antibodies raised against NGF (Livett et al., 1978; Unsicker et al., 1980; Unsicker & Hofmann, 1981, 1983). However, it has been suggested that the process outgrowth in bovine chromaffin cell cultures may depend upon factors which are produced by non-chromaffin cells in culture (Unsicker & Hofmann, 1981, 1983). These neurite-like processes appear to make contacts or synapses with other cell bodies and processes. Recently, electrophysiological experiments have demonstrated that neonatal rat chromaffin cells in culture can be transformed into neuronal cells which possess functional synapses of cholinergic or adrenergic nature depending on the culture condi-

tions used (Ogawa et al., 1984).

Cultured chromaffin cells possess an uptake mechanism for catecholamines which in many respects resembles the neuronal uptake system since it is saturable, follows Michaelis-Menten kinetics and shows a high affinity for noradrenaline. This uptake process is blocked by low concentrations of desipramine and shows an absolute requirement for Na^+. However, this uptake mechanism in chromaffin cells does not exhibit any stereospecificity towards (-)noradrenaline (Kenigsberg & Trifaró, 1980).

Recently, it was demonstrated that cultured chromaffin cells possess neurofilaments, components which are considered to be highly specific neuronal markers. By indirect immunofluorescence, the three characteristic neurofilament subunits were shown to be present in these cells and specifically localized near the nucleus. In addition, using neurofilament subunit antibodies, three proteins of molecular weights of 210,000, 160,000 and 70,000 were specifically immunoprecipitated from solubilized extracts of cultured chromaffin cells (Bader et al., 1984b).

Therefore, from morphological, biochemical and functional studies, it appears that the chromaffin cell in culture is a good model system to study the molecular mechanisms underlying adrenergic neuronal functions.

F. CALMODULIN

The importance of Ca^{2+} as a second messenger in bioregulation is by now well recognized. However, the biochemical mechanisms mediating the Ca^{2+} effects still await further elucidation. Recently, it has become evident that the effects of Ca^{2+} are mediated through the intervention of a family of Ca^{2+}-binding proteins or calciproteins (see Table 1). These Ca^{2+}-binding proteins rapidly and reversibly bind Ca^{2+} in the range of free Ca^{2+} concentrations which are present during physiological stimulation of resting cells.

Among these Ca^{2+}-binding proteins, calmodulin exhibits the widest distribution and greatest versatility. Calmodulin was first discovered as an activator of cyclic nucleotide phosphodiesterase and for several years was solely implicated as a regulator of cyclic nucleotide metabolism (Cheung, 1970; Kakiuchi & Yamazaki, 1970). However, calmodulin is now known to regulate a wide variety of cellular activities such as cyclic nucleotide and glycogen metabolism, Ca^{2+} transport through Ca^{2+}/Mg^{2+}-ATPase, intracellular motility and Ca^{2+}-activated protein phosphorylation, to name just a few (for review see Cheung, 1980, 1981; Klee et al., 1980; Means & Dedman, 1980; Klee & Vanaman, 1982; Kilhoffer et al., 1983; Cox et al., 1984; Manalan & Klee, 1984).

Calmodulin is a ubiquitous protein in eukaryotes; it has been isolated and purified from a wide variety of invertebrate and vertebrate animal species as well as from both plants and fungi but appears to be absent in prokaryotes. The concentration of this protein varies greatly from source to

Table 1 CALCIUM-BINDING PROTEINS

Protein	MW	Distribution	Proposed Function
Calmodulin	16,800	Ubiquitous	Multifunctional
Troponin C	17,900	Skeletal and Smooth Muscles	Regulation of Contraction
Myosin Light Chain (Regulatory)	20,000	Ubiquitous	Regulation of Contraction & Motility
Parvalbumim (α and β)	11,500	Skeletal Muscle Nervous Tissues	Relaxation of Muscle Unknown
S-100 (α and β)	10,500	Nervous Tissues	Regulation of Protein Phosphorylation
Calcineurin B (PPase 2B)	19,200	Brain Muscles	Protein Dephosphorylation
Intestinal CaBP	8,500	Gut, Kidney	Unknown
Skin CaBP	12,000	Skin	Unknown
Caligulin	24,000	Specific Brain Regions	Unknown
Calsequestrin	55,000	Skeletal Muscle	Ca^{2+}-sequestration (Sarcop. Ret.)
Synexin	47,000	Secreting Tissues	Membrane Fusion
Synhibin	68,000	Secreting Tissues	Membrane Fusion
Oncomodulin	11,500	Tumor Tissues	DNA Synthesis, Cell Proliferation

CaBP: Calcium-Binding Protein

source. The highest concentrations of calmodulin have been found in mammalian brain, testes, sperm and eggs and the electroplax of Electrophorus electricus (Childers & Siegel, 1975; Watterson et al., 1976; Dedman et al., 1977; Kakiuchi et al., 1982). In addition, the regional distribution of calmodulin activity was recently determined in 68 discrete areas of the brain. It was found that, in general, the activity of calmodulin was higher in the telecephalon, limbic system and hypothalamus than in the mesencephalon, pons, cerebellum and medulla. However, there were substantial differences in calmodulin activity in discrete nuclei of each region (Zhou et al., 1985).

F.1. Physicochemical Properties of Calmodulin

Calmodulin is an acidic globular heat-stable protein. It consists of a single polypeptide chain of 148 amino acid residues and has a molecular weight ranging from 15,000 to 19,000 as determined by ultracentrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of chelating agents gives slightly higher molecular weight values (Klee et al., 1979), a characteristic which appears to be common to all Ca^{2+}-binding proteins. Aspartate and glutamate constitute one third of the total amino acid residues and account in part for the low isoelectric point of calmodulin (pI 3.5-4.3). The molecule completely lacks cysteine and tryptophan as well as hydroxyproline. It consequently contains no disulfide bridges and is extremely flexible. Its flexibility may very well be essential to its mode of action. The absence of tryptophan and its high phenylalanine/tyrosine ratio (8:2) account for the low extinction coefficient of

calmodulin at 280 nm ($\varepsilon_{276}^{1\%}$=1.8) and its characteristic UV absorption spectrum of multiple peaks at 253, 259, 265, 269 and 277 nm which reveals the fine structure of a phenylalanine absorption band (Stevens et al., 1976; Watterson et al., 1976) (see Table 2). The occurrence of trimethyllysine (position 115) in most, but not all, calmodulins investigated has attracted considerable attention. This unusual amino acid may be essential for biological activity. However, calmodulin from Neurospora crassa and Dictyostelium discoideum, which lacks trimethyllysine, is still fully active (Cox et al., 1982; Marshak et al., 1984).

Calmodulin from phylogenetically diverse sources has similar biological and biochemical properties. The amino acid sequence of the molecule is highly conserved and is invariant in structure in the rat, cow, rabbit and man (Dedman et al., 1978a; Grand & Perry, 1978; Watterson et al., 1980; Grand et al., 1981; Sasagawa et al., 1982) and in the sea pansy, Renilla reniformis (Jamieson et al., 1980), differing by no more than five amino acid residues. However, a more important number of sequence differences can be observed for Neurospora crassa when compared to mammalian calmodulin (Cox et al., 1982).

In addition, calmodulin exhibits a high degree of sequence homology with parvalbumin and troponin C (Dedman et al., 1978a; Watterson et al., 1980). In this regard, Kuo and Coffee (1976a) first described adrenal medullary calmodulin as a troponin C-like protein. There exists 50% direct homology between calmodulin and troponin C which increases even to 78% if one considers conservative amino acid replacements. Like troponin C, calmodulin possesses four internal homologous sequences which constitute the four Ca^{2+}-binding domains. Although the amino acids found in each Ca^{2+}-binding domain

Table 2 PROPERTIES OF CALMODULIN

Distribution:	Ubiquitous
Sequence:	148 Amino Acid Residues Single Polypeptide Chain Highly Conserved
Molecular Weight:	15,000 - 19,000 *
Isoelectric Point:	3.5 - 4.3 *
Thermo-Stability:	$t_{\frac{1}{2}}$ = 7 min./100°C
Extinction Coefficient:	ε_{280} = 1.8
Ca^{2+}-binding Sites:	4 Binding Sites ($K_d \approx 10^{-6}$M)
α-Helical Structure:	High Content (40-60%)**

* : Depending on technique of determination

**: Depending on presence of Ca^{2+}

are similar, the sequence of amino acids is unique. The highest degree of internal homology (70%) exists between Ca^{2+}-binding domains 1 and 3 and domains 2 and 4. The high degree of sequence homology between calmodulin and other calcium-binding proteins, as well as internal homology amongst these proteins, suggests that Ca^{2+}-binding proteins may have evolved from a smaller ancestral precursor by gene duplication (Barker et al., 1977; Iida, 1982; Kilhoffer et al., 1983).

Calmodulin possesses a highly coiled structure. In the absence of metal ions, 40% of the molecule exists in an α-helical configuration and 15% β-pleated sheet structure (Dedman et al., 1977). In addition, the three-dimensional structure of calmodulin has been recently determined by crystallography (Babu et al., 1985). It was found that the molecule consists of two globular lobes connected by a long exposed α-helix and that each lobe binds two Ca^{2+} through helix-loop-helix domains similar to those of other Ca^{2+}-binding proteins. Upon binding of two Ca^{2+}, calmodulin undergoes a large conformational change accompanied by a 5-10% increase in α-helix structure content. Additional conformational changes occur when the last two Ca^{2+} are bound, resulting in a possibility of four different conformations of calmodulin with which Ca^{2+}-dependent enzymes might interact (Bayley et al., 1984; Burger et al., 1984; Olwin et al., 1984). The conformational change of calmodulin has been determined by several methods which include optical rotary dispersion (Liu & Cheung, 1976), circular dichroism (Dedman et al., 1977; Wolff et al., 1977), fluorescence emission (Wang et al., 1975; Dedman et al., 1977), UV absorption spectra (Klee, 1977), nuclear magnetic resonance (NMR) (Seamon, 1980; Ikura et al., 1983, 1984), and altered suscepti-

bility to chemical modifications (Drabikowski et al., 1977; Walsh et al., 1977; Walsh & Stevens, 1977, 1978; Richman, 1978; Richman & Klee, 1978). In addition, it was shown recently that Ca^{2+} binding to calmodulin, which results in a conformational change in the molecule, appears to expose some residues in helix IV (residues 65-92) to proteolytic attack (Babu et al., 1985).

In addition, several studies have been conducted with calmodulin fragments obtained by limited proteolysis with trypsin (Drabikowski et al., 1977), providing new insight concerning the interaction of calmodulin with Ca^{2+} and its target proteins. In the presence of Ca^{2+}, limited proteolysis results in one cleavage in the center of the long central helix (helix IV: residues 65-92) that produces two fragments, 1-77 and 78-148. However, in the absence of Ca^{2+}, three fragments are produced as follows: 1-106, 1-90 and 107-148 (Drabikowski et al., 1977; Andersson et al., 1983; Guerine et al., 1984; Newton et al., 1984). By advanced analytical techniques including two-dimensional proton NMR, it was shown that the proteolytic fragments 1-77 and 78-148 preserved much of the structure they have in the native protein (Dalgano et al., 1984; Ikura et al., 1984; Krebs et al., 1984; Thulin et al., 1984). For instance, they bind divalent cations with high affinity, undergo Ca^{2+}-dependent conformational changes and exhibit a Ca^{2+}-dependent interaction with the calmodulin antagonists, phenothiazines (for review see Manalan & Klee, 1984). In addition, the calmodulin tryptic fragments 1-77, 78-148 and 107-148 coupled to Sepharose 4B have been used recently to test the ability of different calmodulin-regulated enzymes to recognize different domains of calmodulin (Ni & Klee, 1985).

There existed until recently a considerable confusion concerning the ion specificity of the four Ca^{2+}-binding sites. Several cations have been shown to induce conformational changes of calmodulin which appear to be similar but not identical to those seen with Ca^{2+}. In addition, some cations promote enzyme activation with a concentration dependence correlated with their affinity for calmodulin (for review see Cox et al., 1984; Kincaid et al., 1984; Manalan & Klee, 1984). Early studies have shown that certain divalent cations such as Mn^{2+}, Zn^{2+} and Sr^{2+} were able to bind to calmodulin and also inhibit Ca^{2+} binding to calmodulin (Lin et al., 1974; Wolff et al., 1977; Cox et al., 1981; Andersson et al., 1982; Cox & Harrison, 1983). A recent study has shown that in addition to these three cations, others such as Cd^{2+} and Pb^{2+} can produce the same effects (Chao et al., 1984). In addition, some divalent cations such as Mn^{2+}, Sr^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}, but not Mg^{2+}, Be^{2+}, Ba^{2+}, Ni^{2+} or Co^{2+}, are able to effectively substitute for Ca^{2+} in the activation of calmodulin-regulated enzymes (Goldstein & Ar, 1983; Cox & Harrison, 1983; Chao et al., 1984). However, Zn^{2+} and Cd^{2+} appear to be inhibitory at high concentrations (Chao et al., 1984). Furthermore, trivalent cations such as Al^{3+} and the lanthanides La^{3+}, Tb^{3+} and Sm^{3+} have been shown to bind to calmodulin. In the case of Al^{3+}, it appears that it induces an inactive conformation and inhibits the stimulatory effect of calmodulin (Siegel & Haug, 1983; Siegel et al., 1983). In contrast, lanthanides appear to act in a more complex manner. Lanthanides, which bind to calmodulin with high affinity and inhibit Ca^{2+} from binding to it, activate certain calmodulin-regulated enzymes at low concentrations ($<10^{-7}M$) but become inhibitory at higher concentrations ($>10^{-6}M$) (Mazzei et al., 1982; Chao et al., 1984).

Obviously, under physiological conditions, not all of these cations play a crucial role in the calmodulin activity. However, it has been postulated that some of them might interact with calmodulin in pathological conditions caused by metal toxicity (Cox & Harrison, 1983).

Calmodulin appears to possess two classes of Ca^{2+}-binding sites. Early studies using equilibrium techniques have shown the four metal binding sites on the molecule to be Ca^{2+} specific and equivalent, with a K_d of 2.4 μM (Dedman et al., 1977). In contrast, Wolff and collaborators have reported that calmodulin possesses two classes of binding sites for either Ca^{2+}, Mg^{2+} or Mn^{2+}, namely three class A binding sites with dissociation constants of 0.2, 140 and 1.2 μM and one class B binding site with dissociation constants of 1, 20 and 4 μM for Ca^{2+}, Mg^{2+} and Mn^{2+}, respectively (Wolff et al., 1977). These observations have since been substantiated by several studies using techniques such as ^{43}Ca and ^{113}Cd NMR, Tb^{3+}-induced fluorescence and Mn^{2+} paramagnetic resonance spectroscopy (Kilhoffer et al., 1980a, 1980b; Andersson et al., 1982; Wallace et al., 1982; Wang et al., 1982; Xu et al., 1983). There is disagreement about the location of high- and low-affinity Ca^{2+}-binding sites, but most recent studies seem to agree that sites 3 and 4 are the high-affinity sites (Andersson et al., 1983; Yagi et al., 1984; Babu et al., 1985). The exact physiological significance of these two classes of Ca^{2+}-binding sites is still not fully understood. However, it appears that various calmodulin-regulated enzymes might interact with and be activated by distinct conformers of calmodulin containing different amounts of bound Ca^{2+} (for review see Manalan & Klee, 1984). Nevertheless, it is generally accepted that in the resting cell, when intracellular free Ca^{2+} is in the order of

10^{-8} to 10^{-7}M, the sites would be unoccupied, whereas upon stimulation, when intracellular free Ca^{2+} levels may reach 10^{-5}M, there would be partial or complete occupation of the Ca^{2+}-binding sites.

F.2 Mechanism of Action of Calmodulin

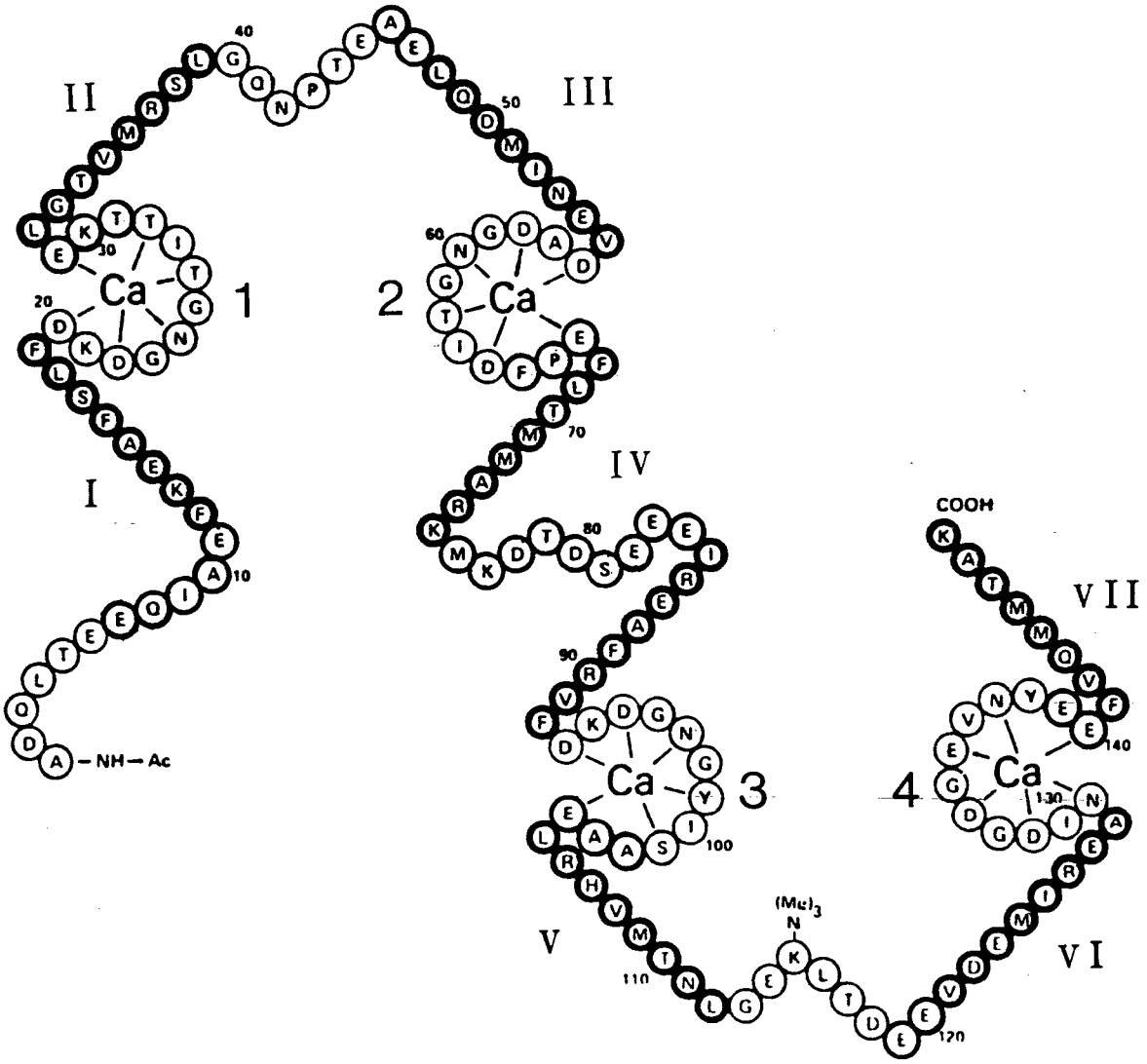
Under normal conditions, calmodulin activity is not regulated by changes in its concentration within the cell but primarily by changes in the concentration of free intracellular Ca^{2+} (Means & Chafouleas, 1982). In the resting cell, intracellular free Ca^{2+} concentrations are relatively low, and the binding of Ca^{2+} to calmodulin is almost negligible. However, upon cell stimulation, intracellular free Ca^{2+} levels rise due to Ca^{2+} movement through plasma membrane channels or due to its release from intracellular Ca^{2+} pools. Consequently, Ca^{2+} binds to calmodulin which undergoes a conformational change, allowing its interaction with an inactive enzyme and thus resulting in the formation of an active complex (see Fig. 3). The intracellular free Ca^{2+} concentration may then be reduced by its extrusion from the cell or by its translocation and possibly sequestration within the cell, which would result in the dissociation of the active Ca^{2+}/calmodulin-enzyme complex, thus terminating the enzymatic reaction.

At present, however, it appears that the mode of action of calmodulin described above is definitely an oversimplification and that many other factors in addition to changes in intracellular free Ca^{2+} concentration are involved in the regulation of the activity of calmodulin (Wang et al., 1983a). In this regard, in addition to the calmodulin-regulated enzymes that bind to

Fig. 3 Amino acid sequence, functional and structural domains of calmodulin. The sequence of bovine brain calmodulin contains 148 amino acid residues. Calmodulin contains four Ca^{2+}-binding domains with noticeable internal homology especially between domains 1 and 3 and also between domains 2 and 4. Calmodulin also contains a high α-helical content. In fact, seven α-helices have been identified recently on this polypeptide (I-VII): I, residues 7-19; II, 29-39; III, 46-55; IV, 68-92; V, 102-112; VI, 119-128; VII, 139-148.

One-letter codes for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

[Taken from Klee et al., 1980 and adapted according to the recent findings of Babu et al., 1985]


II
III
I
IV
V
VI
VII
1
2
3
4
Ca
Ca
Ca
Ca
COOH
-NH-Ac
(Me)3
N

and are consequently activated by calmodulin, other proteins may bind to this modulator protein and in turn regulate its activity (Wang et al., 1980). An example of such a regulatory protein is calcineurin, which has been isolated and characterized from brain (Klee & Krinks, 1978; Klee et al., 1979; Sharma et al., 1979; Klee et al., 1983). Calcineurin can bind to and inactivate calmodulin, thus preventing activation of a variety of calmodulin-regulated enzymes. Calcineurin by itself possesses an enzymatic function since it was shown to be indistinguishable from protein phosphatase 2B (Stewart et al., 1982). Caldesmon is another calmodulin-binding protein which was first isolated from smooth muscles (Sobue et al., 1981). This protein has been shown to be present in several tissues such as the brain and some secretory tissues including adrenal medullary chromaffin cells (Sobue et al., 1983, 1985b). Caldesmon corresponds to a dimer of molecular mass of 147-150kD which was shown to bind to both calmodulin and F-actin (Sobue et al., 1982). The binding of caldesmon to calmodulin and actin filaments alternates depending on the concentration of Ca^{2+} (Ca^{2+}-dependent "flip-flop" binding). In the absence of Ca^{2+} (<1 μM), caldesmon binds to F-actin but not to calmodulin, whereas at higher Ca^{2+} concentrations (>1 μM), it binds to calmodulin and dissociates from actin filaments (Sobue et al., 1983). This "flip-flop" type binding of caldesmon to calmodulin and F-actin depending on the Ca^{2+} concentration has been demonstrated to regulate calmodulin activity in several biochemical events (Sobue et al., 1985a, 1985b).

Another mechanism for the regulation of calmodulin activity may be related to its subcellular localization. Redistribution of calmodulin within the cell appears to be an important factor in the regulation of specific

calmodulin-dependent systems (Conn et al., 1981; Hikita et al., 1984; Wilson & Gillette, 1985). Conversely, calmodulin may promote relocation of some calmodulin-binding proteins within the cell (Saitoh & Schwartz, 1983). Such a mechanism has been described in adrenal chromaffin cells, and it was postulated that such a phenomenon could play a role in the exocytotic process of these cells (Geisow & Burgoyne, 1983).

In addition, calmodulin activity may also be regulated by posttranslational chemical modification of the protein. Such chemical modifications include methylation, carboxymethylation and phosphorylation (Gagnon, 1983; McFadden & Clarke, 1982; Runte et al., 1982; Murtaugh et al., 1983; Plancke & Lazarides, 1983). For instance, carboxymethylation of calmodulin, which occurs in intact cells, reduces the ability of the protein to stimulate cyclic nucleotide phosphodiesterase (Gagnon et al., 1981). Although covalent modification of calmodulin has been described, its exact physiological significance awaits further elucidation (Manalan & Klee, 1984).

Finally, the regulation of calmodulin's activity may also occur at the level of its synthesis or degradation within the cell. Although in normal cells the intracellular levels of calmodulin remain relatively constant, increased synthesis or decreased degradation of this modulator protein, and consequently increased levels of calmodulin, are found in transformed cells (Chafouleas et al., 1980, 1981, 1984; Means et al., 1982; Connor et al., 1983; Takemoto & Jilka, 1983; Veigl et al., 1983). These changes in the level of calmodulin may be important in the manifestation of the transformed state of the cell or in some pathological conditions such as several types of leukemia and carcinoma, diabetes, cystic fibrosis and also in chronic

schizophrenia (Gnegy et al., 1981; Morley et al., 1982; Schreiber et al., 1982; Ganapathi & Grabowski, 1983; Hickie et al., 1983; Moon et al., 1983a, 1983b; Wei et al., 1983; Piascik & McNicholas, 1985).

F.3 Calmodulin and the Secretory Process

Since exocytosis possesses an essential requirement for Ca^{2+}, calmodulin is likely to confer some of the Ca^{2+} sensitivity in the secretory process. In this regard, calmodulin has been found to be present in several secretory systems including platelets, mast cells, endocrine and exocrine pancreas, neurohypophysis, hypothalamus, parathyroid gland, parotid gland, nerve endings and also in the adrenal medulla (Kuo & Coffee, 1976a, 1976b; Thorn et al., 1978; Sudgen et al., 1979; Bartelt & Scheele, 1980; Brown et al., 1981; DeLorenzo, 1981; Henquin, 1981; Sheaves et al., 1982; Ansah & Katz, 1983; Caceres et al., 1983; Yokoyama et al., 1983; Hikita et al., 1984; Chakravarty & Holm-Nielsen, 1985; Murakami et al., 1985; Richardson & Twente, 1985; Watkins & White, 1985).

Moreover, pharmacological agents such as trifluoperazine (TFP) and other potent calmodulin antagonists have been reported to inhibit histamine release from mast cells (Sieghart et al., 1978; Douglas & Nemeth, 1982), serotonin release from platelets (White & Raynor, 1980), protein secretion from polymorphonuclear leukocytes (Elferink, 1979; Naccache et al., 1980; Pfister et al., 1984), theophylline-stimulated release of ACTH, prolactin and growth hormone from anterior pituitary cells (Sand et al., 1983), glucose-mediated insulin release from pancreatic islets and insulinoma cells

(Krausz et al., 1980; Schubart et al., 1980b), protein secretion from lacrimal glands (Mauduit et al., 1983), intestinal ion secretion (Ilundai & Naftalin, 1979) and catecholamine from chromaffin cells (see section F.6). Although the mechanism for such an inhibitory action is not fully understood in all cases, it does not appear to involve a change in the total cell content of calmodulin but may be the result of a redistribution of cellular calmodulin. Such a redistribution of calmodulin has been demonstrated in pituitary gonadotrope before and during gonadotropin-releasing hormone-induced release of luteinizing hormone (Conn et al., 1981).

Since relatively large amounts of calmodulin are present in the adrenal medulla (Sobue et al., 1979; Tirrell & Coffee, 1983; Hikita et al., 1984) and from recent studies performed in cultured chromaffin cells with calmodulin antibodies (Kenigsberg & Trifaró, 1985b), it was suggested that calmodulin may be directly involved in the secretion of catecholamines from the chromaffin cell (see section F.6).

The ability of the Ca^{2+}/calmodulin complex to enhance adenylate cyclase, phosphodiesterase, phospholipase and myosin light chain kinase (MLCK) activities provides evidence for a potentially important link between Ca^{2+} and other known putative mediators (for review see Cheung, 1980; Klee, 1980; Weiss & Wallace, 1980; Bronstrom & Wolff, 1981; Means & Chafouleas, 1981, 1982; Means et al., 1982; Tomlinson et al., 1984). In addition, Ca^{2+}-dependent phosphorylation of certain cellular proteins by protein kinases that require calmodulin for activation may regulate a number of biological processes linked to secretory exocytosis (see section G.6.1).

F.4 Calmodulin Antagonists

F.4.1 Classical Pharmacological Agents

The discovery of the phenothiazine antagonism of Ca^{2+}/calmodulin-activated phosphodiesterase *in vitro* by Weiss and collaborators (1974) was a major development in providing tools for the detection of calmodulin-dependent activities *in vitro* and *in vivo* (see Weiss *et al.*, 1982, 1983; Roufogalis *et al.*, 1983; Van Belle, 1984). For instance, these agents have been used as ligands in affinity chromatography for calmodulin purification (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979; Caldwell & Haug, 1981; Rochette-Egly *et al.*, 1982; Hart *et al.*, 1983) and as tools to examine the possible involvement of calmodulin in various cellular functions.

The mechanism by which the phenothiazine antipsychotics and related agents inhibit the action of calmodulin appears to occur by binding directly to calmodulin in a Ca^{2+}-dependent manner (Levin & Weiss, 1977, 1978, 1979; Weiss & Levin, 1978; Laporte *et al.*, 1980; Tanaka *et al.*, 1983). Calmodulin appears to possess between one and three Ca^{2+}-dependent drug-binding sites per molecule, with dissociation constants for the most potent agents in the range of 1 to 10 μM. Two types of trifluoperazine (TFP)-binding sites were revealed by kinetic analysis. One class of sites bound 2 molecules of TFP per molecule of calmodulin with high affinity (K_d = 1 μM). A second class of sites bound TFP with low affinity (K_d = 5 mM) and high capacity (24 to 27 sites per molecule of calmodulin) (Levin & Weiss, 1977, 1978). The low-affinity binding sites were Ca^{2+}-independent, in contrast to the high-affin-

ity binding sites (Levin & Weiss, 1979). In addition, it was shown that paramagnetic resonance spectra of Ca^{2+}-saturated calmodulin are perturbed by the binding of 2 molecules of TFP. Furthermore, it was demonstrated very recently that the antagonist-binding site on calmodulin resides in helix IV (residues 65-92) which is located between Ca^{2+}-binding domains 2 and 3 and that the primary site of attachment is most probably lysine 75 (Babu et al., 1985).

Different chemical classes of tranquilizers are believed to compete with TFP for the same site of interaction on calmodulin. For example, the butyrophenone penfluridol and the diphenylbutylamine pimozide competitively displaced ^{3}H-labelled TFP from calmodulin.

Several different cations have been shown to promote the binding of TFP to calmodulin. The order of effectiveness of divalent cations in promoting the binding of TFP to calmodulin is as follows: $Ca^{2+} > Sr^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+}$, while Ba^{2+} and Mg^{2+} are ineffective (Levin & Weiss, 1977). The binding of the phenothiazine agents to calmodulin is reversible under normal circumstances but can become irreversible following photoactivation by UV irradiation (Prozialeck et al., 1981).

The ability of various antipsychotic drugs to inhibit calmodulin activity has been attributed to their lipophilic nature (Norman et al., 1979; Landry et al., 1981), which is consistent with the finding that the hydrophobic regions of calmodulin are involved in the binding of the antagonists to the modulator protein (Laporte et al., 1980; Klevit et al., 1981; Jarrett, 1984). The correlation between hydrophobicity and IC_{50} values in the calmodulin inhibition assay is good but not absolute. There is also

evidence for an ionic interaction between a positively charged group on the drug and negatively charged amino acid residues on calmodulin (Weiss et al., 1982). The structure-activity relationship of calmodulin antagonists has been investigated in detail, and it was concluded that, in general, the potent calmodulin inhibitors are for the most part amphiphatic amines that contain large hydrophobic regions and carry a positive charge at neutral pH (Prozialeck & Weiss, 1982; Tanaka et al., 1982; Johnson, 1983; Roufogalis et al., 1983; Inagaki & Hidaka, 1984; Van Belle, 1984).

The calmodulin antagonists described so far belong to several different classes of pharmacological agents. Phenothiazine, butyrophenone and diphenylbutylamine antipsychotics, smooth muscle relaxants and α-adrenergic blockers possess relatively high apparent affinities for calmodulin ($K_{0.5}$ 10^{-6}-10^{-4}M) (Volpi et al., 1981; Prozialeck & Weiss, 1982; Tanaka et al., 1982; Watanabe & West, 1982). Other pharmacological agents, including antidepressants, anesthetics, antihistaminic, antimalarial and cancer chemotherapeutic agents, appear to be weak antagonists (Volpi et al., 1981; Watanabe et al., 1981; Prozialeck & Weiss, 1982; Weiss et al., 1982). In addition, herbicides, fungicides and insecticides have been shown to be also good calmodulin antagonists with apparent affinities in the micromolar or submicromolar concentration range (Hagmann, 1982; Hertel & Marmé, 1983). The two most potent calmodulin antagonists synthesized so far are calmidazolium (R24571) and compound 48/80 (Gietzen et al., 1981, 1983; Van Belle, 1981, 1984; Rossi et al., 1985) (see Table 3). Furthermore, it was found very recently that some Ca^{2+}-channel blockers interact with calmodulin and have been used to detect a cooperative binding of the antipsychotic antagonists

Table 3 CALMODULIN ANTAGONISTS

Pharmacological Classification	Agents
Phenothiazine Antipsychotics	Trifluoperazine
	Chlorpromazine
	Fluphenazine
Butyrophenone Antipsychotics	Penfluridol
	Haloperidol
	Spiroperidol
Diphenylbutylamine Antipsychotics	Pimozide
Antidepressants	Imipramine
	Desipramine
	Amitryptyline
Muscle Relaxants	W Serie Analogues
	(W7, W5, W8, W9, W10, W12)
Local Anesthetics	Dibucaine
	Lidocaine
	Tetracaine
	Mepacrine
Vinca Alkaloids	Vinblastine
	Vincrystine
	Vindesine
	Vindoline
Miscellaneous	Calmidazolium (R24571)
	Compound 48/80

to calmodulin (Janis & Triggle, 1983; Johnson, 1983; Thayer & Fairhurst, 1983; Triggle & Swamy, 1983).

One major problem associated with most, if not all, calmodulin antagonists which belong to several classes of pharmacologically unrelated agents appears to be their lack of specificity. In this regard, it has been demonstrated that other Ca^{2+}-binding proteins which exhibit sequence homology with calmodulin also bind TFP (Levin & Weiss, 1978) and that various other proteins can interact with the phenothiazine antipsychotics (Van Eldik et al., 1980; MacManus, 1981). Phenothiazine antipsychotics, naphthalene sulfonamide derivatives (W5, W7, etc.) and even calmidazolium also interact with the Ca^{2+}-dependent/calmodulin-independent protein kinase with an affinity similar to that for calmodulin (Mori et al., 1980; Vincenzi, 1981; Tanaka et al., 1982; Louis et al., 1983; Schatzman et al., 1983). In addition, inhibition of membrane-bound enzymes as well as calmodulin-regulated enzymes themselves has been observed (Luthra, 1982; Louis et al., 1983). In contrast, compound 48/80 is somewhat more selective in its ability to bind calmodulin as opposed to the enzymes under calmodulin control (Gietzen, 1983). Furthermore, recent evidence suggests that antipsychotic effects of phenothiazines and related agents do not correlate with their ability to interact with calmodulin. For example, clinically inactive enantiomers of butaclamol and flupenthixol as well as calmidazolium have been shown to be potent calmodulin antagonists ($K_{0.5}$ 15 μM) (Norman & Drummond, 1979). On the other hand, efficacious antipsychotics, e.g. haloperidol, have a relatively low affinity for calmodulin (Roufogalis et al., 1983). Phenothiazines have also been used to develop phenothiazine-resistant variant cells such as two

variants of the algae Volvox carteri, which have an abnormal phenothiazine-insensitive calmodulin, and a variant of a macrophage-like cell which has a deficient calmodulin-binding protein (Kurn & Sela, 1981; Speaker et al., 1983). Finally, a covalent adduct of calmodulin/norchlorpromazine, termed $CAPP_1$-calmodulin, has been prepared recently (Newton et al., 1983). $CAPP_1$-calmodulin has the ability to bind to calmodulin-dependent enzymes with high affinity ($K_{0.5}$ 10^{-8} to 10^{-9}M) through its calmodulin moiety. This adduct appears to be a specific and potent antagonist of calmodulin-regulated enzymes (Newton & Klee, 1984).

Despite the limitations due to lack of specificity, most of these pharmacological agents are useful tools for studying the mechanism of action of calmodulin in vitro and in situ and also for characterizing the interaction sites on calmodulin for its target proteins and its antagonists. However, when these agents are used as the unique criterion for evaluating calmodulin-regulated functions, especially in systems where their general effect pattern has not been well established, serious misinterpretations of the cellular mechanisms underlying the event can be obtained. Consequently, other methods to study calmodulin-dependent functions should be used in conjunction with these pharmacological tools.

F.4.2 Endogenous Peptides and Hormones

Recently, in a search for endogenous substances which might interact with calmodulin, it was found that various peptides bind to and also inhibit the action of calmodulin (for review see Barnette & Weiss, 1984).

Opioid peptides such as β-endorphin and dynorphin (1-13) have been shown to bind to and inhibit Ca^{2+}/calmodulin-dependent enzyme activities. In contrast, other opioid peptides such as Met- and Leu-enkephalin as well as α-endorphin show little or no binding to calmodulin and fail to block calmodulin-activated phosphodiesterase (PDE) (Malencik & Anderson, 1982; Barnette & Weiss, 1982). β-endorphin binds directly to calmodulin in a saturable and Ca^{2+}-dependent manner ($K_d \approx 4.6$ μM) and inhibits calmodulin-stimulated PDE activity at concentrations similar to the ones described for the most potent antipsychotic antagonists. This inhibition was not reversed by calcium nor by the opiate antagonist naloxone but was reduced in presence of TFP (Barnette & Weiss, 1982; Giedroc et al., 1983b). In addition to β-endorphin (1-31), some related fragments including β-endorphin (6-31), (1-5/16-31) and (14-31) appear as potent in inhibiting PDE activity (Puett et al., 1983; Barnette & Weiss, 1983). Furthermore, it appears that β-endorphin (14-25) is the important sequence involved in this inhibition and therefore that the region of β-endorphin responsible for opiate activity is not involved (Giedroc et al., 1983a). Dynorphin (1-13) is slightly less effective than β-endorphin in inhibiting calmodulin-stimulated PDE activity. In addition, dynorphin (1-17) was found to be 4-fold more effective than dynorphin (1-13) in inhibiting the enzymatic activity. In none of the above-mentioned cases was the basal PDE activity markedly affected by these peptides (Barnette & Weiss, 1983).

Some neuropeptides such as ACTH (1-24), somatostatin and α-neoendorphin analogue have been shown to bind to calmodulin and to be fairly potent inhibitors of calmodulin. Some other neuropeptides including substance P, the

melanocyte-stimulating hormones (α-MSH, β-MSH and γ-MSH), bombesin and somatostatin can bind to calmodulin but are not effective calmodulin inhibitors. In contrast, angiotensins (I and III), bradykinin and neurotensin show little or no binding to calmodulin and therefore no inhibitory activity (Malencik & Anderson, 1982, 1983; Barnette & Weiss, 1983).

Glucagon and related peptides such as secretin, vasoactive intestinal peptide (VIP) and gastric inhibitory peptide (GIP) have been shown to bind to calmodulin with affinities 10-70 times greater than those of previously described peptides. The order of binding affinity for calmodulin being VIP > GIP > secretin > glucagon correlates with the one described for the binding of these peptides to cell-surface VIP receptors (Malencik & Anderson, 1983). VIP and secretin have been shown recently to be very potent inhibitors of calmodulin activity, having IC_{50} values of 0.5 μM and 2 μM, respectively. By contrast, glucagon failed to inhibit calmodulin-stimulated PDE activity even at high concentrations (100 μM) (Barnette & Weiss, 1985). Very recently, it was shown that glucagon and the antipsychotic antagonist TFP bind to calmodulin at the same sites but produce different conformational changes in the molecule. Therefore, it might explain why glucagon can bind to calmodulin but not inhibit calmodulin activity (Steiner & Marshall, 1985).

Structure-activity studies and sequence homology analyses have shown that for most peptides which bind to calmodulin, two structural characteristics appear to be important determinants of anti-calmodulin activity. These two structural characteristics consist of the presence of a large net positive charge and a hydrophobic surface which is brought about, for peptides, by both the presence of hydrophobic amino acids and also by an ordered sec-

ondary structure such as an amphiphilic α-helix. In some instances, these two characteristics appear to be essential for anti-calmodulin activity whereas in other cases, only one determinant appears to be sufficient for potent antagonistic activity. For example, VIP, secretin and β-endorphin, which are potent calmodulin inhibitors, carry a net positive charge, possess hydrophobic amino acids and contain significant α-helix content. Glucagon, on the other hand, which has been shown to form an amphiphilic α-helix but does not have a net positive charge, does not antagonize the action of calmodulin. In addition, it should be noted that not all peptides that inhibit calmodulin contain α-helices. Two notable exceptions are ACTH (1-24) and dynorphin (1-13), peptides which are potent calmodulin inhibitors and which tend to form a β-pleated sheet structure (for review see Barnette & Weiss, 1984).

In addition, it was found recently that some insect venom peptides such as melittin and mastoparans are potent antagonists of calmodulin (IC_{50} 10^{-9} to 10^{-10}M). These venom peptides are small, highly basic peptides which contain several hydrophobic amino acids and also α-helical structures. These determinants might explain their high potency as calmodulin inhibitors (Comte et al., 1983; Malencik & Anderson, 1983; Maulet & Cox, 1983; Barnette & Weiss, 1984).

F.5 Calmodulin Antibodies

In order to obtain a potent immunochemical tool for the study of the biological significance of calmodulin, many attempts have been made to raise

antibodies against this protein. Although calmodulin has been isolated and purified to homogeneity from various animal and plant species, because of the low species-specificity of this protein with respect to antigenicity, considerable difficulty in producing antibodies against this antigen has been encountered. Several reports have described the preparation of polyclonal antibodies against native calmodulin of rat testis (Dedman et al., 1978b; Chafouleas et al., 1979, 1982) and against chemically modified calmodulin of bovine brain (Wallace & Cheung, 1979; Van Eldik & Watterson, 1981). However, the titer of the antiserum obtained when using chemically modified antigens was often low, and the antibody had to be detected by a sensitive method. More recently, a new method has been developed to obtain highly specific polyclonal antibodies raised against brain calmodulin (Kitajima et al., 1983). Following this procedure, the production of calmodulin antibodies which recognized either Ca^{2+}-replete or Ca^{2+}-free calmodulin has been recently demonstrated (Harper, 1983).

The production of monoclonal antibodies against calmodulin has been reported quite recently (Harper, 1983; Pardue et al., 1983; Wang et al., 1983b). Some of these monoclonal antibodies appear to be very specific in recognizing only one type of calmodulin-activated enzyme whereas others showed cross-reactivity toward several calmodulin-dependent enzymes.

The availability of polyclonal and monoclonal antibodies against calmodulin has permitted several aspects related to this protein to be studied. These antibodies have been used to detect and quantify calmodulin in certain tissues as well as to study the subcellular distribution of this protein in different tissues. They have also been used to purify and characterize

several calmodulin-dependent enzymes and calmodulin-binding proteins. In addition, they have been proven to be very useful in studying the involvement of calmodulin in various cellular processes.

The involvement of calmodulin in the process of stimulus-secretion coupling in various secretory systems has been suggested indirectly with the use of pharmacological agents. Until recently, the only direct evidence for calmodulin involvement in cellular secretion was shown in a cell-free system prepared from sea urchin eggs, where the fusion of cortical granules to isolated cortical surfaces was blocked in the presence of antibodies raised against calmodulin (Steinhardt & Alderton, 1982). However, it was demonstrated very recently that calmodulin is involved in the secretory process of adrenal medullary chromaffin cells. Microinjection of calmodulin antibodies into cultured chromaffin cells using the erythrocyte ghost technique inhibited the stimulation-induced release of catecholamines from these cells (Kenigsberg & Trifaró, 1985b).

F.6 Chromaffin Cell Calmodulin

In 1976, Kuo and Coffee isolated and characterized a troponin C-like protein from the adrenal medulla (Kuo & Coffee, 1976a, 1976b). Based on the physicochemical properties determined for this protein, it was suggested later that this troponin C-like protein corresponds indeed to calmodulin.

Calmodulin has been shown to be present in cultured chromaffin cells. Its concentration in these cells is of 24 ng/10^6 cells, which represents 0.04% of the total protein found in adrenal medulla homogenates (Trifaró *et*

al., 1984a). The subcellular distribution of calmodulin has been determined and shown to be greatly dependent on the free Ca^{2+} concentration present in the cells. It was found that 69% of chromaffin cell calmodulin is present in the cytosol when the cells are homogenized in the presence of EGTA, while the remainder sediments with different membrane-containing fractions including the chromaffin granule fraction (Hikita et al., 1984). In addition, it was determined that secretory granule membranes contain calmodulin at a concentration of 4 pmole/mg protein. However, when the cells were homogenized in the absence of chelating agents, the concentration of calmodulin associated with the granule membranes was of 18 pmole/mg protein (Hikita et al., 1984).

The presence of high-affinity calmodulin-binding sites has been detected in chromaffin granule membranes (Burgoyne & Geisow, 1981; Geisow et al., 1982; Hikita et al., 1984; Bader et al., 1985). The detection of these binding sites appears to be also influenced by the free Ca^{2+} concentration present. In the presence of 10^{-4}M free Ca^{2+}, saturable and high-affinity ^{125}I-labelled calmodulin-binding sites (K_d = 9.8 nM, B_{max} = 25 pmole/mg protein) were detected in granule membranes (Hikita et al., 1984; Bader et al., 1985). In addition, a nonsaturable calmodulin-binding activity was detected at 10^{-7}M free Ca^{2+}. However, calmodulin binding was only observed at 10^{-4}M free Ca^{2+} when the granule membranes were previously delipidated (Bader et al., 1985).

The presence of calmodulin-binding proteins associated with the chromaffin granule membrane was identified by photoaffinity cross-linking and calmodulin affinity chromatography (Bader et al., 1985). Two major calmodulin-

binding proteins of molecular weights of 65,000 and 53,000 were identified in chromaffin granule membranes. However, the 65kD polypeptide was detected in intact membranes in the presence of 10^{-4}M free Ca^{2+} while both the 65kD and the 53kD polypeptide were found when delipidated granule membranes were used. In addition, it was determined that calmodulin binding to granule membranes is inhibited in the presence of an excess of these calmodulin-binding proteins as well as in the presence of calmodulin antibodies or in the presence of TFP (Bader et al., 1985).

It was also demonstrated recently by erythrocyte ghost-mediated microinjection that calmodulin antibodies clearly inhibited catecholamine release from cultured chromaffin cells stimulated by either ACh or a depolarizing K^+ concentration (56 mM) (Trifaró & Kenigsberg, 1983; Kenigsberg & Trifaró, 1985b).

The above-mentioned observations as well as others substantiate the hypothesis that calmodulin may play an important role at certain steps of the secretory process in adrenal chromaffin cells (for review see Trifaró et al., 1985).

G. PROTEIN PHOSPHORYLATION: A REVERSIBLE PROCESS

G.1 Introduction

The presence of phosphorus in proteins has been known for almost a hundred years, but its importance has only been realized since the discovery of enzyme regulation by a reversible phosphorylation process. The "phosphorylation era" began in 1956 when Krebs and Fischer discovered that the neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase (Krebs & Fischer, 1956). Then shortly after, from studies of these investigators and the work of Friedman and Larner, it was demonstrated that two other enzymes, namely phosphorylase kinase and glycogen synthase, were regulated in the same manner (Krebs et al., 1959; Friedman & Larner, 1963). These three enzymes remained the only examples of this type of phenomenon until 1968 when a cyclic AMP-dependent protein kinase was discovered (Walsh et al., 1968) (for review see Krebs & Beavo, 1979; Cohen, 1980a, 1982, 1983; Hardie & Guy, 1982).

The past 15 years have seen an extraordinary and still accelerating growth in the field of protein phosphorylation. About 40 enzymes and countless other proteins are now known to be regulated by a phosphorylation-dephosphorylation mechanism. Protein phosphorylation is clearly the major general mechanism by which intracellular events respond to external physiological stimuli. Such a mechanism has been demonstrated to regulate inter-

mediary metabolism, muscle contractility and protein synthesis and also postulated to be implicated in other cellular events such as receptor activity, ion transport, motility, secretion, cell proliferation and many others (for review see Traugh, 1981; Beavo & Mumby, 1982; Johnson, 1982; Cohen et al., 1983; Demaille & Pechère, 1983; Fischer, 1983). However, more evidence has to be provided to clearly demonstrate the importance of such a mechanism in the latter cellular events mentioned above (for review see Levitan et al., 1983; Robinson & Dunkley, 1983a; Ewald et al., 1985; Marceau & Swierenga, 1985).

More recently, a lot of research in the field of protein phosphorylation has been performed in the nervous system. A great number of the phosphorylation-related events occurring in the nervous system appear to be similar to the ones observed in other cell systems. However, a portion of the phosphorylation process involved in the central nervous system appears to be unique to this one in terms of specific brain regions and specific neuronal pathways. It seems certain that the phosphorylation process in the brain is especially refined and adapted to the specialized functions of the nervous system in the propagation, transmission and processing of messages, although its precise significance is still unclear (for review see Gispen & Routtenberg, 1982; Nestler & Greengard, 1984; Nestler et al., 1984; Browning et al., 1985; Nairn et al., 1985).

Since the literature in the area of protein phosphorylation is extremely vast, and also for reason of simplicity, this section will focus mainly on the phosphorylation-dephosphorylation process occurring in the nervous system and secretory cell systems.

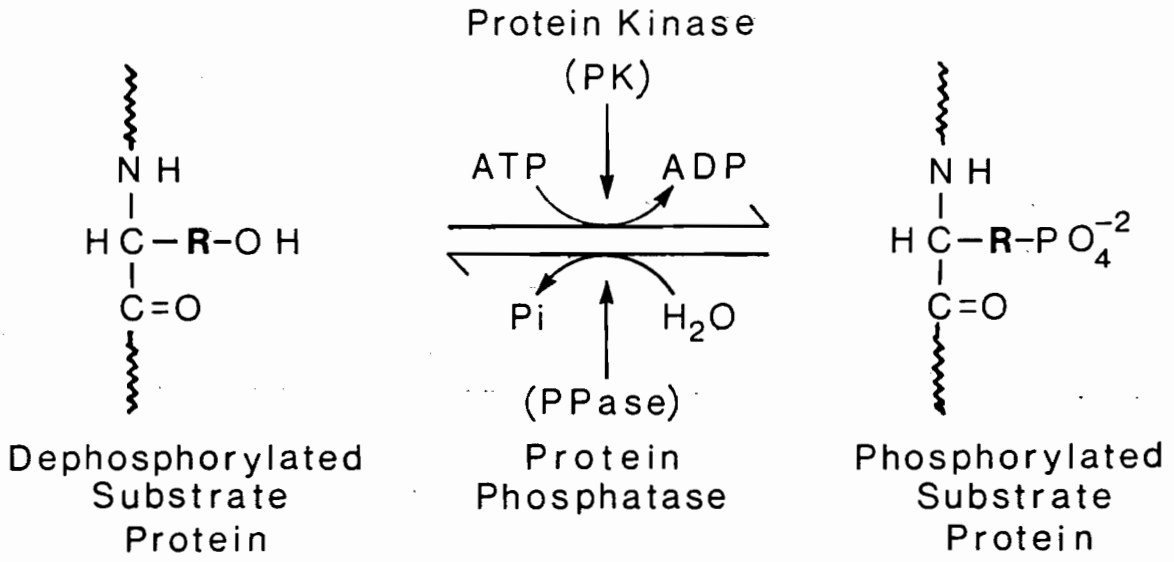
G.2 General Molecular Mechanism of Protein Phosphorylation and Dephosphorylation

Protein phosphorylation is a common mechanism for altering the activity of an enzyme by structural alterations. At the molecular level, it involves the transfer of the terminal phosphate from ATP to the hydroxyl groups of certain amino acid residues of proteins. The system consists of three primary components, namely a substrate protein, a protein kinase and a protein phosphatase. The phosphate transfer to a substrate protein is catalyzed by an enzyme called a protein kinase (PK) and can be reversed by another enzyme called a protein phosphatase (PPase) (see Fig. 4). Such a transfer reaction results in an alteration of the conformation of the substrate protein which often causes an inactive enzyme to become active or the opposite effect. However, many proteins may also become phosphorylated even if no function has been attributed to them yet. The state of phosphorylation of a protein depends on the equilibrium between protein kinase and phosphatase activities, which in turn are regulated by a variety of modulators such as calcium, cyclic nucleotides, phospholipids and proteins (e.g. calmodulin), or by phosphorylation of the enzyme itself (autophosphorylation).

G.3 Protein Kinases and Substrates

Five categories of protein kinase enzymes have been recognized and classified according to the agent which regulates their activity: cAMP, cGMP,

Fig. 4 Molecular mechanisms of the process of protein phosphorylation-dephosphorylation. A given substrate protein can be phosphorylated via the catalytic action of a protein kinase (PK). The opposite reaction (dephosphorylation) is catalyzed by a protein phosphatase (PPase).
R, side chain of serine (CH_2), threonine ($CH\text{-}CH_3$) and tyrosine (Bz) residues; Pi, inorganic phosphate. Other types of phosphate-containing moieties can also be formed (e.g.: acylphosphate, phosphoramidate).


Protein Kinase
(PK)
ATP
ADP
Pi
H_2O
(PPase)
NH
HC—R—OH
C=O
NH
HC—R—PO_4^{-2}
C=O
Dephosphorylated Substrate Protein
Protein Phosphatase
Phosphorylated Substrate Protein

calcium/calmodulin, calcium/phospholipid, or independent (no known agent).

G.3.1 cAMP-Dependent Phosphorylation

Greengard originally postulated that all of the intracellular action of cAMP in mammalian tissues were mediated by cAMP-dependent protein kinase (cAMP-PK), and to date no substantial evidence has challenged this hypothesis (Greengard 1976, 1978, 1979, 1981). cAMP-PK is the most extensively characterized protein kinase. It phosphorylates serine, threonine and tyrosine residues of a large number of proteins of known function *in vitro* and for some also *in vivo* (for review see Beavo & Mumby, 1982; Flockhart & Corbin, 1982; Sharma, 1982; Dunkley *et al.*, 1983; Davis, 1985).

a) cAMP-Dependent Protein Kinase

In 1968, Walsh and collaborators discovered a protein kinase in skeletal muscle that was specifically activated by low concentrations of cAMP. Kuo and Greengard (1969) then demonstrated the presence of cAMP-PK in several tissues and phyla of the animal kingdom, and it has since then been purified to homogeneity from a wide variety of tissues and species. The highest level of cAMP-PK in the organism is localized in the brain and has been shown to be present in both neurons and glia (Cumming *et al.*, 1981).

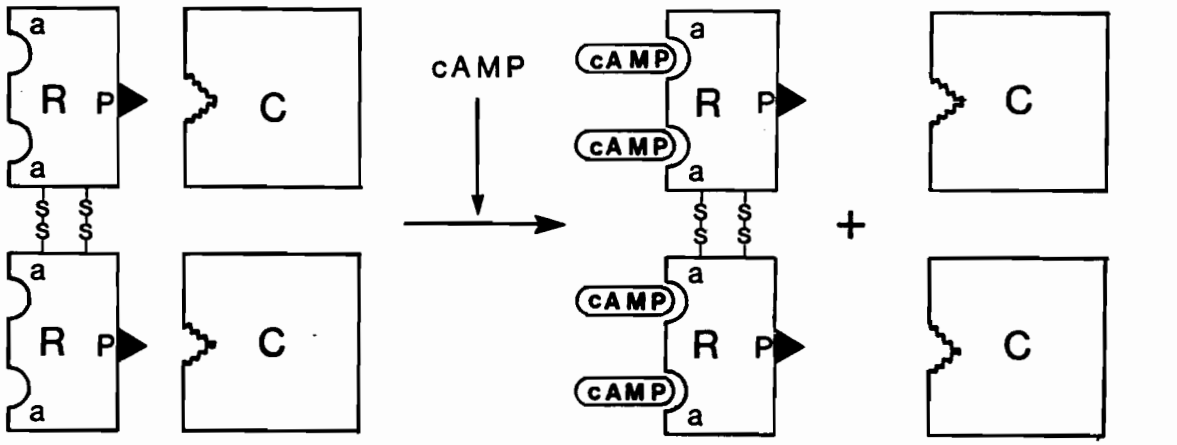
The holoenzyme exists as a tetramer consisting of two regulatory (R) subunits which are joined by disulfide bonds to form a dimeric complex and two catalytic (C) subunit monomers. The holoenzyme form of the kinase is

inactive, and it appears that both R subunits inhibit the C subunits by directly binding to them. cAMP activates the enzyme by binding to the R subunits which possess two cAMP binding sites on each R subunit monomer. Consequently, such binding causes the dissociation of the holoenzyme into two free C subunits and one R subunit dimer bound to cAMP (see Fig. 5). In addition, recent studies have shown that all four cAMP-binding sites are involved in the activation of the enzyme and that the occupation sequence exhibits a positive cooperativity in vitro (Robinson-Steiner & Corbin, 1983; Ogréid et al., 1983; Reed et al., 1985). The R subunits of the kinase are the only known cAMP receptors or binding proteins in eukaryotic cells (Walter et al., 1977; Walter & Greengard, 1982).

Two types of cAMP-PK were first separated from rabbit skeletal muscle (Reimann et al., 1971). These two forms of cAMP-PK, designated type I and type II, possess the same kind of C subunit but are distinguished by different kinds of R subunits. These two types of enzymes differ in their tissue distribution, biochemical properties and immunological reactivities (Corbin et al., 1975; Hofmann et al., 1975; Fleischer et al., 1976). For example, they possess different molecular weights (47,000 for R-I and 54,000 for R-II) and different isoelectric points (Lohmann et al., 1980; Walter & Greengard, 1982). In addition, there exists a microheterogeneity of type R-II subunits which depends on the embryological origin of the tissue and which has been demonstrated by proteolytic peptide mapping (Rubin et al., 1981; Stein et al., 1984). Furthermore, in bovine brain two forms of R-II (referred to as R-II and R-II*) can be distinguished by a small difference in molecular weight, being 58,000 for R-II and 52,000 for R-II*. However

Fig. 5 Schematic representation of the type II cAMP-dependent protein kinase. Following cAMP binding to the inactive holoenzyme, the holoenzyme is dissociated into a dimeric complex of regulatory subunits and two active catalytic subunits. Although only the type II form of cAMP-dependent protein kinase undergoes autophosphorylation, the subunit composition of the type I kinase as well as its dissociation and activation by cAMP is analogous to those shown on this scheme for the type II enzyme.

R, regulatory subunit; a, cAMP-binding site; P, autophosphorylation site; C, catalytic subunit (catalytic site indicated by serrated indentation).


a
R
P
a
C
S
S
S
S
cAMP
cAMP
cAMP
cAMP
+
Inactive
Holoenzyme
Active
Catalytic Subunits

only the R-II form was detected in rat brain (Lohmann et al., 1980).

The catalytic (C) subunit is identical for types I and II cAMP-PK and possesses a molecular weight of 41,000 (Beavo & Mumby, 1982). Regardless of the source of the C subunit, it phosphorylates the same substrates and exerts the same influence on synaptic transmission (Flockhart & Corbin, 1982). In addition to the inhibitory action of the R subunit on the C subunit, the C subunit possesses an endogenous protein inhibitor, the so-called Walsh inhibitor, which exists at about 10-20% of the concentration of the C subunit. The inhibitor is not phosphorylated and may act as a substrate analogue (Beavo & Mumby, 1982; Sharma, 1982; Huszti & Magyar, 1985; Scott et al., 1985).

Type II cAMP-PK can undergo autophosphorylation whereby the C subunit phosphorylates the R-II subunit (Erlichman et al., 1974; Maeno et al., 1974), but in contrast to R-II, R-I is not phosphorylated (Walter et al., 1978). Thus, although both R-I and R-II inhibit the C subunit by binding in the vicinity of the catalytic site, only R-II serves as a substrate for the catalytic site (Rangel-Aldao & Rosen, 1976; Nestler et al., 1981b).

cAMP-PK is found predominantly in the soluble fraction of most tissues, but in contrast, approximately half of the enzymatic activity in the brain is associated with the particulate fraction (Hofmann et al., 1977; Rubin et al., 1979). Despite some evidence for similar regional and subcellular distribution of type I and type II cAMP-PK in the brain, some studies suggest different distributions with type II being predominantly localized in the neuronal cells (Walter et al., 1979; Rubin et al., 1981; for review see Dunkley, 1981).

b) cAMP-Dependent Protein Kinase Substrates

cAMP-PK has a very broad substrate specificity and has been shown to phosphorylate a wide array of substrate proteins in many tissues (for review see Krebs & Beavo, 1979; Beavo & Mumby, 1982; El-Maghrabi et al., 1983).

Neuronal Substrates

Originally, two major endogenous substrates were detected in synaptic fractions of brain and called protein I and protein II (Johnson et al., 1972; Ueda et al., 1973). Since then, about 20 substrates have been detected and localized in different regions of the nervous system (for review see Browning et al., 1985).

Protein I, now called synapsin I, consists of two closely related proteins, synapsin Ia and synapsin Ib, with molecular weights of 86,000 and 80,000, respectively. Synapsin Ia and Ib are almost identical proteins with similar amino acid composition, unusually high pI and elongated structure, and are found in a ratio of 1:2. They contain two domains which are about identical in dimension but different in some respects since one domain is collagenase-insensitive and the other is proline-rich (Ueda & Greengard, 1977; Greengard, 1981). Synapsin I is an effective substrate for at least three distinct protein kinases in the brain and undergoes multisite phosphorylation. cAMP-PK phosphorylates one serine residue in the collagenase-insensitive domain (Huttner et al., 1981). Synapsin I is localized in the

presynaptic terminal of neurons where it is associated with synaptic vesicles. This protein appears to be present in virtually all synapses and localized only in neurons (De Camilli et al., 1983a, 1983b; Huttner et al., 1983; Schiebler et al., 1983). Synapsin I has been studied extensively since it is a very good substrate for several protein kinases although its function remains unknown (for review see Nestler & Greengard, 1984; Browning et al., 1985). However, it was reported recently that synapsin I is immunologically related to the erythrocyte protein 4.1 and that synapsin I may correspond in fact to a spectrin-binding protein in the brain (Baines & Bennett, 1985).

Another substrate which is immunologically related to synapsin I (protein I) has been named protein III. Protein III was first detected in rat brain slices and has since been purified to near homogeneity from bovine brain and partially characterized (Forn & Greengard, 1978; Huang et al., 1982). It consists of two related proteins, which have been termed protein IIIa and IIIb, with molecular weights of 74,000 and 55,000, respectively. Protein III contains one phosphorylation site on a serine residue and is an effective substrate for cAMP-PK and also other protein kinases. Protein III is present throughout the central and peripheral nervous system, and its regional and subcellular localizations in the brain closely parallel that of synapsin I (Browning et al., 1982).

DARPP-32 (Dopamine and cAMP-Regulated PhosphoProtein-32) is an endogenous substrate which was first detected in homogenates of rat neostriatum (Walaas et al., 1983b). This substrate has since been purified from bovine caudate nucleus and extensively characterized (Hemmings et al., 1984a,

1984b, 1984c; Walaas & Greengard, 1984; Ouimet et al., 1984). DARPP-32 is an acidic, elongated and heat-stable protein with a molecular weight of 32,000. cAMP-PK catalyzes the incorporation of up to 1 mole of phosphate into a single threonine residue. DARPP-32 is present only in the nervous system, concentrated in brain regions that are richly innervated by dopaminergic neurons and specifically those that possess D_1-dopamine receptors (Ouimet et al., 1984).

Several other endogenous substrates have been identified in different brain regions. Some of them, such as synapsin I (Ia and Ib), protein III (IIIa and IIIb), microtubule-associated protein MAP2 and R subunits (R-II and R-II*) of the cAMP-PK (type II) appear to be widely distributed throughout all regions of the brain while other neuronal substrates are not usually detected in fractions of cerebral cortex (Walaas et al., 1983b, 1983c). For example, tyrosine hydroxylase is known to be present but a low concentration in the brain does not permit its detection by standard autoradiographic techniques (Walaas et al., 1983c; see also section G.6.2). Detailed regional studies have recently revealed the presence of other new substrates with specific brain distributions, including a 260kD particulate fraction protein which is unique to the cerebellum (Purkinje cells) and seven cytosolic proteins of molecular weights ranging from 98,000 to 21,000 which are localized in regions of the basal ganglia (Walaas et al., 1983a, 1983b, 1983c; Rauch & Roskoski, 1984). In addition, it was shown recently that cAMP-PK phosphorylates specific subunits of neurotransmitter receptors and of ion channels such as the γ and δ subunits of nicotinic acetylcholine receptor and the α subunit of voltage-dependent sodium channels (Huganir & Greengard, 1983;

Huganir et al., 1983; Costa & Catterall, 1984a).

G.3.2 cGMP-Dependent Phosphorylation

By analogy with cAMP, Kuo and Greengard (1970) have proposed that the role of cGMP-dependent protein kinase (cGMP-PK) was to mediate some, and possibly all, of the physiological effects of cGMP. However, this protein kinase hypothesis for cGMP has been much more difficult to test than that for cAMP since there are fewer possible physiological roles for cGMP and they are less well described than those for cAMP. Direct evidence for this hypothesis in the case of cGMP requires studies in which cGMP-PK is injected into cells and the physiological effects of the injection are compared to those of cGMP. Such evidence has been recently obtained in the case of retinal rod outer segments (Shimoda et al., 1983; for review see Greengard, 1981; Nairn & Greengard, 1983; Drummond, 1983; Browning et al., 1985).

a) cGMP-Dependent Protein Kinase

In 1970, Kuo and Greengard discovered a protein kinase in lobster tail muscle that was specifically activated by low concentrations of cyclic GMP. Its presence was then demonstrated in a wide variety of species including vertebrates, mollusks, arthropods and protozoa (Greengard & Kuo, 1970; Kuo et al., 1971; Murofushi, 1974; Kuo & Shoji, 1982; Lincoln & Corbin, 1983). Some tissues, such as cerebellum, smooth muscle and lung, contain high levels of cGMP-PK whereas other tissues, such as liver, skeletal muscle and

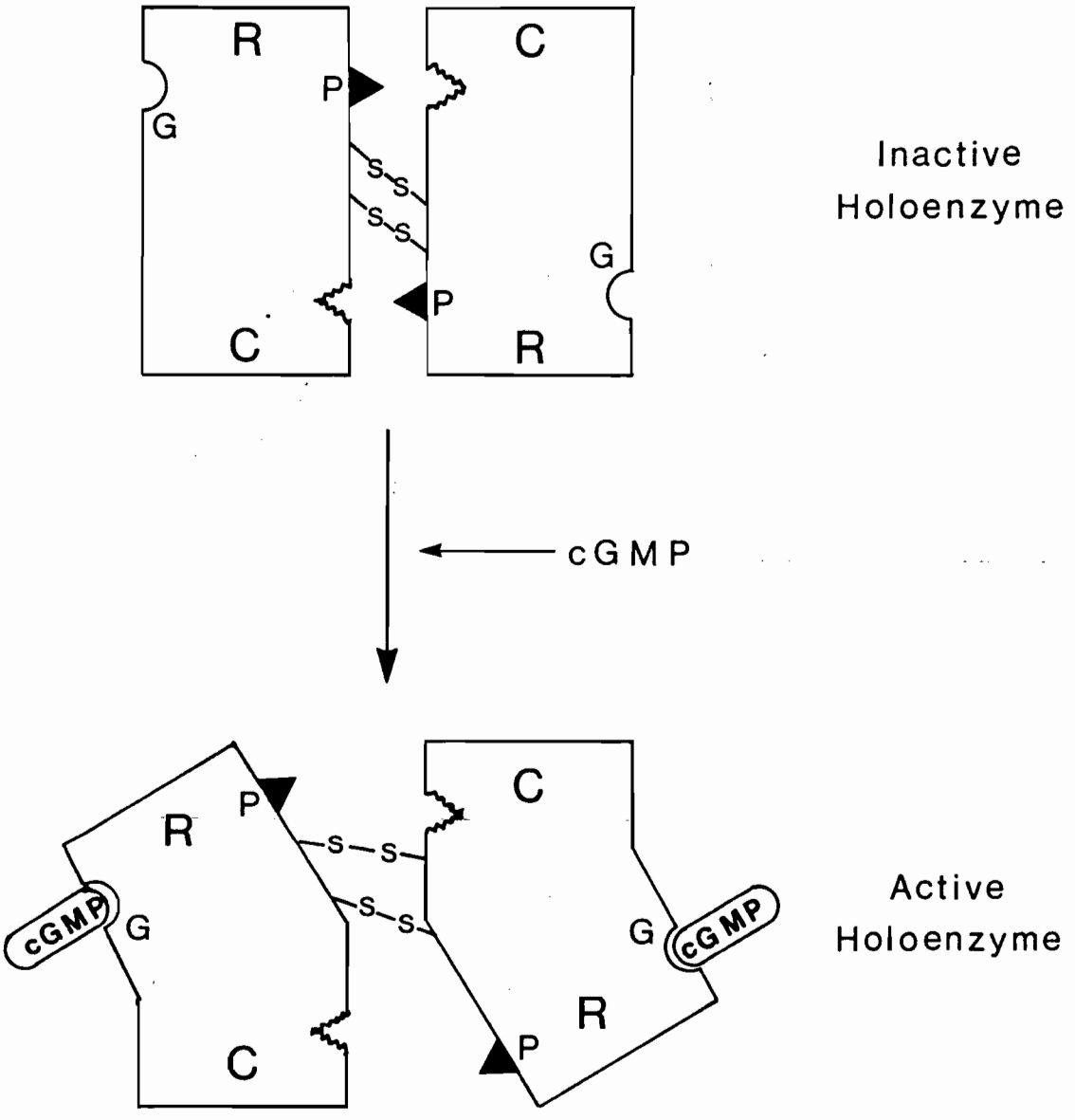
adipose tissue, contain low levels of the kinase (Walter, 1981; Lincoln & Corbin, 1983; Lincoln & Johnson, 1984).

The holoenzyme represents a dimer of identical proteins of approximately 75kD that are joined by disulfide bonds. The protein kinase holoenzyme is inactive in the absence of cGMP. cGMP activates the enzyme by binding to specific sites, but in contrast to cAMP-PK, the cGMP activation does not involve the dissociation of the holoenzyme. Similarly to cAMP-PK, cGMP-PK possesses four cGMP-binding sites which are all involved in the activation of the enzyme and exhibit a positive cooperativity in vitro (Kuo & Shoji, 1982; Lincoln & Corbin, 1983). Each monomer of the enzyme contains three spatially and functionally distinct domains: a catalytic domain, a cGMP-binding domain and a phosphorylation domain. The catalytic domain contains the active site of the enzyme. The cGMP-binding domain contains the two sites for cGMP binding and thereby activates the catalytic domain. The phosphorylation domain contains both the interchain disulfide bonds and the active site where the kinase undergoes autophosphorylation. This domain probably functions as the regulatory domain; this region binds to the catalytic domain where it serves as a substrate for the active site and inhibits the phosphorylation of the other substrate proteins (Hofmann et al., 1983). It appears that the two monomers of cGMP-PK are arranged in an antiparallel fashion such that the regulatory domain of one subunit inhibits the catalytic activity of the other subunit (for review see Gill, 1977; Lincoln & Corbin, 1983) (see Fig. 6).

In contrast to cAMP-PK, only one form of cGMP-PK has been detected in vertebrate and invertebrate tissues. The cGMP-PK purified from different

Fig. 6 Schematic representation of the cGMP-dependent protein kinase. Following cGMP binding to the inactive holoenzyme, the enzyme undergoes conformational changes and consequently the catalytic sites of the kinase become activated.

R, regulatory domain; G, cGMP-binding site; P, autophosphorylation site; C, catalytic domain (catalytic site indicated by serrated indentation).


R
C
P
G
S
S
S
S
G
P
C
R
Inactive
Holoenzyme
cGMP
P
R
S
S
S
S
C
cGMP
G
G
cGMP
R
C
P
Active
Holoenzyme

tissues exhibits similar biochemical and immunological properties. However, one group has reported that the cGMP-PK of intestinal epithelial cells appeared to be somewhat different in terms of biochemical properties when compared to other tissues (de Jonge & Van Dommelen, 1981).

cGMP-PK is present in every brain region studied, although its concentration in this tissue shows marked regional differences. For example, it is present in very high levels in cerebellar Purkinje cells and in cerebellar cortex, at moderate levels in choroid plexus and at low levels in other brain regions. Subcellular fractionation studies have shown that cGMP-PK is soluble in most tissues studied. In the brain, it was shown that the kinase is present throughout the cytoplasm of cell bodies, dendrites, axons and nerve terminals for both cerebellar Purkinje cells and striatal neurons (Greengard & Kuo, 1970; Schlichter et al., 1978; Lohmann et al., 1981; Ariano, 1982; Lincoln & Corbin, 1983).

b) cGMP-Dependent Protein Kinase Substrates

Endogenous substrate proteins for cGMP-PK are fewer and present at lower concentrations compared to those for cAMP-PK. However, some substrates for this kinase have been identified and characterized in smooth muscle, in intestinal brush border epithelium and in cerebellum (Casnellie & Greengard, 1974; Casnellie et al., 1980; de Jonge, 1981; Aswad & Greengard, 1981a, 1981b; Aitken et al., 1981). In addition, more recently, endogenous substrates have been detected in platelets, retina, heart, brain and preparations of ribosomal and nuclear proteins, but these proteins await further

characterization (Kuo & Shoji, 1982; Farber, 1982; Schlichter, 1982; Lincoln & Corbin, 1983; Matsumoto & Pak, 1984).

G-substrate is to date the only physiological substrate protein for cGMP-PK which has been purified and characterized from the brain. This protein was first detected in the cerebellum as an endogenous substrate for cGMP-PK but not for cAMP-PK (Schlichter et al., 1978). This heat-stable substrate of molecular weight of 23,000 is phosphorylated on two threonine residues which can accept up to 1 mole of phosphate on each residue (Aswad & Greengard, 1981b). G-substrate is exclusively localized in Purkinje cells of the cerebellum, and the phosphorylated form of the protein appears to play an important role in the regulation of cellular functions in this tissue (Nairn et al., 1982; Dolphin et al., 1983).

G.3.3 Calcium/Calmodulin-Dependent Phosphorylation

Calcium-stimulated phosphorylation was first demonstrated in the brain (DeLorenzo, 1976, 1977; Schulman & Greengard, 1978a). Around the same period, the ubiquitous calcium-binding protein calmodulin was found to play an essential role as mediator in calcium-stimulated phosphorylation (Dabrowska et al., 1978; Yagi et al., 1978; Schulman & Greengard, 1978b). The discovery of calcium/calmodulin-dependent protein kinase (CaM-PK) has generated intensive research in the field of protein phosphorylation. It is now known that CaM-PK is the dominant phosphorylating system in the brain in terms of protein kinase activity and the number of substrates phosphorylated (Walaas et al., 1983b) and also occurs in every tissue examined so far. Recently, it

was found that several distinct species of CaM-PK's exist in the brain and in several other tissues (for review see Klee & Newton, 1983). These enzymes phosphorylate a large array of substrate proteins, some of which are also substrates for cAMP-PK and calcium/phospholipid-dependent protein kinase (Cohen, 1982; Walaas et al., 1983b, 1983c; McGuinness et al., 1984; Schulman, 1984a; Browning et al., 1985).

a) Calcium/Calmodulin-Dependent Protein Kinase

Various types of CaM-PK activities have been described in association with numerous tissues, subcellular fractions and substrate proteins. Originally the CaM-PK activity was resolved into three enzymes with different substrate specificities (Yamauchi & Fujisawa, 1980). Later, Kennedy and Greengard (1981) demonstrated that at least four different types of kinase existed at least in the brain. These included phosphorylase kinase, myosin light chain kinase (MLCK) and two other CaM-PK's, referred to as CaM-PK I and CaM-PK II. Shortly after, other investigators also reported the existence of MLCK and CaM-PK II (Miyamoto et al., 1981; Fukunaga et al., 1982a, 1982b; Yamamoto et al., 1983). In addition, these other CaM-PK's have been detected and have been called tubulin kinase (Burke & DeLorenzo, 1981; Goldenring et al., 1982), glycogen synthase kinase (Payne & Soderling, 1980; Ahmad et al., 1982) and phospholamban kinase (Demaille & Pechère, 1983). These three last enzymes might represent variants of already known CaM-PK's as indicated by several studies. Recently, a new type of CaM-PK was demonstrated in neuronal nuclei (Sahyoun et al., 1984).

Although phosphorylase kinase and MLCK are abundant in the brain (0.1% of total brain protein), it appears that these two enzymes do not account for a large portion of the brain CaM-PK activity (Yamauchi & Fujisawa, 1980; Kennedy & Greengard, 1981; Hathaway et al., 1981; Cohen, 1982; Ververken et al., 1982; Fukunaga et al., 1982a, 1982b). CaM-PK II constitutes as much as 0.6% of total brain protein and accounts for most of the CaM-PK activity in the brain (McGuinness et al., 1983a).

Calcium/Calmodulin-Dependent Protein Kinase I

CaM-PK I was first identified in brain as an enzyme that catalyzed the phosphorylation of synapsin I at the same site as the one for cAMP-PK (site 1) (Kennedy & Greengard, 1981). This enzyme is distinct from other CaM-PK's and is found only in brain cytosol where it may be associated with synaptic vesicles (Kennedy & Greengard, 1981; Moskowitz et al., 1983). More recently, it was shown that synapsin I appears to be the best endogenous substrate for this kinase, and consequently, it was suggested that this enzyme might be of physiological importance in phosphorylating site 1 of synapsin I in nerve terminals (Kennedy et al., 1983b; Nairn & Greengard, 1983).

The enzyme has been purified recently from bovine brain and consists of two subunits of molecular weights of 37,000 and 35,000. Both subunits can bind calmodulin and can undergo autophosphorylation (Kennedy, 1983). In brain coated vesicles, it was shown that when antibodies raised against the kinase were used, calmodulin-dependent phosphorylation was abolished. There-

fore, it was suggested that the enzyme and its substrates may be tightly associated with synaptic function (Moskowitz et al., 1983).

Calcium/Calmodulin-Dependent Protein Kinase II

CaM-PK II was first identified in brain as a kinase that catalyzed the phosphorylation of sites 2 and 3 of synapsin I (Kennedy & Greengard, 1981). The enzyme has since been purified from rat brain and partially characterized (Kennedy et al., 1983b; Lai et al., 1983; Huttner et al., 1983).

The enzyme is composed of a 50kD and a 60kD protein subunit that bind calmodulin, undergo autophosphorylation and appear to possess catalytic activity (Kennedy et al., 1983b; Lai et al., 1983; Kuret & Schulman, 1985; Levine et al., 1985). In addition, a 58kD subunit has been detected in brain preparations, but this subunit appears to be a proteolytic fragment of the 60kD subunit (McGuinness et al., 1983a). The 50kD subunit is identical to the major postsynaptic density protein described by several investigators (Grab et al., 1981a; Kelly et al., 1984; Rostas et al., 1983; Kennedy et al., 1983a; Goldenring et al., 1984).

In addition, the purified enzyme is almost identical to the previously described enzyme in rat liver referred to as glycogen synthase kinase (Payne & Soderling, 1980; Soderling & Payne, 1981; Ahmad et al., 1982; Fukunaga et al., 1982b; Yamamoto et al., 1983). However, glycogen synthase kinase is composed of 51kD and 53kD subunits, and therefore both enzymes (glycogen synthase kinase and CaM-PK II) are composed of calmodulin-binding phosphoproteins of similar but not identical molecular weights. Both enzymes have

as major substrates synapsin I, and also, although to a lesser extent, microtubule-associated protein MAP2 and myosin light chain but no activity towards tubulin or phosphorylase (McGuinness et al., 1983a; Bennett et al., 1983). However, the two enzymes differ in specificity for glycogen synthase. In addition, a glycogen synthase kinase has been isolated from skeletal muscle, and its properties are similar to that exhibited by liver glycogen synthase kinase and brain CaM-PK II (Woodgett et al., 1982; McGuinness et al., 1983b). Greengard has proposed that CaM-PK II and glycogen synthase may be isozymes of a "multifunctional CaM-PK" (McGuinness et al., 1983b).

Tubulin Kinase

Another CaM-PK was isolated from brain cytosol and appeared distinctive from other CaM-PK's reported, since one of its substrate was tubulin, and in addition, it did not phosphorylate synapsin I (Burke & DeLorenzo, 1981; Goldenring et al., 1982, 1983; Nosé & Schulman, 1982). However, the structure and some properties of the enzyme might be related to another variant of Greengard's "multifunctional CaM-PK". The purified enzyme has a native molecular weight of 600,000 and is composed of two subunits of 63kD and 52kD. These two subunits have significant structural homology, and the 52kD subunit might correspond to the microtubule-associated component tau factor (Goldenring et al., 1983). The enzyme copurifies with microtubules and possesses as major substrates the microtubule-associated protein MAP2 and tubulin (α and β) (Goldenring et al., 1982). In addition, a CaM-PK which possesses properties almost identical to the ones of tubulin kinase was found

to be associated to neurofilament preparations (Vallano et al., 1984). Further studies are required to determine if tubulin kinase and CaM-PK are similar or different kinase entities.

Phosphorylase Kinase

Phosphorylase kinase catalyzes the phosphorylation of phosphorylase, the enzyme responsible for glycogen breakdown, and phosphorylase is activated upon such phosphorylation. The activation of phosphorylase kinase is part of the mechanism by which calcium mediates the hormonal and neural regulation of glycogen metabolism in skeletal muscle and also in other tissues (for review see Cohen, 1978; Krebs & Beavo, 1979; Krebs, 1981; Fischer, 1983).

The holoenzyme consists of a multi-subunit tetrameric complex of a molecular weight of about 1.3 million $(\alpha\beta\gamma\delta)_4$. Each monomer possesses four subunits which are present in equal ratio and have molecular weights of 145,000 (α), 128,000 (β), 45,600 (γ) and 17,000 (δ). In contrast to the other CaM-PK's, phosphorylase kinase possesses calmodulin as an integral subunit (δ). The γ subunit and possibly the β subunit also appear to be the catalytic subunits of the enzyme. The calmodulin-binding sites are believed to be located in the γ subunit although recent studies have raised the possibility that the α and β subunits might also act as calmodulin-binding subunits. The kinase can undergo autophosphorylation on α and β subunits. In addition, the enzyme can be phosphorylated by cAMP-PK on its α and β subunits, which results in the stimulation of the kinase activity (Cohen, 1978; Krebs & Beavo, 1979; Krebs, 1981; Schulman, 1982). This activation can be

partially blocked by ADP-ribosylation of the enzyme (Tsuchiya et al., 1985). Furthermore, it was shown recently that the γ subunit of phosphorylase kinase possesses a great homology with the R subunit of cAMP-PK I (Reimann et al., 1984).

Phospholamban kinase is a CaM-PK which was isolated from the sarcoplasmic reticulum of cardiac and skeletal muscles and believed to be related to phosphorylase kinase (Le Peuch et al., 1979; Varsanyi & Heilmeyer, 1979; Demaille & Pechère, 1983). This enzyme catalyzes the phosphorylation of phospholamban, a low molecular weight sarcoplasmic reticulum protein associated with the ATPase (Tada et al., 1983; Wegener & Jones, 1984). The enzyme has a native molecular weight of 1.25 million and is composed of a 52kD protein. Both phospholamban kinase and phosphorylase kinase are able to phosphorylate phospholamban and phosphorylase. Therefore, it was proposed that they might be related enzymes, although they have been distinguished on the basis of their affinity for calmodulin and their immunoreactivity (Varsanyi & Heilmeyer, 1979; Demaille & Pechère, 1983).

Myosin Light Chain Kinase

Myosin light chain kinase (MLCK) catalyzes the phosphorylation of myosin light chain. This enzyme has been studied extensively; it has been isolated, purified and characterized from numerous species and tissues including skeletal muscles, smooth muscles and non-muscle cells (for review see Hartshorne & Persechini, 1980; Adelstein & Eisenberg, 1980; Hartshorne, 1982; Klee & Vanaman, 1982). A lot of evidence indicates that MLCK mediates

the second messenger action of calcium in triggering contractility in smooth muscle and in non-muscle cells and may mediate some other second messenger action of calcium in modulating contractile activity in skeletal muscle (Adelstein, 1982; Adelstein et al., 1982; Malencik et al., 1982; Demaille & Pechère, 1983; Mayr & Heilmeyer, 1983; Stull et al., 1983; Kamm & Stull, 1985).

The enzyme consists in a monomer of molecular weight of about 130,000, although in contrast to other protein kinases, its molecular weight varies greatly (ranging from 90,000 to 150,000) depending on the tissue and the species of origin. The enzyme can undergo autophosphorylation, a process in which both Ca^{2+} and calmodulin appear to be essential (Wolf & Hofmann, 1980; Foyt & Means, 1985). This kinase can be phosphorylated by the C subunit of cAMP-PK which results in the inactivation of kinase towards its endogenous substrate myosin light chain and appears to greatly influence the binding capacity of the enzyme for calmodulin (Conti & Adelstein, 1980; Edelman & Krebs, 1982; Bhalla et al., 1982). Recently, the identification of the amino acid sequence of the phosphorylation site of myosin light chain has resulted in the production of a synthetic peptide substrate which activates MLCK in vitro (Kemp et al., 1982). In addition, the calmodulin-binding domain of the kinase was recently characterized in skeletal muscle (Blumenthal et al., 1985).

b) Calcium/Calmodulin-Dependent Protein Kinase Substrates

A great number of substrates for the CaM-PK's have been detected in numerous systems. This abundance of substrates seems evident since several types of CaM-PK activities have been detected in vitro, in situ and in vivo. Certain CaM-PK's such as phosphorylase kinase and MLCK exhibit very narrow substrate specificities: the only demonstrated physiological substrates being phosphorylase and myosin light chain, respectively (Krebs, 1981; Demaille & Pechère, 1983). Phosphorylase kinase also phosphorylates glycogen synthase, but the physiological significance of this process remains to be determined (Krebs, 1981). A detailed knowledge of the substrate specificity of the other CaM-PK's is not yet available and awaits the purification and further characterization of each enzyme.

However, in the nervous system, several substrates for CaM-PK have been recognized and partially characterized. Some of these substrates, especially CaM-PK subunits and its proteolytic fragment as well as synapsin I (a and b), have been described by many groups of investigators as major substrates in this system (Schulman & Greengard, 1978a, 1978b; DeLorenzo, 1981; Dunkley, 1981; Gower & Rodnight, 1982; Sorenson & Mahler, 1983; Fukunaga et al., 1984b; Patton et al., 1985a, 1985b). The phosphorylation of synapsin I by CaM-PK has been reviewed extensively (Greengard, 1981; Schulman, 1982; Huttner et al., 1983). Extensive studies on the phosphoproteins of molecular weights ranging from 50,000 to 60,000 have allowed the identification of some major substrates as being tubulin (α and β) (Burke & DeLorenzo, 1981, 1982a, 1982b), phosphodiesterase and calcineurin (Carlin et al., 1981; Grab

et al., 1981a, 1981b; Cohen et al., 1982), tyrosine hydroxylase and tryptophan hydroxylase (Yamauchi & Fujisawa, 1983; Vulliet et al., 1984). Many other proteins are phosphorylated by CaM-PK, including the microtubule-associated protein MAP2 (Walaas et al., 1983b, 1983c; Schulman, 1984b), neurofilament subunits (Vallano et al., 1984), myelin basic proteins (Agrawal et al., 1982; Ulmer & Braun, 1985) and GABA-modulin (Wise et al., 1983) although most substrates remain unidentified. The regional distribution of all the substrates has been extensively studied by Walaas and collaborators (1983b, 1983c).

G.3.4 Calcium/Phospholipid-Dependent Phosphorylation

In 1979, Nishizuka and colleagues discovered a new class of calcium-dependent protein kinase that is activated by Ca^{2+} in conjunction with phospholipid rather than calmodulin (Takai et al., 1979a, 1979b). This enzyme, referred to as calcium/phospholipid-dependent protein kinase or protein kinase C, has been found in a wide variety of animal tissues and phyla, although the highest concentrations of the enzymes were observed in brain and peripheral nervous tissues (Kuo et al., 1980; Minakuchi et al., 1981). It was suggested that the physiological role of protein kinase C is to mediate some of the second messenger actions of calcium in cell function (Takai et al., 1979a, 1979b, 1981).

The enzyme is thought to be activated by the binding of hormones and neurotransmitters to specific receptors which stimulates the turnover of phosphatidylinositol (PI). PI breakdown results in the production of 1,2-

diacylglycerol (DAG) and inositol phosphates, which are required in addition to calcium for the activation of protein kinase C (Nishizuka & Takai, 1981; Takai et al., 1982a, 1982b; Nishizuka, 1983; Berridge & Irvine, 1984). Since the enzyme appears to require more than one activator, it was postulated that the activity of this protein kinase might be physiologically regulated by more than one messenger; in other words, that its activity in vivo may reflect the concerted effects of calcium, 1,2-DAG and phospholipids in stimulating the enzyme (Takai et al., 1981; McPhail et al., 1984; Hokin, 1985).

a) Calcium/Phospholipid-Dependent Protein Kinase

Protein kinase C has been purified from several sources including nervous and non-nervous tissues and always consists of a single subunit. However, there is little agreement concerning the size of the enzyme, the molecular weight being 83,000-100,000 in heart (Wise et al., 1982b), 68,000 in spleen (Schatzman et al., 1983) and 70,000-88,000 in brain (Kuo et al., 1980; Zwiers et al., 1980; Miyamoto et al., 1981). More recently, the enzyme has been purified from the brain, and molecular weights of 77,000 following sucrose density centrifugation and of 82,000 by electrophoresis were reported (Kikkawa et al., 1982). In addition, it was reported that the polypeptide chain is composed of two functionally different domains. One is a hydrophobic domain that may bind to membranes, and the other is a hydrophilic domain that carries the catalytically active center (Kikkawa et al., 1982). Protein kinase C can undergo autophosphorylation in the presence of Ca^{2+} and

phospholipids onto both serine and threonine residues, but the significance of such a reaction is still unknown (Kikkawa et al., 1982; Nishizuka, 1983).

Protein kinase C possesses no affinity towards calmodulin but is profoundly inhibited by many phospholipid-interacting agents such as dibucaine and some phenothiazines (relatively high concentrations) (Mori et al., 1980; Miyamoto et al., 1981; Wise et al., 1982a; Donnelly & Jensen, 1983). Two mechanisms of activation for this kinase have been postulated and quite extensively substantiated (for review see Takai et al., 1982a; Nishizuka, 1983, 1984).

The first proposed mechanism involves the activation of the kinase in presence of high Ca^{2+} concentrations, which results in an irreversibly activated enzyme due to proteolysis (Mori et al., 1980). It appears that such a proteolytic reaction takes place by cleavage of the two domains of the enzyme and produces an enzyme fragment of molecular weight of 51,000 (Kikkawa et al., 1982). At first, the physiological significance of such a proteolytic-activated mechanism was overlooked since millimolar concentrations of Ca^{2+} were required to activate the protease (Inoue et al., 1977). However, since the discovery of a new class of Ca^{2+}-dependent thiol proteases which are active at micromolar concentrations of Ca^{2+}, the significance of this mechanism has been reevaluated (see Nishizuka, 1983; Anderson et al., 1985). Furthermore, proteolytic activation of enzymes has been demonstrated for some other kinases such as cAMP-PK, cGMP-PK, MLCK and phosphorylase kinase, and in each case, partial proteolysis appears to destroy the regulatory subunit or domain of the protein kinase and thereby produces a free catalytic subunit or domain that exhibits non-regulatable protein kinase activity

(Schulman, 1982; Flockhart & Corbin, 1982; Beer et al., 1984).

The second proposed mechanism involves the activation of the kinase in presence of 1,2-DAG, which reduces the Ca^{2+} requirement to micromolar levels and does not involve proteolysis (Kishimoto et al., 1980). DAG has little stimulatory effect on its own but even at low concentrations sharply increases the affinity of the enzyme for Ca^{2+} as well as for phospholipids, which renders the enzyme fully active. Among various phospholipids tested, phosphatidylserine appears to be indispensable, but other phospholipids show positive or negative cooperativity in the activation of the enzyme. For instance, in the presence of phosphatidylethanolamine as an additional lipid component, the full enzymatic activity is obtained at lower Ca^{2+} concentrations, whereas phosphatidylcholine and other less common phospholipids (e.g. sphingo-myelin) appear to be inhibitory (Kaibuchi et al., 1981). Thus, the asymmetric distribution of various phospholipids in the lipid bilayer appears to favor the activation of protein kinase C. In the presence of 1,2-DAG, protein kinase C becomes reversibly attached to membranes where it exhibits enzymatic activity. However, the precise nature of the lipid-enzyme interaction is still unknown (Nishizuka, 1983, 1984; Hokin, 1985). In addition, it was recently demonstrated that apart from Ca^{2+}, both Sr^{2+} and Ba^{2+} were able to partially activate protein kinase C (Schatzman et al., 1983) and that besides 1,2-DAG, other membrane-accessible diacylglycerols could cause activation of protein kinase C (Mori et al., 1982; Malaisse et al., 1985). Furthermore, the activation of protein kinase C by phospholipids and Ca^{2+} appears to be a stereospecific event (Rando & Young, 1984).

Protein kinase C-activated phosphorylation can be inhibited in vitro by

calmodulin, S-100 protein and troponin C (Albert et al., 1984b). In addition, a novel Ca^{2+}-binding protein was recently isolated and characterized from bovine brain and found to be clearly distinct from calmodulin and other known calciproteins on the basis of amino acid composition analysis (Walsh et al., 1984). This 17kD Ca^{2+}-binding protein was found to be the most potent inhibitor of protein kinase C activity when compared to other calciproteins such as calcineurin, calmodulin, troponin C, S-100 protein, intestinal Ca^{2+}-binding protein and a novel 21kD brain Ca^{2+}-binding protein. Furthermore, this 17kD protein appears to inhibit protein kinase C by binding to it directly rather than by interaction with phospholipids or the brain basic protein histone III-S (McDonald & Walsh, 1985).

It was reported recently that certain tumor-promoting phorbol esters increase protein kinase C activity in vitro (Castagna et al., 1982; Kraft & Anderson, 1983). Consequently, numerous studies have utilized these agents in an attempt to understand the actions of protein kinase C in several systems, both in situ and in vitro. The most utilized phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, also referred to as TPA or PMA, can substitute for 1,2-DAG at extremely low concentrations and directly activates protein kinase C in vitro in the presence of both Ca^{2+} and phospholipid. In addition, a number of studies seem to indicate that these phorbol esters have specific cell surface receptors which are present in a wide variety of tissues and that one of these receptors might correspond to the protein kinase C-phospholipid complex (Ashendel et al., 1983; Leach et al., 1983; Niedel et al., 1983; Sharkey et al., 1984).

Protein kinase C appears to be ubiquitous in the animal kingdom. In most

nonnervous tissues the enzyme activity appears to be associated predominantly with the soluble fraction (Kikkawa et al., 1982). In the adrenal medulla, protein kinase C activities have been found to be equally distributed between the soluble and membrane fractions (Wise & Costa, 1985). In addition, a protein kinase C activity has been shown to be associated to the chromaffin granule membrane fraction (Summers & Creutz, 1985). In contrast, only one third of the total activity of the enzyme in brain is associated with the soluble fraction and the remainder with the particulate fraction. In addition, the subcellular distribution of protein kinase C in brain is similar to the distribution of cAMP-PK (Kikkawa et al., 1982). The soluble and particulate enzymes isolated from brain have been shown to be indistinguishable on the basis of their biochemical properties and substrate specificity, suggesting that the enzyme in the two fractions is the same (Kikkawa et al., 1982; Walaas et al., 1983b, 1983c).

b) Calcium/Phospholipid-Dependent Protein Kinase Substrates

Like cAMP-PK, protein kinase C appears to be multifunctional in that it exhibits a broad substrate specificity. Protein kinase C and cAMP-PK can often use the same phosphate-acceptor proteins (Nishizuka, 1980). However, in vitro analysis of the phosphorylation sites indicates that the two kinases show distinctly different catalytic properties, and each appears to recognize the respective serine or threonine residues in common substrates (Kikkawa et al., 1982). Phospholipids in the presence of Ca^{2+} have been shown to stimulate the phosphorylation of several endogenous substrates in a

wide variety of tissues, including the brain (Wrenn et al., 1980; Kuo et al., 1980; Wu et al., 1982; Walaas et al., 1983b, 1983c). One of the best characterized protein kinase C substrates corresponds to a platelet polypeptide of molecular weight of 47,000-49,000. This polypeptide, often referred to as the 40kD platelet protein, has been shown to be heavily phosphorylated following platelet stimulation by a wide variety of agents including thrombin, collagen, divalent cation ionophore A23187 and phospholipase C (Haslam et al., 1980; Kawahara et al., 1980). It has also been demonstrated that secretion of platelet granule constituents is closely associated with the phosphorylation of this cytosolic polypeptide (Imaoka et al., 1983).

The substrates for protein kinase C detected in the nervous system include myelin basic protein (Turner et al., 1982), tyrosine hydroxylase (Raese et al., 1981; Albert et al., 1984a), microtubule-associated protein MAP2 (Walaas et al., 1983c; Deshmukh et al., 1984), nicotinic acetylcholine receptor subunits (α and δ) (Huganir et al., 1983), sodium channel subunit (α) (Costa & Catterall, 1984b), GABA-modulin (Wise et al., 1984), the synaptic membrane protein B-50 (Aloyo et al., 1982, 1983) and a 87kD cytosolic protein (Wu et al., 1982; Juskevich et al., 1983), although most of the other substrates have not yet been determined.

B-50 was first detected as a phosphoprotein whose phosphorylation was decreased by ACTH in particulate synaptic fraction of brain (Zwiers et al., 1978). It is now known that this protein is a major substrate for protein kinase C in the brain (Aloyo et al., 1982, 1983). B-50 has an apparent molecular weight of 47,000-48,000 and an isoelectric point of 4.5 (Dosemeci

& Rodnight, 1982; Wu et al., 1982). B-50 is present only in nervous tissues and is concentrated in nerve terminals primarily in association with presynaptic membranes (Sorenson et al., 1981; Oestreicher et al., 1981; Kristjansson et al., 1982). Increases in the phosphorylation of B-50 have been reported to decrease lipid kinase activity (Jolles et al., 1980; Zwiers et al., 1982). In addition, it was suggested that the phosphorylated and dephosphorylated forms of B-50 modulate the activity of phosphatidylinositol 4-phosphate kinase in the brain (Van Dongen et al., 1985).

The "87kD" substrate was first detected, in synaptosomes of rat cerebral cortex as one of several phosphoproteins whose phosphorylation state was stimulated by depolarizing agents in a calcium-dependent manner (Wu et al., 1982). It was also determined that this substrate seems to be specific for protein kinase C since neither CaM-PK nor cAMP-PK could stimulate the phosphorylation of this substrate in synaptosomal cytosol. In addition, calmodulin was found to inhibit the phosphorylation of the "87kD" protein produced by protein kinase C (Wu et al., 1982; Albert et al., 1984b). The "87kD" substrate has been detected in numerous brain regions. However, within synaptosomes, it was detected in the cytosol but not in particulate fractions (Wu et al., 1982; Walaas et al., 1983b, 1983c). This substrate has a molecular weight similar to that of protein kinase C, and it was postulated that this protein could correspond to the autophosphorylated form of the kinase. However, this hypothesis has been abandoned, since it has recently been demonstrated by column chromatography that these two proteins are different kinases (Albert et al., 1984b).

G.4 Protein Phosphatases and Substrates

The only known mechanism for protein dephosphorylation involves the action of enzymes called protein phosphatases (for review see Li, 1982; Ingebritsen & Cohen, 1983b; Nestler & Greengard, 1984). Very little was known about protein phosphatases until recently when Cohen and colleagues studied the phosphatases involved in glycogen metabolism extensively (for review see Cohen, 1982).

G.4.1 Classification

Protein phosphatases have been recently classified by Cohen and collaborators (for review see Ingebritsen & Cohen, 1983b). Two main categories of protein phosphatases (PPase), PPase 1 and PPase 2, have been distinguished by their sensitivity to inhibition by specific proteins called inhibitor 1 and inhibitor 2. PPase 2 can be subdivided into 3 groups, and all four groups (PPase 1 and PPase 2 (A, B, C)) together account for virtually all the phosphatase activities described up to now (for review see Ingebritsen & Cohen, 1983a, 1983b; Ingebritsen *et al.*, 1983a, 1983b, 1983c; Pato *et al.*, 1983; Stewart *et al.*, 1983). However, some protein phosphatases do not fit into any of these categories, such as pyruvate dehydrogenase phosphatase and phosphotyrosyl-protein phosphatases (Pratt *et al.*, 1982; Foulkes *et al.*, 1983; Chernoff & Li, 1985).

Protein Phosphatase 1

PPase 1 is an important regulator of glycogen metabolism. The catalytic subunit of PPase 1 has been purified to homogeneity from skeletal muscle and has a molecular weight of 35,000 (Ingebritsen et al., 1983b). The enzyme has a high activity and a broad substrate specificity. The enzyme is important in glycogen metabolism since it dephosphorylates specifically the β subunit of phosphorylase kinase and also, to a certain extent, glycogen synthase (site 2). An inactive form of the enzyme, termed MgATP-dependent PPase, has also been isolated and consists of a 1:1 complex between the 35kD catalytic subunit and inhibitor 2. Activation of this species requires prior incubation with MgATP and glycogen synthase kinase-3 and involves the phosphorylation of inhibitor 2 (Hemmings et al., 1982; Resink et al., 1983). In addition, the presence of a glycogen-bound form of PPase 1 was very recently demonstrated in skeletal muscle (Stralfors et al., 1985). The activity of PPase 1 is regulated by three proteins, namely inhibitor 1, inhibitor 2 and glycogen synthase kinase-3, which have been characterized extensively. The enzyme is inactivated by the binding of inhibitor 2 and reactivated by phosphorylation of inhibitor 2 by cAMP-PK. Inhibitor 1 will inhibit the enzyme if it is itself phosphorylated by cAMP-PK before binding (for review see Ingebritsen & Cohen, 1983b).

Inhibitor 1 and inhibitor 2 were discovered by Huang and Glinsmann in 1975 (see Huang & Glinsmann, 1975, 1976a, 1976b). Inhibitor 1 is a heat-stable protein purified from skeletal muscle which possesses a molecular weight of 20,000 (Nimmo & Cohen, 1978a, 1978b). In addition, the complete

primary structure and active fragments of the protein have been isolated and characterized (Aitken et al., 1982; Aitken & Cohen, 1982). Inhibitor 2 is a heat-stable protein purified from skeletal muscle which has a molecular weight of 22,500 and possesses an amino acid composition distinctly different from that of inhibitor 1 (Foulkes & Cohen, 1980).

Protein Phosphatase 2

PPase 2 comprises three distinct enzymes termed PPase 2A, 2B and 2C (Pato et al., 1983; Stewart et al., 1983).

Three forms of PPase 2A have been resolved by anion exchange chromatography, and these species, which have been called $2A_0$, $2A_1$ and $2A_2$, have apparent molecular weights of 210,000, 210,000 and 150,000, respectively. The three of them have a common catalytic subunit of 38kD (Ingebritsen et al., 1983b). PPase $2A_0$ has not yet been purified, but PPase $2A_1$ is composed of three subunits of 60kD, 55kD and 38kD, whereas PPase $2A_2$ contains only the 60kD and 38kD subunits. The mechanism for regulating PPase 2A is not understood at present. However, PPase $2A_0$ is catalytically inactive, and its activity is only expressed after dissociation of the 38kD catalytic subunit. In contrast, PPase $2A_1$ and PPase $2A_2$ are spontaneously active, although dissociation of 38kD catalytic subunit results in a severalfold activation of these enzymes (Ingebritsen & Cohen, 1983b). PPase 2A has a high activity and a broad substrate specificity which is very similar to the one of PPase 1 with the exception that it dephosphorylates the α subunit of phosphorylase kinase.

PPase 2B is a Ca^{2+}-dependent enzyme whose activity is activated tenfold by calmodulin. The enzyme has been purified to homogeneity from rabbit skeletal muscle and is composed of three components termed A, A' and B with apparent molecular weights of 61,000, 58,000 and 15,000 (Stewart et al., 1982, 1983; Yang et al., 1982). However, the enzyme isolated from the brain possesses only the 61kD and 15kD subunits, although it is indistinguishable from the skeletal muscle enzyme in terms of specific activity and substrate specificity (Yang et al., 1982). In addition, this enzyme was recently isolated from platelets and found to be almost identical to the enzyme isolated in brain (Tallant & Wallace, 1985). This enzyme is also referred to as calcineurin. PPase 2B has a narrow substrate specificity and has significant activity on the inhibitor 1, the α subunit of phosphorylase kinase and the phosphorylated light chain of myosin (Ingebritsen & Cohen, 1983b) (see also next section).

PPase 2C is distinguished by its dependence on Mg^{2+}. The enzyme isolated from skeletal muscle, liver and gizzard is composed of only one subunit with molecular weight of 45,000 (Hiraga et al., 1981; Pato & Adelstein, 1980; Ingebritsen et al., 1983b). This enzyme possesses low activity and broad substrate specificity.

G.4.2 Protein Phosphatases in Nervous Tissues

Less is known about protein phosphatases in nervous tissues than in non-nervous tissues. Only recently, some systematic investigations of protein phosphatases in brain have been undertaken and have shown that brain con-

tains multiple forms of protein phosphatases (Parsadanian et al., 1982; Ingebritsen et al., 1983c; Foulkes et al., 1983). Cohen and collaborators have found that each one of the four categories of PPase described in skeletal muscle are also present in brain (Ingebritsen et al., 1983c). The first detailed study conducted by Maeno and Greengard (1972) showed that most cortical phosphatase activity was localized in synaptosomes. This activity was shown to be present in all primary subfractions of brain, with the highest specific activity being present in the cytosol and little or no phosphatase activity in brain membrane or synaptic junctional fractions (Maeno & Greengard, 1972; Thérien & Mushynski, 1979).

By analogy with nonnervous tissues, brain protein phosphatase activities appear to be regulated by second messengers indirectly through the regulation of phosphatase inhibitor proteins. Brain contains phosphatase inhibitor 1 and cAMP, which, through the phosphorylation and activation of inhibitor 1, presumably decreases the activity of PPase 1 in brain (Parsadanian et al., 1982). In addition, recent studies have indicated that cGMP and cAMP may also regulate PPase activity in brain indirectly through the regulation of the phosphorylated form of phosphatase inhibitors G-substrate and DARPP-32, respectively (Schlichter et al., 1980; Walaas et al., 1983a; Ouimet et al., 1984).

Protein phosphatases that dephosphorylate G-substrate, and endogenous substrate proteins for cGMP-PK, appear to be distinct from calcineurin (PPase 2B) and PPase 1 but very similar to phosphatases 2A and 2C described for skeletal muscle (Nestler & Greengard, 1984).

In addition to the typical protein phosphatase activities described in

skeletal muscles, some other protein phosphatase activities have been detected in various preparations of tissues that dephosphorylate endogenous substrate proteins. Protein phosphatase activity has been detected in preparations of myelin basic proteins (Miyamoto & Kakiuchi, 1975; McNamara & Appel, 1977; Wu et al., 1980), rhodopsin (Goridis & Weller, 1976), nicotinic acetylcholine receptor (Gordon et al., 1979) and microtubule-associated protein MAP2 (Coughlin et al., 1980; Prus & Wallin, 1983).

Calcineurin

Calcineurin, or PPase 2B, is the best characterized protein phosphatase in the brain. Calcineurin was first identified as a major soluble calmodulin-binding protein in brain (Klee et al., 1979; Wallace et al., 1980) and, more recently, has been shown to be a calcium/calmodulin-dependent protein phosphatase (Stewart et al., 1982; Yang et al., 1982). The brain enzyme is almost identical to the platelet enzyme (Stewart et al., 1983; Tallant & Wallace, 1985). The enzyme isolated from the brain has an apparent molecular weight of 80,000 and is composed of two subunits of 61kD and 15kD which are present in equimolar ratios. The 60kD subunit is a calmodulin-binding protein which appears to act as the catalytically active center, and the 15kD subunit appears to be a Ca^{2+}-binding protein that may be similar to calmodulin (Stewart et al., 1982; Yang et al., 1982; Manalan & Klee, 1983; Tonks & Cohen, 1983). In fact, the complete primary structure of the 15kD subunit (B subunit) has been recently elucidated, and it appears that this Ca^{2+}-binding protein belongs to the same family as calmodulin and troponin C but is rela-

tively different (Aitken et al., 1984).

Calcineurin appears to be ubiquitous; it has been detected by radioimmunoassay in a wide variety of tissues and phyla of the animal kingdom (Wallace et al., 1980; Tallant & Cheung, 1982). The brain contains much higher levels of calcineurin than do nonnervous tissues such as liver or skeletal muscle (Wallace et al., 1980). The highest concentrations of the enzyme were found in the cerebral cortex and particularly in the striatum, and its subcellular localization was determined to be mainly within neurons, primarily in association with postsynaptic regions of cell bodies and dendrites (Wood et al., 1980). In addition, it has been reported that calcineurin levels increase in brain during synaptogenesis and thus, suggests that this enzyme might be implicated in some synaptic functions (Tallant & Cheung, 1983).

Calcineurin activity can be regulated by calcium via two mechanisms. In the absence of calmodulin, calcineurin is activated by calcium via the action of the 15kD subunit. The interaction of Ca^{2+} with the 15kD subunit appears to be essential for substrate binding onto the 61kD catalytic subunit and shows no activity in presence of Ca^{2+} chelators (Yang et al., 1982; Tonks & Cohen, 1983). The 15kD subunit possesses four Ca^{2+}-binding sites with affinities in the micromolar range but is not inhibited by calmodulin antagonists. Interaction of calmodulin with the 61kD subunit, which only occurs in the presence of Ca^{2+}, results in a 8- to 10-fold increase of the enzyme activity without a significant effect in the K_m for substrates (Yang et al., 1982; Stewart et al., 1983; King & Huang, 1984). The calmodulin-stimulated activity of calcineurin is inhibited by the phenothiazine trifluopera-

zine. In addition, since calcineurin is activated equally by Ca^{2+}, Ba^{2+} and Sr^{2+} in the presence of calmodulin, it was suggested that these divalent cations may act directly on the phosphatase rather than on calmodulin itself (King & Huang, 1983). Furthermore, it was demonstrated that Mg^{2+} or other divalent cations such as Ni^{2+}, Mn^{2+} and Co^{2+} can further activate calcineurin in the presence of Ca^{2+} and calmodulin (King & Huang, 1983; Gupta et al., 1984; Li, 1984; Pallen & Wang, 1984). Similarly to other calmodulin-stimulated enzymes, calcineurin is activated by limited proteolysis, which results in a 45kD fragment that is then calmodulin independent (Manalan & Klee, 1983).

It has been suggested that calcineurin exhibits a narrow substrate specificity in contrast to other phosphatases, dephosphorylating specifically substrates such as myosin light chain, phosphatase inhibitor 1, phosphorylase kinase and the R subunits of cAMP-PK II (Stewart et al., 1982; Blumenthal & Krebs, 1983; Ingebritsen & Cohen, 1983a; Manalan & Klee, 1983). However, several additional brain substrates, including G-substrate, DARPP-32, synapsin I, protein K-F, microtubule-associated protein MAP2, tau factor and tubulin, have recently been found to be dephosphorylated by calcineurin (King et al., 1983, 1984; Goto et al., 1985).

Several lines of evidence suggest that calcineurin might regulate second messenger actions by interacting with them at several steps in the cascade of events in which they are involved in producing their effects. For instance, in the case of calmodulin and cAMP, calcineurin could produce its effect at different levels such as in the dephosphorylation of proteins which are substrates for CaM-PK or cAMP-PK, in binding and sequestering cal-

modulin, and in controlling cytoplasmic calcium and cAMP levels (for review see Nestler & Greengard, 1984). Calcineurin was demonstrated to inhibit the calmodulin-mediated activities of adenylate cyclase, phosphodiesterase and Ca^{2+}-ATPase, although without affecting their basal activities (Wallace et al., 1980). Two mechanisms for the calcineurin inhibition of these enzymes are possible, being either sequestration of calmodulin or dephosphorylation and consequently, inactivation of the enzymes (Cheung, 1980; Wallace et al., 1980; Cheung & Storm, 1982). In addition, it was shown that calcineurin can bind Ca^{2+} with a 100-fold higher affinity than for calmodulin especially in the second phase following Ca^{2+} influx into the cells. Therefore, by such a mechanism, calcineurin could act as an inhibitor of calcium signals during nerve transmission by lowering intracellular calcium concentrations (Klee & Haiech, 1980).

Pyruvate Dehydrogenase Phosphatase

Pyruvate dehydrogenase phosphatase appears to be an intramitochondrial enzyme for which the only known substrate protein is pyruvate dehydrogenase (Krebs & Beavo, 1979; Reed, 1981; Randle, 1981; Li, 1982). The multienzyme complex of brain mitochondria contains the enzyme pyruvate dehydrogenase which possesses a specific pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase that regulate the activity of the enzyme. Phosphorylation of the α subunit (42kD) of pyruvate dehydrogenase by pyruvate dehydrogenase kinase inactivates the complex. Activation occurs by dephosphorylation (Randle, 1976; Li, 1982; Robinson & Dunkley, 1983b) and is due to sti-

mulation of pyruvate dehydrogenase phosphatase rather than inhibition of pyruvate dehydrogenase kinase (Popp et al., 1980; Reed, 1981).

Pyruvate dehydrogenase phosphatase is composed of two subunits of molecular weights of 97,000 and 50,000 (Reed & Pettit, 1981; Pratt et al., 1982). The enzyme is a Mg^{2+}-dependent phosphatase whose activity is greatly stimulated by Ca^{2+} (Weller, 1979; Sheu et al., 1983). In addition, a small heat-stable peptide of about 1.5kD appears to be required for stimulation of the enzyme (Larner et al., 1979). Furthermore, pyruvate dehydrogenase phosphatase was shown to be distinct from calcineurin since the enzyme possesses only a single Ca^{2+}-binding site and does not require calmodulin for maximal interaction (Pratt et al., 1982).

G.5 Interactions among Protein Phosphorylation Systems

Many individual molecular pathways involving protein phosphorylation have been elucidated over the past several years and appear to mediate diverse biological responses to a wide variety of extracellular and intracellular messengers in various tissues. Since these pathways play important roles in cell function, it is not surprising that many interactions exist both within individual pathways and among different pathways. The existence of numerous individual pathways and of numerous interactions between these pathways supports the view that protein phosphorylation is a final common pathway of great importance in cell regulation (Greengard, 1978; Nestler & Greengard, 1983, 1984).

G.5.1 Interactions within a Single Protein Phosphorylation System

Many physiological stimuli, or first messengers, appear to produce diverse biological effects in target tissues by regulating the state of phosphorylation of specific substrate proteins in those tissues. Such regulation appears to be achieved primarily through the activation of protein kinases. Some first messengers activate protein kinases directly, such as in the case of epidermal growth factor and insulin, by binding to plasma membrane receptors for these factors (Cohen et al., 1982; Roth & Cassell, 1983; Shia & Pilch, 1983). In contrast, many first messengers activate protein kinases indirectly via second messengers such as cAMP, cGMP and Ca^{2+}. Numerous hormones, neurotransmitters and other regulatory agents activate cAMP-PK, cGMP-PK or calcium-dependent protein kinases (CaM-PK and protein kinase C) indirectly by increasing the intracellular levels of cAMP, of cGMP or of Ca^{2+} (for review see Nestler & Greengard, 1984). Still other first messengers appear to produce biological responses by regulating the total amount of specific protein kinases such as in the case of interferon and some oncogenic viruses (Sen, 1982; Hunter & Sefton, 1982).

The next step in the molecular pathway through which first messengers produce biological responses is the phosphorylation of specific substrate proteins via these protein kinases. These substrates could represent the immediate physiological effectors, so that their phosphorylation results directly in the biological response, or they could be several steps removed from the effectors, so that their phosphorylation leads to the biological response through other mediating steps. Some first messengers activate

protein kinases that phosphorylate only one substrate, for instance MLCK activation following membrane depolarization which results in the phosphorylation of only one protein, namely myosin light chain (Adelstein et al., 1982; Demaille & Pechère, 1983). In contrast, other first messengers activate protein kinases that phosphorylate several substrates. The phosphorylation of numerous substrates by a single protein kinase represents one mechanism by which many cellular processes can be regulated in a coordinated fashion by a given physiological stimulus.

Some first messengers stimulate the phosphorylation not only of these substrates involved in the generation of a particular biological response per se, but also of other substrates which are involved in negative and positive feedback pathways which regulate the biological response, in the mobilization of cellular energy which sustains the biological response, and finally, in the control of protein synthesis which is involved in long-term regulation of cellular functions. For example, such a mechanism has been demonstrated for cAMP-PK activity in the intermediary metabolism of different types of cells and also in the secretory process of some exocrine tissues (Beavo & Mumby, 1982; Cohen, 1982; Freedman & Jamieson, 1982a; Jahn & Söling, 1983). Thus, the phosphorylation of numerous substrates by a single protein kinase represents one level of interaction that occurs within individual protein phosphorylation systems.

Protein phosphatases are also regulated by intracellular messengers in a variety of tissues. In some cases, second messengers regulate protein phosphatases directly. For example, calcium and calmodulin activate calcineurin, or calcium alone stimulates pyruvate dehydrogenase phosphatase (Randle,

1981; Reed, 1981; Stewart et al., 1982). It is also the case for cAMP which activates a cAMP-PK which in turn stimulates the phosphorylation of a protein phosphatase that dephosphorylates aminoacyl-tRNA synthase in liver and smooth muscle (Berg, 1978). In other cases, second messengers regulate protein phosphatases indirectly via the phosphorylation of phosphatase inhibitors such as inhibitor 1 and DARPP-32, through the activation of cAMP-PK, and G-substrate, through the activation of cGMP-PK (for review see Cohen, 1982; Nestler & Greengard, 1984). Regulation of protein phosphatases may play a negative or a positive feedback role. In the case of negative feedback, a first messenger, acting through the same second messenger, rapidly activates a protein kinase and more slowly activates a protein phosphatase (see Fig. 7A). In contrast, in the case of positive feedback, a first messenger activates a protein kinase that phosphorylates effector substrate proteins as well as phosphatase inhibitors (see Fig. 7B).

Interactions within individual protein phosphorylation systems are also manifested at the level of the total amount of substrate proteins. Thus, first and second messengers, acting through protein kinases, can regulate the total amount of substrate proteins such as in the cases of synapsin I and tyrosine hydroxylase (Nestler et al., 1982; Costa & Guidotti, 1978).

G.5.2 Interactions between Different Protein Phosphorylation Systems

Second messengers such as cAMP, cGMP and calcium appear to regulate many of the same cellular processes. These second messengers can interact with each other at different steps in their molecular pathways and can produce

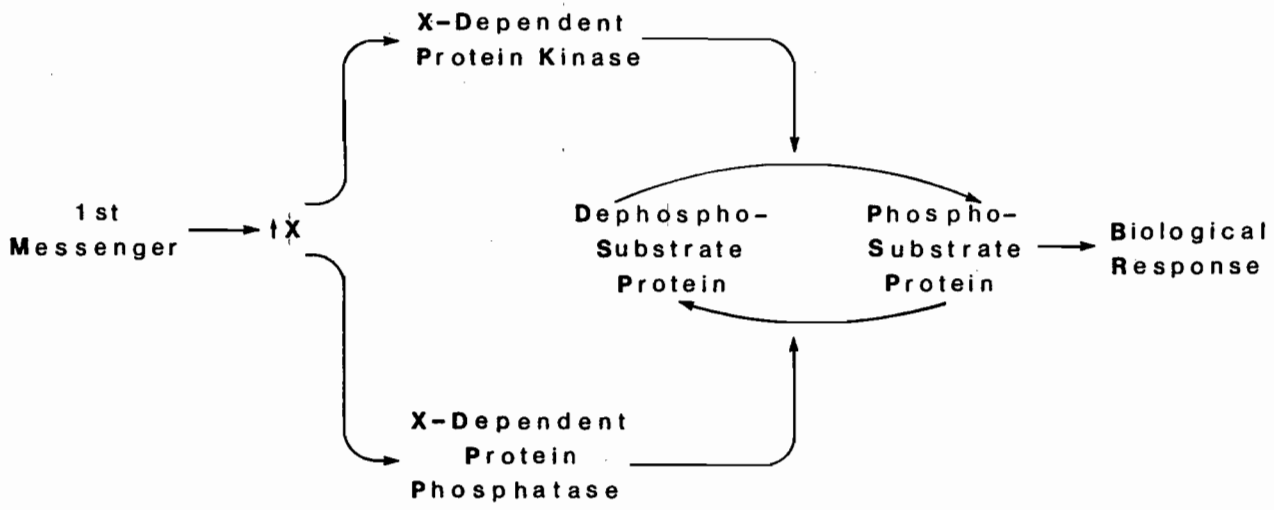
Fig. 7 Schematic representation of negative and positive feedback roles for protein phosphatases.

A) Negative feedback: A first messenger would activate both a protein kinase and a protein phosphatase, but at different rates. In this case, the first messenger would produce the biological response through the elevation of intracellular levels of a second messenger, the rapid activation of a protein kinase and the phosphorylation of a specific substrate protein. On the other hand, the first messenger would also initiate a negative feedback pathway that could antagonize the same biological response. This would occur via the slow-activation of a protein phosphatase and the dephosphorylation of the substrate protein.

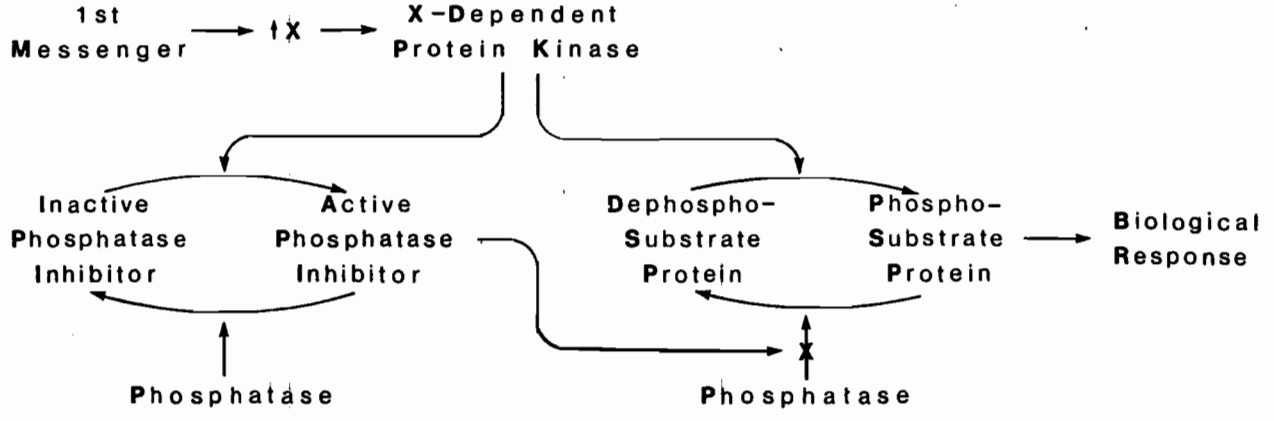
B) Positive feedback: A first messenger would activate a protein kinase as well as inactivate a protein phosphatase. For instance, the first messenger would produce the biological response through the elevation of intracellular levels of a second messenger which would then activate a specific protein kinase. In turn, this protein kinase would act through two different pathways: one which would phosphorylate a substrate protein and a second one which would phosphorylate a phosphatase inhibitor that could then inhibit a specific phosphatase. Therefore, both reactions would favor the phosphorylation of the substrate protein which is responsible for the biological response.

X: Ca^{2+}, cAMP or cGMP.

A) **NEGATIVE FEEDBACK**



B) **POSITIVE FEEDBACK**



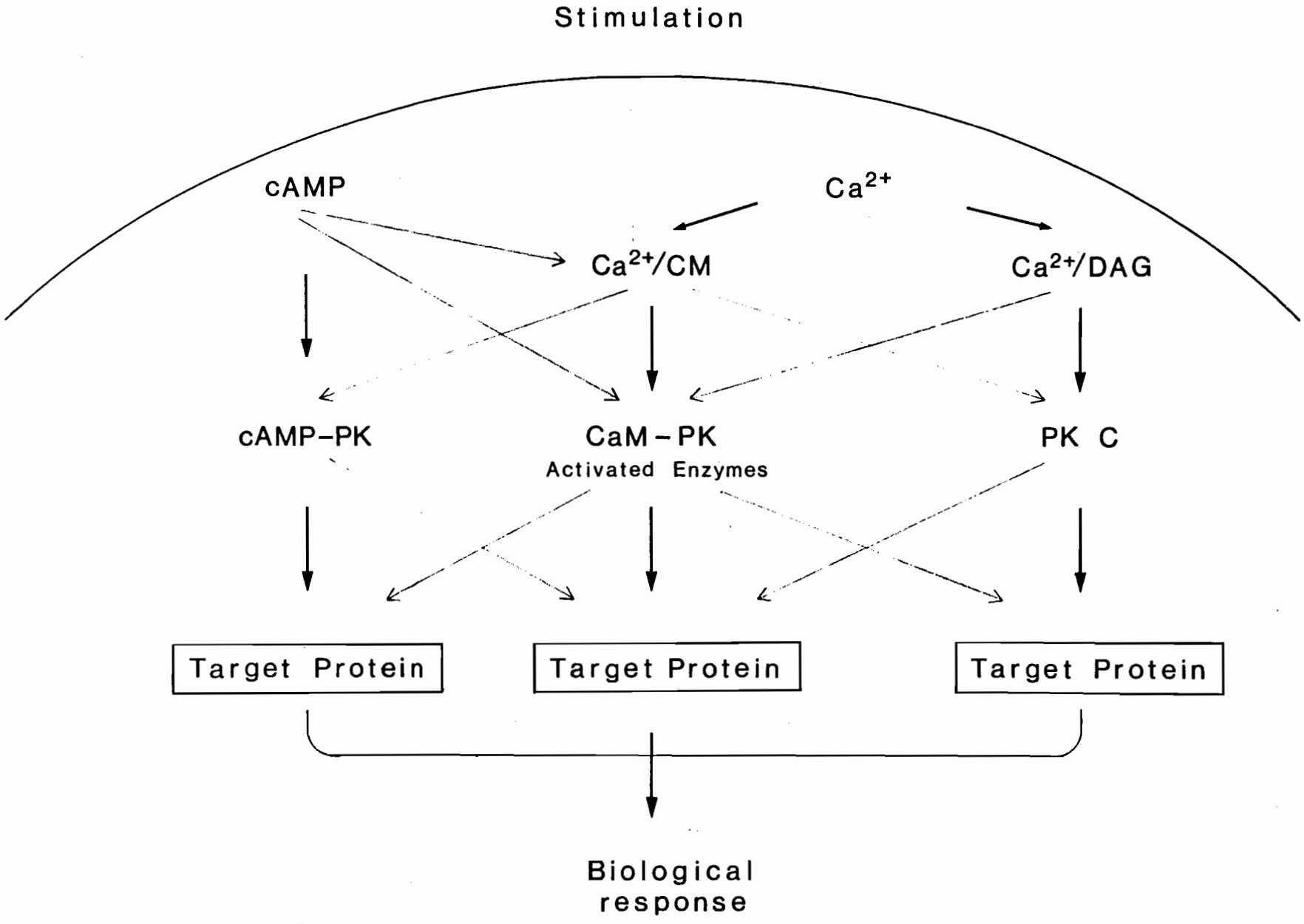
convergence or divergence in their physiological actions.

Interactions between different pathways can occur at the level of the regulation of the intracellular concentration of second messengers. In other words, one of the second messengers regulates the intracellular concentration of another messenger (Berridge, 1975; Rasmussen, 1981). For example, cAMP and first messengers increasing cAMP levels can regulate through cAMP-PK's the intracellular Ca^{2+} concentration such as in molluscan neurons by decreasing K^+ channel conductance which results in an increased Ca^{2+} entry during action potentials (Castellucci et al., 1980, 1982), in heart muscle by increasing the voltage-dependent Ca^{2+} channel conductance which results in increased intracellular Ca^{2+} levels (Osterrieder et al., 1982), in heart and skeletal muscles by stimulating the activity of Ca^{2+}/Mg^{2+}-ATPase (Demaille & Pechère, 1983) and in sarcoplasmic reticulum by activation of Ca^{2+}/Mg^{2+}-ATPase through the phosphorylation of phospholamban (for review see Demaille & Pechère, 1983; Loutzenhiser et al., 1985). In addition, cAMP may regulate cGMP levels through the phosphorylation and activation by cAMP-PK of guanylate cyclase (Zwiller et al., 1982). Conversely, cGMP may regulate cAMP levels by activating cGMP-dependent phosphodiesterase (Lincoln & Corbin, 1983). Finally, calcium can regulate cAMP and cGMP levels via Ca^{2+}/calmodulin-dependent enzymes such as adenylate cyclase, guanylate cyclase and phosphodiesterase (Cheung, 1980, 1982a, 1982b; Fukunaga et al., 1984a).

Interactions can also take place at the level of different protein kinases including cAMP-PK, cGMP-PK, CaM-PK and protein kinase C (for review see Nestler & Greengard, 1984; Nairn et al., 1985; Pallen et al., 1985) (see Fig. 8). Important physiological interactions between cAMP and Ca^{2+} have

Fig. 8 Schematic diagram of the interactions between different protein phosphorylation pathways. Stimulation of the cell by either neurotransmitters, hormones or ions can result in an increase of one or several second messengers (e.g. cAMP and Ca^{2+}). At a first level, a certain second messenger may regulate the level of another second messenger. At a second level, a certain second messenger can regulate by itself the activity of a protein kinase of another pathway or via the interaction of a kinase of one pathway on another pathway. Finally, the interaction can occur at the level of the same target protein.

CM, calmodulin; DAG, 1,2-diacylglycerol; cAMP-PK, cAMP-dependent protein kinase; CaM-PK, Ca^{2+}/calmodulin-dependent protein kinase; PK C, protein kinase C.


Stimulation
cAMP
Ca2+
Ca2+/CM
Ca2+/DAG
cAMP-PK
CaM-PK
Activated Enzymes
PK C
Target Protein
Target Protein
Target Protein
Biological response

been shown to occur at the level of protein kinase at which cAMP-PK phosphorylates and thereby regulates the activity of Ca^{2+}-dependent protein kinases. For example, cAMP-PK phosphorylates and activates phosphorylase kinase and also other enzymes involved in triggering glycogen breakdown (for review see Krebs & Beavo, 1979; Cohen, 1982; Schulman, 1982; Camici et al., 1984). Furthermore, cAMP-PK phosphorylates and inhibits myosin light chain kinase which results in an inhibition of the development of Ca^{2+}-activated tension in smooth muscle and non-muscle cells (for review see Adelstein, 1982; Adelstein et al., 1982; Demaille & Pechère, 1983; Kamm & Stull, 1985). Three types of kinases, namely cAMP-PK, CaM-PK and protein kinase C, have been implicated in the regulation of monoamine biosynthesis. This biosynthetic pathway appears to be regulated by the three above-mentioned kinases at the level of tyrosine hydroxylase and tryptophan hydroxylase (Yamauchi & Fujisawa, 1980; Albert et al., 1984a; Fujisawa et al., 1984; Zigmond, 1985). In addition, it was shown that CaM-PK may regulate the activity of protein kinase C in the brain through the dephosphorylation of the brain "87kD" protein (Albert et al., 1984b) and also through the phosphorylation of diphosphoinositide (DPI) (Hayashi & Amakawa, 1985). Furthermore, it was demonstrated very recently that cAMP-PK might play an important role in the regulation of protein kinase C, in growth and proliferation of tumor cells (Anderson et al., 1985) and in the generation of calcium action potentials of aplysia bag cell neurons (DeRiemer et al., 1985).

Interactions between the second messengers cAMP, cGMP and calcium can also occur at the level of protein phosphatases since the latter have been found recently to be regulated by these second messengers. Thus, the physio-

logical effects of one second messenger, achieved through the activation of a protein kinase, could be enhanced by one or more other second messengers through the inhibition of protein phosphatase(s) (Kuo et al., 1985). Such a mechanism has been proposed recently for the well-known substrate, synapsin I (Nestler & Greengard, 1984; Nairn et al., 1985; Pallen et al., 1985). In this case, Ca^{2+} could activate CaM-PK and protein kinase C, and cAMP could activate cAMP-PK, which, in both instances, would result in the phosphorylation of the substrate. On the other hand, cAMP could modulate the phosphorylation of the phosphatase inhibitor DARPP-32 through the activation of cAMP-PK, and cGMP could modulate the phosphorylation of the phosphatase inhibitor G-substrate through the activation of cGMP-PK, which, in both cases, would result in the inhibition of protein phosphatase.

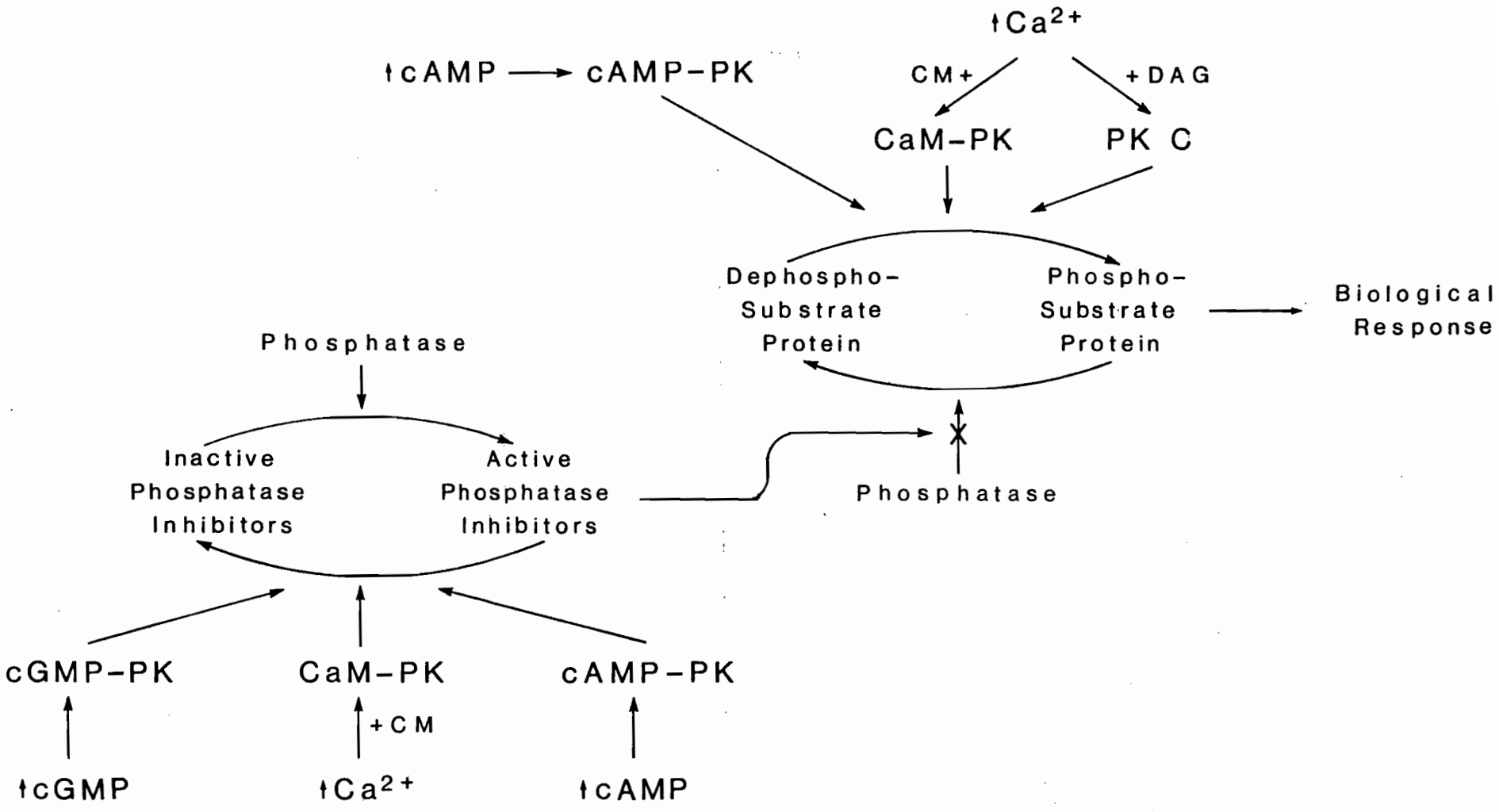
Finally, the interactions can take place at the level of the same substrate protein. Multisite phosphorylation of substrate proteins, that is, the phosphorylation of more than one amino acid residue in the same protein, now appears to be quite frequent rather than the exception. Numerous substrates are phosphorylated on more than one site by the same protein kinase such as MLCK by cAMP-PK, G-substrate by cGMP-PK and synapsin I by CaM-PK II, for example (Conti & Adelstein, 1981; Aitken et al., 1981; Huttner et al., 1981). However, the precise physiological significance of this phenomenon remains obscure. Substrates that are phosphorylated by more than one protein kinase can be phosphorylated either on the same site or in different sites by those kinases. Obviously, protein kinases that phosphorylate the same site of a substrate produce the same functional change in the protein. In contrast, protein kinases that phosphorylate different sites of a substrate

can produce the same or different functional changes in that protein. Therefore, the phosphorylation of these different sites could result in equal, additive, synergistic or antagonistic effects on the final response produced by the phosphoprotein in the system considered. The best characterized substrate that undergoes multisite phosphorylation by more than one protein kinase is glycogen synthase. This enzyme contains seven serine residues that are differentially phosphorylated by at least five different protein kinases. Phosphorylation of any one of these sites is associated with inhibition of the enzyme, and phosphorylation of more than one site is associated with additive inhibition of the enzyme (Cohen, 1982; Picton et al., 1982; Woodgett & Cohen, 1984). Numerous other substrates, in addition to glycogen synthase, have been shown to undergo multisite phosphorylation by more than one protein kinases, although in most cases the precise functional role of each phosphorylation reaction has not been elucidated. These other substrates include synapsin I, tyrosine hydroxylase, microtubule-associated protein MAP2, tau factor, myosin light chain, phospholamban, ribosomal protein S6 and many other proteins (for review see Nestler & Greengard, 1984; Nairn et al., 1985; see also Goldenring et al., 1985).

Therefore, the interactions between the different protein phosphorylation systems appear to be regulated in a rather complex manner since these interactions can take place at several levels of the molecular pathways. However, it appears that these different phosphorylation systems interact in a coordinate fashion which is important in initiating, sustaining and terminating the biological response desired (see Fig. 9).

Fig. 9 Interactions between different protein kinases and protein phosphatases. Different first messengers could potentially result in the intracellular elevation of the levels of different second messengers. In turn, these second messengers could act coordinately to favor the phosphorylation of a specific substrate protein either directly by increasing the phosphorylation of the substrate protein via protein kinases or indirectly by inhibiting a specific protein phosphatase.

CM, calmodulin; DAG, 1,2-diacylglycerol; cAMP-PK, cAMP-dependent protein kinase; CaM-PK, Ca^{2+}/calmodulin-dependent protein kinase; PK C, protein kinase C; cGMP-PK, cGMP-dependent protein kinase.


↑Ca2+
↑cAMP
cAMP-PK
CM+
+DAG
CaM-PK
PK C
Dephospho-
Substrate
Protein
Phospho-
Substrate
Protein
Biological
Response
Phosphatase
Inactive
Phosphatase
Inhibitors
Active
Phosphatase
Inhibitors
Phosphatase
cGMP-PK
CaM-PK
cAMP-PK
+CM
↑cGMP
↑Ca2+
↑cAMP

G.6 Phosphorylation Process of the Adrenal Chromaffin Cells and of other Secretory Cell Systems

G.6.1 Protein Phosphorylation and Stimulus-Secretion Coupling

Protein phosphorylation has been implicated in the stimulus-secretion coupling of several secretory cells including the adrenal chromaffin cells. This implication is based on the fact that stimulation of secretion also activates the phosphorylation of specific proteins. In some cases, the time courses of protein phosphorylation and of secretion appear to be correlated whereas in others they appear to be dissociated. However, the evidence currently linking protein phosphorylation to secretion is circumstantial. It is based exclusively on four sets of observations: i) the requirements for secretion are intracellular Ca^{2+}, calmodulin and Mg-ATP, which are identical to the components required for Ca^{2+}/calmodulin-stimulated phosphorylation; ii) in several secretory cells where a stimulus evokes secretion, there is a concomitant increase in protein phosphorylation; iii) in several of these systems, calmodulin antagonists have been found to inhibit both secretion and protein phosphorylation; iv) in several secretory systems where contractile proteins have been implicated in secretion, these proteins have been shown to be phosphorylated.

Therefore, according to the above-mentioned observations, protein phosphorylation has been implicated in the secretory process of adrenal chromaffin cells as well as in other secretory systems such as platelets (Haslam & Lynham, 1977; Nishikawa et al., 1980; Wallace & Bensusan, 1980; Takai et

al., 1981; Feinstein & Hadjian, 1982; Siess et al., 1983), neutrophils (Andrews & Babior, 1983), mast cells (Sieghart et al., 1978, 1981; Theoharides et al., 1980; Hempstead et al., 1981; Winslow & Austen, 1982; Katakami et al., 1984), pancreatic islets (Suzuki et al., 1981; Colca et al., 1983a, 1983b, 1985), insulinoma cells (Schubart et al., 1980a, 1980b), exocrine pancreas (Freedman & Jamieson, 1982a, 1982b, 1982c; Roberts & Butcher, 1983), lacrimal glands (Jahn & Söling, 1981a; Dartt et al., 1982), parotid gland (Jahn et al., 1980; Baum et al., 1981; Jahn & Söling, 1981b; Dowd et al., 1981), submandibular gland (Quissell et al., 1983) and GH3 and GH4C1 pituitary cells (Drust & Martin, 1982, 1984; Sobel & Tashjian, 1983; Fearon & Tashjian, 1985).

G.6.2 Protein Phosphorylation Process of the Adrenal Chromaffin Cells

Protein phosphorylation in the adrenal medulla and in isolated chromaffin cells has been examined by several investigators. However, most of these studies have focused either on a specific polypeptide, namely tyrosine hydroxylase or phosphoproteins related to specific subcellular fractions. Furthermore, the enzymes involved in protein phosphorylation and dephosphorylation, namely protein kinases and protein phosphatases, have not been isolated and characterized in chromaffin cells yet. Only recently has a Ca^{2+}/phospholipid-dependent protein kinase activity been detected and characterized in soluble and membrane fractions of the bovine adrenal medulla (Wise & Costa, 1985).

Two groups of investigators have reported that stimulation of bovine chromaffin cells with different secretagogues resulted in a Ca^{2+}-dependent increase of phosphorylation of two specific polypeptides, namely the ones with molecular masses of 56-60kD and 95-99kD, and that this phenomenon occurred prior to the onset of catecholamine secretion (Holz et al., 1980; Amy & Kirshner, 1981). Recently, it has been shown that the phorbol ester PMA produces an increase in the phosphorylation of a 56kD polypeptide and a concomitant increase in catecholamine secretion (Pocotte et al., 1985). It is believed that both effects are mediated via the activation of protein kinase C. Furthermore, in chromaffin cells permeabilized by exposure to electric fields, the so-called "leaky" cells, there is an increased phosphorylation of a 59kD polypeptide which closely parallels catecholamine release with respect to time, Ca^{2+} and Mg-ATP concentrations (Baker et al., 1982). On the other hand, the phosphorylation of the 59kD polypeptide could also be stimulated by cAMP in the absence of Ca^{2+}, a condition which does not result in catecholamine release from "leaky" cells (Baker et al., 1982). The 56-60kD polypeptide was identified as the phosphorylated subunit of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (Haycock et al., 1982a). Consequently, it has been suggested that this phosphoprotein is probably not directly implicated in the exocytotic process. At the present time, the function and subcellular localization of the 95-99kD phosphoprotein are unknown.

Other phosphoproteins have been identified or detected in subcellular fractions of chromaffin cells (Burgoyne & Geisow, 1982; Konings & De Potter, 1983a, 1983b; Treiman et al., 1983; Haycock et al., 1984). In the presence

of Ca^{2+}, cholinergic stimulation of chromaffin cells has been shown to increase the phosphorylation of the two well-known synaptic vesicle-associated proteins, protein IIIa (74kD) and protein IIIb (58kD) (Haycock et al., 1984). However, the subcellular localization of these proteins in chromaffin cells has not been determined yet. Konings and De Potter have used the fusion of isolated chromaffin granules with plasma membrane as a model to study exocytosis and protein phosphorylation (Konings & De Potter, 1981a, 1981b, 1982, 1983a, 1983b). Consequently, upon co-incubation of these fractions, it was found that a polypeptide of apparent molecular mass of 45.5kD was preferentially phosphorylated under these conditions. On the other hand, in the plasma membrane fraction, two polypeptides of apparent molecular masses of 20kD and 60kD have been shown to be phosphorylated preferentially. In addition, it was shown that only the phosphorylation of the plasma membrane polypeptides appeared to be Ca^{2+} dependent (Konings & De Potter, 1983a). Furthermore, it was determined that when a non-hydrolyzable ATP-analogue was used in this system, a decrease in protein phosphorylation and an inhibition of the plasma membrane-induced chromaffin granule-content release occurred (Konings & De Potter, 1983a, 1983b). Similar results have been observed in chemically skinned chromaffin cells (Brooks et al., 1984). Very recently, it was shown that a chromaffin granule membrane-binding protein of 37kD, termed chromobindin 9, was phosphorylated by protein kinase C since Ca^{2+}/phospholipids and TPA were demonstrated to activate the phosphorylation of this polypeptide in chromobindin preparations (Summers & Creutz, 1985).

H. CONTRACTILE PROTEINS AND THE CYTOSKELETON

H.1 Contractile Proteins

The contractile proteins are a group of proteins which were first identified in skeletal muscles as components involved in the mechanism of excitation-contraction coupling. This group of components includes the well-characterized proteins actin, myosin, α-actinin, tropomyosin and the troponin complex (troponin T, troponin I and troponin C) and also some other components such as titin, nebulin and C protein. Contractile proteins have been isolated, characterized and their functional roles extensively investigated in a wide variety of tissues including skeletal muscles, smooth muscles and nonmuscle cells. The phylogenetical evolution of these proteins has been extensively documented (for review see Omodeo et al., 1982).

In skeletal and smooth muscles, the contractile proteins possess an obvious specific role, which is to provide the essential structural and biochemical machinery for excitation-contraction coupling (for review see Schaub & Watterson, 1981; Squire, 1983; Garfield & Somlyo, 1985; Murakami & Uchida, 1985; Wang, 1985). In contrast, the exact functional roles of some of these proteins are not completely understood in certain nonmuscle cell systems. However, they have been shown to mediate some cellular and subcellular processes requiring movement such as cell locomotion, cell shape changes, capping of ligand-induced patches, endocytosis, exocytosis and intracellular organelle transport (for review see Trifaró, 1978; Buckley, 1983; Albertini & Herman, 1984; Lewis, 1984; Schliwa, 1984).

H.1.1 Actin

Actin is one of the most abundant unitary protein in eukaryotic cells. Actins isolated from different sources including skeletal muscles, smooth muscles and nonmuscle cells have similar physicochemical properties. Actin has a subunit molecular weight of 42,000 and can exist in two structural states, namely G-actin, the globular monomer, and F-actin, a filamentous polymer. In skeletal muscles, F-actin is the double helical polymeric structure which makes up the thin filaments (see Fig. 10). These actin filaments have a diameter of 4-6 nm and possess a definite polarity as indicated by heavy meromyosin arrowhead formation. G-actin undergoes self-assembly into filamentous F-actin at physiological ionic strengths. The mechanisms underlying actin self-assembly have been studied extensively (for review see Hill & Kirschner, 1982, 1983; also Carlier et al., 1984, 1985; Miki & Wahl, 1985; Pantaloni et al., 1984, 1985).

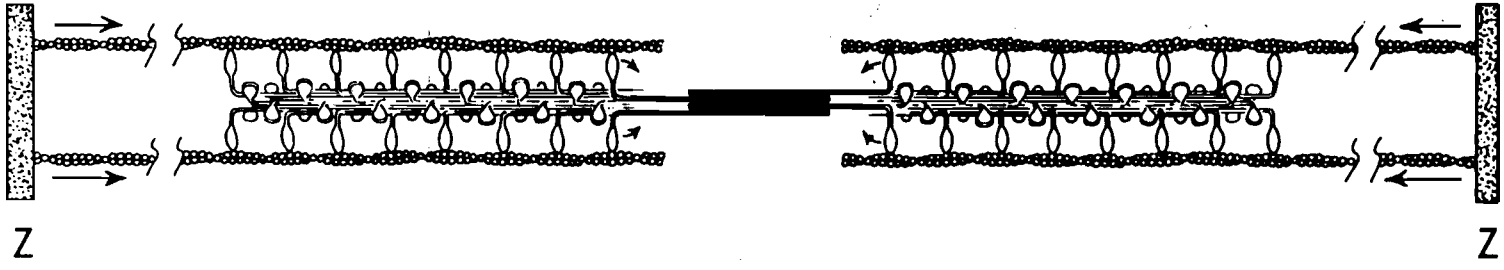
The amino acid sequence of actins from phylogenetically diverse sources is highly conserved and contains an unusual amino acid, namely N^{τ}-methylhistidine (Vandekerckhove & Weber, 1978a, 1978b, 1978c). By studying actin from different vertebrate sources, it was determined that at least six different genes exist, which following translation, result in the expression of six different actin species. There exist four muscle actins (α family) consisting of two sarcomeric actins, one specific for skeletal muscle and one for cardiac muscle, and two smooth muscle actins, one found mainly in visceral smooth muscle and the other related to vascular smooth muscle. In the case of nonmuscle cells, they contain two generally occurring cytoplasmic actins

Fig. 10 Schematic representation of a sarcomere from striated muscle. Bipolar myosin filaments (head-to-tail polarity) interact with actin filaments (F-actin) which results in the movement (contraction) of the sarcomere.

Z: Z-line structures of the striated muscle.

[Taken from Spudich, 1974]

STRIATED MUSCLE
CONTRACTILE APPARATUS



termed β- and γ-actin (Vandekerckhove & Weber, 1979). β-Actin and γ-actin are very similar to each other (only 4 amino acid substitutions) and closely related to the actin found in lower eukaryotes (Vandekerckhove & Weber, 1980).

In skeletal muscles, the thin actin filaments are attached to the sarcomeric Z-line structure via the anchorage protein α-actinin. In smooth muscles, the actin filaments are inserted into α-actinin-containing dense bodies which are associated with the plasma membrane. In nonmuscle cells, it has been shown recently that actin filaments are associated to the plasma and subcellular organelle membranes via the interaction of α-actinin (Burridge & Feramisco, 1982; Bloch & Hall, 1983; Geiger et al., 1984; Wu & Stracher, 1985).

Although the functional significance of actin in nonmuscle cells is not fully understood, several passive and active roles have been ascribed to this protein. The passive roles include a cytoskeletal role which is involved with structural support for cell attachment and a role in the maintenance of cytoplasmic viscosity (Pollard, 1976; Byers et al., 1984; Tucker et al., 1985). The active roles are more diversified and include axonal transport (Lasek & Hoffman, 1976; Lasek, 1982), chromosome segregation involved in cell division (McIntosh, 1982; Nagata & Ichikawa, 1984), changes in cell shape (Albertini & Herman, 1984), regulation of the topographical distribution of membrane proteins (Ash & Singer, 1976; Oliver & Berlin, 1983), endocytosis (Buckley, 1983) and exocytosis (Poisner & Trifaró, 1967; Trifaró, 1978; Fox, 1985; Trifaró et al., 1985).

Recently, a new actin-like protein called anthrin has been isolated and

characterized from insect flight muscle. This protein has a molecular weight of 55,000 and possesses several of the characteristics related to actin. However, its function is still unknown (Bullard et al., 1985).

H.1.2 Myosin

Myosin is a ubiquitous protein which has been detected in almost every tissue and phylum of the animal kingdom examined so far. It has been isolated and characterized extensively from numerous tissues including skeletal muscles, smooth muscles and nonmuscle cell systems. Myosin is a hexameric molecule which is composed of two heavy chains (200-215kD), two regulatory light chains (19-23kD) and two essential light chains (15-18kD). The molecular weight of the different components (heavy and light chains) vary greatly depending on the origin of the myosin. The overall configuration of the native molecule consists of a coiled-coil tail region and two protruding, randomly coiled head regions which are associated with the light chains (see Fig. 10).

Proteolytic cleavage of native myosin with trypsin yields two fragments called light meromyosin (LMM) and heavy meromyosin (HMM). HMM, which contains the two head regions of myosin, can be further digested with papain or chymotrypsin, producing three subfragments designated S-1A, S-1B and S-2. These fragments have been used extensively to localize and characterize the actin-binding and ATPase-activity domains of myosin. For example, HMM has been shown by electron microscopy to decorate actin microfilaments, producing arrowhead patterns (see recent findings Siemankowski & White, 1984; Mil-

ler & Reisler, 1985; Mornet et al., 1985; Stafford, 1985; Wagner et al., 1985).

Myosin has been shown to form thick bipolar filaments where the tail regions of one myosin molecule interact with the tail regions of other myosin molecules. The protruding head regions of myosin bipolar filaments bind to thin actin filaments (see Fig. 10). Myosin filament self-assembly has been studied extensively in vitro. It appears that this process is extremely sensitive to small changes in pH and ionic strength and also to the nature of the ionic species involved. In addition, synthetic filament populations obtained are very heterogeneous and are not associated to non-myosin components which are usually present in vivo (Reisler et al., 1980; Kendrick-Jones et al., 1983; Starr & Offer, 1983). However, it was shown recently that MgATP specifically controls in vitro myosin filament self-assembly in physiological pH range (Pinset-Härström, 1985).

In 1954, Hanson and Huxley were first to propose the two-filament sarcomere model which has provided a structural basis of the well-known sliding-filament theory of muscle contraction. This model states that tension generation and muscle shortening (contraction) occur when two sets of discontinuous, inextensible filaments move or slide across each other, varying the degree of overlap while undergoing no change in filament length themselves (i.e., I bands shorten while A bands remain constant) (for review see Huxley, 1972; Huxley, 1974; Harrington, 1979; Pollack, 1983). According to this model, structural continuity between a series of sarcomeres in the myofibril is transient and occurs only when myosin crossbridges on the thick filaments are engaged to thin actin filaments. In the past five years, this

theory has greatly evolved since the presence of a third type of filament, called a gap filament, extra bands, namely N_1 and N_2 lines, and new contractile elements such as titin and nebulin has been demonstrated (for review see dos Remedios, 1983; Murphy et al., 1983; Cooke, 1985; Wang, 1985).

The discovery that the 20kD light chain of myosin could be phosphorylated and that the phosphorylation of myosin is associated with an increase in its actin-activated Mg^{2+}-ATPase activity has led to a phenomenal number of investigations concerning the regulatory role of myosin phosphorylation in a wide variety of tissues including skeletal muscles, smooth muscles and nonmuscle cells. Myosin light chain phosphorylation appears to be one of the major regulatory mechanisms which is involved in the contractile events of smooth muscles and nonmuscle cells (for review see Adelstein & Eisenberg, 1980; Stull, 1980). In these systems, myosin light chain phosphorylation appears to possess a dual function: it increases actomyosin ATPase activity and promotes myosin assembly (for review see Demaille & Pechère, 1983; Kamm & Stull, 1985). In smooth muscles and nonmuscle cells, myosin light chain phosphorylation has been shown to be regulated by a Ca^{2+}/calmodulin-dependent kinase, namely myosin light chain kinase (MLCK) (see section G.3.3). In addition, it has been shown more recently that myosin light chain can also be phosphorylated by protein kinase C (Endo et al., 1982; Naka et al., 1983). However, the sites of phosphorylation on the 20kD light chain are different for protein kinase C and MLCK.

In contrast to the observations described above for smooth muscles and nonmuscle cells, it appears that myosin phosphorylation in skeletal muscle is not an essential prerequisite but rather plays a modulatory role in the

actin-myosin interaction (Adelstein, 1980; Schaub & Watterson, 1981).

In nonmuscle cells, myosin is present in much smaller quantities (relative to actin) than in skeletal muscle, and thick filaments of myosin analogous to those seen in muscle are rarely observed. Nonmuscle cell myosin is found associated under certain conditions with the cytoskeleton microfilaments (actin filaments). Like actin, myosin has been implicated in these cells in cell locomotion and subcellular organelle movement (for review see Trifaró, 1978; Weatherbee, 1981).

The presence of myosin isoenzymes has been observed in several tissues including skeletal muscles, cardiac muscle and nonmuscle cells. These myosin isoenzymes have been related exclusively to changes at the level of myosin heavy chains. They have been extensively studied and characterized in ventricular cardiac muscles where they are associated to some pathologies (Hoh et al., 1978; D'Albis et al., 1979; Swynghedauw et al., 1983; Dillmann, 1985; Malhotra et al., 1985). Until recently, little was known about the molecular basis for differential gene expression during muscle development and about the functions of myosin isoenzymes in different cell systems. However, extensive myosin gene cloning studies have recently been performed in the nematode Caenorhabditis elegans, where it was confirmed that myosin gene modifications are located at the level of myosin heavy chains (for review see Karn et al., 1985).

H.1.3 α-Actinin

α-Actinin was first described by Ebashi and collaborators as a structural component of skeletal muscle and as a factor promoting actomyosin superprecipitation in vitro (Ebashi et al., 1964; Ebashi & Ebashi, 1965). α-Actinin has been isolated and characterized from several tissues including skeletal muscles, smooth muscles, cardiac muscle and nonmuscle cells. This protein is a dimer which is composed of two very similar subunits of molecular weight of 95,000-105,000. The protein possesses a relatively high α-helical content, and it appears as a rod-shaped molecule when observed at the electron microscope. α-Actinin cross-links actin filaments and enhances actomyosin ATPase activity (Suzuki et al., 1976; Feramisco & Burridge, 1980).

In skeletal muscles, α-actinin is localized exclusively in Z-discs (Z-lines) of myofibrils whereas in smooth muscles, it is concentrated both in intracellular dense bodies and in membrane-associated dense plaques, structures which are the sites of attachment of thin actin filaments. In nonmuscle cells, α-actinin is associated with bundles of actin filaments, both along the length of the bundles and also in the points of attachment of bundles to the plasma and subcellular organelle membranes (for review see Weatherbee, 1981; also Meyer et al., 1982; Fay et al., 1983).

Comparative studies performed in various tissues have recently shown that several types of α-actinin were present in these tissues and that they were in most cases biochemically and immunologically different. In skeletal muscles, three types of α-actinin were characterized and were found to be preferentially localized in either fast-twitch (type I and IIa) or slow-

twitch (type IIb) muscles (Bretscher et al., 1979; Kobayashi et al., 1983a). Smooth muscle α-actinin possesses several of the characteristics of skeletal muscle and cardiac muscle α-actinin forms, but it has a distinct amino acid sequence and interacts differently with F-actin (Endo & Masaki, 1982). In nonmuscle cells such as platelets, fibroblasts, kidney cells, chromaffin cells, brain tissues and tumor cells, α-actinin appears to be somewhat different than skeletal muscle isoproteins with respect to physicochemical properties, immunological reactivity and Ca^{2+} dependency of F-actin interaction (Rosenberg et al., 1981; Sixma et al., 1982; Kobayashi & Tashima, 1983; Lando & Olomucki, 1983; Fox, 1985; Trifaró et al., 1985).

H.1.4 Tropomyosin

Tropomyosin is present throughout evolution both as a major contractile protein and as an important component of the microfilaments maintaining the cellular cytoarchitecture. This protein was first isolated by Bailey (1948) from skeletal muscle myofibrils. Tropomyosin has since been isolated from numerous tissues including skeletal muscles, smooth muscles and more recently, several nonmuscle cell systems (for review see Côté G.P., 1983; Payne & Rudnick, 1985).

Skeletal muscle tropomyosin is a dimeric molecule consisting of two polypeptide chains of 284 amino acid residues arranged in an α-helical coiled-coil configuration. Although both tropomyosin subunits (α and β) contain the same number of amino acid residues, they have different apparent molecular weights on SDS-PAGE being of 34,000 and 36,000 for α and β sub-

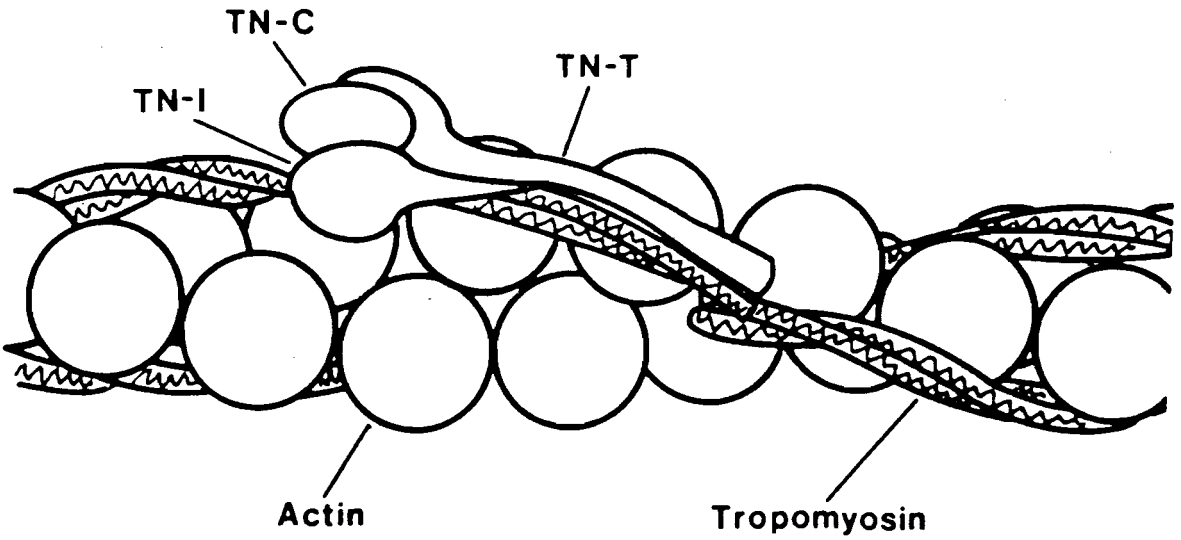
units, respectively. Under physiological conditions, skeletal muscle tropomyosin subunits form a dimer of about 68kD which has a length of ~40 nm and can interact with seven actin monomers. Tropomyosin is present in skeletal muscles as approximately 7% of the total structural protein and is a key component in the actin-linked Ca^{2+} regulatory mechanism that controls muscle contraction and relaxation. Tropomyosin filaments are formed by dimers polymerizing in a head-to-tail linkage producing a tropomyosin filament that spans the entire length of the thin filament along each of the grooves formed on the sides of the F-actin helix (Cohen & Szent-Gyorgyi, 1957; Sodek et al., 1972, 1978; McLachlan & Stewart, 1975; Stone & Smillie, 1978; Lewis & Smillie, 1980; Mak et al., 1980; see also reviews Côté, 1983; Gillis, 1985; Payne & Rudnick, 1985) (see Fig. 11).

A new tropomyosin-like protein, called paratropomyosin, has recently been isolated and characterized from skeletal muscle myofibrils. This protein resembles tropomyosin in several physicochemical properties and possesses the same molecular weight as skeletal muscle α-tropomyosin as determined by SDS-PAGE. However, it differs from skeletal tropomyosin in its amino acid sequence and in its ability to interact with actomyosin (Nakamura & Takahashi, 1985; Takahashi et al., 1985).

Cardiac muscle and smooth muscle tropomyosins have also been examined quite extensively. Cardiac muscle tropomyosin has been shown by electrophoretic techniques and sequence analysis to correspond to striated muscle α-tropomyosin isoform in several animal species (Cummins & Perry, 1973; Mak et al., 1979; Lim et al., 1983). However, in big mammals, it was demonstrated that both α- and β-tropomyosin isoforms (α-α and α-β dimers) were present

Fig. 11 Model for the interaction of tropomyosin with F-actin. In striated muscle, tropomyosin molecules can interact with seven actin monomers which are included in thin actin filaments. In addition, the troponin (TN) complex can interact with different regions of skeletal muscle tropomyosin, either near the Cys-190 region of tropomyosin or at the end-to-end region between tropomyosin molecules.

[Taken from Côté G.P., 1983]


TN-C
TN-I
TN-T
Actin
Tropomyosin

although the β isoform content was only of 15-20% (Leger et al., 1975, 1976; Ookubo et al., 1975). In addition, phosphorylated species of α-tropomyosin have been detected by two-dimensional electrophoresis (Izant & Lazarides, 1977; Segura et al., 1982).

Adult smooth muscles appear to contain both α- and β-tropomyosin isoforms as observed by electrophoretic techniques. However, smooth muscle tropomyosins migrate on SDS-PAGE to molecular weights higher than that of skeletal muscles (Izant & Lazarides, 1977; Dabrowska et al., 1980; Hirai & Hirabayashi, 1983). Although smooth muscle does not contain troponins similar to skeletal muscle, tropomyosin appears to be necessary for full activation of smooth muscle actomyosin and can fully substitute for skeletal muscle tropomyosin in skeletal muscle actomyosin (Pearlstone & Smillie, 1982; Sobieszek, 1982; see also review Hartshorne & Mrwa, 1982).

Proteins with structural characteristics similar to skeletal muscle tropomyosin have been isolated from a variety of nonmuscle systems such as brain (Fine et al., 1973; Fine & Blitz, 1975), pancreas (Fine & Blitz, 1975), platelets (Cohen & Cohen, 1972; Côté & Smillie, 1981a; der Terrosian et al., 1981), fibroblasts (Fine & Blitz, 1975; Giometti & Anderson, 1981), macrophages (Fattoum et al., 1983), thyroid (Kobayashi et al., 1982) and kidney (Kobayashi et al., 1983b). In these systems, isolated nonmuscle tropomyosins have an apparent molecular weight of 30,000, which is significantly less than that of skeletal muscle tropomyosin (for review see Côté, 1983).

The physiological role of tropomyosin in nonmuscle tissues is not clearly understood (for review see Côté, 1983). However, Nosaka and collaborators

have recently reported that any tropomyosin enhances the ATPase activity of any actomyosin when actin and myosin are in the state of "rigor" (tight) complex. On the other hand, the skeletal tropomyosin is capable of inhibiting the ATPase activity of the skeletal actomyosin under a MgATP concentration and a myosin/actin ratio which do not allow the formation of a "rigor" complex but under which the troponin complex exerts its effects of Ca^{2+}-sensitive regulation (Nosaka et al., 1984).

Another striking feature is that the expression of several tropomyosin isoforms has been reported in normal and transformed nonmuscle cells in culture (Schloss & Goldman, 1980; Hendricks & Weintraub, 1981; Leonardi et al., 1982; Matsumura et al., 1983a, 1983b; Talbot & MacLeod, 1983; Lin et al., 1984). In these systems, molecular weights as high as 36,000 to 40,000 have been reported for some of the tropomyosin isoforms. Thus, important cytoplasmic roles have been postulated by Payne and Rudnick for those multiple tropomyosin isoforms having possibly different functional properties (for review see Payne & Rudnick, 1984, 1985).

H.2 Cytoskeleton

During the last ten years, the term cytoskeleton has become very popular. The cytoskeleton is described as a framework of cytoplasmic fibers which are retained together with the nucleus and display very good ultrastructural preservation when cells are extracted under controlled conditions by non-ionic detergents. In a broader sense, the cytoskeleton corresponds to the three-dimensional network formed by the nucleus, organelles, fibrous

systems and membranes with the underlying assumption that these structures function coordinately. The term cytoskeleton often implies a rather static function. However, the well-known dynamic aspects of the organization should not be neglected.

Several aspects related to the organization and functions of the cytoskeletal filament networks and their associated proteins have been extensively reviewed (Stossel, 1978; Weber & Osborn, 1979, 1981a, 1981b, 1982a, 1982b; Weatherbee, 1981; Brinkley, 1982a, 1982b; Denk & Krepler, 1982; Lazarides, 1982; de Brabander, 1983; Franke et al., 1983; Albertini & Herman, 1984; Birchmeier, 1984; Bourguignon & Bourguignon, 1984; Ben-Ze'ev, 1985; Marceau & Swierenga, 1985; Oliver et al., 1985). In addition, some specific cell systems such as fibroblasts, platelets and erythrocytes have been very useful in studying various aspects related to the cytoskeleton (see recent reviews Lévy-Tolédano et al., 1982; Fox & Phillips, 1983; Byers et al., 1984; Lewis, 1984; Fox, 1985).

The cytoskeleton is composed basically of three distinct filament systems, namely microfilaments, microtubules and intermediate filaments, which are distinguished principally by their size and localization. In addition, several specific proteins associated with these filamentous networks have been described.

H.2.1 Microfilaments

Microfilaments are among the earliest cellular organizations which have been identified by electron microscopy. These submembranous structures are

composed of G-actin subunits which are helically arranged in a double-stranded filament (F-actin) with a diameter of 4-7 nm.

The microfilament network has often been associated with structural (static) roles in maintaining the cytoarchitecture and cytoplasmic consistency. However, the often dominant microfilament bundles observed in many cell types are not solely an F-actin storage form since they also contain myosin. Consequently, it was proposed that these filaments may correspond to some complex "cytomuscular structures". This proposal has been supported by several microscopy studies which have revealed that several proteins including α-actinin, vinculin, tropomyosin, filamin and fimbrin are associated with these filaments and ATP-dependent shortening of fibrous structures (for review see Weber & Osborn, 1982a; and also Masuda et al., 1983).

The presence of functionally distinct microfilament systems within the same cell has been documented in several cell systems including skin and intestinal epithelial cells, fibroblasts, astrocytes, platelets, erythrocytes and leukocytes (Matsudaira & Burgess, 1979; Green & Goldman, 1983; Nachmias, 1983; Oliver & Berlin, 1983; Byers et al., 1984; Goldman & Chiu, 1984; Cohen & Nemhauser, 1985; Fox, 1985). Therefore, in addition to the core microfilament system, there exist some cross-bridging microfilament systems.

At present, more than forty microfilament-associated proteins have been isolated and characterized from various nonmuscle cell systems and some of them also in muscles (for review see Craig & Pollard, 1982; Cooke, 1985; Shear & Bloch, 1985). These proteins have been shown to regulate the assembly of microfilament structures, to mediate microfilament reorganization and to be involved in the interaction of microfilaments with cytoplasmic and

subcellular organelle membranes. Consequently, the numerous microfilament-associated proteins were classified into three functional groups:

1) proteins that promote microfilament network formation such as actinogelin, gelactin (I-IV), filamin, fimbrin, fascin and villin;
2) proteins that disrupt microfilament formation or existing microfilament structures such as gelsolin, brevin, fragmin, villin, 60kD-capping protein, β-actinin, profilin and γ-actinin;
3) anchorage proteins such as α-actinin, spectrin, globin, ankyrin, vinculin, 110kD-microvillus protein and talin.

The functional activity of some microfilament-associated proteins has been found to be regulated by different factors such as Ca^{2+} concentrations, pH, ionic strength and temperature. Calcium appears to regulate the association of some of these proteins with the microfilaments since the action of actinogelin, villin, 60kD-capping protein, gelsolin, brevin, fragmin and nonmuscle α-actinin is inhibited by Ca^{2+} concentrations higher than 10^{-6}M (for review see Craig & Pollard, 1982; also Ebisawa et al., 1985). In particular, villin, which produces opposite effects (promoting and disrupting) on microfilament systems, is highly influenced by calcium. Villin possesses two distinct actin-binding sites which are differentially regulated by Ca^{2+}. Therefore, at less than 10^{-6}M Ca^{2+}, villin promotes microfilament association whereas at greater than 10^{-6}M Ca^{2+}, villin inhibits microfilament formation (Glenney et al., 1981). Physiological changes in pH, ionic strength and temperature indirectly affect the association of spectrin and filamin with microfilaments through the regulation of their subunits' self-association (Ungewickell & Gratzer, 1978; Davies et al., 1980; Goodman & Shiffer,

1983; Stokke et al., 1985). In addition, changes in ionic strength appear to modulate indirectly the interaction of filamin with microfilaments via its binding to myosin (Szpacenko et al., 1984; Dabrowska et al., 1985; Weihing, 1985).

Spectrin is among the microfilament-associated proteins which have been most investigated in nonmuscle cells. This protein was first described as a component of the erythrocyte membrane skeleton. In 1981, spectrin and related proteins (fodrin) were found to be expressed in a wide variety of non-erythroid cells (Goodman & Kulikowski, 1981; Goodman et al., 1981). Erythrocyte spectrin is composed of two distinct large polypeptide chains (α (240kD) and β (220kD) subunits) that form an elongated flexible heterodimer. The prevalent and physiologically relevant spectrin arrangement appears to be a tetramer $(\alpha\beta)_2$ which is formed by head-to-head association of two heterodimers (for review see Goodman & Shiffer, 1983). Brain spectrin, also referred to as calspectin, has been extensively characterized (for review see Goodman & Zagon, 1984). Brain spectrin possesses many of the characteristics of both erythrocyte spectrin and brush border TW 260/240 protein. However, the molecular weights of its α and β subunits are of 240,000 and 235,000, respectively. In addition, peptide-mapping analysis and gene-cloning studies have shown that both α and β subunits of brain spectrin are significantly different than those of erythrocyte spectrin. Nevertheless, the functions of these two spectrin species appear to be very similar and regulated in a similar fashion.

H.2.2 Intermediate Filaments

Several years ago, electron microscopy studies revealed the presence of a major 10-nm filamentous system in the cytoplasm of higher eukaryotic cells. In immature skeletal muscle, 10-nm filaments were first called "intermediate" since their diameter was between that of thin actin and thick myosin filaments (Ishikawa et al., 1968). In nonmuscle cells, "intermediate" stands for a diameter between that of actin microfilaments and microtubules. The term "intermediate filaments" is now applied to all 10-nm filaments (7-11 nm) regardless of their location.

The intermediate filaments were initially regarded as disaggregation products of either microtubules or myosin filaments and thus, until recently, attracted little attention. Current biochemical and immunofluorescence methods have established the intermediate filaments as a distinct class of cytoplasmic proteins that differ with respect to the physical properties of their subunits. In contrast to the major structural protein subunits of microfilaments and microtubules, the intermediate filament proteins do not appear to be highly conserved and exhibit a relatively high degree of tissue specificity (for review see Lazarides, 1980, 1982; Weatherbee, 1981; Franke et al., 1983; Dahl & Bignami, 1985).

The intermediate filaments have been divided into five different subclasses on the basis of biochemical and immunochemical observations, and their constituent proteins have been named accordingly. These subclasses at present include: (1) keratin (tono) filaments found in epithelial cells and cells of epithelial origin; (2) desmin (skeletin) filaments identified pre-

dominantly in smooth, skeletal and cardiac muscle cells; (3) vimentin (decamin) filaments found in mesenchymal cells and cells of mesenchymal origin; (4) neurofilaments described in neurones and cells of neuronal origin; and (5) glial filaments identified in astrocytes and also in some other types of glial cells. In several cases, it has been shown that more than one of these intermediate filament subclasses coexist in the same cell type. This phenomenon has been shown to occur for all subclasses of intermediate filaments in certain cell types (for review see Lazarides, 1980, 1982; Fusuler et al., 1981; Steinert et al., 1984; Dahl & Bignami, 1985).

The exact functional significance of these different intermediate filament systems which are present in various cell types is still not fully understood, although they have been investigated extensively. However, they seem to serve a cytoskeletal function and in fact may represent the true cytoskeleton of several cell types, although they are not present in certain embryonic cells (for review see Weber & Osborn, 1982a, 1982b). Cytokeratin filaments appear to interact extensively with membranes through desmosomes (for review see Weihing, 1979; Jones et al., 1982; also Jones et al., 1984; Jones & Goldman, 1985). They may also be involved in nuclear anchorage and act in muscle cells as "mechanical integrators of cellular space" (for review see Lazarides, 1980; Zackroff et al., 1981). In addition, many intermediate filaments seem to exist in a state of interdependence with microtubule expression (for review see McIntosh, 1982). Furthermore, it was shown more recently that these filaments interact with microfilaments through specific proteins such as spectrin, ankyrin and syndeins (for review see Goodman & Zagon, 1984).

Although the protein components of the different intermediate filaments have been shown to be distinct in several respects, there is an increasing number of studies which support the idea that these polypeptides possess certain common ultrastructural features. It appears that these polypeptides share a common linear sequence segment called a rod domain. This segment is a highly α-helical middle domain of about 310 residues which is flanked by non-α-helical terminal domains that correspond to the head and tail segments located at the amino and carboxyl end respectively. These protofilament units are arranged in tetrameric structures which possess an antiparallel orientation of two double-stranded coiled-coil structures (see Fuchs & Hanukoglu, 1983; Steinert et al., 1984; Geisler et al., 1985; Ip et al., 1985).

In addition, all intermediate filament proteins described so far appear to be phosphorylated in vivo and can be phosphorylated in vitro. However, the role of phosphorylation of these polypeptides in the regulation of assembly or other functions of intermediate filaments is still only partially understood (for review see Lazarides, 1980; Nestler & Greengard, 1984; Marceau & Swierenga, 1985).

Subclasses of Intermediate Filaments

Cytokeratin Filaments

Cytokeratin filaments have been detected by immunological techniques in a wide variety of epithelia including all stratified squamous and lining

epithelia, myoepithelial cells of various glands, thymus reticular epithelium and keratinocytes (Franke et al., 1979a, 1979b, 1979c). At present, more than 30 keratin polypeptides have been isolated and characterized from different cytokeratin filaments. These polypeptides possess different isoelectric points and molecular weights ranging between 40,000 and 70,000, but they appear to be immunologically related (Moll et al., 1982; Woodcock-Mitchell et al., 1982; Sun et al., 1983).

The assembly of different keratins into filaments has been investigated extensively both in vivo and in vitro. Most combinations of keratin polypeptides (2-7) polymerize in vitro into filaments of the same general structure as native cytokeratin filaments. The presence of two specific keratin polypeptides, one acidic (46kD) and one basic (56kD), has been demonstrated throughout vertebrate epithelia by cDNA-recombinant studies. In addition, other keratin polypeptides have been shown to be expressed in various normal and tumor cells (for review see Steinert et al., 1984). Thus, keratins consist of a highly complex multigene family of proteins which are expressed differentially in various epithelia.

Desmin Filaments

Desmin filaments are composed of polypeptide subunits of molecular weight of 50,000-55,000 which have been called desmin (also referred to as skeletin). Desmin filaments were first described in smooth muscles as the dominant 10-nm filament system. They have also been described in skeletal and cardiac muscle cells. In these three muscle types, these filaments rep-

resent a cytoskeleton which holds the components (Z-discs, N and M lines) of myofibrils in place (for review see Lazarides, 1980; also Ip et al., 1983). In addition, these filaments have been detected in some nonmuscle cells such as embryonic fibroblasts and kidney cell lines. However, in these cells, desmin filaments are present in low amounts and appear to be heteropolymers with vimentin (Tuszynski et al., 1979; Ip et al., 1983). The significance of the expression of desmin filaments in these cells remains unknown.

Vimentin Filaments

Vimentin filaments are the major intermediate filament system found in various mesenchymal cells including endothelial cells, fibroblasts, lymphocytes, macrophages and chondrocytes (Franke et al., 1978). These filaments have also been detected in some vascular smooth muscles and in epithelial and cortical fiber cells of the ocular lens (Berner et al., 1981; Ellis et al., 1984; Bagchi et al., 1985). Vimentin filaments are also expressed in a wide variety of cultured cells which does not only include mesenchymally derived cells (Traub et al., 1983; Holthöfer et al., 1984; Dellagi et al., 1985). However, in these cells, they are often co-expressed with other intermediate filament classes.

The ultrastructure, organization, biosynthesis and turnover of vimentin filaments have been extensively documented in various cell types. These filaments consist of vimentin subunits of molecular weight of 52,000-58,000 which possess an α-helical coiled-coil domain and are arranged in protofilament structures (Geisler et al., 1983; Quax-Jeuken et al., 1983; Ip et al.,

1985). Vimentin assembly appears to be regulated by the ionic environment (Nelson & Traub, 1982; Traub & Vorgias, 1984). Vimentin expression in the different cell types appears to be regulated by a single copy gene, but its degree of expression in these cells is related to their developmental stage and some regulatory factors (Connell & Rheinwald, 1983; Houle & Fedoroff, 1983; Laszlo & Bissell, 1983; Zehner & Paterson, 1983; Ngai et al., 1984). Vimentin filament turnover is regulated by a Ca^{2+}-activated proteolytic mechanism (McTavish et al., 1983; Nelson & Traub, 1983; Roy et al., 1983; Ciesielski-Treska et al., 1984). In addition, vimentin phosphorylation has been reported in several cell systems and appears to be related to some mitotic events in these cells (Ben-Ze'ev, 1983; Celis et al., 1983; Spruill et al., 1983a, 1983b; Evans, 1984).

Neurofilaments

Mammalian neurofilaments are formed by a triplet of polypeptides which have molecular weights of about 200,000, 145,000 and 65,000 (Hoffman & Lasek, 1975; Anderton et al., 1978; Liem et al., 1978; Micko & Schlaepfer, 1978; Schlaepfer & Freedman, 1978). Peptide-mapping studies have shown no major similarities in the primary sequence of the three neurofilament proteins (Dahl, 1979; Chiu et al., 1980; Brown et al., 1981). These three polypeptides do not appear to be derived from a large precursor protein since they are all synthesized in cell-free systems (Strocchi et al., 1982). Contrary to earlier studies which suggested that only the 68kD-subunit constituted the neurofilament core while the other two subunits represented as-

sociated components, a recent study has shown that both the 145kD- and the 200kD-components can also self-assemble into filaments (Gardner et al., 1984). The distribution of neurofilaments in different types of neurons as well as their subcellular localization has been extensively documented (see Drake & Lasek, 1984; Dahl & Bignami, 1985). Neurofilaments are not uniformly distributed in different types of neurons. In addition, it appears that neurofilaments differ in polypeptide composition according to their location and that some subunits undergo posttranslational modifications (Nixon et al., 1982; Bennett & DiLullo, 1985).

The phosphorylation of the neurofilament proteins has been investigated extensively both in vitro and in vivo. Phosphorylation of neurofilament subunits was first demonstrated in squid and Myxicola both in vitro and in vivo (Pant & Yoshioka, 1977; Pant et al., 1978; Schecket & Lasek, 1979). Then, their phosphorylation was demonstrated in rat and rabbit CNS both in vitro and in vivo (Julien & Mushynski, 1981, 1983; Honchar et al., 1982). In addition, it was observed that a protein kinase copurified with neurofilament preparations, and it was suggested that it may be involved in their in vivo phosphorylation (Julien & Mushynski, 1981). Furthermore, it has been suggested that aberrant neurofilament phosphorylation and specific protein kinase imbalance might be implicated in Alzheimer's disease (see new findings Sternberger et al., 1985).

Glial Filaments

Glial filaments are characterized by the presence of a polypeptide of molecular weight of 50,000-53,000 termed glial fibrillary acidic protein (GFAP). GFAP can be separated by electrophoresis into three isoforms that differ slightly in isoelectric points (Dahl & Bignami, 1983). Glial filaments are present in astrocytes and in some specialized cells of neuroglia. They have also been detected in Müller glia of the retina under certain pathological conditions and also in some immature glial cells where they disappear during glial development (for review see Dahl & Bignami, 1985; Noetzel & Agrawal, 1985). In addition, these filaments have been detected in several cultured cells including differentiated astrocytes and glioma cell lines (Eng, 1985; Pruss, 1985). Changes in GFAP expression appear to be related to CNS development and certain pathological states (Chiu & Goldman, 1985). Although the exact functions of these filaments are still obscure, a putative function has been ascribed to glial filaments, which is its role as a component of the cytoskeleton in defining and maintaining the shape of the astrocyte.

H.2.3 Microtubules

Microtubules are distinct structures found in all eukaryotic cells. They appear as tubular structures of indefinite length with an approximate outer diameter of 25-30 nm when examined under the electron microscope. In cross-section, microtubules appear as hollow tubules with walls approximately 5 nm

thick. Microtubule structure, assembly, disassembly and organization have been extensively reviewed (Burton, 1981; Gunning & Hardham, 1982; Linck, 1982; Correia & Williams, 1983; Schultheiss & Mandelkow, 1983; Hesketh, 1984, 1985; McKeithan & Rosenbaum, 1984; Purich & Kristofferson, 1984; Chen & Hill, 1985).

Microtubules are highly ordered polymers of the globular protein tubulin which has a native molecular weight of about 110,000. This protein is composed of two similar but not identical subunits (α and β) with molecular weights of about 53,000. Native singlet microtubules are composed of about thirteen protofilaments which are elongated tubulin ($\alpha\beta$) heterodimers arranged end to end. In addition to monomer concentrations, several factors appear to regulate the assembly-disassembly process of microtubules. These factors include guanine nucleotides, divalent cations (Mg^{2+}, Ca^{2+}), pH, temperature and the presence of microtubule-associated proteins and also their phosphorylation. However, temperature is not a critical factor for all populations of microtubules since a substantial subpopulation of microtubules are cold-stable. In addition to the usual tubular microtubule structures, tubulins have been shown to assemble into numerous aberrant structures such as sheets, ribbons, spirals and twisted ribbons. Although the presence of these aberrant structures has been reported quite frequently in different cells, their differential cellular functions are not fully understood.

Microtubules appear to be central elements of cellular organization. Microtubules have been implicated in several cellular functions such as change in cell shape, cell motility, axonal transport, subcellular organelle motility, DNA synthesis and cell membrane-related events (for review see de

Brabander, 1982; also Brimijoin, 1982; Busson-Mabillot et al., 1982; Otto, 1982; Suchard & Goode, 1982; Schliwa, 1984; Vale et al., 1985a, 1985b). The best documented function of microtubules is their role in cell membrane-related events. These filamentous structures have been demonstrated to modulate the response of cells to receptor triggering at multiple sites. Microtubules are involved in the distribution and motility of cell surface receptors. However, microtubules may be involved indirectly since usually no direct connections with transmembrane proteins are observed. Surface receptors may be linked through the membrane with microfilament networks, the activity and distribution of which could in turn be coordinated by a microtubule-dependent mechanism. This type of interaction could take place through microfilament-associated proteins such as spectrin or calmodulin-dependent "linker" proteins (Fach et al., 1985; Sobue et al., 1985a). In addition, microtubules have been shown to interact with intermediate filaments through specialized "coupler" structures or microtubule-associated proteins (Leterrier et al., 1982; Nagele & Roisen, 1982; Bloom & Vallee, 1983; Bloom et al., 1984a, 1984b). Although the molecular interactions of microtubules with other cellular structures are not completely understood, microtubules appear to transfer information through the cytoplasm in a bidirectional fashion.

Microtubule-Associated Components

In addition to tubulin (α and β subunits), microtubules contain several species of protein that bind to and copurify with them when isolated in vitro. These components generally comprise about 15% of the total protein

content. These proteins can be divided into two main categories: 1) proteins of high molecular weights (~350,000-180,000) which have been termed "microtubule-associated proteins" (MAP1, MAP2 and MAP3); 2) proteins of intermediate molecular weights (~62,000-55,000) which are collectively referred to as tau factor.

MAP1 and MAP2 obtained from brain microtubule preparations can be resolved by electrophoretic techniques into several polypeptide bands. MAP1 corresponds to a closely spaced triplet of molecular weight of about 350,000 (MAP1A, MAP1B, MAP1C) and MAP2 corresponds to a doublet of molecular weights of 285,000 (MAP2.1) and 270,000 (MAP2.2). Some of these MAPs represent fine filamentous projections arranged regularly on the microtubule surface, and consequently, it has been suggested that they are involved in mediating the interaction of microtubules with other cell components (for review see McKeithan & Rosenbaum, 1984). In addition, it was determined that the MAPs and tau factor are components which promote the assembly of tubulin into microtubules (for review see Correia & Williams, 1983; McEwen et al., 1983).

The distribution of the various MAPs in the nervous system appears to be quite different. MAP1 (especially MAP1A, the most abundant MAP1 polypeptide) is widely distributed in the nervous system while MAP2 is specifically enriched in dendrites and perikarya of neurons (Bloom et al., 1984b). MAP3 is a newly discovered microtubule-associated protein of molecular weight of about 180,000. In the brain, MAP3 is present in both neurons and glia. In nerve cells, its distribution is mainly restricted to neurofilament-rich axons (Huber et al., 1985).

Among all the microtubule-associated components, MAP2 is the most exten-

sively characterized protein (for review see Vallee, 1984). MAP2 is a large, highly asymmetric molecule consisting of two subunits (MAP2.1 and MAP2.2) which are quite similar in size (15kD difference), shape and charge. MAP2 is extremely susceptible to proteolytic enzymes, although they do not appear to attack the binding domain involved in promoting microtubule assembly. Both MAP2 subunits are phosphorylated by an associated kinase which corresponds to a type II cAMP-dependent kinase. MAP2 phosphorylation appears to be an important phenomenon in nerve cells since these cells contain large concentrations of MAP2, MAP2 possesses several phosphorylation sites and the kinase involved in this phosphorylation process is highly concentrated in these cells. Several studies indicate that MAP2 phosphorylation affects microtubule assembly. In addition, phosphorylation sites are present on the projection portion of MAP2 suggesting that the interaction of MAP2 with other cellular structures may also be regulated by phosphorylation. Although the exact significance of MAP2 phosphorylation is still unclear, it has been postulated that it might define the functional state of MAP2.

H.3 Contractile Proteins and Cytoskeleton of Chromaffin Cells

Several contractile proteins and cytoskeletal components have been isolated from chromaffin cells and characterized. Their possible roles in the secretory process of these cells have also been investigated extensively. In addition, some studies concerning the presence of tropomyosin in chromaffin cells are presented in this thesis.

H.3.1 Chromaffin Cell Actin

Actin has been isolated and characterized in adrenal chromaffin cells (Lee et al., 1979; Lee & Trifaró, 1980, 1981; Aunis et al., 1980; Hesketh et al., 1981). Chromaffin cell actin possesses an apparent molecular weight of 42,000 as determined by SDS-PAGE. It was revealed by two-dimensional gel electrophoresis that chromaffin cells contain two isomeric forms of actin, namely β-actin and γ-actin. These two actin isoforms have identical molecular weights but different isoelectric points. In addition, it appears that 10% more β-actin than γ-actin is present in chromaffin cells (Lee et al., 1979).

Immunocytochemical studies using adrenal actin antibodies have shown that actin is widely distributed in chromaffin cells and associated with many subcellular structures. However, the cellular distribution of actin in cultured chromaffin cells appears to depend on the age of the cells maintained in culture. In one-day-old cells in culture, a strong membrane and a weak cytosolic fluorescence was observed by indirect immunofluorescence using adrenal actin antibodies (Lee & Trifaró, 1981). In contrast, four- to seven-day-old cultured cells exhibited a much weaker fluorescence in the cytosol of cell bodies, neurites and terminal cones (Aunis et al., 1980; Lee & Trifaró, 1980, 1981; Hesketh et al., 1981). In addition, it was shown that the granular distribution pattern for actin was similar to the one observed for dopamine β-hydroxylase (Lee & Trifaró, 1980, 1981). Furthermore, it was found by electron microscopy using the protein A-gold technique that electron-opaque gold particles were localized in the vicinity of the dense cores

of chromaffin granules (Bendayan et al., 1982).

Several biochemical and immunocytochemical studies have shown that actin interacts with some chromaffin cell organelles such as secretory granules and mitochondria under certain conditions (Burridge & Phillips, 1975; Wilkins & Lin, 1981; Fowler & Pollard, 1982; Trifaró et al., 1982; Bader & Aunis, 1983; Aunis & Perrin, 1984; Kao & Westhead, 1984; Perrin & Aunis, 1985). The binding of actin to chromaffin granule membranes appears to be a Ca^{2+}-regulated phenomenon. It has been determined that when chromaffin granule membranes were isolated under high ionic strength conditions, more actin binds to these membranes than to those isolated at low ionic strength, a condition that displaces α-actinin from the membranes (Burridge & Phillips, 1975; Aunis et al., 1980; Trifaró et al., 1982; Kao & Westhead, 1984). The presence of Ca^{2+} in the medium partially abolishes actin binding to granule membranes, thus suggesting that a component of the binding is Ca^{2+} independent (Aunis & Perrin, 1984). In addition, in vitro experiments have shown that chromaffin granule membranes induce actin polymerization and gel formation and that increased Ca^{2+} concentrations inhibit both of these events (Burridge & Phillips, 1975; Wilkins & Lin, 1981; Fowler & Pollard, 1982; Aunis & Perrin, 1984). Electron microscopy studies of chromaffin granule-actin mixtures have revealed the presence of chromaffin granule-associated actin filaments (Burridge & Phillips, 1975; Wilkins & Lin, 1981).

In addition to actin, the presence of a polypeptide of higher molecular weight (92,000; pI = 6.1-6.2) was also observed in the column eluate when chromaffin cell actin was isolated by DNAse I affinity chromatography (Lee et al., 1979). When this procedure was carried out in the presence of a

EGTA-containing buffer, this polypeptide was selectively recovered in the column eluate as determined by SDS-PAGE. In addition, this 92kD protein was recognized by a rabbit lung macrophage gelsolin antibody as observed by immunoblotting (Trifaró et al., 1985). Therefore, it was concluded that this chromaffin cell 92kD polypeptide corresponds to a gelsolin-like protein.

The above-mentioned observations have demonstrated the presence of actin in chromaffin cells and its interaction with chromaffin granules. In addition, the presence of a gelsolin-like protein was also observed in chromaffin cells. Consequently, it was suggested that actin may play an important role in some steps of the secretory process in adrenal chromaffin cells (for review see Trifaró et al., 1985).

H.3.2 Chromaffin Cell Myosin

The presence of myosin in chromaffin cells was first suggested by the observation that an actomyosin-like protein was present in these cells (Trifaró & Ulpian, 1975; Ulpian, 1977). This protein possessed an ATPase activity that was stimulated by both Ca^{2+} and Mg^{2+} and decreased by the presence of high concentrations of K^{+}, which is a characteristic typical of muscle actomyosin. In addition, increases in viscosity and superprecipitation of adrenal actomyosin were observed when incubated in the presence of KCl, Mg^{2+} and increasing concentrations of ATP, similarly to muscle actomyosin (Trifaró & Ulpian, 1975). Several bands were observed when adrenal actomyosin was analyzed by SDS-PAGE, some of which corresponded in terms of molecular weight to known contractile proteins such as actin, myosin (heavy and

light chains) and tropomyosin (Trifaró & Ulpian, 1975).

Chromaffin cell myosin was purified from adrenal actomyosin by a classical salting-out procedure followed by gel filtration chromatography (Trifaró & Ulpian, 1976). This protein was shown to possess characteristic myosin ATPase activities (high K^+- and Ca^{2+}-dependent ATPase activities and ATPase activity inhibited by Mg^{2+}). Chromaffin cell myosin is composed of a heavy chain of molecular weight of 200,000 and two light chains of molecular weights of 22,000 and 16,500 as revealed by SDS-PAGE (Trifaró & Ulpian, 1976). The apparent molecular weights of myosin light chains reported by Trifaró and Ulpian were similar to the ones observed by other investigators: 20,000 and 17,000 (Creutz, 1977) and 23,000 and 20,000 (Hesketh et al., 1978). The amino acid composition of chromaffin cell myosin was found to be similar to rabbit skeletal muscle and Plasmodium physarum myosins (Trifaró & Ulpian, 1976).

Adrenal myosin has been shown to assemble into filaments in vitro. These filaments were characterized by a bare central segment and tapered ends. Large myosin arrangements which possessed order and periodicity were also observed and corresponded in size to small bipolar filaments (Hesketh et al., 1978). The adrenal myosin molecule morphologically resembles muscle myosin since it is 160 nm long and consists of two globular heads which are joined flexibly to a tail segment (Trifaró & Ulpian, 1976). In addition, adrenal myosin was shown by electron microscopy to bind to the skeletal F-actin filaments (6 nm) with periodicity of 30-35 nm in the characteristic arrowhead formation.

The presence in chromaffin cells of antigenic sites for myosin antibod-

ies has been demonstrated by immunohistochemical techniques (Creutz, 1977; Trifaró et al., 1978; Aunis et al., 1980). Indirect immunofluorescence studies have shown that chromaffin cells treated with myosin antibodies exhibited an intense fluorescence localized in the cytosol, a pattern which was absent under control conditions (Trifaró et al., 1978; Aunis et al., 1980; Bader et al., 1983). In addition, when myosin antibodies were added to freshly isolated chromaffin cells, some antigenic sites appeared to be localized on or close to the cell surface as determined by immunofluorescence (Trifaró et al., 1978). This latter observation substantiated earlier studies concerning the subcellular distribution of adrenal myosin which showed a parallel distribution between K^+-ATPase activity and plasma membrane markers but an absence of such activity in secretory granule fraction (Hesketh et al., 1977, 1979). Therefore, these biochemical and immunocytochemical studies seem to suggest that some myosin is associated with the chromaffin cell plasma membrane.

H.3.3 Chromaffin Cell α-Actinin

The presence of α-actinin in chromaffin cells has been demonstrated by indirect fluorescence using both bovine and chicken skeletal muscle α-actinin antibodies (Aunis et al., 1980; Trifaró et al., 1984a). α-Actinin was shown to be distributed over the entire cell body. Some diffuse fluorescence was also detected in the cytoplasm around the nucleus but not on the nucleus itself. In addition, a punctuate staining pattern was observed in the cytoplasm, along the processes and in neurite endings where it was particularly

intense (Aunis et al., 1980; Bader et al., 1984a; Trifaró et al., 1984a). The staining pattern observed for α-actinin appears to correlate those of DβH and chromogranin A (for review see Trifaró et al., 1985). Thus, according to these immunocytochemical studies, it was suggested that α-actinin may be present in chromaffin granules.

The presence of an α-actinin-like protein has been shown in chromaffin granule membranes by SDS-PAGE (Trifaró et al., 1982; Bader & Aunis, 1983). This protein corresponds to a minor component of the granule membranes that can be removed from them under conditions described for α-actinin extraction. In addition, this component appears to be localized on the external surface of the granule membrane as determined by pronase treatment of these granules (Aunis et al., 1980; Bader & Aunis, 1983). This protein, which possesses a molecular weight of 97,000 and an isoelectric point of 6.4, was specifically recognized by α-actinin antibodies (Aunis et al., 1980; Trifaró et al., 1985).

The above-mentioned studies have demonstrated the presence of α-actinin in chromaffin cells and its association with chromaffin granule membrane. Thus, it was suggested that α-actinin may be involved in the interaction of actin with the chromaffin granules (for review see Trifaró et al., 1985).

H.3.4 Chromaffin Cell Spectrin (Fodrin)

The presence of a spectrin-like protein has been shown in chromaffin granule membranes, and it appears to be localized on the cytoplasmic surface of the granule. This protein is composed of two subunits of molecular

weights of 240,000 (α) and 235,000 (β). However, by two-dimensional immunoblotting, only the 240kD-subunit (pI = 5.5) was recognized by a human erythrocyte spectrin antibody (Aunis & Perrin, 1984).

Indirect immunofluorescence studies have demonstrated the presence of a spectrin-like protein in cultured chromaffin cells (Aunis & Perrin, 1984; Perrin & Aunis, 1985). When a human erythrocyte spectrin antibody was used, this protein was shown to be distributed close to the cell membrane and also in the cytoplasm (Aunis & Perrin, 1984). Using immunoaffinity-purified bovine brain membrane α-fodrin antibody, an intensely stained, continuous ring in the subplasmalemmal region of chromaffin cell was observed. In contrast, when a β-fodrin antibody was used, no staining of chromaffin cells was detected (Perrin & Aunis, 1985). In addition, fodrin patch formation was observed at the cell surface when the cells were stimulated with different secretagogues in the presence of calcium. Furthermore, this aggregation was inhibited by trifluoperazine, and thus, it was suggested that the initiation of patch formation following cell depolarization is a calmodulin-dependent event (Perrin & Aunis, 1985).

Viscometry studies have revealed that a spectrin-like protein may be involved in the binding of F-actin to chromaffin granule membranes. The actin-binding capacity of the secretory granules was partially inhibited (50%) when granule membranes were treated with a nonionic detergent (Kyro EOB), a condition which was shown to remove the spectrin-like protein from these membranes (Aunis & Perrin, 1984).

These biochemical and immunohistochemical studies have demonstrated the presence of a spectrin-like protein in chromaffin cells, its association

with chromaffin granule membranes and the formation of fodrin patches. Consequently, it was suggested that a spectrin-like protein may be involved in the interaction of actin with the chromaffin granules (Trifaró et al., 1985) and also that this protein might be involved in granule movement in chromaffin cells (Perrin & Aunis, 1985).

H.3.5 Microtubules in Chromaffin Cells

The presence and distribution of tubulin subunits in cultured chromaffin cells have been determined by indirect immunofluorescence using brain tubulin antibodies. Chromaffin cells that have just recently been maintained in culture (one- or two-day-old) appear to possess a microtubule network localized in the cell periphery. In addition, a great amount of immunoreactive material was also present around the nucleus and in some cases at the level of the plasma membrane as revealed by an intense fluorescence staining. In older cultured chromaffin cells (~1-week-old), a partly different distribution pattern was observed with staining in neurites and their endings, although intense fluorescence persisted around the nucleus. Therefore, it was suggested that microtubules extend from the nuclear region towards the neurites where the microtubules form a network along the neurites up to their terminal structures (Bader et al., 1981, 1984a).

Immunohistochemical studies conducted in cultured chromaffin cells have demonstrated that following treatment with colchicine, a known microtubule-depolymerizing agent, there is a complete disappearance of the microtubule network which is accompanied by a concomitant cell process retraction. In

parallel, chromaffin granule distribution studies performed by indirect immunofluorescence using chromogranin A antibodies have shown that following treatment with this agent, there is an accumulation of granules in the cell body, in contrast to their usual localization along the neurites and particularly in the neurite endings (Bader et al., 1981, 1984a). Therefore, it was suggested that microtubules play an important role in secretory granule transport and also in neurite outgrowth and maintenance.

The presence of high affinity tubulin-binding sites has been demonstrated on chromaffin granule membranes by radiolabelling techniques. The binding of radioactive tubulin was shown to be temperature dependent, reversible and saturable. In addition, tubulin-binding sites were also observed on plasma and mitochondria membranes (Bernier-Valentin et al., 1983; Bader et al., 1984a). These observations suggest that there may exist an association between microtubules and chromaffin granules.

Recently, MAP2, a microtubule-associated protein, has been shown to be present in cultured chromaffin cells by immunocytochemistry using a monoclonal MAP2 antibody. MAP2 immunoreactivity was found to be distributed in the cell body, processes and varicosities of the chromaffin cells. In addition, MAP2 polypeptides were detected by immunoblotting in adrenal medullary homogenates but not in chromaffin granule membranes (Burgoyne & Norman, 1985).

These immunological studies have demonstrated the presence of microtubules and the microtubule-associated protein MAP2. It has been suggested that microtubules are probably involved in the transport of chromaffin granules to the cell periphery. However, they do not appear to be implicated in

the final stages of catecholamine secretion from chromaffin cells (for review see Trifaró et al., 1985; also Trifaró et al., 1972).

H.3.6 Neurofilaments in Chromaffin Cells

The presence of neurofilaments in chromaffin cells has been investigated by immunological techniques using specific antibodies raised against the 200kD-, 145kD- and 68kD-subunits of rat neurofilament preparation.

Indirect immunofluorescence studies in cultured chromaffin cells have revealed the presence of the three neurofilament subunits which appear to be assembled into intact filaments since similar staining patterns were observed for the three of them. In addition, very thin and highly ramified filaments were also observed around the nucleus and specifically localized on one side of the nuclear region. However, no staining was observed on the nucleus itself nor at the level of the plasma membrane and almost none in the neurites and their endings (Bader et al., 1984b). Therefore, it was suggested that intact neurofilaments elongate from one side of the nuclear region and form a network around the nucleus.

Immunoprecipitation experiments performed on solubilized extracts of ^{35}S-labelled cultured chromaffin cells have shown that the three neurofilament antibodies specifically immunoprecipitate three polypeptides with molecular weights of 210,000, 160,000 and 70,000 as determined by SDS-PAGE. These polypeptides correspond in terms of molecular weight to the neurofilament triplet of bovine brain. Two-dimensional gel electrophoresis separation of chromaffin cell cytoskeleton has also revealed the presence of the

210kD-, 160kD- and 70kD-polypeptides which possess isoelectric point values of 5.5, 5.4 and 5.3, respectively, pI values which correspond to the ones of rat neurofilament subunits (Bader et al., 1984b).

The presence of a 70kD protein in freshly isolated chromaffin cells has also been observed by immunoblotting using a specific 68kD-neurofilament subunit antibody. Thus, it was suggested that neurofilaments are not only present in cultured chromaffin cells but also in the adrenal gland in vivo (Bader et al., 1984b).

H.3.7 Possible Involvement of Contractile Proteins in the Secretory Process of Chromaffin Cells

Several contractile proteins and cytoskeletal components have been detected in chromaffin cells and in certain cases isolated and characterized extensively. Their distribution has been examined in these cells by various techniques. Some of these components are widely distributed in the cell while others appear to be specifically localized in certain cellular areas such as the nuclear region, subplasmalemma, cell processes and growth cone-like structures or found associated to some cytoskeletal networks. In addition, some of these contractile proteins have been shown to be components of, associated to or interacting with subcellular organelle membranes or the plasma membrane.

The presence of contractile proteins and cytoskeletal components as well as the regulatory protein calmodulin in chromaffin cells and, in particular, their interaction with chromaffin granules have led several investigators to

postulate that these components may be involved in the secretory process of chromaffin cells. However, most of the studies where this hypothesis was discussed dealt with the possible involvement of only some specific components in this process. It was only recently that Trifaró and collaborators have published an extensive review concerning the chromaffin cell cytoskeleton and its possible role in secretion (Trifaró et al., 1985).

In this recent review, two molecular mechanisms of action for the cytoskeletal proteins possibly involved in the secretory process were discussed. The leading hypothesis was that exocytosis is a true contractile event and therefore, that cytoskeletal components and their regulatory proteins might be involved in the mechanism of transport of secretory granules to the plasma membrane, in the fusion process or in the extrusion phenomenon itself. The two mechanisms of action proposed for the intervention of contractile proteins are described briefly:

i) The first mechanism of action involves the contractile proteins actin, myosin and α-actinin as well as the regulatory protein calmodulin. Similarly to muscles, a sliding filament system would be operative in chromaffin cells. Therefore, myosin should be arranged in bipolar filaments and actin-binding sites should be present on both granule and plasma membranes. In this case, α-actinin present in granule membranes would provide binding sites for actin while an anchorage protein (?) would play a similar role in the plasma membrane. As demonstrated previously, cell depolarization induces Ca^{2+} entry into the cell which results in a transient increase in intracellular Ca^{2+}. These events, in turn, would activate the sliding filament mechanism via a calmodulin-regulated activity. At the molecular level in

nonmuscle cells, this implies the activation of myosin ATPase by actin via the phosphorylation of the 20kD-myosin light chain which is regulated by calmodulin.

ii) The second mechanism of action is based on the viscosity properties of actin and does not necessarily involve myosin (see Fig. 12). In this case, actin would control cytosol viscosity through the formation of a microfilament network which would be cross-linked and stabilized by the chromaffin granule α-actinin and spectrin. These events appear to be regulated by Ca^{2+} as demonstrated in recent studies. Therefore, under resting conditions (10^{-8}M free Ca^{2+}), the cytosolic actin network would oppose the movement of secretory granules towards the release sites at the level of the plasma membrane (see Fig. 12A). In contrast, upon cell stimulation which results in an increase of intracellular Ca^{2+} (10^{-6}M free Ca^{2+}), there would be a partial disassembly of the actin filament network via a direct action of Ca^{2+} or through the activation of a specific protein (gelsolin?). In addition, actin filaments would dissociate from secretory granule and plasma membrane spectrins under these conditions. Consequently, there would be a decrease in cytosolic viscosity which would allow the movement of chromaffin granule towards the plasma membrane (see Fig. 12B). The regulatory protein calmodulin could be involved in the modulation of these events or alternatively, may be involved in the granule-plasma membrane fusion process itself since high affinity calmodulin-binding sites appear to be present in granule membranes (Burgoyne et al., 1982; Trifaró et al., 1985).

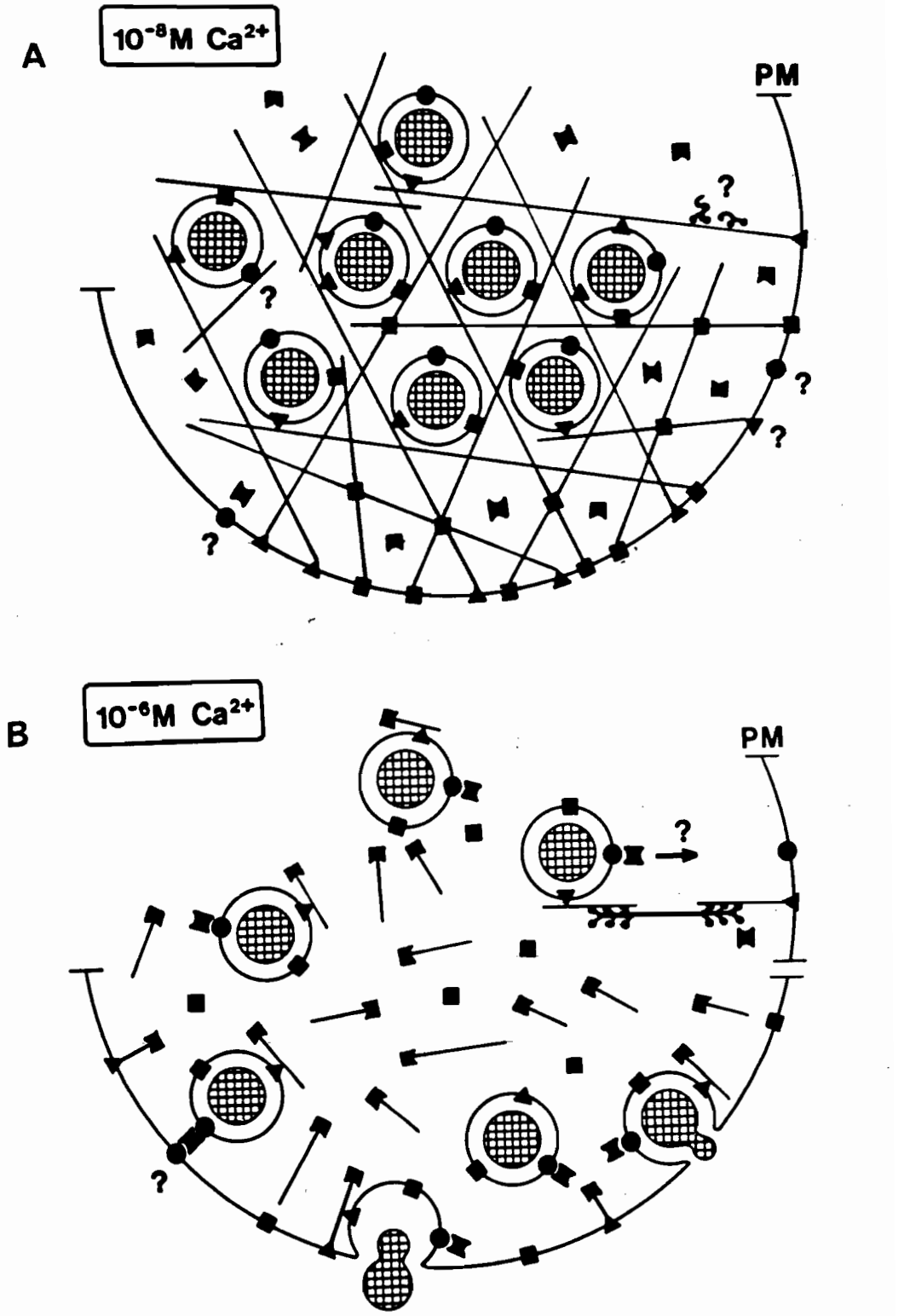
The two mechanisms of action described above are substantiated by numerous studies conducted in chromaffin cells. However, they do not exclude the

Fig. 12 Schematic representation of a possible mechanism in which cytoskeletal and regulatory proteins may be involved in chromaffin cell secretion.

A) Under resting conditions ($Ca_i^{2+} \sim 10^{-8}M$), actin would control the cell viscosity via the formation of a microfilament network. This network would be cross-linked and stabilized by both spectrin (cytosolic and membrane-bound) and by α-actinin present in granule membranes and possibly in the plasma membrane. In addition, the chromaffin granules contain a calmodulin-binding protein which might also be present in the plasma membrane. Furthermore, most of the myosin would be present in a nonfilamentous and nonphosphorylated form.

B) Following cell stimulation ($Ca_i^{2+} \sim 10^{-6}M$), Ca^{2+} would produce a dissociation of actin from spectrin and the activation of gelsolin which would result in the capping and shortening of actin filaments. However, the binding of actin to granule membrane α-actinin does not appear to be affected. Consequently, the cytosolic viscosity should decrease and allow the free movement of granules towards the plasma membrane. In addition, under these conditions, Ca^{2+} could activate some calmodulin-dependent processes such as binding of calmodulin to granule membranes and myosin phosphorylation (actin-myosin sliding mechanism?).

[Taken from Trifaró, Bader & Doucet, 1985]


A
10⁻⁸M Ca²⁺
PM
?
?
?
?
?
B
10⁻⁶M Ca²⁺
PM
?
?
α-actinin
spectrin
calmodulin-binding protein
myosin (nonphosphorylated)
myosin (phosphorylated bipolar filaments)
calmodulin
gelsolin
actin

possibility that other cytoskeletal and regulatory components might be involved in some steps of the secretory process. For instance, an ankyrin-like protein could be involved in the anchorage of actin microfilaments at the level of the plasma membrane, or the contractile protein tropomyosin might interact with the microfilament network and in some way modulate the association of some cytoskeletal components with this filamentous system (see discussion section of this thesis). In addition, the phosphorylation of some cytoskeletal components may provide a regulatory mechanism for their interaction with other cellular components or structures (see discussion section of this thesis). Furthermore, other regulatory proteins such as caldesmon, which has been recently detected in chromaffin cells, might provide an alternative regulatory mechanism ("flip-flop" mechanism) for the interaction of calmodulin-binding proteins with microfilaments or the actin-myosin interaction (Sobue et al., 1985b, 1985c).

I. STATEMENT OF THE PROBLEM

The process of protein phosphorylation and dephosphorylation appears to be a major general mechanism by which intracellular events respond to external physiological stimuli. In almost all tissues and cell systems, this general process has been shown to regulate numerous cellular events and has been implicated in several others. Changes in the state of phosphorylation of specific proteins has been implicated in the secretory process of nervous tissues as well as in numerous secretory cell systems including the adrenal medullary chromaffin cells.

The process of protein phosphorylation in the adrenal medulla and in isolated chromaffin cells has been examined by a certain number of investigators. However, most of these studies have focused either on a specific polypeptide (tyrosine hydroxylase) or phosphoproteins related to specific subcellular fractions. In the two previous phosphorylation studies carried out in cultured chromaffin cells, only two polypeptides (56-60kD and 95-99kD) were shown to be affected in their degree of phosphorylation in response to different secretagogues (Holz et al., 1980; Amy & Kirshner, 1981). In view of the facts that protein phosphorylation is a general mechanism which is involved in several cellular events and possibly in the secretory process of chromaffin cells, it would be of great interest to determine the general pattern of phosphorylation in chromaffin cells both under resting conditions and following cell stimulation. By using appropriate experimental protocols and analytical techniques, these phosphorylation studies could reveal the molecular characteristics and possibly the identity of chromaffin

cell phosphoproteins as well. Such studies may also provide some insights concerning the polypeptides which may be implicated in the secretory processes of this cell system. In addition, since Ca^{2+} and the regulatory protein calmodulin have been shown to regulate several cellular functions and also appear to be involved in the secretory process of chromaffin cells (Trifaró et al., 1985), it would be important to determine whether the phosphorylation of some of the chromaffin cell proteins is regulated by a Ca^{2+}/calmodulin-dependent system.

The process of secretion shares many common characteristics with the process of excitation-contraction coupling of muscle and consequently was termed stimulus-secretion coupling (Douglas & Rubin, 1961). It has been postulated that some type of contractile events might be involved in the secretory process of adrenal medullary chromaffin cells (Poisner & Trifaró, 1967; Douglas, 1968). Some contractile proteins and cytoskeletal components have been isolated and characterized from adrenal chromaffin cells. In addition, some of these components have been found to be associated with the chromaffin granules. These findings as well as others support the view that some contractile elements may be involved at some steps of the secretory process of chromaffin cells (for recent reviews see Trifaró et al., 1984a, 1985).

Another contractile protein, namely tropomyosin, has been shown to be present in a wide variety of tissues including several nonmuscle cell systems. The presence of tropomyosin has been expected in adrenal chromaffin cells (Trifaró, 1978) although no study has demonstrated so far that this contractile protein is indeed present in this cell system. In view of the fact that tropomyosin was shown to interact with actin filaments, components

which are present in chromaffin cells, and from studies in nonmuscle cells which indicate that tropomyosin may modulate the interaction of actin with other cytoskeletal components (for reviews see Côté, 1983; Payne & Rudnick, 1984, 1985), it would be of interest to establish if tropomyosin is indeed present in adrenal chromaffin cells and if so, to determine its molecular characteristics as well as its functional properties. In addition, since the phosphorylation of some cytoskeletal components appears to regulate their interaction with other proteins and in view of the fact that tropomyosin has been shown to be phosphorylated in some cell systems (Payne & Rudnick, 1985), it would be interesting to determine whether or not tropomyosin is phosphorylated in this cell system.

The studies described in this thesis were carried out for the following purposes:

1. To establish the general patterns of phosphorylation in cultured chromaffin cells under resting conditions and following cell stimulation.
2. To determine some of the biochemical characteristics (MW, pI) of these chromaffin cell phosphoproteins in an attempt to establish their nature or relate them to previously described proteins in chromaffin cells and also in other systems.
3. To provide some insights concerning the enzymes which might affect the state of phosphorylation of these chromaffin cell polypeptides. In this regard, the effects of Ca^{2+} deprivation and calmodulin antagonists were examined.

4. To examine if the process of phosphorylation of some of the chromaffin cell polypeptides might be temporarily associated with the onset of catecholamine secretion.
5. To determine if tropomyosin is indeed present in adrenal medulla and in chromaffin cells.
6. If tropomyosin is present in this system, to determine its molecular and functional properties in order to compare adrenal tropomyosin to its counterpart in muscle and nonmuscle cell systems.
7. To examine the intracellular distribution of tropomyosin in cultured chromaffin cells by immunohistochemistry.
8. To determine if tropomyosin is phosphorylated in cultured chromaffin cells and if its level of phosphorylation is affected by cell stimulation.

Large portions of the results presented in this thesis have been communicated at meetings (abstracts) or published in the form of full papers:

Papers

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Côté A., Doucet J.P. and Trifaró J.M. (1986) Phosphorylation and dephosphorylation of chromaffin cell proteins in response to stimulation. Neuroscience (in press).

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Côté A., Doucet J.P. and Trifaró J.M. (1984) Adrenal medullary tropomyosin. Int. Symp. Mol. Biol. Periph. Catechol. Stor. Tiss., 100.

Trifaró J.M., Bader M.F., Côté A., Hikita T. and Kenigsberg R.L. (1984) Chromaffin cell calmodulin: Does it play a role in secretion? Int. Symp. Mol. Biol. Periph. Catechol. Stor. Tiss., 90.

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CHAPTER 2

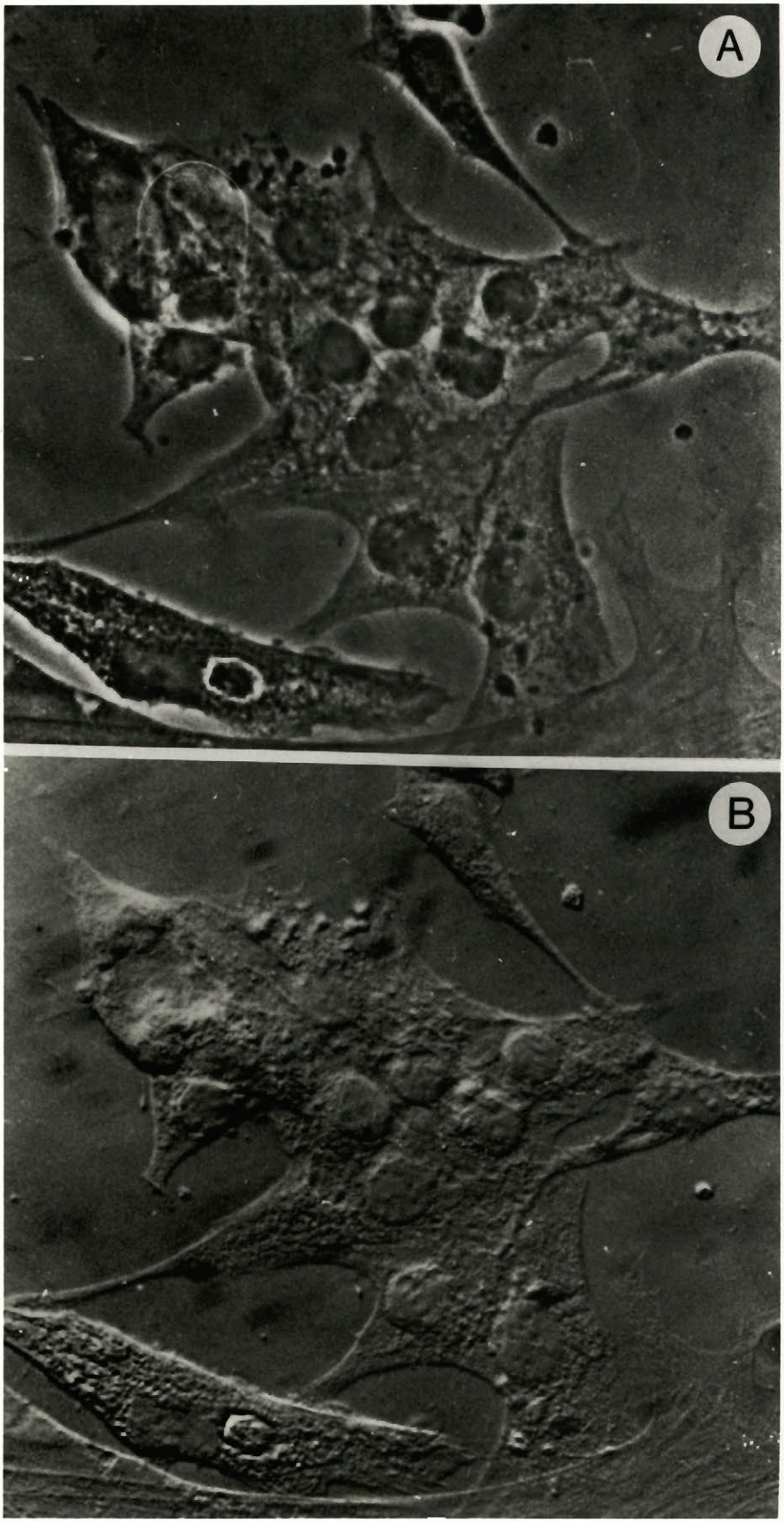
EXPERIMENTAL PROCEDURES

A. CHROMAFFIN CELL CULTURES

Bovine adrenal glands were obtained from a local slaughterhouse, freed from their cortices and perfused with Ca^{2+}/Mg^{2+}-free Locke's solution as described by Trifaró et al. (1967). The glands were subsequently perfused with Locke's solution containing 0.05% collagenase and 0.001% DNAse I, and then chromaffin cells were isolated as described by Trifaró et al. (1978). The isolated cells were further purified by centrifugation on a self-generating Percoll density gradient. Purified chromaffin cells were plated on collagen-coated plastic dishes at densities of 1×10^6 cells/35 mm or 5×10^6 cells/85 mm diameter dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 14.7 mM HEPES (pH 7.4), 5.6 mM glucose, penicillin (100 µg/ml), streptomycin (100 µg/ml), gentamycin (50 µg/ml) and mycostatin (25 units/ml). 5-Fluorodeoxyuridine (10^{-5}M) and cytosine arabinoside (10^{-5}M) were included in the medium to prevent fibroblast proliferation. Cell cultures were maintained at 37°C under a CO_2 + air (5% + 95%) atmosphere in a humidified incubator (National).

In all these studies, the viability and morphology of the cultured chromaffin cells were assessed by examining the cultures under the light microscope by phase contrast (see Fig. 13). The catecholamine content of these chromaffin cells was well maintained even after keeping them in culture for relatively long periods. In addition, their ability to accumulate exogenous amine and the release pattern of [^{3}H]noradrenaline, which parallels the one of endogenous amine, were as reported previously (Kenigsberg & Trifaró, 1980; Trifaró & Lee, 1980).

Fig. 13 Seven-day-old bovine adrenal medullary chromaffin cells maintained in culture. A group of several chromaffin cells is seen in the center. Several of these cells show long processes which in some cases have established contact with other cells. Phase contrast (A), Nomarski optics (B). Magnification: × 560.


A
B

B. PHOSPHORYLATION STUDIES IN CULTURED CHROMAFFIN CELLS

B.1 Phosphorylation in Response to Acetylcholine

Nine-day-old to 13-day-old cultured chromaffin cells (5×10^6 cells/dish) were incubated with 7.5 ml of a low phosphate (0.4 mM) DMEM supplemented with 10% fetal calf serum, 14.7 mM HEPES (pH 7.3) and 5.6 mM dextrose containing 250 µCi [^{32}P]orthophosphate (NEN) for 1 h at 37°C under a CO_2 + air (5% + 95%) atmosphere. The medium was removed and the cells were washed twice rapidly (30 sec/wash) with 7.5 ml of a low phosphate Locke's solution containing (mM): NaCl, 154; KCl, 5.2; K_2HPO_4, 0.287; KH_2PO_4, 0.113; $MgCl_2$, 1.2; $CaCl_2$, 2.2; HEPES, 10; dextrose, 5.6 and phenol red 15 µg/ml; pH 7.2 (buffer A). Then the cells were incubated with 7.5 ml of buffer A containing ACh (10^{-4}M) and 250 µCi [^{32}P]orthophosphate for 3 min at room temperature. Finally, the buffer solution was removed, and the cells were scraped off the culture dish with 1 ml of an incubation-stopping solution containing 2 mM (-)p-bromotetramisole oxalate, 1 mM EDTA, 0.2 mM PMSF and 6% SDS. The extract was heated at 100°C for 2 min and centrifuged at 12,000 g (Microfuge, Brinkmann) for 10 min at room temperature. Following centrifugation, the DNA aggregate was removed and the sample frozen immediately at -80°C.

In the case of the ACh stimulation time course experiments, the protocol was basically as described above though incubation periods in the presence of ACh were of 30 sec, 1 min, 2 min and 3 min. Control experiments were as those described above with the exception that ACh was omitted during the 3 min incubation period.

B.2 Phosphorylation in Response to a Depolarizing Concentration of K^+

Basically, the phosphorylation protocol was as that described above for the phosphorylation in response to ACh. However, in this case, the 3 min incubation period was carried out in the presence of a high potassium (56 mM) low phosphate Locke's solution. In this 56 mM K^+-Locke's solution, the NaCl was reduced by an equivalent amount (50.4 mM).

B.3 Phosphorylation in the Presence of Trifluoperazine

Cultured chromaffin cells were incubated with 7.5 ml of low phosphate DMEM containing 250 µCi [^{32}P]orthophosphate for 40 min at 37°C under a CO_2/air atmosphere. The medium was removed and the cells were washed twice rapidly with 7.5 ml of low phosphate Locke's solution (buffer A, described in section B.1). Then, the cells were incubated with 7.5 ml of buffer A containing TFP (2×10^{-7}M) and 250 µCi [^{32}P]orthophosphate for 20 min at 37°C. After this pre-incubation period, the solution was replaced and the cells incubated with 7.5 ml of buffer A containing TFP (2×10^{-7}M), ACh (10^{-4}M) and 250 µCi [^{32}P]orthophosphate for 3 min at room temperature. The rest of the procedure was identical as that described above under "Phosphorylation in Response to Acetylcholine" (section B.1). Control experiments were performed the same way as described above. However, in this case, the ACh was omitted during the 3 min incubation period.

B.4 Phosphorylation in the Presence of a Low Calcium Concentration

Cultured chromaffin cells were incubated with 7.5 ml of low phosphate DMEM containing 250 μCi [^{32}P]orthophosphate for 45 min at 37°C under a CO_2/air atmosphere. The medium was removed, and the cells were washed twice rapidly with 7.5 ml of low phosphate Locke's solution (buffer A, described in B.1). Then the cells were incubated with 7.5 ml of low Ca^{2+}/high Mg^{2+}-low phosphate Locke's solution containing (mM): NaCl, 154; KCl, 5.2; K_2HPO_4, 0.287; KH_2PO_4, 0.113; $MgCl_2$, 15; $CaCl_2$, 0.5; HEPES, 10; dextrose 5.6 and phenol red 15 μg/ml; pH 7.2 (buffer B) for 15 min at 37°C. Then, the cells were incubated with 7.5 ml of buffer B containing ACh (10^{-4}M) and 250 μCi [^{32}P]orthophosphate for 3 min at room temperature. The rest of the procedure was identical to that described under "Phosphorylation in Response to Acetylcholine" (section B.1). Control experiments were performed as those described above. However, in this case, ACh was omitted during the 3 min incubation period.

B.5 Detection and Quantitation of Phosphoprotein Bands

Dried gels were placed in Cronex cassettes equipped with Dupont Lightning Plus screens against Kodak X-Omat AR (XAR-5) film for 2 to 4 days at -80°C. The intensity of the bands in the autoradiograms was measured by scanning each well with a transmission densitometer (model EC910, E-C Apparatus Corp., Philadelphia, PA). Scans of gels from control and treated cells were obtained (e.g. Fig. 16), and the height above the baseline was

measured for 23 distinct peaks in the region of molecular weights between 313,000 and 14,000. The ratio of the height of each peak (control and treated cell preparations) to the sum of the 23 peak heights (100%) was determined for each well to correct for minor variations in the amount of sample applied on the gel (experiments performed on the same day included at least triplicates of each condition). The numbers shown in the tables represent the differences (Δ) between control and treated cells expressed as Δ% control.

C. ISOLATION AND CHARACTERIZATION OF TROPOMYOSINS

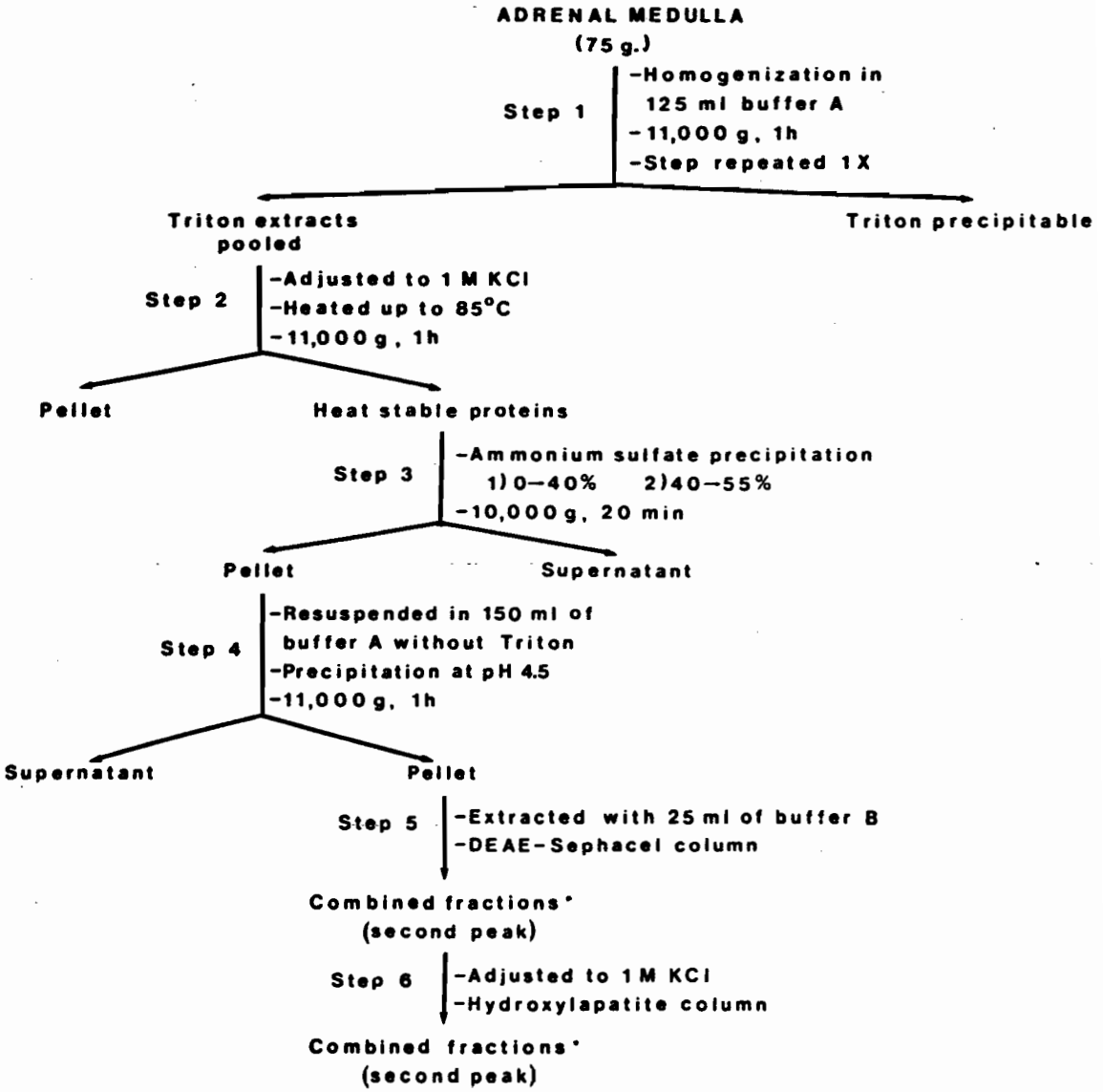
C.1 Purification of Tropomyosins

C.1.1 Adrenal Medullary Tropomyosins

The isolation and purification procedure of bovine adrenal medullary tropomyosins, which is a combination of two previously described techniques (Côté & Smillie, 1981a; Barylko & Sobieszek, 1983), is outlined in Fig. 14.

Bovine adrenal glands were obtained from a local slaughterhouse, and the medullae were freed from their cortices. All subsequent steps were carried out at 4°C unless otherwise mentioned in the text. Approximately 75 g of medulla were washed rapidly twice with 125 ml of 150 mM NaCl containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The medullae were cut in small pieces and suspended in 125 ml of buffer A containing (mM): KCl, 40; $MgCl_2$, 1; dithiothreitol (DTT), 5; imidazole, 10; PMSF, 0.2 and 1% (wt/vol) Triton X-100, pH 6.8. The resulting material was homogenized at high speed in a Waring blender for 30 s followed by disruption with a Potter homogenizer with a type C pestle. The first extract was obtained by centrifugation at 24,000 g for 1 h. The extraction procedure was repeated once more with the same volume of buffer A, and the two supernatants were combined. After addition of solid KCl to 1.0 M, the pooled extracts were heated in a boiling water bath until the temperature of the sample reached 85°C. After cooling of the preparation on an ice bath, the precipitated proteins were removed by centrifugation at 24,000 g for 30 min. The soluble fraction was brought to

Fig. 14 Outline of the procedure followed in the purification of bovine adrenal medullary tropomyosins. Step 1: washed adrenal medullae (about 75 g) were homogenized in 125 ml of 40 mM KCl, 1 mM $MgCl_2$, 5 mM DTT, 0.2 mM PMSF, 10 mM imidazole and 1% Triton X-100, pH 6.8 (buffer A) in a Waring blender. This was followed by further disruption of the material in a Potter homogenizer. This detergent extraction step was repeated one more time. Step 2: the Triton X-100 extracts were adjusted to 1 M KCl and heated to 85°C in a boiling water bath. Step 3: the supernatants were submitted to two successive ammonium sulfate fractionations (0-40% and 40-55%). Step 4: the pellets obtained after the second ammonium sulfate cut were resuspended in 250 ml of buffer A devoid of Triton X-100 and a crude tropomyosin fraction was obtained by precipitation at pH 4.5, 0°C for 1 h. Step 5: this fraction was first extracted with 25 ml of 150 mM KCl, 5 mM DTT, 10 mM imidazole, 0.2 mM PMSF, pH 7.0, then diluted to a final concentration of KCl of 100 mM. This was followed by fractionation on a DEAE-Sephacel column using a two step KCl gradient (250 mM KCl and 350 mM KCl). Step 6: the fractions enriched in tropomyosins (350 mM KCl eluted peak) were combined, adjusted to 1 M KCl and finally fractionated on a hydroxylapatite column using a linear phosphate gradient (1 to 200 mM phosphate). All the steps were performed at 4°C.


ADRENAL MEDULLA
(75 g.)
Step 1
-Homogenization in 125 ml buffer A
-11,000 g, 1h
-Step repeated 1X
Triton extracts pooled
Triton precipitable
Step 2
-Adjusted to 1 M KCl
-Heated up to 85°C
-11,000 g, 1h
Pellet
Heat stable proteins
Step 3
-Ammonium sulfate precipitation
1) 0–40% 2) 40–55%
-10,000 g, 20 min
Pellet
Supernatant
Step 4
-Resuspended in 150 ml of buffer A without Triton
-Precipitation at pH 4.5
-11,000 g, 1h
Supernatant
Pellet
Step 5
-Extracted with 25 ml of buffer B
-DEAE-Sephacel column
Combined fractions*
(second peak)
Step 6
-Adjusted to 1 M KCl
-Hydroxylapatite column
Combined fractions*
(second peak)

40% saturation by addition of solid ammonium sulfate and was allowed to stir for 20 min. The precipitate was removed by centrifugation at 20,000 g for 20 min, and the supernatant was then brought to 55% saturation by addition of solid ammonium sulfate. The precipitate recovered by centrifugation at 20,000 g for 20 min was redissolved in 250 ml of buffer A but devoid of Triton X-100. The pH of the solution was adjusted to 4.5, and the suspension was then kept on ice for at least 1 h (or overnight). The resulting precipitate was collected by centrifugation at 20,000 g for 1 h. The pH 4.5 precipitate was then extracted with 25 ml of buffer B containing (mM): KCl, 150; DTT, 5; imidazole, 10 and PMSF, 0.2; pH 7.0. After centrifugation at 10,000 g for 1 h the supernatant was adjusted to 100 mM KCl in buffer C containing (mM): DTT, 5; imidazole, 10 and PMSF, 0.2; pH 7.0. After applying the sample to the DEAE-Sephacel column (5.0 × 1.6 cm), the column was washed with 25 ml of 100 mM KCl in buffer C, and the proteins were then eluted with a stepwise gradient consisting of 60 ml of 250 mM KCl in buffer C followed by 40 ml of 350 mM KCl in buffer C. The fractions eluted with the 350 mM KCl buffer solution were combined, and the concentration of KCl was then adjusted to 1.0 M. The preparation thus obtained was applied to a column (3.0 × 1.6 cm) of hydroxylapatite (Bio-Gel HTP) which had been previously equilibrated with buffer D containing (mM): KCl, 1,000; DTT, 2; imidazole, 10 and PMSF, 0.2; pH 7.0. The column was then eluted with a linear phosphate gradient generated from solutions consisting of 50 ml of 1 mM sodium phosphate and 50 ml of 200 mM sodium phosphate, both in buffer D.

C.1.2 Cultured Chromaffin Cell Tropomyosin

Chromaffin cell tropomyosin was partially purified from cultured cells basically as described for the isolation of adrenal medullary tropomyosin (section C.1.1). However, a protein-carrier fraction was added to this preparation in order to maximize the recovery of protein.

Nine-day-old cultured chromaffin cells (5×10^6 cells/dish) were washed twice with 7.5 ml of Locke's solution. Then the cells were scraped off the culture dish with 1 ml of an ice-cold extraction solution containing 1 mM EDTA, 0.2 mM PMSF and 1% (vol/vol) Triton X-100 in 20 mM sodium phosphate buffer (pH 7.0).

After a 20 min incubation period on ice in the extraction solution, each cell suspension (5×10^6 cells) was centrifuged at 12,000 g (Microfuge) for 60 s at 4°C. All the supernatants were combined (20 culture dishes, 100×10^6 cells) and adjusted to 1 M KCl. Then the combined supernatant was proceeded for tropomyosin isolation as described in section D.1. However, a protein-carrier fraction was added to this preparation before the isoelectric precipitation step. This protein-carrier was obtained from 30-40kD polypeptide-free fractions of the first peak eluted from the DEAE-Sephacel column during adrenal medullary tropomyosin isolation.

The partially purified chromaffin cell tropomyosin fraction obtained from DEAE-Sephacel chromatography was then applied in parallel with purified adrenal medullary tropomyosins onto an SDS-polyacrylamide gel. Following electrophoresis, the gel was processed for silver staining.

C.2 Interaction of Adrenal Medullary Tropomyosins with F-Actin

Purified actin was obtained from a crude actin preparation by gel filtration chromatography on a Sephadex G-200 column according to previously described procedures (Carsten & Mommaerts, 1963; Rees & Young, 1967).

Hydroxylapatite-purified tropomyosin fraction was dialyzed overnight at 4°C against 6 mM β-mercaptoethanol, 0.02% NaN_3, 0.2 mM PMSF and 2 mM Tris-HCl (pH 7.6). Purified chicken gizzard actin was clarified by centrifugation and polymerized for 2 h at 22°C in a buffer containing 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM ATP, 0.15 mM $CaCl_2$, 6 mM β-mercaptoethanol and 2 mM Tris-HCl (pH 7.6). The tropomyosin fraction (0.40 mg/ml) was mixed with F-actin (0.60 mg/ ml) to give final concentrations of 0.36-2.47 μM of tropomyosin fraction and 4.5 μM of actin in a 50 μl volume in a buffer containing 50 mM KCl, 0.25 mM ATP, 0.075 mM $CaCl_2$, 6 mM β-mercaptoethanol, 0.1 mM PMSF, 0.01% NaN_3, 2 mM Tris-HCl (pH 7.6) in the presence or the absence of 10 mM $MgCl_2$. The adrenal medullary tropomyosin fraction concentrations were calculated considering the actual molar ratio of the different tropomyosins. The mixture was incubated 2 h at 22°C and then centrifuged 2 h at 20,000 g at room temperature to sediment the F-actin-tropomyosin complex. The resulting precipitates and supernatants were analyzed by 10% SDS-PAGE. Quantitative densitometric measurements were made with an E-C densitometer apparatus on gels stained with Coomassie blue as described in section E.1.4. The quantity of adrenal medullary tropomyosins was determined by densitometry of the Coomassie blue stained gels on the basis of standardization with the same purified medullary tropomyosin fraction used for the interaction study.

C.3 Phosphorylated Form of Tropomyosin in Cultured Chromaffin Cells

Ten-day-old cultured chromaffin cells (5×10^6 cells/dish) were labelled with [^{32}P]orthophosphate as described in section B.1. At the end of the labelling period, the cells were scraped off the culture dish with 1 ml of an ice-cold extraction solution containing 2 mM (-)p-bromotetramisole oxalate, 10 mM Na_2MoO_4, 40 mM NaF, 5 mM EGTA, 2 mM NaN_3, 20 mM β-glycerophosphate and 1% (vol/vol) Triton X-100 in a 20 mM sodium phosphate buffer (pH 7.0).

After a 1 min treatment with the extraction solution, each cell suspension (5×10^6 cells) was centrifuged at 12,000 g (Microfuge) for 60 s at 4°C. The supernatant and the sediment obtained were quickly frozen in a dry ice-acetone bath. All the supernatants were thawed rapidly, combined (12 culture dishes, 60×10^6 cells) and adjusted to 1 M KCl. Then, the combined supernatant was proceeded for tropomyosin isolation as described in section C.1. However, the two ammonium sulfate fractionations and the isoelectric precipitation were omitted to speed up the procedure in order to avoid loss of ^{32}P labelling.

The partially purified chromaffin cell tropomyosin fraction obtained from DEAE-Sephacel chromatography was then applied in parallel with purified adrenal medullary tropomyosins onto a first SDS-polyacrylamide gel. Following Coomassie blue staining, drying and autoradiography, the appropriate bands were cut out of the gel, swollen and applied onto a second SDS-polyacrylamide gel. Following electrophoresis, this second gel was processed for staining, drying and autoradiography.

C.4 Immunological Techniques

C.4.1 Tropomyosin Antibody

The antibody used in these double immunodiffusion (C.4.2) and immunofluorescence microscopy studies (C.4.3) was raised against smooth muscle (chicken gizzard) tropomyosin. The rabbit immune sera obtained were purified by affinity chromatography on a smooth muscle tropomyosin-conjugated column. The affinity-chromatography purified antibody obtained was characterized by double immunodiffusion, SDS-PAGE and immunoblotting (Talian et al., 1983). This antibody was shown previously to produce a characteristic tropomyosin staining pattern in chick embryo fibroblasts (Talian et al., 1983).

C.4.2 Immunodiffusion Studies

The Ouchterlony double immunodiffusion technique used was a modification of a previously described procedure adapted for contractile proteins (Ulpian, 1977). The procedure was carried out in petri dishes (5.5 cm diameter) containing an agarose matrix of the following composition: 1% agarose, 25 mM Tris-HCl (pH 7.4), 0.1% NaN_3 and 0.01% trypan blue. The different proteins were either dialyzed against or dissolved in a buffer containing 25 mM Tris-HCl (pH 7.4) and 0.1% NaN_3 and were adjusted at a concentration of 1 mg/ml. Tropomyosin antibodies were placed in the central well whereas the different antigens were applied in the peripheral wells. The dishes were kept for 48 h in a humidified atmosphere at room temperature until the precipitation lines

were clearly visible. Then the agarose gel was stained as described below.

C.4.3 Immunofluorescence Microscopy

Cells grown on collagen-coated glass coverslips (2.5×10^5 cells/coverslip) were washed with Locke's solution (mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 2.15; KH_2PO_4, 0.85; $MgCl_2$, 1.2; $CaCl_2$, 2.2; dextrose, 10.0 and HEPES, 5) several times and fixed for 20 min with Locke's solution containing 3.7% formaldehyde and 0.3% Triton X-100. The cells were subsequently permeabilized with ice-cold acetone solutions (50%, 100%, 50% in water) as previously described (Lee & Trifaró, 1981). The coverslips were then washed with PBS (25 mM phosphate buffer containing 150 mM NaCl, pH 7.4) and incubated for 15 min at room temperature in PBS containing normal goat IgG (2 mg/ml). The coverslips were then washed with PBS ten times, and 70 µl of affinity chromatography-purified smooth muscle tropomyosin antibody were applied. The coverslips were incubated for 60 min in a humid atmosphere at 37°C and washed extensively with PBS. Then, 70 µl of affinity chromatography-purified FITC-conjugated goat anti-rabbit IgG diluted 1:35 with PBS were added, and the coverslips were incubated as before for 60 min. After the incubation, the coverslips were washed ten times with PBS, and they were mounted cell side down in PBS containing 50% (vol/vol) glycerol, 0.05 M Tris buffer (pH 8.0) and 0.02 M DTT. In control experiments, the antiserum was replaced by either normal rabbit serum or antiserum preabsorbed with an excess of adrenal medullary tropomyosins. The preparations were examined with a Leitz Ortholux II microscope fitted with a 200 W/4 high pressure mercury lamp and

a Ploemopack incident light illuminator housing three exciting filters (one K455 filter and two KP490 filters), a TK510 dichroic beam-splitting mirror and a TK515 suppression filter. A X63 oil immersion objective was used and photographs were taken with Kodak Tri-X pan film (400 ASA).

D. CATECHOLAMINE RELEASE AND CALCIUM MOVEMENT STUDIES

D.1 Catecholamine Release

Catecholamine output was monitored basically as described previously (Kenigsberg & Trifaró, 1980; Trifaró & Lee, 1980) although some modifications were added in these studies.

Isotopic incubations

Six-day-old to 10-day-old cell cultures were used. The culture medium (DMEM) was removed, and cells were incubated for 10 min with 1 ml of the same medium but devoid of amino acids and antibiotics. Following this preincubation period, the cells were incubated for 5 min with 1 ml of the amino acid-free, antibiotic-free DMEM containing 10^{-7}M [^{3}H]noradrenaline ([^{3}H]NA: specific activity 3 μCi/0.1 mmole). Each dish (10^6 cells) was subsequently washed 6 times. The first 2 washes were carried out with 1 ml of the aforementioned amino acid-free, antibiotic-free DMEM, while the final washes were with 1 ml of regular Locke's solution of the following composition (mM): NaCl, 154; KCl, 2.6; K_2HPO_4, 2.15; KH_2PO_4, 0.85; $MgCl_2$, 1.2; $CaCl_2$, 2.2; and dextrose, 10; pH 7.2. Wash intervals were set constant at 10 min. Incubations and washings were carried out at room temperature.

In the case of the time course of catecholamine release in response to ACh stimulation, the protocol was as described above, although the last 4 washes were carried out with 1 ml of a low phosphate Locke's solution of the

following composition (mM): NaCl, 154; KCl, 5.2; K_2HPO_4, 0.287; KH_2PO_4, 0.113; $MgCl_2$, 1.2; $CaCl_2$, 2.2; HEPES, 10; dextrose, 5.6 and phenol red 15 µg/ml; pH 7.2.

Release of [^{3}H]noradrenaline

[^{3}H]NA output from the chromaffin cells was monitored in the following incubation media, (a) regular Locke's solution and (b) "high potassium" Locke's solution which contained 56 mM KCl and in which NaCl was reduced by an equivalent amount (50.4 mM). Catecholamine release was monitored from chromaffin cells that were stimulated by either ACh (10^{-4}M) or 56 mM K^+ in the absence or presence of TFP. The incubation medium was collected every 3 min. At the end of the incubation periods, the cells were harvested with 2 ml of 0.4 M perchloric acid, the cell suspension was spun at 10,000 g for 10 min and then the supernatant was collected. [^{3}H]NA was measured in both effluents and total cell extracts in a liquid scintillation spectrometer (SL 40 Intertechnique) using 15 ml of Aquasol (NEN) per vial as scintillation cocktail.

In the case of the time course of catecholamine release, [^{3}H]NA output from the chromaffin cells was monitored in incubation media collected from cells incubated in the presence or absence of ACh (10^{-4}M). In these studies, incubation medium was collected for 2 consecutive periods of the same duration (30 sec, 1 min, 2 min or 3 min).

D.2 Calcium Flux and Uptake Studies

Cultured chromaffin cells were washed with amino acid-free, antibiotic-free DMEM for 10 min followed by two 5 min washes with Ca^{2+}-free (20 mM) Hepes-buffered Locke's solution in the absence or presence of TFP or Ni^{2+}. The cells were subsequently incubated with radiolabelled calcium (^{45}Ca: 5 μCi/dish) in 2.2 mM Ca^{2+}-Locke's solution containing 56 mM K^+ in the absence or presence of TFP or Ni^{2+} for 5 min. The cells were then washed with Ca^{2+}-free Hepes-buffered Locke's solution containing 2 mM EDTA. Each wash interval was set constant at 5 min. The washes were collected and radioactivity within assayed in vials containing 15 ml of Aquasol by a liquid scintillation spectrometer. Once the washing was completed, the cells were collected by scraping them from the culture dishes with 2 ml of H_2O. One ml aliquots of the cell scrapings were assayed for radioactivity as in section D.1. Cellular calcium uptake was calculated by the peeling method (Aguirre et al., 1977; Kenigsberg & Trifaró, 1980). Extracellular calcium is removed in the first few washes, while in the latter washes intracellular calcium that had been taken up by the cells appeared in the wash (for details, see Aguirre et al., 1977). The actual amount of calcium that had been accumulated by the cells at the end of the labelling period can be obtained by extrapolating to zero time from the values obtained for the last seven washes (35-65 min).

In order to investigate whether TFP could perturb calcium efflux or exchange, the following experiment was carried out. Cultured chromaffin cells were loaded with ^{45}Ca in the presence of 56 mM K^+ as described above.

Subsequent to this loading period, the cells were washed for 75 min with Ca^{2+}-free (2 mM EDTA) Hepes-buffered Locke's solution. Wash intervals were set constant at 5 min. For the last 20 min of washing, one third of the dishes were exposed to TFP, one third to Ni^{2+} and the rest served as a control. Following these washings, Ca^{2+}-containing Locke's solution was applied for an additional 25 min followed again by Ca^{2+}-free Locke's solution in the absence or presence of TFP or Ni^{2+}. Radioactivity in the washing and cells was determined as described previously. The ^{45}Ca efflux rate coefficient for each wash interval was obtained from the following formula:

$$RC = \frac{C \times 100}{\bar{c}t}$$

where RC = rate coefficient; C = radioactivity collected in the medium during collection interval t and $\bar{c}$ = average ^{45}Ca content of cells during collection interval t.

E. GENERAL METHODS

E.1 Electrophoretic Techniques

E.1.1 One-Dimensional Gel Electrophoresis

Slab SDS-PAGE was carried out by a modification of a method described previously for continuous disc electrophoresis (Porzio & Pearson, 1977). Briefly, the running gel (12.5 cm long × 14 cm × 1.5 mm) consisted of 10% acrylamide and 0.1% bisacrylamide (ratio 100:1) [in 100 mM Tris, 300 mM glycine, 0.1 mM EDTA, 5% (vol/vol) glycerol and 0.1% SDS, pH 8.8], and the stacking gel (1.5 cm) consisted of a 4% acrylamide and 0.04% bisacrylamide [in 62.5 mM Tris-HCl, 4 mM EDTA, 5% (vol/vol) glycerol and 0.6% SDS, pH 6.7]. The stacking gel was polymerized by addition of 10.5 mM ammonium persulfate and 2.64 mM tetraethyl methylethylene diamine. The running buffer contained 50 mM Tris, 150 mM glycine and 0.1% SDS, pH 8.8. The samples were applied to the gel in a modified mixture used for contractile proteins (Yates & Greaser, 1983) which contained 8 M urea, 2 M thiourea, 50 mM Tris-HCl (pH 6.7), 100 mM DTT, 3% SDS and 0.01% bromophenol blue. The gel was then run at 50 V (constant voltage) overnight.

The urea/SDS-PAGE technique followed was identical to that used for the regular SDS-PAGE (see above) except that both running and stacking gels contained 8 M urea (Saborío et al., 1979).

E.1.2 Two-Dimensional Gel Electrophoresis

The two-dimensional gel electrophoresis technique used was a modification of previously described procedures (O'Farrell, 1975; Perdew et al., 1983; Bravo, 1984). Briefly, SDS-protein extracts were diluted with 1 volume of cold water and 9 volumes of cold acetone and cooled at -20°C overnight, then centrifuged at 12,000 g (Microfuge) at 4°C for 10 min and the pellets dried at 40°C for 20-30 min. The pellets were dissolved in a urea-detergent buffer consisting of 9.8 M urea, 2% CHAPS, 100 mM DTT, 1.5% ampholine (pH 3.5-10 LKB) and 0.5% ampholine (pH 5.0-8 LKB) (volume 70 µl). A sample of the protein extract was overlaid with a urea buffer containing 8 M urea, 5% β-mercaptoethanol and the same ampholine composition as above and then separated in the first dimension by electrofocusing on a pre-run slab gel containing 3.31% acrylamide, 0.19% bisacrylamide, 9.16 M urea, 2% CHAPS, 1.5% ampholine (pH 3.5-10) and 0.5% ampholine (5.0-8). Upper and lower tank buffers contained 0.1 M NaOH and 0.017 M H_3PO_4 respectively. The gel was run at 800 V (constant voltage) overnight and at 1100 V for the last hour. After the first dimensional PAGE, the slab gel was cut into thin slices, each containing all the proteins originally loaded into a single well of the gel. The appropriate slice was equilibrated for 10 min in the sample mixture for regular SDS-PAGE, then loaded for second dimension electrophoresis on a 10% SDS-polyacrylamide gel (see section E.1.1).

E.1.3 Limited Proteolytic Digestion in Gel Slices

Wet gel pieces from a 10% SDS-PAGE stained with Coomassie blue were digested with Staphylococcus aureus V8 protease according to Cleveland et al. (1977) with a few modifications. The gel pieces were first equilibrated in 0.1% SDS, 1 mM EDTA and 125 mM Tris-HCl (pH 6.8) for 20 min at room temperature. The solution was replaced by a minimum volume of a solution containing 0.1% SDS, 5% sucrose, 1 mM EDTA and 62.5 mM Tris-HCl (pH 6.8), and then the gel pieces were heated to 100°C for 2 min. Each gel piece with the accompanying incubation solution was transferred into the appropriate well of the stacking gel. Finally, 250 ng of protease were added to each well for proteolytic digestion.

For these experiments, the SDS-PAGE system described in section E.1.1 was used except that the stacking gel was 3 cm long and that the SDS concentration was reduced to 0.1%. The running gel was shortened to 10 cm, and the concentrations of acrylamide and bisacrylamide were increased to 17% and 0.17% respectively.

E.1.4 Gel Staining Procedures

Coomassie Blue Staining Procedure

The gels were fixed in 25% 2-propanol and 10% acetic acid for 1 h and then incubated with 0.1% Coomassie brilliant blue (R-250) in 25% methanol and 10% acetic acid for 1 h. Following appropriate staining, the gels were

destained with several changes of a solution containing 25% methanol and 10% acetic acid and then dried with a slab gel drier (BioRad).

In the case of two-dimensional electrophoresis gels, the protocol was slightly different. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid (TCA), 5% sulfosalicylic acid and 30% methanol for 1 h. Then the gels were washed in several changes of 30% ethanol and 12.5% TCA and finally stained as indicated above.

Silver Staining Procedure

The silver staining procedure was carried out basically as described previously by Morrissey (1981) although the fixation step was modified. Gels were fixed in 25% 2-propanol and 10% acetic acid for 1 h. Then, the gels were washed with several changes of distilled water over a minimum period of 2 h and stained following the original procedure.

E.2 Protein Assay

The method of Markwell et al. (1978) was used most of the time to determine protein concentrations. However, the protein concentration in some of the tropomyosin samples was determined according to the method of Bradford (1976) using a commercially available dye reagent (BioRad). Protein content determination of the different samples was performed in triplicate. Bovine serum albumin (RIA grade, Sigma) or bovine skeletal muscle tropomyosin (Sigma) was used as standards.

F. MATERIALS

All the electrophoresis chemicals, MW standards and Bio-Gel HTP were purchased from BioRad Laboratories Ltd. (Mississauga, ON). Most of the tissue culture media and reagents were obtained from GIBCO (Grand Island, NY). PMSF, Triton X-100, Nonidet P-40, CHAPS, thiourea, silver nitrate, skeletal muscle tropomyosin (rabbit and bovine), DNAse, collagenase and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). Sucrose, urea and ammonium sulfate (all Ultra Pure) were purchased from Schwarz/Mann Inc. (Montréal, QC). ATP and dithioerythritol were supplied by Boehringer-Mannheim GmbH (Montréal, QC). DEAE-Sephacel, Sephadex G-200, Percoll and high MW standards were obtained from Pharmacia Fine Chemicals (Montréal, QC). DTT was purchased from Calbiochem-Behring Corp. (San Diego, CA). S.A. V8 protease was obtained from Miles Laboratories Inc. (Elkhart, IN). [^{3}H]noradrenaline, $^{45}CaCl_2$ and [^{32}P]orthophosphate were purchased from New England Nuclear (Montréal, QC). Acetylcholine was obtained from Hoffmann-La Roche & Co. AG (Basel, Switzerland). Other chemicals were of reagent grade and obtained from Sigma Chemical Co. or local suppliers.

Trifluoperazine hydrochloride was a generous gift of Dr. H.A. Sheppard of Smith Kline and French, Canada Ltd. (Mississauga, ON). The tropomyosin antibody was a generous gift of Prof. S. Puszkin, Mt. Sinai School of Medicine (New York, NY).

CHAPTER 3

RESULTS

A. PHOSPHORYLATION AND DEPHOSPHORYLATION OF CHROMAFFIN CELL PROTEINS IN RESPONSE TO STIMULATION

A.1 Protein Phosphorylation under Resting Conditions

When chromaffin cells in culture were incubated in the presence of ^{32}P for 1 h as indicated in Chap. 2 section C.1, numerous polypeptides were labelled as revealed by the autoradiography of the SDS-polyacrylamide gels of cell extracts. From the beginning of these studies the need for an SDS-PAGE system of such resolution that would allow quantitation of the phosphorylated bands within a wide molecular weight range was apparent. Different proportions of acrylamide and bisacrylamide were used in the preparation of the gels which were tested with phosphorylated chromaffin cell extracts. A mixture of 10% acrylamide plus 0.1% bisacrylamide (see Chap. 2 section E.1) proved to be, under these experimental conditions, the best combination in terms of resolution of the chromaffin cell polypeptides. Gels prepared with these proportions of acrylamide and bisacrylamide were then used in all the studies described here.

Figure 15 shows that under resting conditions, ^{32}P was incorporated into numerous polypeptides of molecular weights ranging from 500,000 to 14,000. Scanning of the autoradiogram revealed that polypeptides of molecular weights of 63,000 or greater were heavily phosphorylated under resting conditions (Fig. 16). Moreover, autoradiography of the two-dimensional electrophoresis gels of chromaffin cell extracts revealed that at least 70 polypeptides were phosphorylated under resting conditions (Fig. 18) although only a

Fig. 15 Effect of ACh stimulation on ^{32}P incorporation into polypeptides of cultured adrenal medullary chromaffin cells. The cells were labelled with ^{32}P for 1 h and then incubated in Locke's solution in the absence (control) or presence of ACh (10^{-4}M) for 3 min as described in Chap. 2 section B.1.

A) Coomassie blue staining of a typical SDS-PAGE (10% acrylamide, 0.1% bis-acrylamide) of SDS extracts of ^{32}P labelled chromaffin cells. Lanes a-c correspond to samples of protein extracts of different culture dishes containing cells labelled under control conditions and lanes d-f to samples of different culture dishes containing cells stimulated with ACh. Each sample contained 100 µg protein. The first three lanes correspond to MW standards (thyroglobulin subunit, ferritin subunit, myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme). The origin (Or) and dye front (Fr) are indicated by the arrows.

B) Autoradiogram of the Coomassie blue stained gel shown in A. As in A, lanes a'-c' correspond to cultured cells labelled under control conditions and lanes d'-f' to cultured cells stimulated with ACh.

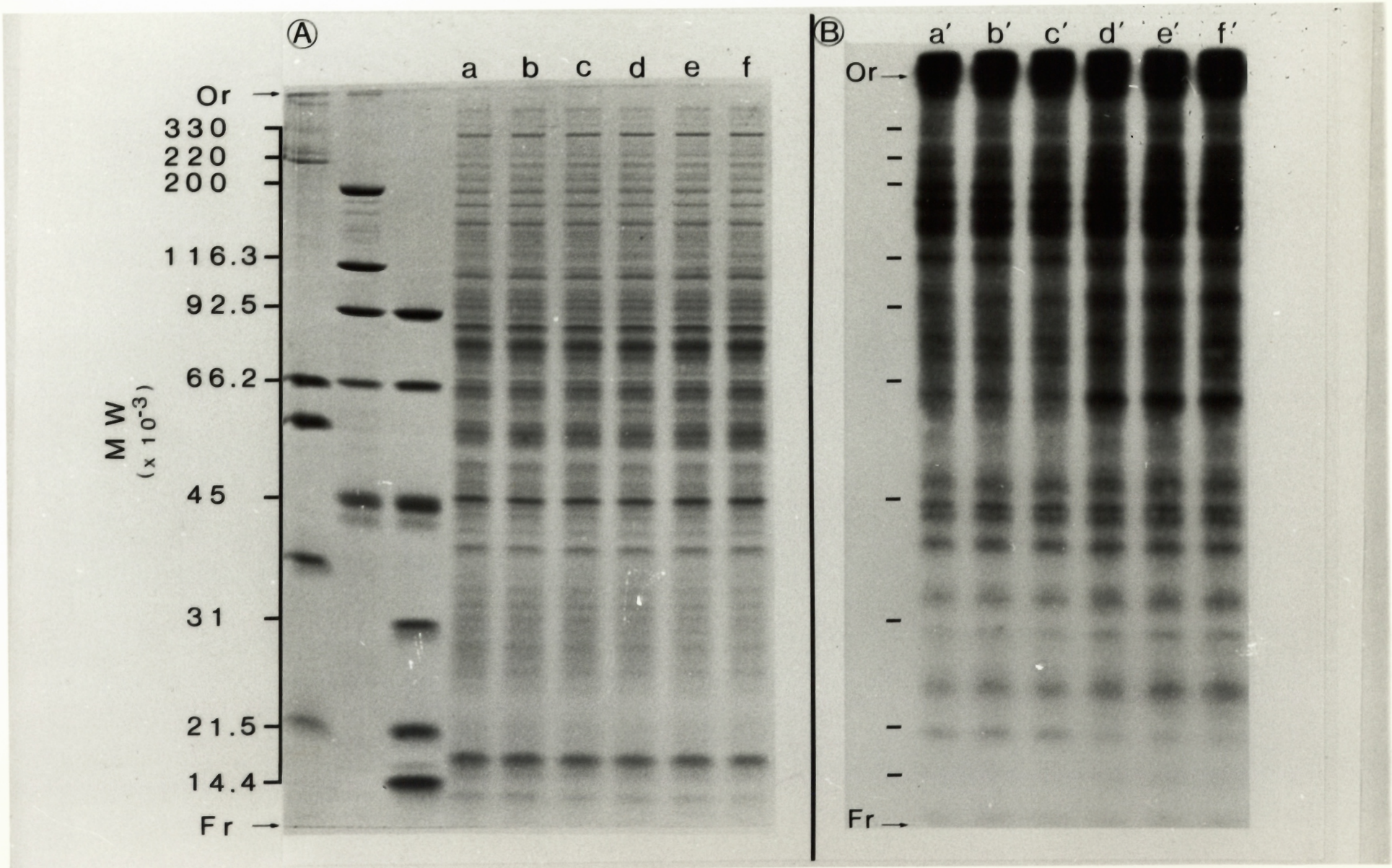
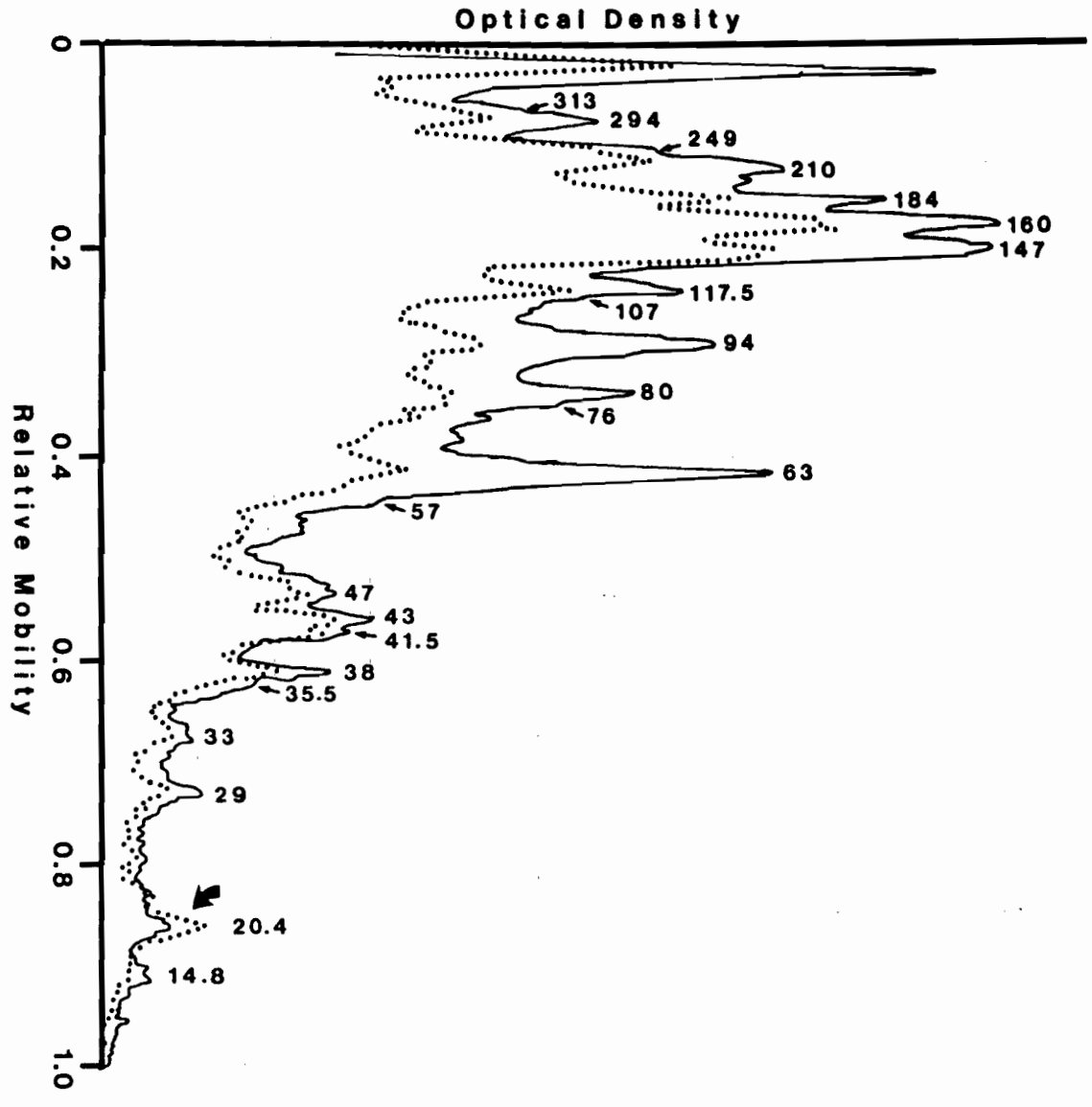

Ⓐ
a b c d e f
Or
330
220
200
116.3
92.5
66.2
45
31
21.5
14.4
Fr
MW
(x 10⁻³)
Ⓑ
a′ b′ c′ d′ e′ f′
Or
Fr

Fig. 16 Representative densitometric scannings of autoradiograms of gel patterns of ^{32}P-labelled chromaffin cell polypeptides from control and ACh-stimulated cells. The tracings correspond to two of the autoradiograms shown in Fig. 15. The scanning of the phosphoprotein pattern obtained from cells stimulated with ACh ($10^{-4}M$) for 3 min (——) was compared to the pattern obtained for cells labelled with ^{32}P under control conditions (···). Twenty three (23) distinct phosphoproteins were resolved by the SDS-PAGE system used; these polypeptides ranged from 313 to 14.8kD. The curved arrow (➔) corresponds to the polypeptide (20.4kD) which was consistently dephosphorylated upon stimulation. The first peak corresponds to a group of high MW polypeptides which are phosphorylated and that have run too close together to be resolved by densitometric scanning.


Optical Density
Relative Mobility
0
0.2
0.4
0.6
0.8
1.0
313
294
249
210
184
160
147
117.5
107
94
80
76
63
57
47
43
41.5
38
35.5
33
29
20.4
14.8

limited number of polypeptides were visualized by Coomassie blue staining of the corresponding gels (Fig. 17).

A.2 Effect of Acetylcholine Stimulation on Chromaffin Cell Protein Phosphorylation

Chromaffin cells previously incubated with ^{32}P for 60 min were stimulated in the presence of ^{32}P for an additional period of 3 min with $10^{-4}M$ ACh. Under these experimental conditions, 22 polypeptides of molecular weights between 313,000 and 14,800 showed an increased ^{32}P incorporation of 21 to 85% (Table 4, Figs. 15 and 16). Only one protein, on one-dimensional gel (MW 20,400, pI=6.7) showed a consistent decrease in labelling in response to ACh stimulation (Table 4, Figs. 15-18). The two polypeptides (MW 94,000 and 63,000 in this electrophoresis system) found in a previous study (Amy & Kirshner, 1981) to be phosphorylated in response to nicotine were found to respond also to ACh stimulation with an increase in ^{32}P incorporation (Table 4, Fig. 16). The increases in the labelling of the 94kD and 63kD polypeptides were of 41% and 59% (n=20) respectively. Two-dimensional electrophoresis analysis showed that the 94kD protein was resolved in three polypeptide forms of pIs 4.2, 4.3 and 4.9 whereas the 63kD protein, in the same system, showed three polypeptide spots with pIs of 6.1, 6.2 and 6.3 (Fig. 18). The greatest increase in ^{32}P incorporation in response to ACh stimulation was observed in a 14.8kD (pI=5.0) polypeptide (Table 4, Fig. 18).

The time course of ^{32}P incorporation into chromaffin cell proteins in response to ACh stimulation was also studied. The phosphorylated chromaffin

Table 4

ACh or high K^+-stimulated phosphorylation or dephosphorylation of adrenal medullary chromaffin cell proteins.

Phosphorylated Polypeptides (M.W. × 10^{-3})	Δ as % of control	
	10^{-4}M ACh	5.6 × 10^{-2}M [K^+]
313	30.9 ± 3.3*	37.6 ± 5.7*
294	26.3 ± 3.4	34.0 ± 7.2
249	21.3 ± 3.9	27.6 ± 5.3
210	26.9 ± 3.4	26.5 ± 2.7
184	22.5 ± 3.0	17.2 ± 2.8
160	22.1 ± 2.6	17.2 ± 3.1
147	23.7 ± 2.7	19.0 ± 3.8
117.5	23.1 ± 3.4	14.0 ± 3.4
107	30.0 ± 4.1	18.5 ± 3.0
94	41.3 ± 4.1	30.1 ± 4.0
80	29.7 ± 4.0	18.0 ± 3.4
76	24.8 ± 4.3	16.1 ± 3.6
63	59.1 ± 7.7	64.7 ± 3.2
57	38.6 ± 4.8	22.4 ± 2.6
47	26.9 ± 3.7	12.3 ± 2.3
43	21.3 ± 3.6	12.2 ± 3.3
41.5	25.2 ± 6.3	9.3 ± 3.3
38	23.8 ± 3.7	21.2 ± 5.8
35.5	33.7 ± 9.0	53.1 ± 6.5
33	36.6 ± 4.2	35.1 ± 3.6
29	43.3 ± 5.5	46.8 ± 6.0
20.4	-30.5 ± 2.5	-39.2 ± 3.6
14.8	85.5 ± 14.3	124.2 ± 13.3

The incorporation of ^{32}P into individual polypeptides was determined from the densitometric scans of autoradiograms (i.e. Fig. 16) (for details see Chap. 2 section B). The numbers in the table represent the differences (expressed as Δ% of control) between control and ACh-stimulated cells or between control and high K^+-stimulated cells. Each value represents the mean ± S.E. (*) obtained from 17-20 different culture dishes treated with ACh and 6 different culture dishes treated with high K^+.

Fig. 17 Two-dimensional gel electrophoresis of SDS extracts of cultured chromaffin cells. Seven-day-old cultured chromaffin cells were first washed with Locke's solution and then scraped off the culture dishes with a 6% SDS-extraction buffer. Samples from the cell extracts were first submitted to isoelectric focusing, and then corresponding bands were equilibrated against the sample mixture for regular SDS-PAGE (see Chap. 2 section E.1.1) and loaded onto 10% SDS-polyacrylamide gels. The protein spots were visualized by Coomassie blue staining.

A limited number of polypeptides could be identified among the numerous protein spots present in the two-dimensional electrophoregram. Actin (A), tubulin (T) subunits and chromogranin A (Chg A) were localized according to their molecular weights and pI values. In addition, a polypeptide spot with molecular characteristics similar to chromogranin B was also detected (►).

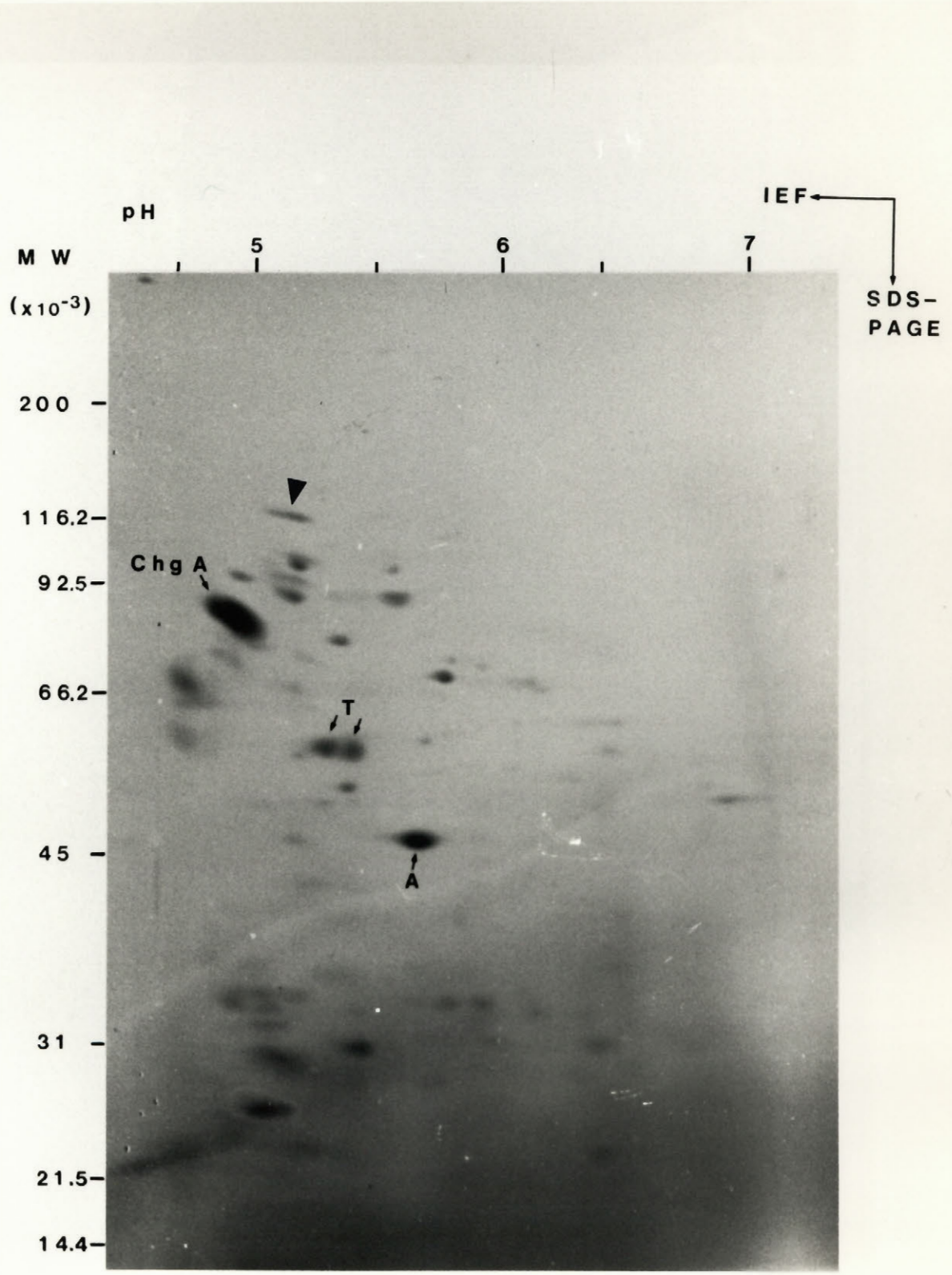
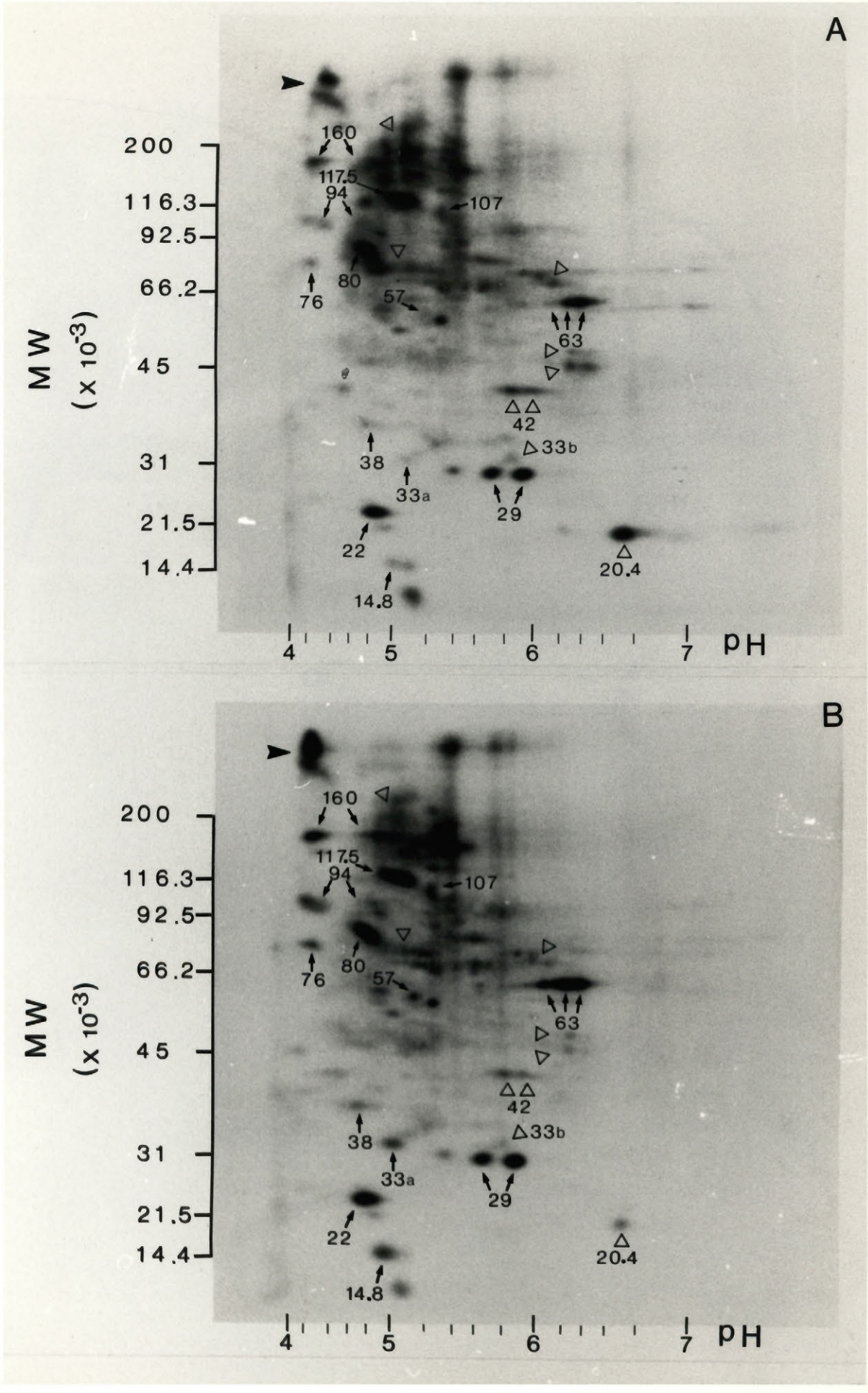

pH
IEF
MW
(x10-3)
5
6
7
SDS-
PAGE
200
116.2
92.5
66.2
45
31
21.5
14.4
Chg A
T
A

Fig. 18 Two-dimensional gel electrophoresis of SDS extracts of ^{32}P-labelled chromaffin cells. Cultured chromaffin cells were labelled with ^{32}P for 1 h and incubated in Locke's solution in the absence (control) or presence of ACh ($10^{-4}M$) for 3 min as described in Chap. 2 section B.1. Samples from the cell extracts were first submitted to isoelectric focusing and then corresponding bands were equilibrated against sample mixture for regular SDS-PAGE (see Chap. 2 section E.1.1) and loaded on 10% SDS-polyacrylamide gels. The radioactive protein spots were visualized by autoradiography. Similar results were obtained in 5 other experiments.

A) Two-dimensional pattern of chromaffin cells labelled with ^{32}P under control conditions.

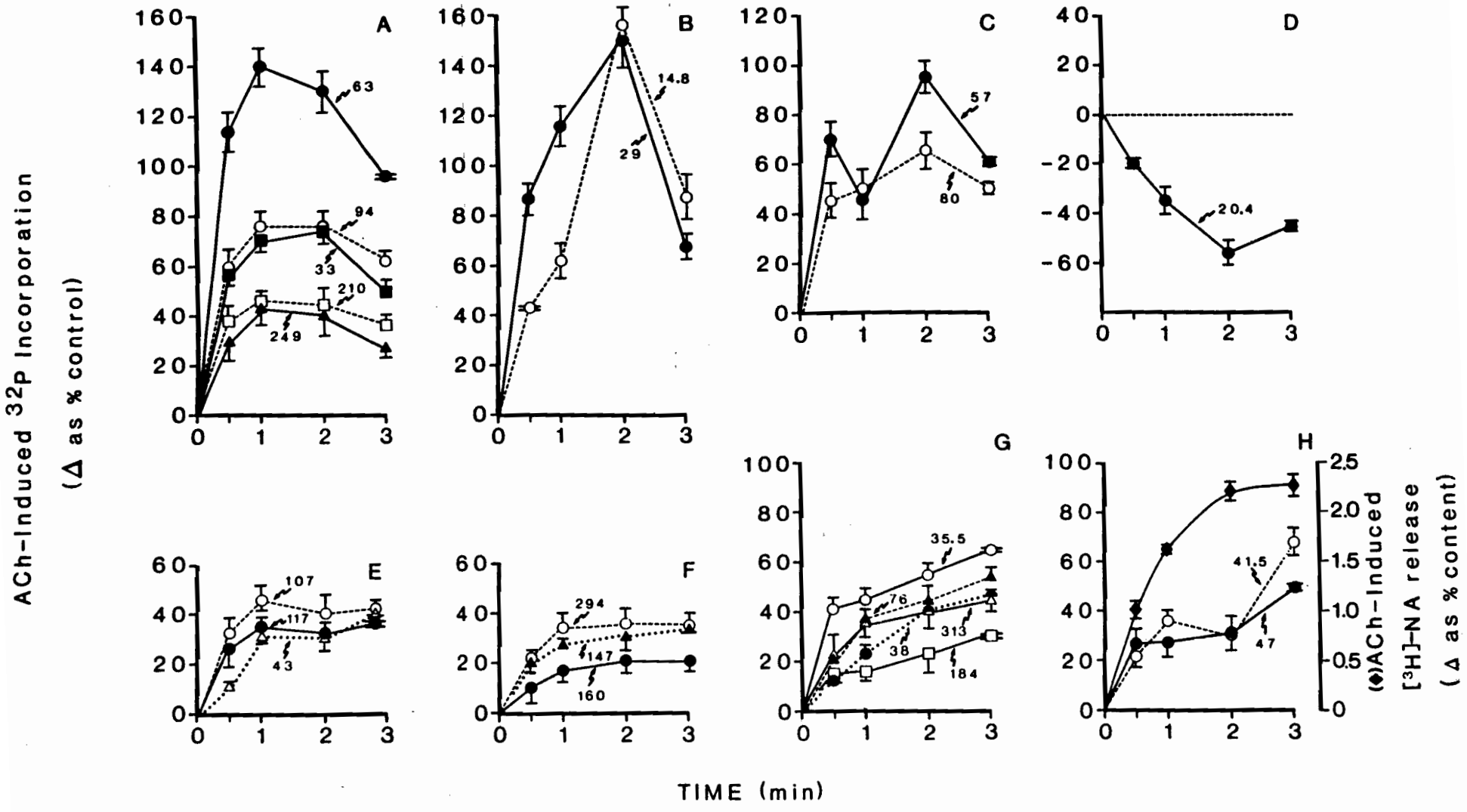
B) Two-dimensional pattern of those cells labelled upon stimulation with $10^{-4}M$ ACh. The arrowhead (➤) indicates a group of high MW phosphopeptides which showed an increase in ^{32}P incorporation upon stimulation. The open arrowheads (▷) correspond to polypeptides which are dephosphorylated upon ACh stimulation. The straight arrows (→) indicate the positions of some of the phosphopeptides detected on two-dimensional SDS-PAGE system. The numbers correspond to the molecular masses (kD) of these polypeptides.


A
MW (x 10-3)
200
116.3
92.5
66.2
45
31
21.5
14.4
160
117.5
94
107
80
76
57
63
42
33b
38
33a
29
22
20.4
14.8
4
5
6
7
pH
B
MW (x 10-3)
200
116.3
92.5
66.2
45
31
21.5
14.4
160
117.5
94
107
80
76
57
63
42
33b
38
33a
29
22
20.4
14.8
4
5
6
7
pH

cell polypeptides can be divided into at least five categories (Fig. 19): i) A group of polypeptides that reached maximum phosphorylation levels 1 min after ACh stimulation and showed lower values of ^{32}P incorporation after 3 min (Fig. 19A); ii) a series of polypeptides in which the stimulation-induced phosphorylation reached a plateau after 1 min of stimulation (Figs. 19, E and F); iii) polypeptides that reached maximum phosphorylation 2 min after initiating the stimulation and showed much lower phosphorylation values at 3 min (Figs. 19, B and C); iv) a group of polypeptides that showed steady increase in ^{32}P incorporation during the entire 3 min of cholinergic stimulation (Figs. 19, G and H); v) finally, a protein (20.4kD) which was dephosphorylated upon stimulation. The maximum level of dephosphorylation was observed 2 min after application of the stimulus (Fig. 19D). The largest levels (Δ=140-150%) of phosphorylation in response to cell stimulation were observed in three polypeptides of molecular weights 14,800, 29,000 and 63,000 (Figs. 19, A and B).

The results obtained from the time course of ^{32}P incorporation were also compared to the time course of catecholamine release from chromaffin cells in response to ACh stimulation. Catecholamine secretion showed a steady increase up to about 2 min after the addition of ACh and appeared to reach a plateau between 2 and 3 min (Fig. 19H). This pattern of secretion was consistent with the time course of phosphorylation of the 29, 147, 160, 184 and 313kD polypeptides as well as with the time course of dephosphorylation of the 20.4kD polypeptide (Figs. 19B, D, F and G). However, exposure of chromaffin cells to ACh resulted in a faster stimulation of phosphate incorporation into the 33, 63, 80, 94, 107, 117.5, 210, 249 and 294kD polypeptides

Fig. 19 Effects of ACh on the time course of ^{32}P incorporation into chromaffin cell proteins and on the time course of catecholamine secretion from chromaffin cell cultures. Cultured chromaffin cells were labelled with ^{32}P for 1 h and incubated with Locke's solution in the presence or absence of 10^{-4}M ACh for 30 sec to 3 min as indicated on the abscissa. The incubations were stopped at the indicated times with SDS-stopping solution. The ^{32}P-labelled protein bands of different molecular masses were detected by autoradiography and the increments in ^{32}P labelling calculated from the densitometric scannings. The results were analyzed as described in the legend to Table 4. The results obtained for the time course of ACh-induced ^{32}P incorporation were divided into five categories: polypeptides that reach maximum phosphorylation levels 1 min (A) or 2 min (B and C) after stimulation and show a subsequent loss of label during longer incubation times; polypeptides that reach a plateau after 1 min of stimulation (E and F); polypeptides that show a steady increase of ^{32}P incorporation during the entire 3 min period of stimulation (G and H); finally, a polypeptide which is dephosphorylated upon stimulation (D). Each point represents the average of 3 experiments. The numbers represent the molecular weights ($\times 10^{-3}$) of the different polypeptides. The time course of ACh-stimulated catecholamine secretion is also shown in (H). Chromaffin cells were prelabelled with [^{3}H]NA and were incubated with Locke's solution in the presence or absence of 10^{-4}M ACh. Catecholamine secreted in response to stimulation minus the secretion observed under control conditions (also expressed as percent of total catecholamine content) is shown in H. Each point represents the mean ± S.E. of 5 experiments.

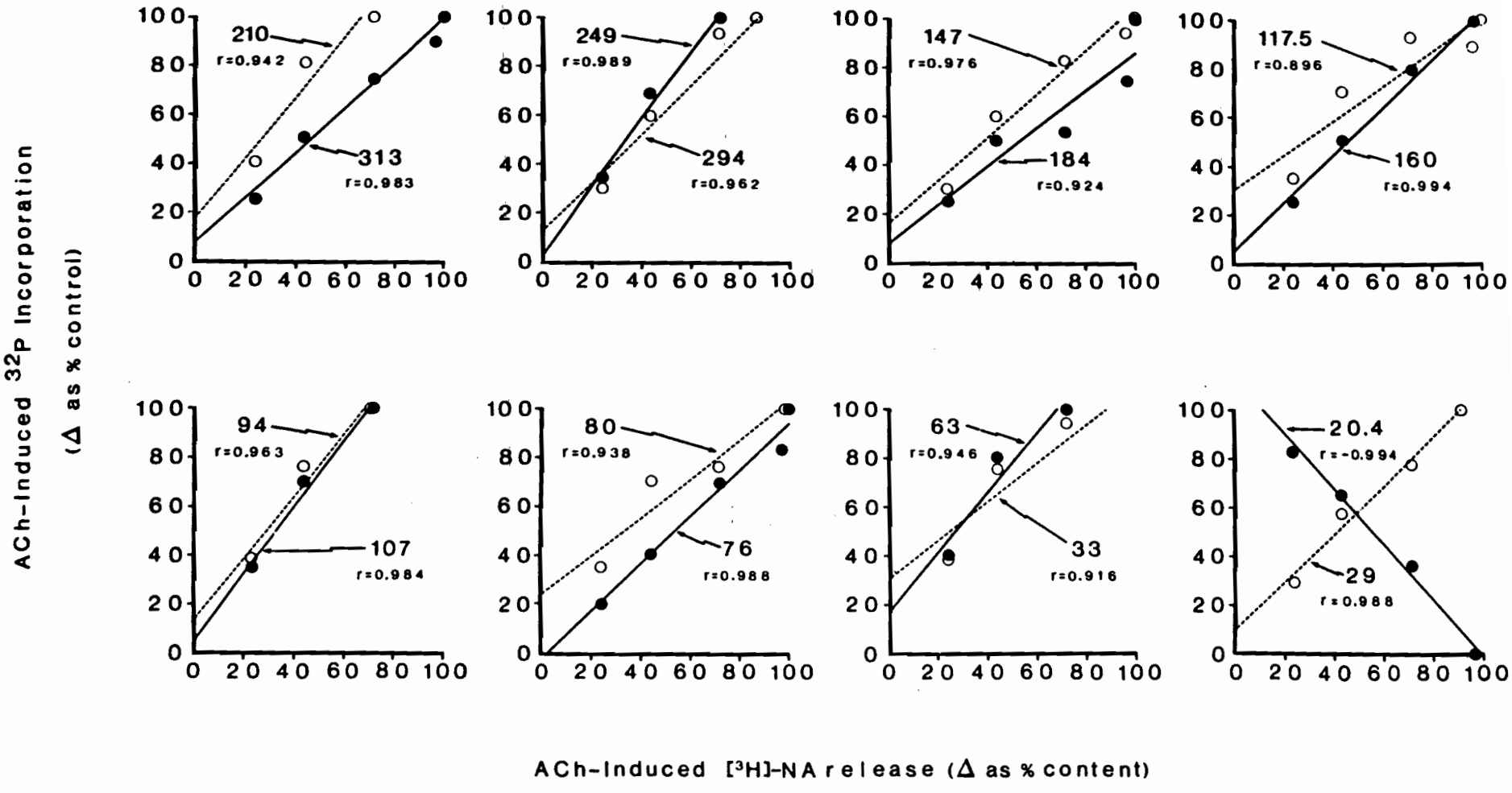

ACh-Induced ^{32}P Incorporation
(Δ as % control)
A
B
C
D
E
F
G
H
63
94
33
210
249
14.8
29
57
80
20.4
107
117
43
294
147
160
35.5
76
38
313
184
41.5
47
TIME (min)
(♦)ACh-Induced
[^{3}H]-NA release
(Δ as % content)

(Figs. 19A, C, E, F and G). This stimulation was already evident at 30 sec and reached a maximum level for most of them after 1 min. In the case of the 14.8, 35.5, 38, 41.5, 43 and 47kD polypeptides, phosphorylation reactions following the addition of ACh proceeded more slowly (Figs. 19B, E, G and H). For those proteins in which the change in phosphorylation occurred rapidly enough to be temporally associated with the onset of secretion, the correlation between catecholamine secretion and protein phosphorylation is shown in Fig. 20. A least-squares linear regression line was determined for each individual polypeptide, and in each instance, there appeared to be a good correlation between secretion and phosphorylation.

A.3 Effect of K^+-Induced Depolarization on Chromaffin Cell Protein Phosphorylation

The same chromaffin cell proteins that showed increased ^{32}P labelling in response to ACh were found to be phosphorylated after exposure to a depolarizing concentration (56 mM) of K^+ (Table 4). Again, the 14.8kD (pI=5.0) polypeptide showed the largest increase in ^{32}P labelling. Moreover, and as with ACh stimulation, high K^+ induced the dephosphorylation of the 20.4kD (pI=6.7) polypeptide (Table 4).

Fig. 20 Correlation between ACh-evoked protein phosphorylation and [^{3}H]NA release from chromaffin cells. Correlation coefficients (r) and computer fitted lines for all the individual polypeptides have been obtained from the results described in Fig. 19. The numbers represent the molecular weights ($\times 10^{-3}$) of the polypeptides. The figure shows only the correlations for those phosphoproteins which could be temporally associated with the onset of catecholamine secretion. The inverse correlation between the dephosphorylation of the 20.4kD chromaffin cell polypeptide and [^{3}H]NA release is also shown.


ACh-Induced ^{32}P Incorporation (Δ as % control)
ACh-Induced [^{3}H]-NA release (Δ as % content)
210
r=0.942
313
r=0.983
249
r=0.989
294
r=0.962
147
r=0.976
184
r=0.924
117.5
r=0.896
160
r=0.994
94
r=0.963
107
r=0.984
80
r=0.938
76
r=0.988
63
r=0.946
33
r=0.916
20.4
r=-0.994
29
r=0.988

A.4 Effect of Low Ca^{2+} and High Mg^{2+} Concentrations on Stimulation-Induced ^{32}P Labelling of Chromaffin Cell Proteins

In these experiments, the extracellular concentration of Ca^{2+} was reduced to 0.5 mM and that of Mg^{2+} was increased to 15 mM. The responses to ACh in low Ca^{2+} and high Mg^{2+} medium were found to be different in 18 of the 23 chromaffin cell polypeptides examined (Table 5). The ACh-induced increase in ^{32}P protein labelling was completely inhibited in 15 polypeptides and inhibited by 50% in 3 others (Table 5), thus, indicating that Ca^{2+} entry followed by a Ca^{2+}-dependent process is required during stimulation-induced increase in ^{32}P protein labelling. On the other hand, the ACh-induced dephosphorylation of the 20.4kD polypeptide was not affected (Table 5), suggesting that extracellular Ca^{2+} is not required in this case.

A.5 Effect of Trifluoperazine on Stimulation-Induced ^{32}P Labelling of Chromaffin Cell Proteins

In these experiments, chromaffin cells in culture were incubated in the presence or absence of 2×10^{-7}M TFP and were stimulated by 10^{-4}M ACh. TFP is a calmodulin antagonist which has been used extensively in numerous cell systems and which appears to produce various effects depending on the conditions used in those systems. Therefore, a series of experiments were conducted in cultured chromaffin cells to first determine the effects of this agent on catecholamine release and Ca^{2+} movements (for details, see appendix section D).

Table 5

Calcium dependency of the effects of ACh on ^{32}P incorporation into chromaffin cell proteins.

Phosphorylated Polypeptides (M.W. × 10^{-3})	Δ as % of control 10^{-4}M ACh	
	2.2 mM Ca^{2+} + 1.2 mM Mg^{2+}	0.5 mM Ca^{2+} + 15 mM Mg^{2+}
313	28.6 ± 3.4*	29.9 ± 10.3*
294	25.6 ± 3.8	16.4 ± 2.6
249	21.0 ± 4.7	23.4 ± 7.0
210	26.3 ± 4.0	21.2 ± 8.3
184	18.6 ± 3.9	18.3 ± 7.5
160	19.7 ± 3.2	8.7 ± 8.5
147	21.3 ± 3.3	1.8 ± 4.8
117.5	19.1 ± 4.4	7.3 ± 7.4
107	31.0 ± 4.9	-2.2 ± 1.5
94	41.9 ± 4.7	8.5 ± 7.1
80	30.0 ± 4.7	5.8 ± 8.3
76	26.6 ± 5.1	-23.9 ± 4.5
63	57.5 ± 8.9	14.2 ± 10.4
57	40.9 ± 5.7	6.7 ± 8.1
47	25.9 ± 4.0	-4.6 ± 6.5
43	17.3 ± 4.0	0.8 ± 9.2
41.5	26.4 ± 7.5	-9.4 ± 5.2
38	18.5 ± 4.7	-12.1 ± 9.4
35.5	25.4 ± 10.1	-10.9 ± 11.1
33	32.6 ± 4.2	-2.7 ± 11.2
29	42.7 ± 6.4	-1.8 ± 11.4
20.4	-29.3 ± 2.8	-35.6 ± 9.0
14.8	56.5 ± 14.1	-5.9 ± 11.7

Incorporation of ^{32}P into individual polypeptides following stimulation of chromaffin cells with ACh in either regular (2.2 mM Ca^{2+}) or low Ca^{2+} (0.5 mM)-high Mg^{2+}(15mM) Locke's solutions. In the experiments using low Ca^{2+} medium, a 15 min preincubation step with modified Locke's solution preceded the stimulation with ACh. The results were analyzed as described in the legend to Table 4. Each value represents the mean ± S.E. as obtained from 3 different culture dishes.

At the concentration used in these phosphorylation studies, TFP produces 50% inhibition in the secretory response to ACh but has no effect on Ca^{2+} entry (see section D). TFP inhibited the ACh-induced ^{32}P labelling by 50% or more in 20 of the 23 chromaffin cell polypeptides studied (Table 6). The ^{32}P labelling of the 63kD polypeptide was decreased by 38% (Table 6). Moreover, exposure to TFP did not modify either the ^{32}P labelling of the 14.8kD polypeptide or the degree of dephosphorylation of the 20.4kD polypeptide (Table 6).

Table 6

Effect of TFP on the ACh-stimulated phosphorylation or dephosphorylation of chromaffin cell proteins.

Phosphorylated Polypeptides	Δ as % of control	
(M.W. × 10^{-3})	10^{-4}M ACh	10^{-4}M ACh + 2 × 10^{-7}M TFP
313	26.2 ± 2.0*	13.6 ± 0.5*
294	31.5 ± 1.2	16.3 ± 1.1
249	27.5 ± 3.7	12.2 ± 0.0
210	23.6 ± 2.4	11.8 ± 0.3
184	12.8 ± 1.1	7.8 ± 0.2
160	13.1 ± 1.5	8.5 ± 0.0
147	14.3 ± 1.1	9.2 ± 0.9
117.5	16.9 ± 1.7	10.4 ± 0.6
107	49.4 ± 2.2	17.0 ± 0.7
94	42.3 ± 1.9	25.7 ± 1.8
80	30.0 ± 3.0	12.8 ± 0.0
76	26.2 ± 0.7	9.2 ± 1.2
63	64.0 ± 1.3	39.6 ± 0.8
57	51.3 ± 8.0	6.4 ± 0.0
47	25.7 ± 2.8	6.0 ± 0.0
43	15.1 ± 3.5	1.2 ± 1.2
41.5	23.0 ± 2.8	3.6 ± 0.0
38	21.5 ± 3.1	2.8 ± 1.6
35.5	26.2 ± 2.4	-7.7 ± 0.0
33	49.0 ± 3.8	17.7 ± 2.7
29	67.1 ± 5.7	36.2 ± 5.6
20.4	-43.8 ± 2.3	-47.9 ± 2.1
14.8	73.5 ± 9.1	75.2 ± 0.0

Incorporation of ^{32}P into individual polypeptides following stimulation of chromaffin cells with ACh in presence or absence of TFP. In the experiments using the calmodulin antagonist, a 20 min preincubation step with modified Locke's solution preceded the stimulation with ACh in presence of the inhibitor. The results were analyzed as described in the legend to Table 4. Each value represents the mean ± S.E. from 3 different culture dishes.

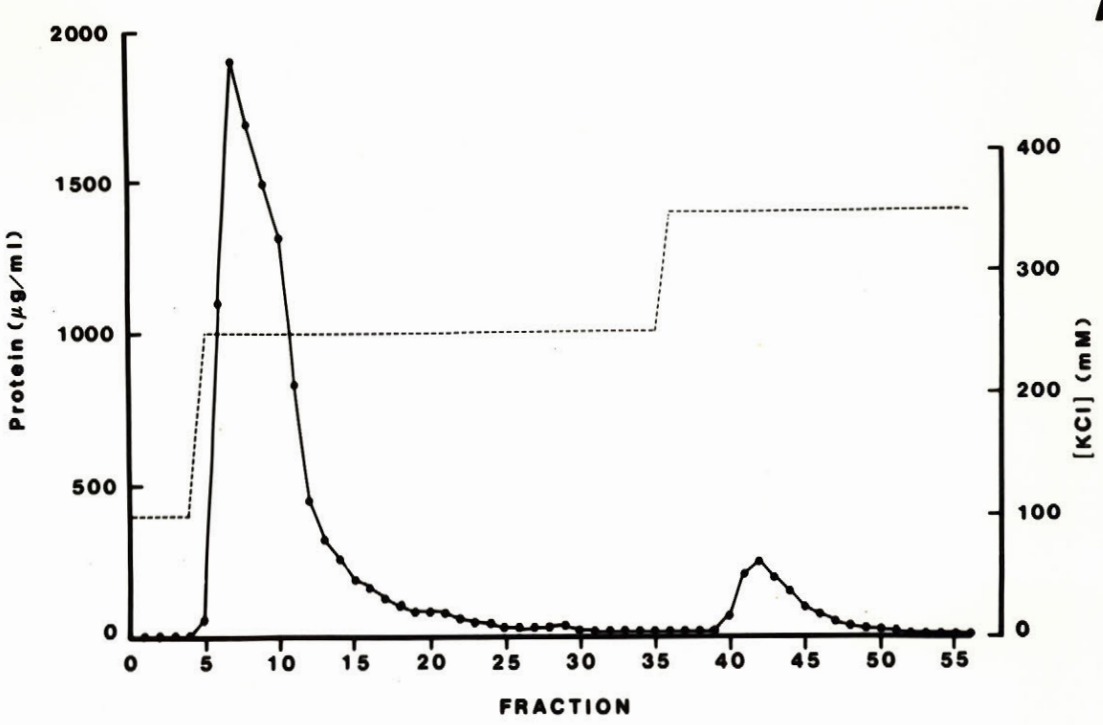
B. ADRENAL MEDULLARY TROPOMYOSINS: PURIFICATION AND BIOCHEMICAL CHARACTERIZATION

B.1 Purification of Bovine Adrenal Medullary Tropomyosins

The protocol followed in the purification of tropomyosins from bovine adrenal medulla is shown in Fig. 14. The first steps of purification were derived from the technique of Barylko and Sobieszek (1983) originally developed for the purification of brain tropomyosin. These first steps which are described in detail in Chap. 2 section C.1.1 involve two successive extractions with 1% Triton X-100 at pH 6.8, a heating step in boiling water, two ammonium sulfate fractionations and one isoelectric point precipitation. Further purification of adrenal medullary tropomyosins was achieved by the addition of two column chromatography steps (ion exchange on DEAE-Sephacel and hydroxylapatite) used under conditions similar to those described by Côté and Smillie (1981a) for the purification of equine platelet tropomyosin. Fig. 21 shows the elution profile of the DEAE-Sephacel column (Fig. 21A) and a silver staining pattern of the corresponding fractions analyzed by SDS-PAGE (Fig. 21B). While several bands (20 to 220kD) were detected in the first peak eluted from the DEAE-Sephacel column, two bands with molecular weights of 66,000 and 86,000 were largely prominent and consistently seen (Fig. 21B). The second peak of the ion-exchange column was enriched in tropomyosins but still many higher molecular weight proteins were present (Fig. 21B). The final purification of adrenal medullary tropomyosins on a hydroxylapatite column is shown in Fig. 22. This includes the elution pat-

Fig. 21 Partial purification of adrenal medullary tropomyosins by chromatography on a DEAE-Sephacel column. The precipitate obtained by lowering the pH of the adrenal preparation at 4.5 (see Fig. 14) was extracted with 25 ml of 150 mM KCl in 2 mM DTT, 0.2 mM PMSF, 10 mM imidazole, pH 7.0. After centrifugation at 10,000 g for 1 h to remove insoluble material, the supernatant was first diluted to a final KCl concentration of 100 mM in the same buffer, and then it was applied (about 7 mg protein) to a DEAE-Sephacel column (5.0 × 1.6 cm). The column was washed at a flow rate of 30 ml/h with 20 ml of 100 mM KCl-buffer. Panel A shows a representative chromatogram of the fractions eluted from the ion-exchange column using a KCl step gradient (---). Two ml fractions were collected. The first peak (fractions 5 to 20) was eluted with 60 ml of 250 mM KCl buffer, the second (fractions 39 to 50) enriched in tropomyosins (see panel B) was eluted with 40 ml of 350 mM KCl buffer. Panel B: aliquots (20 µl) of the fractions collected from the eluates were concentrated by TCA precipitation, analyzed by SDS-PAGE on a 10% acrylamide, 0.1% bisacrylamide gel and then subjected to the silver staining procedure. The apparent MWs ($\times 10^{-3}$), as obtained from marker proteins are shown in lane a. Lane b, contains commercial rabbit skeletal muscle tropomyosin isoforms (3 µg); lane c, shows the pattern obtained with a 20 µl aliquot of the pre-column sample, also concentrated by TCA precipitation.

A


2000
1500
1000
500
0
Protein (μg/ml)
400
300
200
100
0
[KCl] (mM)
0 5 10 15 20 25 30 35 40 45 50 55
FRACTION

B

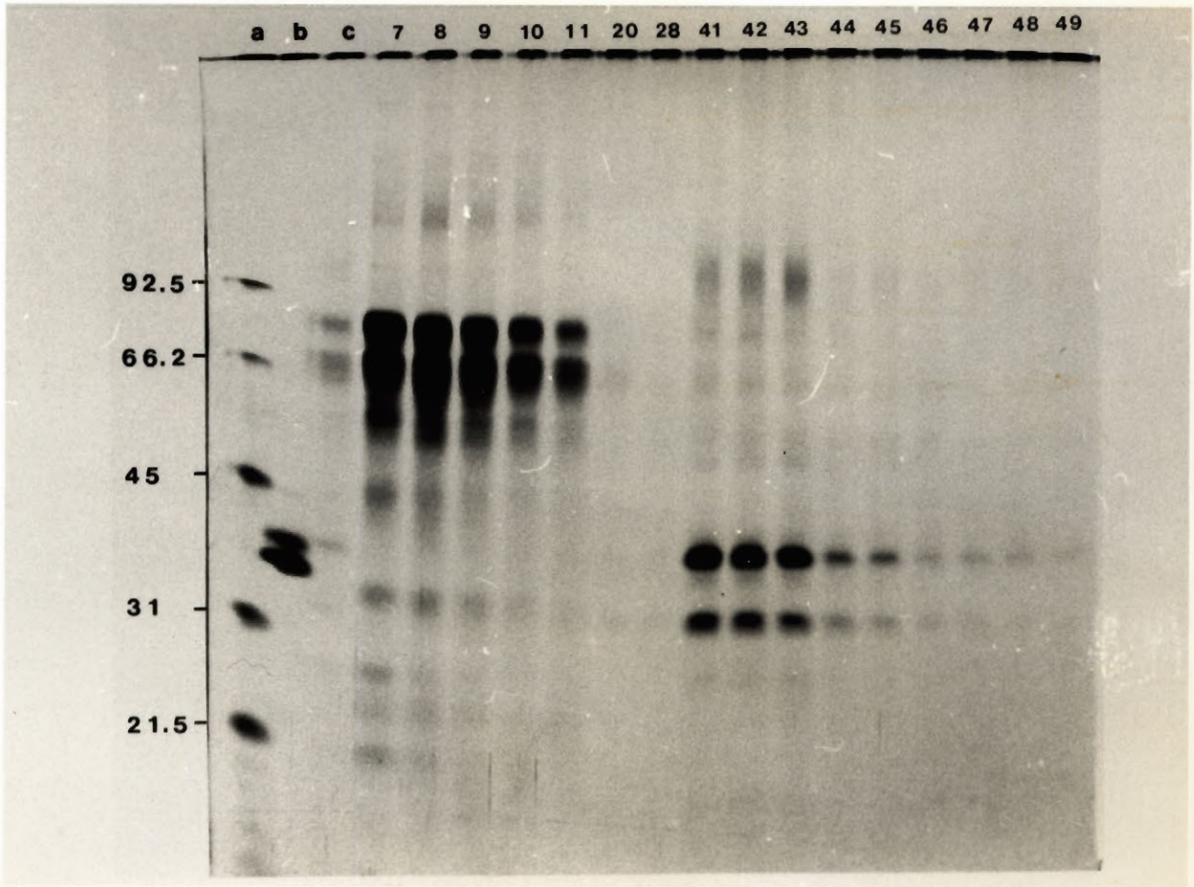
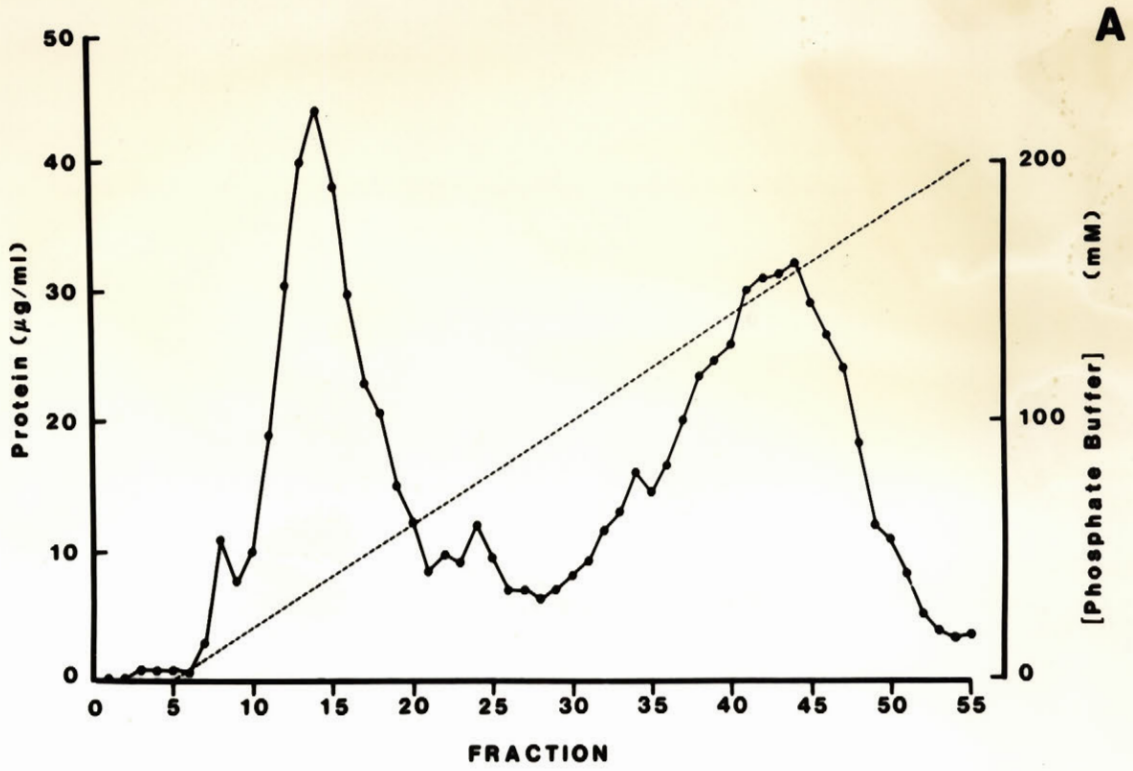
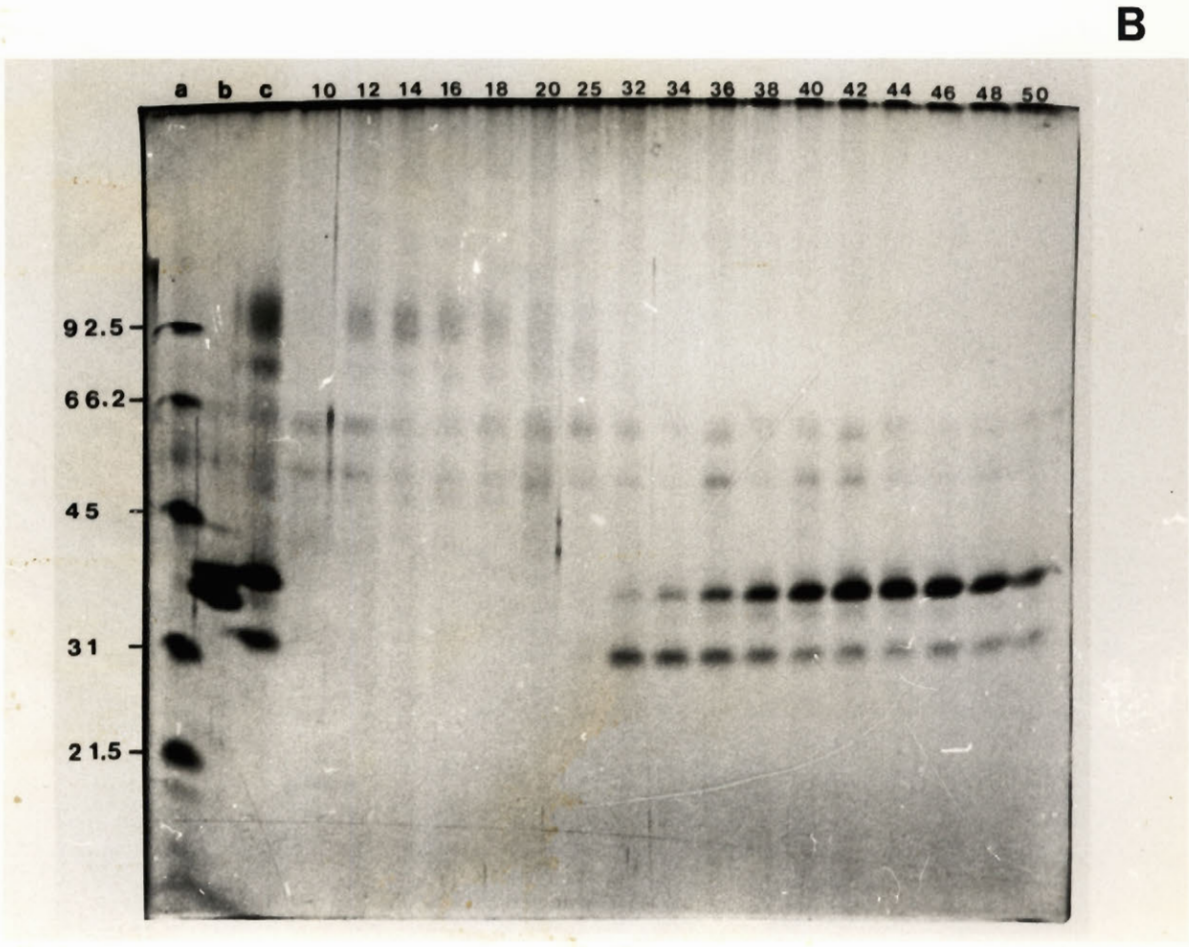

a b c 7 8 9 10 11 20 28 41 42 43 44 45 46 47 48 49
92.5
66.2
45
31
21.5

Fig. 22 Purification of adrenal medullary tropomyosins by chromatography on a hydroxylapatite column. The fractions enriched in tropomyosins obtained from the DEAE-Sephacel column (second peak) were combined and adjusted to 1 M KCl. The preparation (about 1.4 mg protein) was then applied to a hydroxylapatite column (3.0 × 1.6 cm) previously equilibrated with 1 M KCl, 2 mM DTT, 0.2 mM PMSF, 10 mM imidazole, pH 7.0. Panel A shows a representative chromatogram of the fractions eluted from the hydroxylapatite column at a flow rate of 30 ml/h with a 100 ml linear gradient of 1-200 mM sodium phosphate (---) dissolved in the same buffer. Two ml fractions were collected. Panel B: aliquots (50 µl) from the fractions were concentrated by TCA precipitation, analyzed by SDS-PAGE and then visualized by the silver staining procedure. The apparent MWs ($\times 10^{-3}$), as obtained from the marked proteins, are shown in lane a. Lane b shows the pattern obtained with commercial rabbit skeletal muscle tropomyosin isoforms (3 µg); lane c shows the pattern obtained with a 50 µl aliquot of the pre-column sample precipitated with TCA.


A
Protein (μg/ml)
[Phosphate Buffer] (mM)
FRACTION
0
10
20
30
40
50
0
100
200
0 5 10 15 20 25 30 35 40 45 50 55


B
a b c 10 12 14 16 18 20 25 32 34 36 38 40 42 44 46 48 50
92.5
66.2
45
31
21.5

tern (Fig. 22A) and a silver-stained SDS-PAGE pattern of the purified fractions (Fig. 22B).

The purified tropomyosins isolated from bovine adrenal medulla have the same chromatographic patterns (Figs. 21 and 22) as equine platelet tropomyosin components (Côté & Smillie, 1981a) and were purified under very similar conditions on anion-exchange and hydroxylapatite columns. Chromatography on a hydroxylapatite column led to the partial separation of the two predominant forms of adrenal medullary tropomyosin (Fig. 22B). Although the tropomyosins were eluted as a single broad peak from the hydroxylapatite column (Fig. 22A), the leading edge of the peak was always enriched in the faster moving form, while the trailing edge contained predominantly the slower moving form (Fig. 22B). This desorption behavior has also been reported for tropomyosins from both equine platelets (Côté & Smillie, 1981a) and erythrocyte membranes (Fowler & Bennett, 1984). The final yield of adrenal medullary tropomyosins was between 0.6 and 0.7 mg. Contrary to other systems previously described (Côté & Smillie, 1981a; Fowler & Bennett, 1984), when the adrenal medulla was used as the source of material, it was not feasible to measure the tropomyosin content, by densitometric scanning of SDS-polyacrylamide gels, at each of the purification steps. In fact, two-dimensional PAGE analysis of various subcellular fractions, in particular the soluble one, has revealed the presence of many proteins co-migrating with the tropomyosin forms.

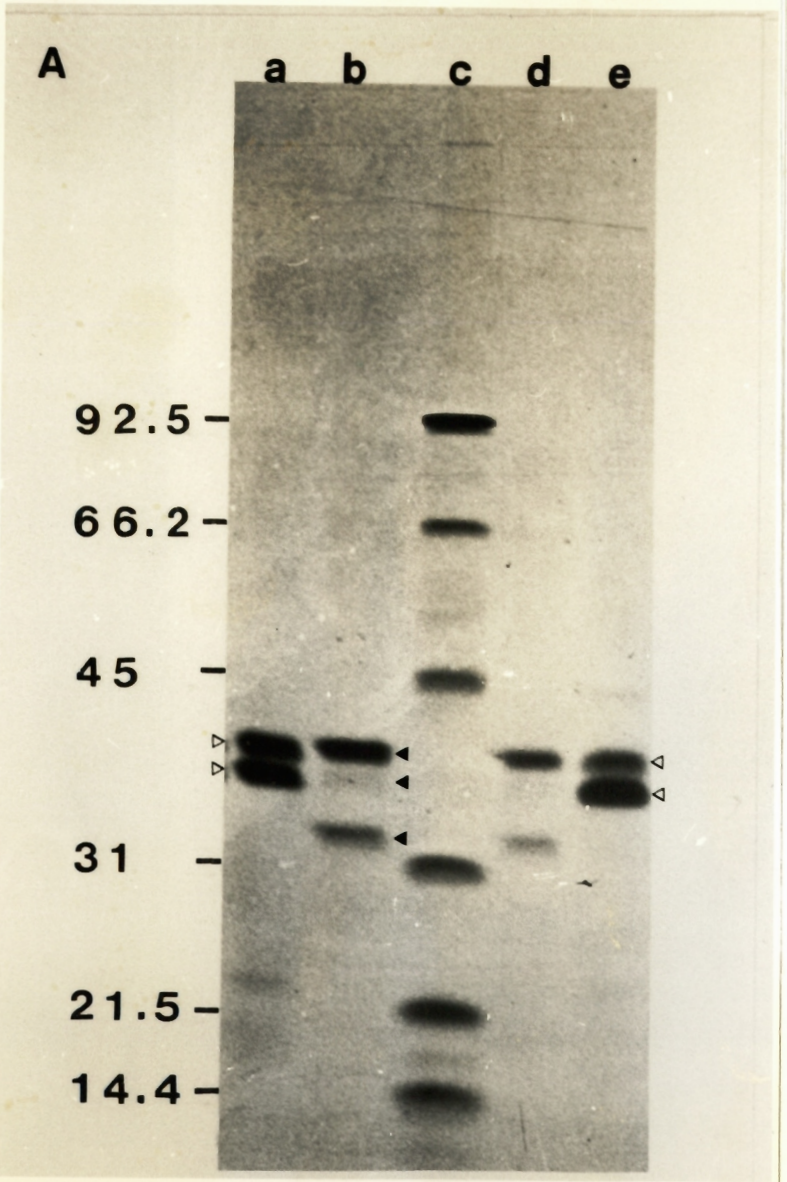
All nonmuscle tropomyosins isolated so far, except those purified from cultured cells, have been distinguished by having apparent molecular weight values smaller than those of muscle tropomyosins. The apparent molecular

weights of purified adrenal medullary tropomyosins were determined by SDS-PAGE and apparent molecular weights of 38,000, 35,500 and 32,000 were then calculated (Figs. 23, A and B). When Coomassie blue staining was used, the 38kD form sometimes appeared as a closely spaced doublet. For a comparative purpose, commercial bovine (Fig. 23, lane a) and rabbit (Fig. 23, lane e) skeletal muscle tropomyosins were run in parallel with purified adrenal medullary tropomyosins. These muscle proteins had, in this SDS-PAGE system, apparent molecular weight values of 38,000 (β form) and 35,500 (α form). The molar ratio of the 38kD to the 32kD tropomyosin polypeptides present in the adrenal medullary extracts was 2:1 (Fig. 23, lanes b and d). The presence of a small quantity of high molecular weight proteins in the tropomyosin fractions eluted from the hydroxylapatite column as detected by the sensitive silver staining technique was somewhat variable from one experiment to another (Fig. 22B, fractions 32 to 50). However, when present, these high molecular weight polypeptides represented a very low proportion (≤1%) of the total amount of material obtained in the final tropomyosin fraction.

B.2 Two-Dimensional Gel Electrophoresis

The preparation of adrenal medullary tropomyosins was further analyzed by two-dimensional gel electrophoresis. Under the conditions used, an actual pH gradient ranging from pH 3 to 9 was determined and a similar pI value of about 4.7 was obtained for the three tropomyosins (Fig. 24).

Fig. 23 Determination of the apparent MWs of purified adrenal medullary tropomyosins and their comparison to those MWs obtained for skeletal muscle tropomyosins. SDS-PAGE was carried out as described in Chap. 2 section E.1.1 on a 10% acrylamide, 0.1% bisacrylamide slab gel. Purified samples and MW standards were applied in the wells at the center of the gel to overcome any problem which might result from current field distortion at both ends. Panel A shows the electrophoretic patterns of gel stained with Coomassie brilliant blue. The lanes correspond to (a) 5 μg of commercial bovine skeletal muscle tropomyosin isoforms; (b) and (d) 7.5 μg and 5 μg respectively of purified adrenal medullary tropomyosins; (e) 5 μg of commercial rabbit skeletal muscle tropomyosin isoforms; and (c) 2 μg of each of the MW markers. Panel B: the ordinate represents the apparent MWs (log scale) and the abscissa represents the relative mobilities for the protein standards used in the electrophoresis system. The protein markers used were: a, phosphorylase B (92.5kD); b, bovine serum albumin (66.2kD); c, ovalbumin (45kD); d, carbonic anhydrase (31kD) and e, soybean trypsin inhibitor (21.5kD). The open arrowheads (▷) in panel A and arrows (—▷) in panel B correspond to the β and α skeletal muscle tropomyosin isoforms from bovine or rabbit muscles and the closed ones (▶,—▶) to the adrenal medullary tropomyosins.


A
a
b
c
d
e
92.5
66.2
45
31
21.5
14.4

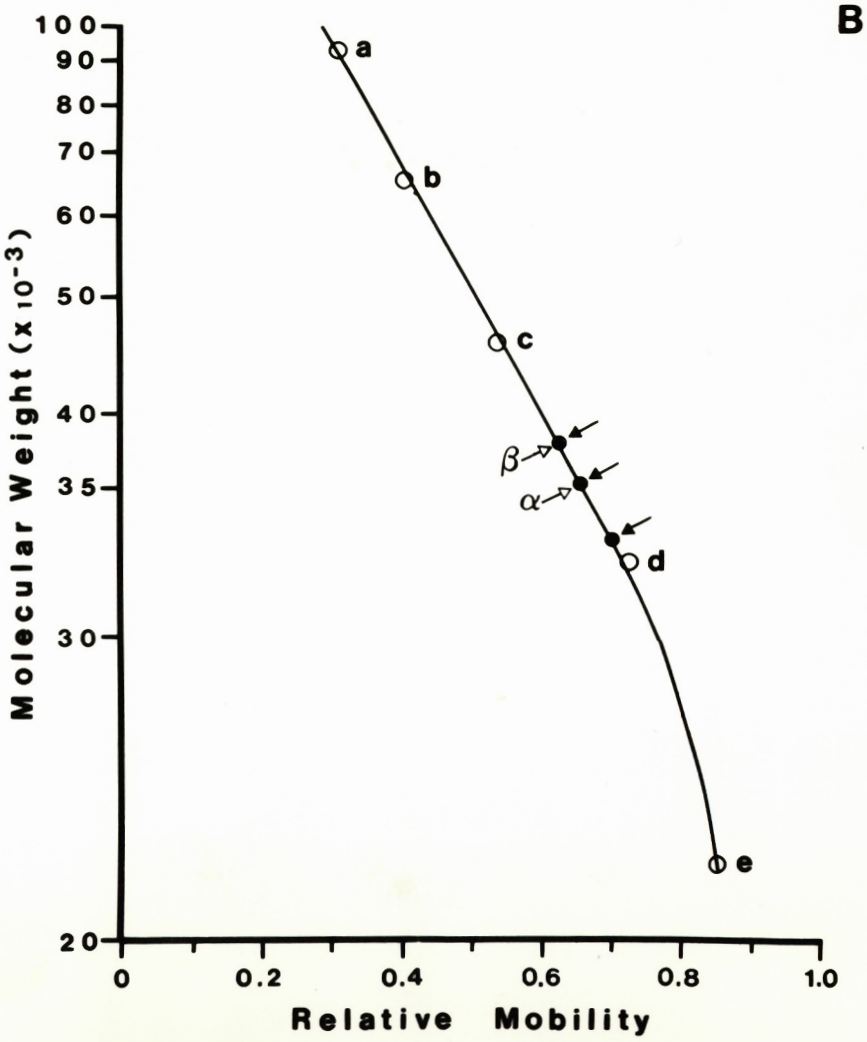
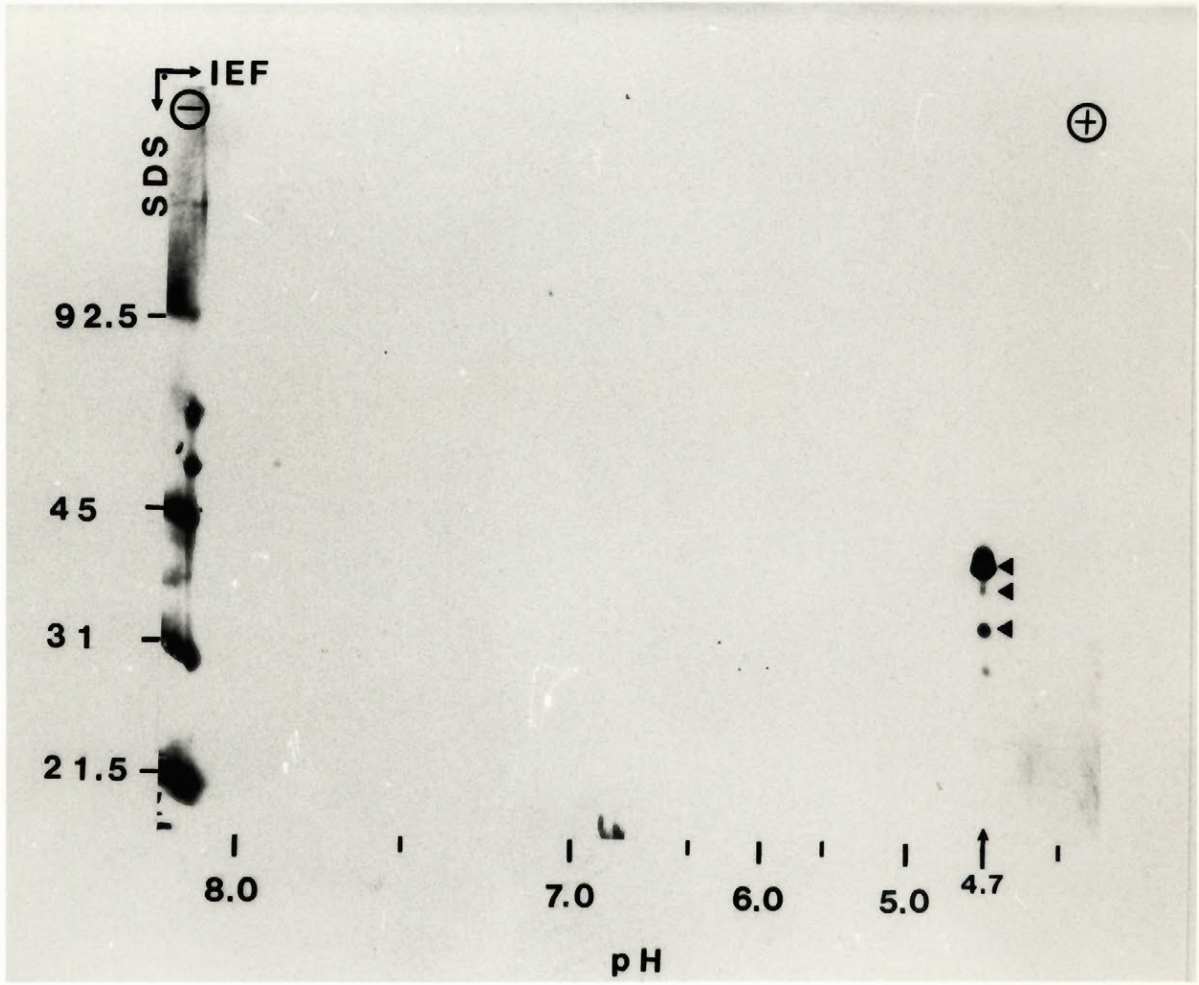

B
Molecular Weight (x10⁻³)
100
90
80
70
60
50
40
35
30
20
a
b
c
d
e
β
α
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility

Fig. 24 Two-dimensional gel electrophoresis of tropomyosins purified from bovine adrenal medulla. The hydroxylapatite combined fraction (0.5 ml, 10 µg) preparation was dialyzed at 4°C against 0.2 mM PMSF in an Eppendorf tube with an open cap which was covered with a piece of dialysis membrane. After lyophilyzation, the sample was first submitted to isoelectric focusing as described in Chap. 2 section E.1.2, and the corresponding band previously equilibrated in the SDS sample mixture was then loaded on a 10% SDS-polyacrylamide gel. The electrophoregram shown was obtained after silver staining. The pH scale indicated on the figure was obtained by cutting into 0.5 cm slices a band located at the center of the first dimension. The slices were subsequently equilibrated for 2 h at room temperature in 1 ml of a degassed solution of 10 mM KCl. The arrowheads (▸) indicate the positions of the adrenal medullary tropomyosins.


IEF
SDS
⊖
⊕
92.5
45
31
21.5
8.0
7.0
6.0
5.0
4.7
pH

B.3 Characteristic Shift in the Mobility of Adrenal Medullary Tropomyosins in Urea/SDS-PAGE

The adrenal medullary tropomyosins as well as the commercial rabbit skeletal muscle tropomyosins migrate faster than purified chicken gizzard actin (45kD) in a regular SDS-polyacrylamide gel but slower than that protein in an SDS-polyacrylamide gel containing 8 M urea (Fig. 25). This phenomenon is thought to be a common feature for all tropomyosins (Storti et al., 1976).

B.4 Specific Interaction of the 38kD Adrenal Medullary Tropomyosin with F-Actin

The binding of adrenal medullary tropomyosins to chicken gizzard F-actin was determined by the combination of centrifugation and SDS-PAGE with quantitative densitometry as described in Chap. 2 section C.2. The preparation of adrenal medullary tropomyosins used consisted of a heterogeneous population of three forms of 38kD, 35.5kD and 32kD polypeptide chains (homodimers and possibly heterodimers as well). Fig. 26A (lanes a-g) shows the electrophoretic pattern of the pellets (P) and their corresponding supernatants (S) obtained after the incubation of various amounts of the tropomyosin fraction and gizzard F-actin (at low salt concentration (50 mM KCl) in the presence of 10 mM $MgCl_2$). Under these conditions, the 38kD tropomyosin interacts strongly with F-actin in a concentration-dependent manner (Fig. 26A) while the 32kD and 35.5kD tropomyosins bind very poorly to F-actin. At a low salt

Fig. 25 Characteristic shift of purified adrenal tropomyosins to a higher apparent MW after SDS-PAGE in presence of 8 M urea. The protein preparations were separated by electrophoresis in polyacrylamide gel containing SDS and 8 M urea. After the electrophoresis, the gel was stained with Coomassie blue. Lane (a) corresponds to 15 μg of rabbit skeletal muscle tropomyosin isoforms; (b) to 10 μg of chicken gizzard actin; (c) to 10 μg of the final combined fraction of adrenal tropomyosins; (d) to 10 μg of the trailing fraction (fraction 51, Fig. 22a) eluted from the hydroxylapatite column and containing only the 38kD adrenal tropomyosin, and (e) to 2 μg of the leading fraction (fraction 31, Fig. 22a) eluted from the hydroxylapatite column and enriched in the 32kD adrenal tropomyosin. Similar results were obtained in 3 other experiments.

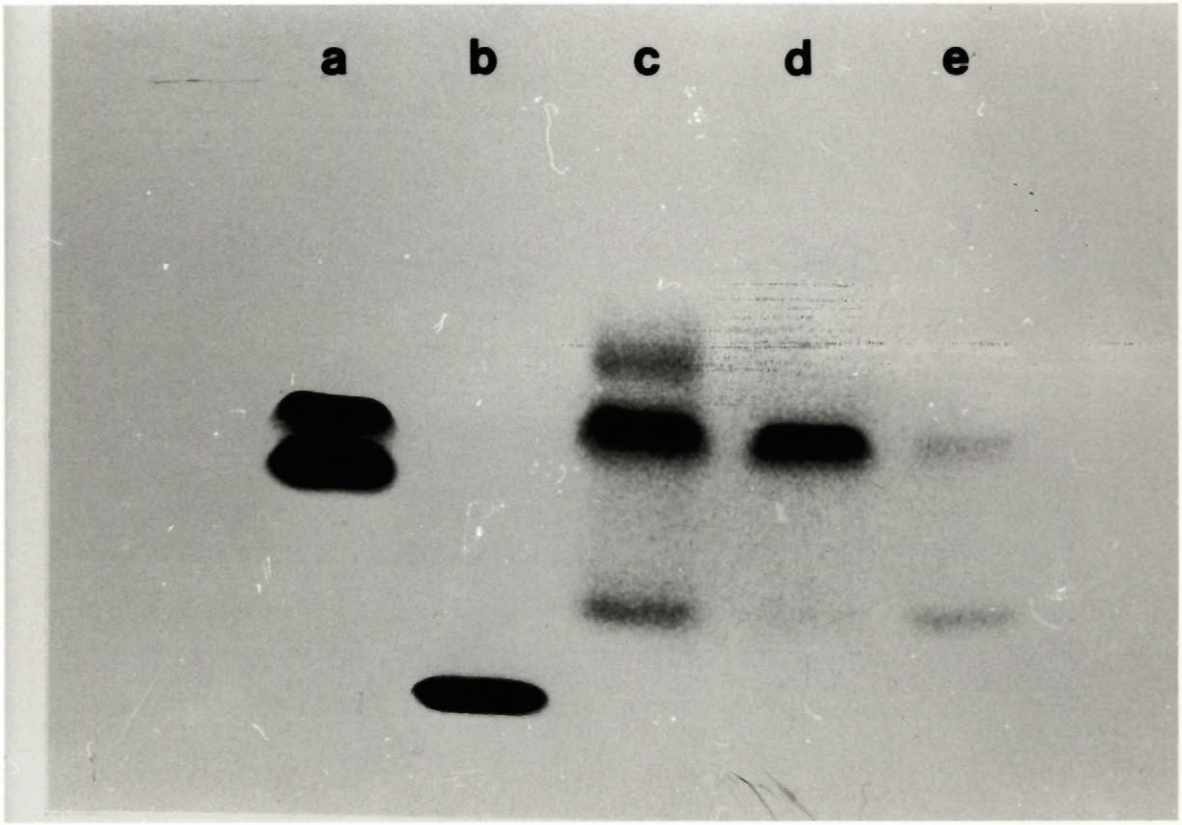
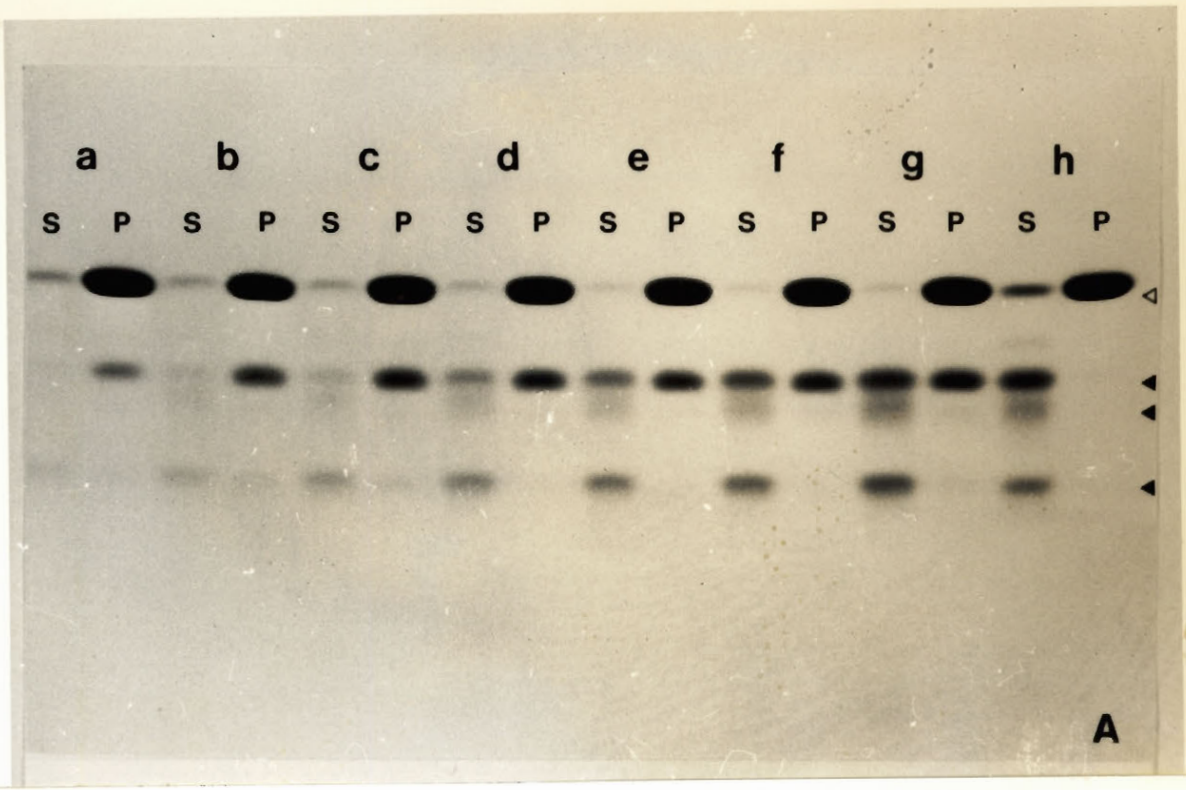
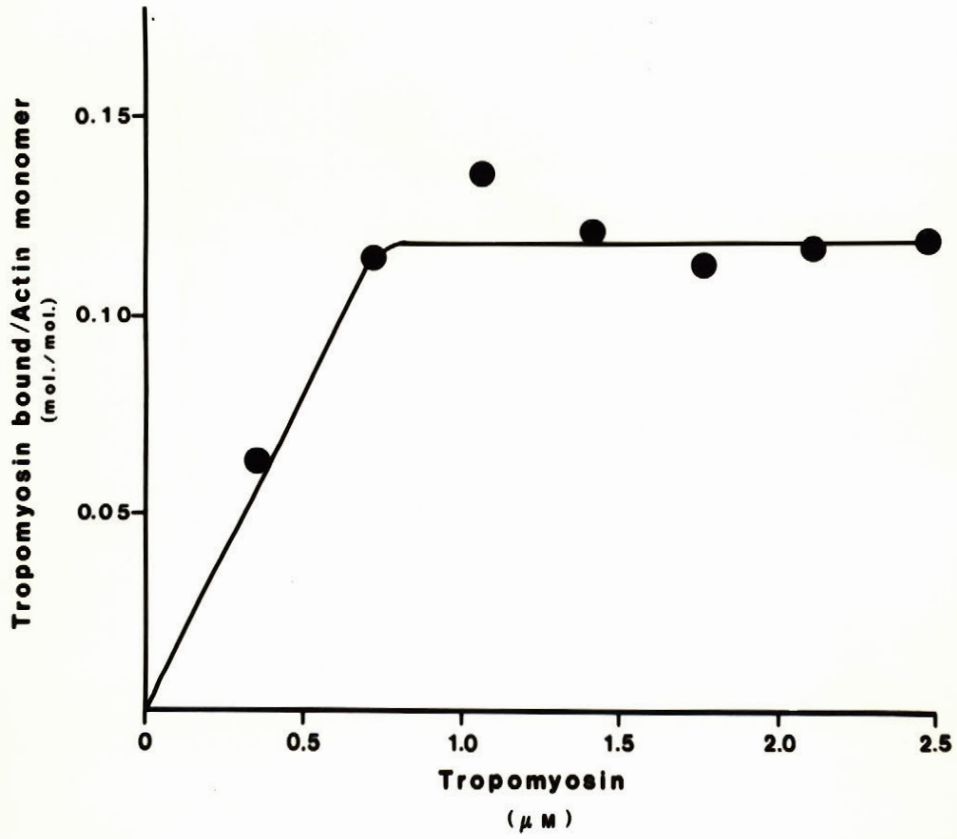

a
b
c
d
e

Fig. 26 Binding of adrenal medullary tropomyosins to chicken gizzard F-actin in the presence of 10 mM $MgCl_2$. Purified adrenal medullary tropomyosin fraction at various concentrations (0.36-2.47 μM) was incubated for 2 h at 22°C with chicken gizzard F-actin (4.5 μM) as described in Chap. 2 section C.2, and then centrifuged for 2 h at 20,000 g. Panel A shows the Coomassie blue staining of 10% SDS-polyacrylamide gel used to analyze the supernatants (S) and pellets (P) obtained from the interaction experiment with different concentrations of tropomyosins in presence (a-g) or absence (h) of 10 mM $MgCl_2$. The tropomyosin concentrations (μM) were: a, 0.36; b, 0.72; c, 1.06; d, 1.41; e, 1.77; f, 2.11; g, 2.47 and h, 1.41. The open arrowhead (▷) indicates the position of actin and the closed arrowheads (▶) indicate the positions of the adrenal medullary tropomyosins (38, 35.5 and 32kD). The protein concentration of the tropomyosin fraction was determined by the dye reagent method using bovine skeletal muscle tropomyosin as standard. The adrenal medullary tropomyosin concentrations were then calculated considering the actual molar ratio of the isoforms present. Panel B shows the corresponding plot of tropomyosin bound per actin monomer versus the tropomyosin concentrations. The amount of actin (90%) which sedimented under the experimental conditions was determined by densitometry. The quantity of tropomyosin in each pellet was also determined by densitometry but on the basis of standardization with the same adrenal medullary tropomyosin preparation used for the binding experiment. The molar ratio of 38kD tropomyosin to actin was calculated assuming a MW = 45,000 for gizzard actin and a MW = 76,000 for tropomyosin homodimer. Similar results were obtained in another experiment done under identical conditions and in another 2 experiments carried out in presence of 100 mM KCl and 1 mM $MgCl_2$.


a b c d e f g h
S P S P S P S P S P S P S P S P
A


Tropomyosin bound/Actin monomer
(mol./mol.)
0.15
0.10
0.05
0
0.5
1.0
1.5
2.0
2.5
Tropomyosin
(μM)

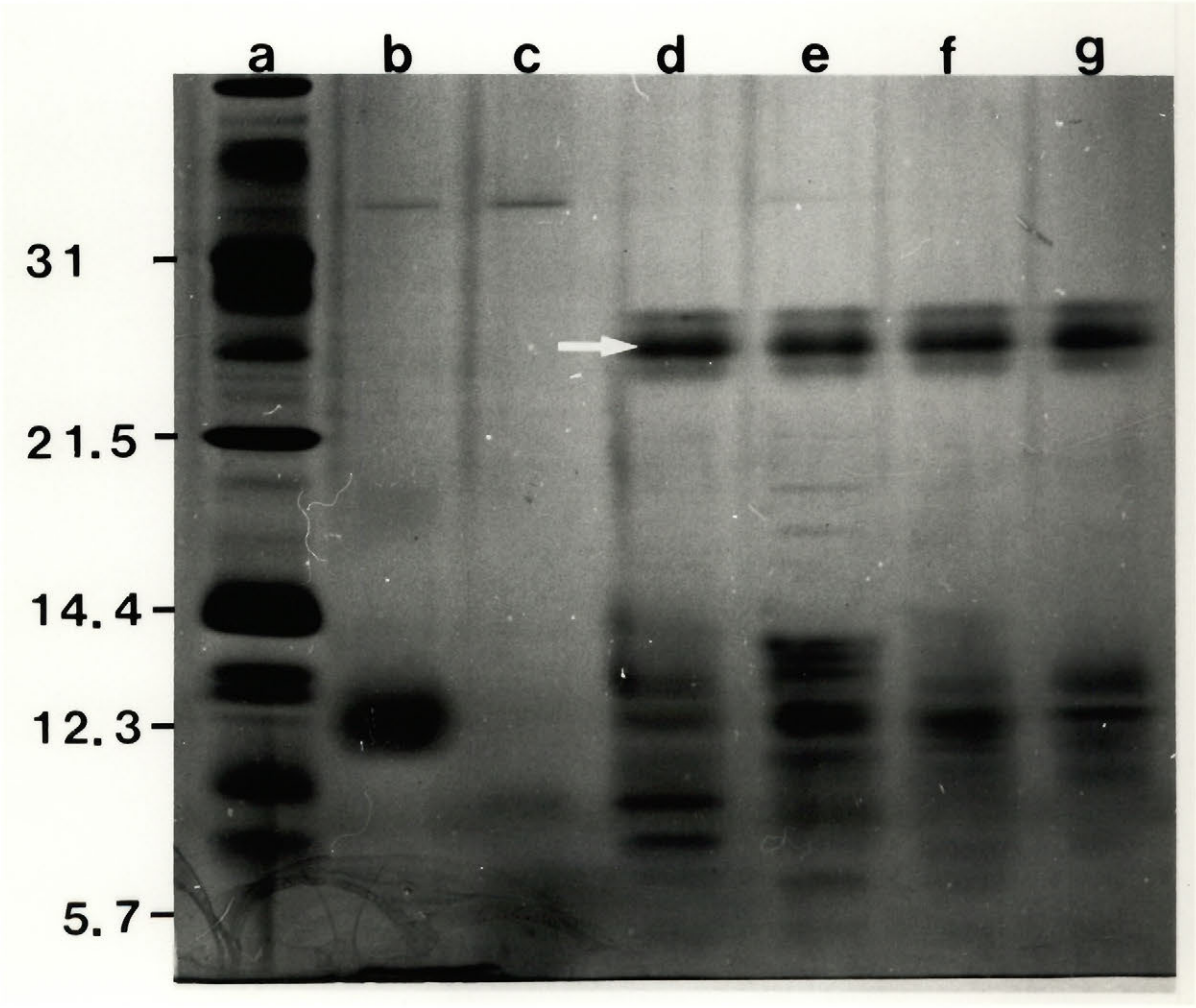
B

concentration (50 mM KCl) in the absence of $MgCl_2$, no interaction with F-actin was observed for any of the tropomyosin forms present (Fig. 26A, lane h). Identical results as those described in Fig. 26 were obtained when the interaction study was done in the presence of 100 mM KCl plus 1 mM $MgCl_2$ (data not shown). In some experiments, the tropomyosin preparation was first depleted of its 38kD component by interaction with F-actin, the remaining supernatant which was enriched in the 32kD and 35.5kD tropomyosins was used for the study of F-actin binding. Even under these experimental conditions, the 32kD and 35.5kD forms failed to bind significantly to actin filaments (data not shown). From the above results and the plot of the tropomyosin bound per actin monomer versus the tropomyosin concentration (Fig. 26B), a molar ratio of 0.12 mole of 38kD adrenal medullary tropomyosin per mole of actin monomer is obtained.

B.5 Limited Proteolytic Digestion of the Different Adrenal Medullary Tropomyosins

Limited proteolysis using Staphylococcus aureus V8 protease was performed to determine if any similarities existed between the peptide profiles of different adrenal medullary tropomyosins and those of β and α forms of bovine skeletal muscle tropomyosin. Most of the peptides obtained had molecular weight values ranging from 16,000 to 6,000 (Fig. 27). Although the digestion patterns were distinctly different from each other, there was some similarity between the different adrenal medullary tropomyosins and also between these and the skeletal muscle tropomyosin β and α forms (Fig. 27).

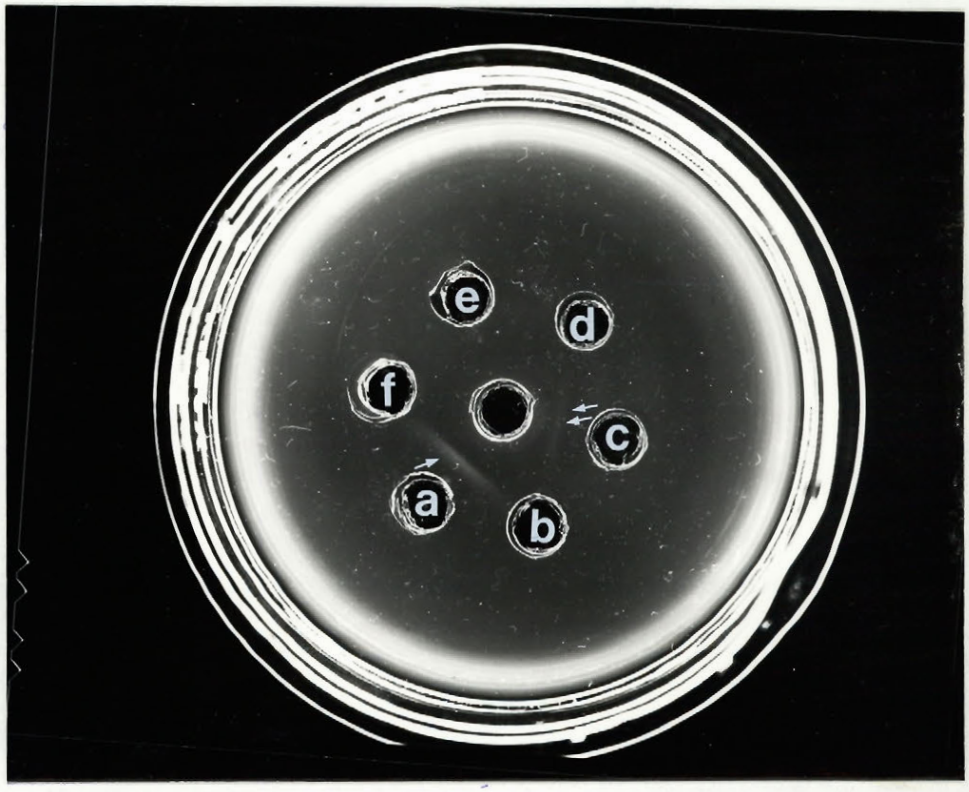
Fig. 27 Limited proteolytic digestion with V8 protease of bovine adrenal medullary tropomyosins and bovine skeletal muscle tropomyosin isoforms. The protein preparations were first separated on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue and after destaining equilibrated in distilled water. The different protein bands were cut out and incubated for 20 min in 0.1% SDS, 1 mM EDTA and 125 mM Tris-HCl, pH 6.8. The medium was replaced by a minimum volume of 0.1% SDS, 5% sucrose, 1 mM EDTA and 62.5 mM Tris-HCl, pH 6.8, and the bands were heated at 100°C for 2 min. The content in each case (band plus medium) was applied to a discontinuous gel composed of 4% stacking gel (3 cm) and a 17% running gel (10 cm), and aliquots (250 ng) of V8 protease were added to each well just before the electrophoresis was started. The electrophoregram shown was obtained after silver staining. The first 3 lanes correspond to the calibration standards: 2 μg each of carbonic anhydrase (31kD), soybean trypsin inhibitor (21.5kD), lysozyme (14.4kD) (lane a); 3 μg of cytochrome c (12.3kD) (lane b); and 3 μg of insulin (5.7kD) (lane c). The other different lanes correspond to the peptide profiles of bovine skeletal muscle β (d) and α (e) isoforms, and of bovine adrenal medullary 38kD (f) and 32kD (g) tropomyosins. The arrow indicates the position of the V8 protease (28kD). Similar results were obtained in 2 other experiments.


a
b
c
d
e
f
g
31
21.5
14.4
12.3
5.7

B.6 Immunological Characterization of Adrenal Medullary Tropomyosins

Double immunodiffusion studies using a modified Ouchterlony procedure were performed to determine if adrenal medullary tropomyosins would react with tropomyosin antibodies. Under the conditions used (see Chap. 2 section C.4.1), the hydroxylapatite-purified adrenal medullary tropomyosins were shown to be recognized by a smooth muscle tropomyosin antibody as revealed by the presence of a strong precipitation line (Fig. 28, well a). This antibody reacted also with bovine skeletal muscle tropomyosin but to a much lesser extent (Fig. 28, well c). The immunological reaction was specific for tropomyosins since no precipitation lines were detected for the other antigens present, namely troponins, actin, α-actinin and BSA (Fig. 28, wells b, d, e and f).

Fig. 28 Immunological characterization of adrenal medullary tropomyosins by double immunodiffusion. The central well contained an aliquot of a smooth muscle tropomyosin antibody and the peripheral wells the different antigens at a concentration of 1 mg/ml. Well a, adrenal medullary tropomyosin preparation; b, troponins; c, bovine skeletal muscle tropomyosin; d, actin; e, α-actinin and f, BSA. Following the immunological incubation (see Chap. 2 section C.4.1), a strong precipitation line was observed between the central well and well a which contained adrenal medullary tropomyosins (indicated by an arrow). In addition, a very faint precipitation line was also observed between the central well and well c which contained skeletal muscle tropomyosin (indicated by a double arrow).


e
d
f
c
a
b

C. CHROMAFFIN CELL TROPOMYOSINS: ISOLATION, PHOSPHORYLATION AND IMMUNOCYTOCHEMICAL LOCALIZATION

C.1 Chromaffin Cell Tropomyosins

Chromaffin cell tropomyosins were partially purified from cultured cells basically as described for the isolation of adrenal medullary tropomyosins (for details see Chap. 2 section C.1.2).

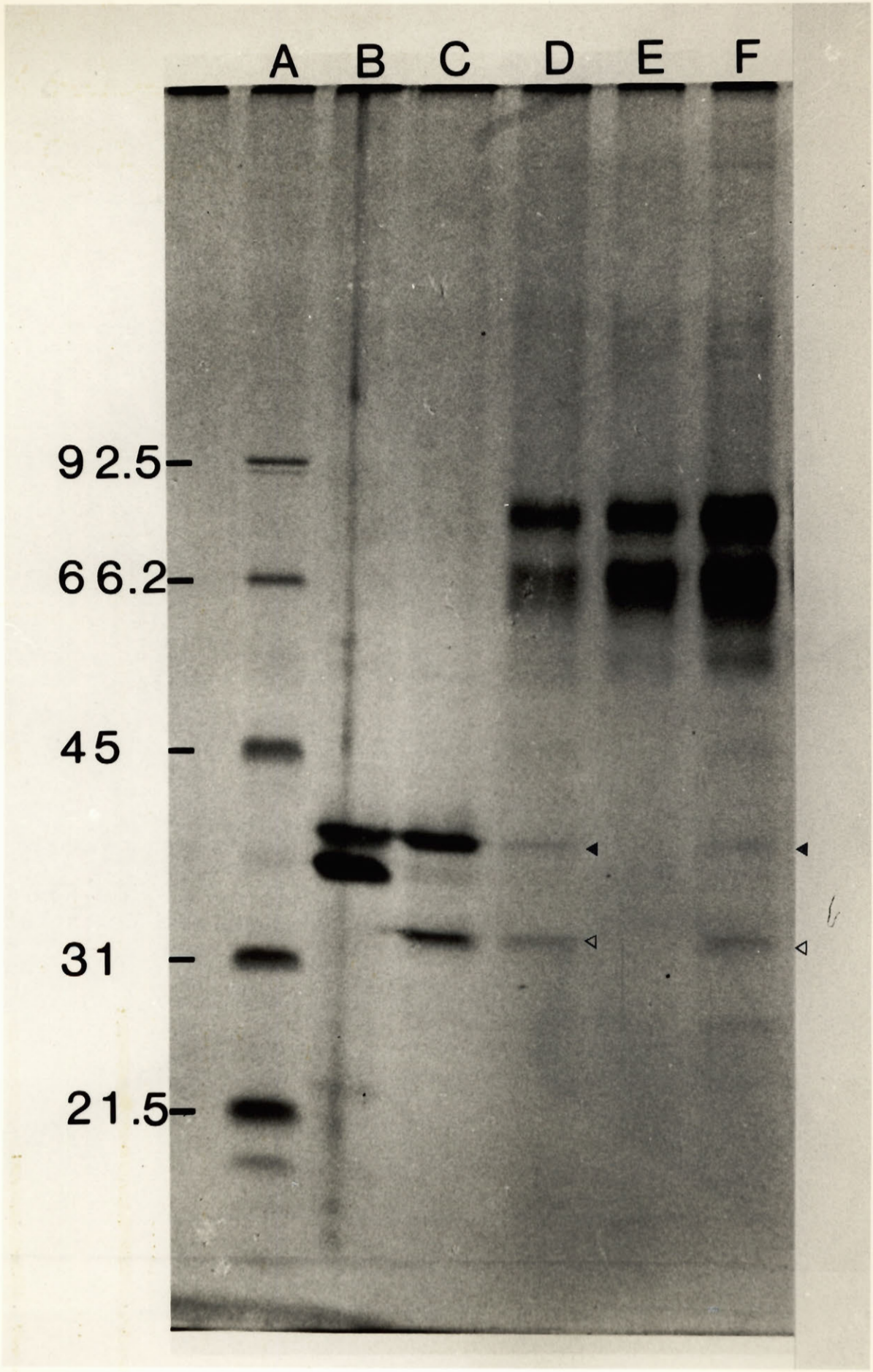
The partially purified chromaffin cell tropomyosin preparation obtained was analyzed by gel electrophoresis (Fig. 29). Following silver staining of the gel, it was observed that two polypeptide bands comigrated with the 38kD and the 32kD components of adrenal medullary tropomyosins (Fig. 29, lanes D and F). As previously described for adrenal medullary tropomyosins, these two polypeptides were enriched in the DEAE-Sephacel column fraction following elution with 0.35 M KCl (Fig. 29, lane D). However, in this case, the tropomyosin preparation was still contaminated by high molecular weight proteins which correspond to the protein-carrier fraction (Fig. 29, lane E) (for details see Chap. 2 section C.1.2). It should be noted also that the ratio of the 38kD to the 32kD polypeptide of cultured chromaffin cells appears to be much lower than the one determined for adrenal medullary tropomyosin components.

Fig. 29 SDS-polyacrylamide gel of cultured chromaffin cell tropomyosins.

Chromaffin cell tropomyosins were partially purified from cultured cells as described in Chap. 2 section C.1.2.

This figure shows the silver staining of a 10% SDS-polyacrylamide gel of some of the fractions obtained during the purification procedure. Lane F corresponds to an aliquot of the preparation which was applied to the DEAE-Sephacel column (pre-column fraction). Lane D corresponds to the fraction eluted from the DEAE-Sephacel column with 0.35 M KCl. This fraction contains the two forms of chromaffin cell tropomyosins (32kD and 38kD). However, this preparation is still contaminated by the protein-carrier fraction (shown in lane E). The position of the 38kD and 32kD forms of tropomyosin are indicated by closed arrowheads and opened arrowheads, respectively.

The first three lanes (A-C) corresponds to MW standards (A), bovine skeletal muscle tropomyosin (B) and purified adrenal medullary tropomyosins (C).


A
B
C
D
E
F
92.5
66.2
45
31
21.5

C.2 Phosphorylated Form of Chromaffin Cell Tropomyosin

Cultured chromaffin cells were labelled with ^{32}P for 1 h as described previously in these studies (Chap. 2 section B). The labelled chromaffin cell extracts obtained were then treated in a similar way as described for the isolation of adrenal medullary tropomyosins although some steps of the procedure were omitted to minimize phosphatase activity (for details see Chap. 2 section C.3).

The partially purified chromaffin cell tropomyosin preparation obtained was analyzed by gel electrophoresis (Fig. 30). Coomassie blue staining and autoradiography of the gel revealed that several polypeptides were present in this fraction (Fig. 30A) and that some of them were phosphorylated (Fig. 30B). Three of these protein bands had relative mobilities similar to purified adrenal medullary tropomyosins (Fig. 30, bands B, C and D). Consequently, those three bands were cut out of the gel, swollen and applied onto a second SDS-polyacrylamide gel.

Following Coomassie blue staining and autoradiography of the second gel, it was observed that in fact two of the bands (B and C) comigrated respectively with the 38kD (B) and 32kD (C) components of adrenal medullary tropomyosins while band D does not appear to correspond to any of the adrenal tropomyosin polypeptides (Fig. 31A). In addition, the 38kD component (band B) was phosphorylated under these experimental conditions (Fig. 31B). However, no phospholabelled bands appear to correspond to the 35.5kD or 32kD adrenal medullary tropomyosins (Fig. 31B, lanes C and D).

Fig. 30 SDS-polyacrylamide gel of ^{32}P-labelled chromaffin cell tropomyosin preparation.

Cultured chromaffin cells were labelled with ^{32}P for 1 h at 37°C as described in Chap. 2 section B.1. The chromaffin cell extracts obtained were treated in a similar way as outlined in Fig. 14 although some purification steps were omitted (for details see Chap. 2 section C.3).

The partially purified ^{32}P-labelled tropomyosin preparation was analyzed on a 10% SDS-polyacrylamide gel. Coomassie blue staining (A), autoradiogram (B).

As observed by electrophoresis, this partially purified tropomyosin preparation contained several polypeptides, some of which were phosphorylated. However, only three proteins (bands B, C, D) had apparent molecular weights similar to adrenal medullary tropomyosins. (This preparation was further analyzed by electrophoresis; see Fig. 31).

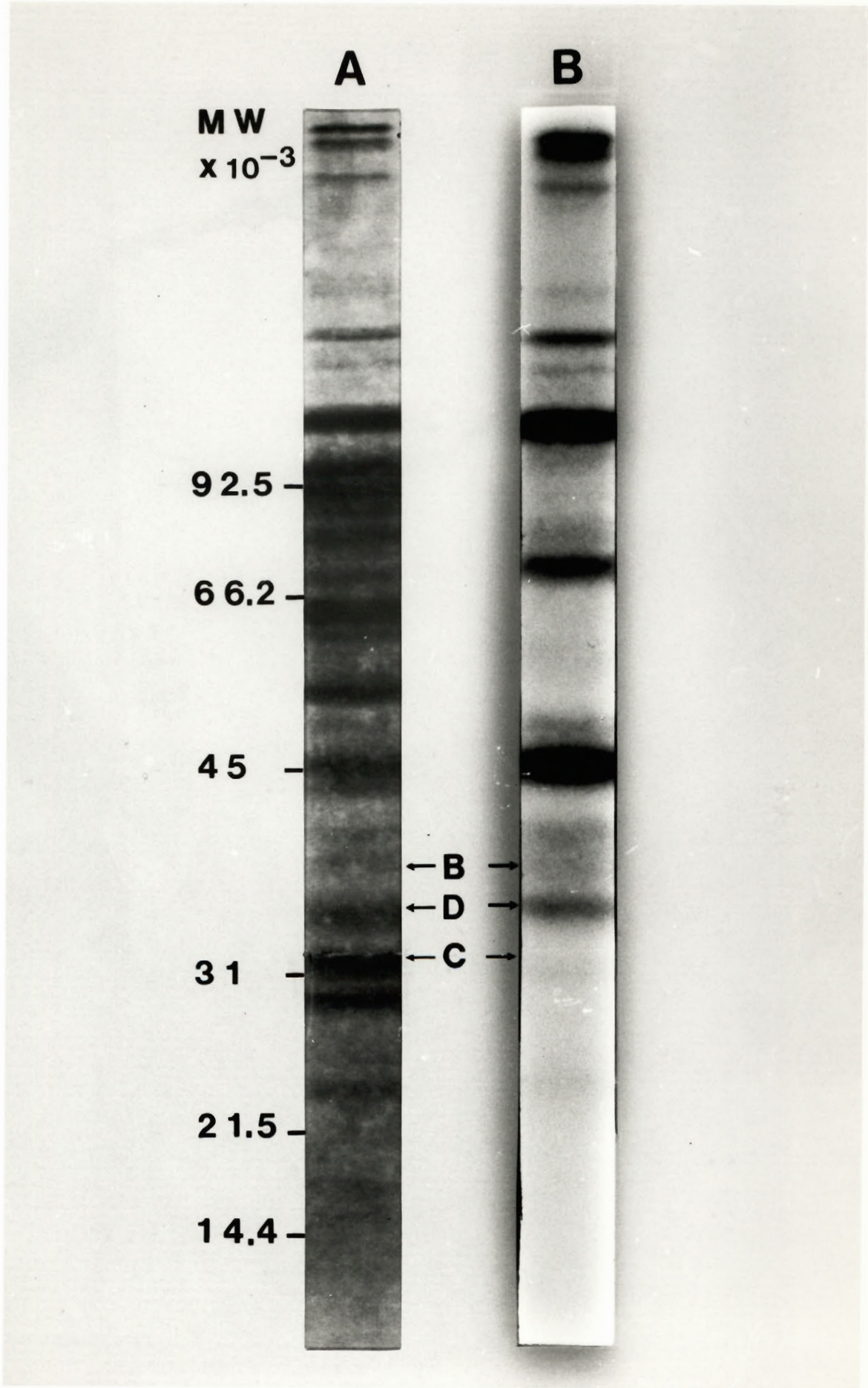
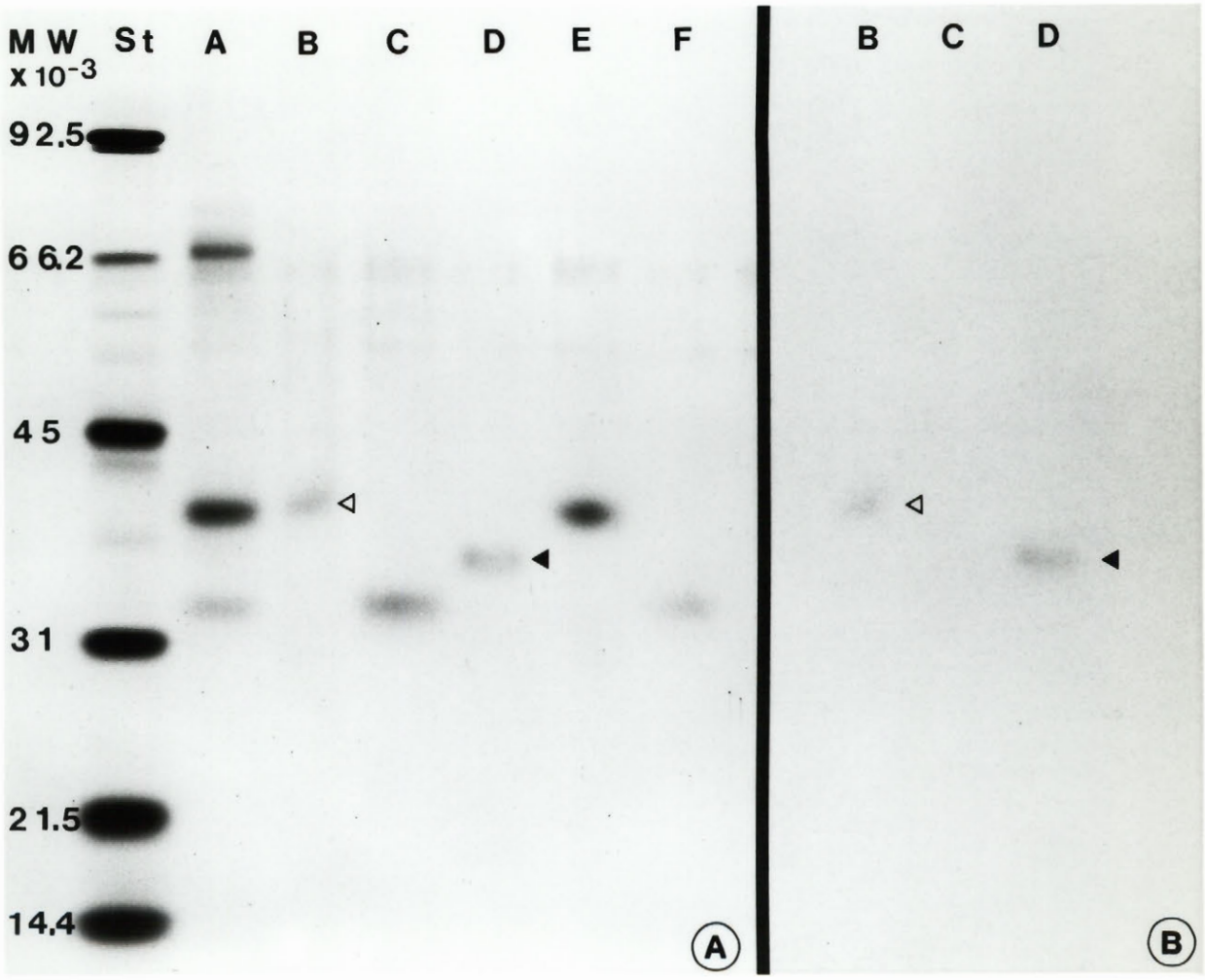

A
B
MW
x 10⁻³
92.5
66.2
45
31
21.5
14.4
B
D
C

Fig. 31 SDS-polyacrylamide gel of the phosphorylated form of chromaffin cell tropomyosin. Three polypeptides of apparent molecular weights of 30-40kD which were present in the ^{32}P-labelled chromaffin cell tropomyosin preparation (see Fig. 30, bands B, C, D) were further characterized by SDS-PAGE. These three protein bands (B, C, D) separated on a first SDS-polyacrylamide gel (shown in Fig. 30) were cut out of the gel, equilibrated and re-run on a second 10% SDS-polyacrylamide gel in parallel with purified adrenal medullary tropomyosins. Panel A shows the Coomassie blue staining of the gel and panel B the corresponding autoradiogram. Lanes B, C and D correspond to the three protein bands obtained from the first gel. Lane A corresponds to a sample of adrenal medullary tropomyosins while lanes E and F correspond to the 38kD and 32kD tropomyosin polypeptides previously separated by SDS-PAGE. Following staining and autoradiography, it was observed that band B (▷) co-migrated with the 38kD form of adrenal medullary tropomyosins and that band C co-migrated with the 32kD adrenal tropomyosin polypeptide. It was also observed that bands B (▷) and D (▶) were phosphorylated.


MW St A B C D E F
x 10-3
92.5
66.2
45
31
21.5
14.4
A
B C D
B

C.3 Immunocytochemical Localization of Chromaffin Cell Tropomyosin

Cultured chromaffin cells were stained by a smooth muscle tropomyosin antibody (for details see Chap. 2 section C.4.2). A combined pattern of fluorescence was observed in these cells and consisted of a certain number of fluorescent granules distributed preferentially in areas close to the cell nucleus and of fluorescent filaments distributed over the entire area of the cell (Figs. 32, a, b and c). In many cases, especially in bipolar chromaffin cells, these fine fluorescent filaments were arranged in parallel (Figs. 32, b and c). This was better observed at a large magnification (Fig. 32c'). Fluorescent filaments were also seen to extend from a point of cell attachment to the next and to run parallel to each other and to the plasma membrane (Fig. 32a).

A small percentage of fibroblasts contaminated these preparations. Therefore, those cells were also examined in order to see if the antibody used in these experiments would stain these fibroblasts as those described in previous studies using antibodies raised against skeletal muscle tropomyosin (Lazarides, 1976). Thus, a bright fluorescent staining was observed on the stress fibers (Fig. 33a). High resolution fluorescence microscopy determined that the stress fibers stained with the tropomyosin antibody show periodic fluorescence (Fig. 33b).

Control experiments were performed by incubating the cultured cells with a tropomyosin antibody preparation previously absorbed with an excess of adrenal medullary tropomyosins. Under those experimental conditions, the characteristic fluorescent patterns described above for both chromaffin

Fig. 32 Immunocytochemical staining of cultured bovine adrenal chromaffin cells using a smooth muscle tropomyosin antibody.

Chromaffin cells cultured on collagen-coated coverslips for 7 days were fixed and stained with an affinity-purified smooth muscle tropomyosin antibody (for details see Chap. 2 section C.4.2).

The preparations were examined by incident light fluorescent microscopy (epifluorescence). A combined pattern of fluorescence was observed in these cells. Some fluorescent granules were distributed preferentially in the perinuclear region (a, b, d) whereas fluorescent filaments (⇐) were observed over the entire area of the cell (a, b, c, c').

Magnification: × 600 (a,b), × 750 (c) and × 2000 (c').

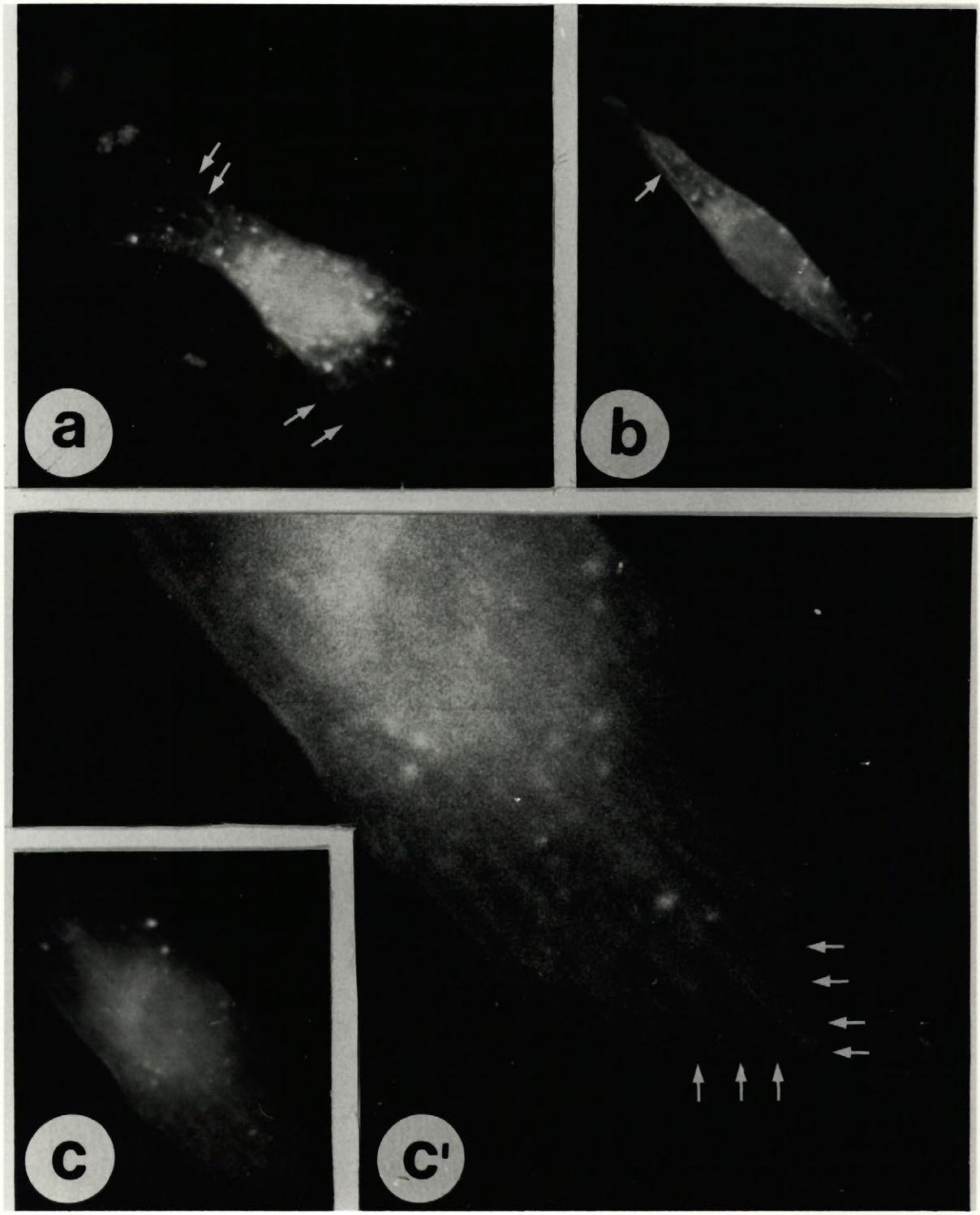
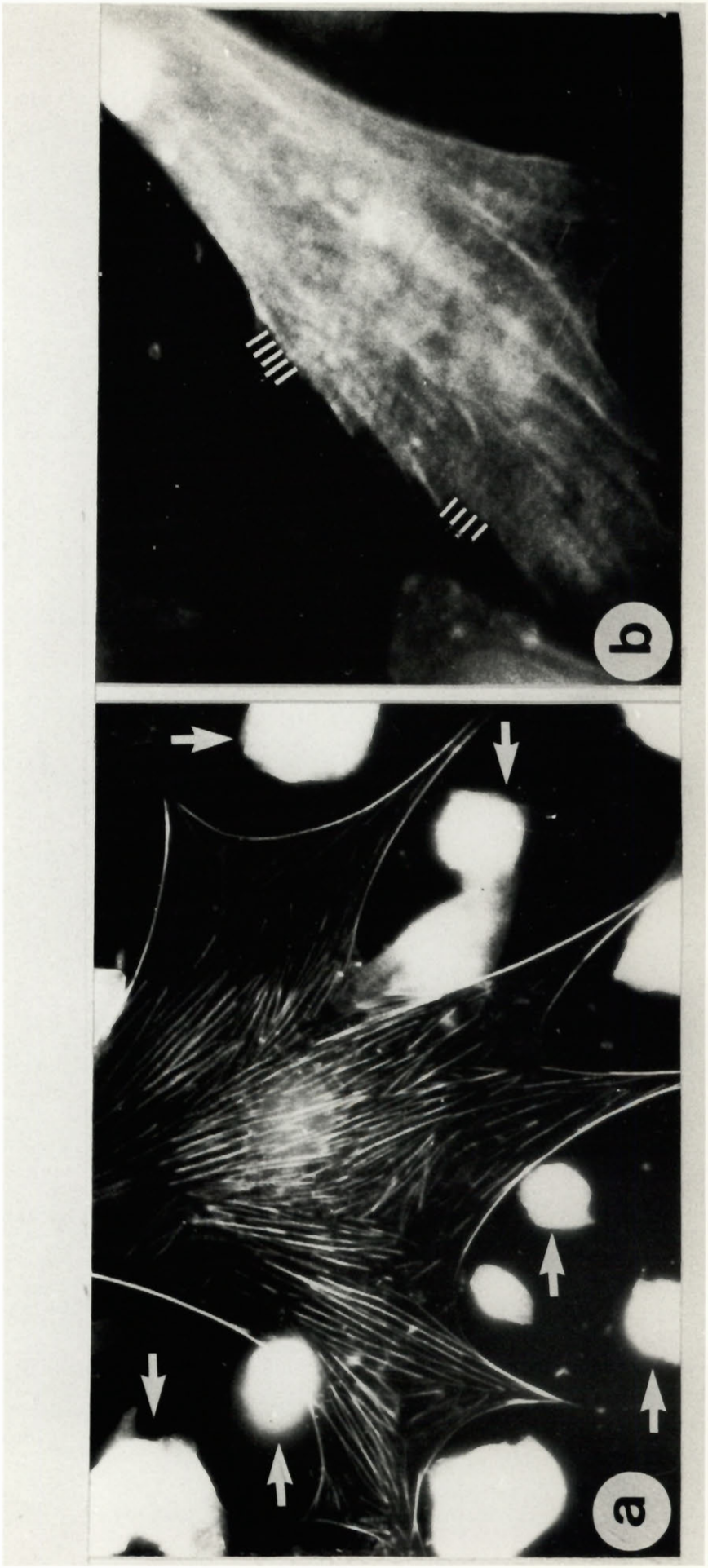

a
b
c
c'

Fig. 33 Immunocytochemical staining of fibroblasts with a smooth muscle tropomyosin antibody. The chromaffin cell cultures were contaminated by a small percentage of fibroblasts which were also examined under the same conditions as described in Fig. 32. In this case, a bright fluorescent staining was observed on the stress fibers of the fibroblasts (a). In addition, a periodic fluorescent staining pattern (▭) was observed on stress fibers using high resolution fluorescence microscopy (b). The arrows (:⊐) indicate the position of chromaffin cells seen at another focusing plane.

Magnification: × 950 (a) and × 3,500 (b).


b
a

cells and fibroblasts were not observed, and only background fluorescence was detected in those control preparations (Figs. 34, b and d).

Fig. 34 Control experiments for immunocytochemical staining.

In these experiments, the smooth muscle tropomyosin antibody was pre-absorbed with an excess of purified adrenal medullary tropomyosin preparations. Under these conditions, only background fluorescence was detected for both chromaffin cells (b) and fibroblasts (d).

Phase contrast (a, c), Epifluorescence (b, d). Magnification: × 900 (a, b) and × 600 (c, d).

a
b
c
d

D. APPENDIX: EFFECTS OF TRIFLUOPERAZINE ON CATECHOLAMINE SECRETION AND RELATED EVENTS IN CULTURED CHROMAFFIN CELLS

The calmodulin antagonist trifluoperazine (TFP) was utilized in some of the phosphorylation studies presented in this thesis (section A.5). This agent has been used extensively in numerous cell systems and has been reported to produce different effects depending upon the experimental conditions utilized. Consequently, prior to the studies on the effect of TFP on ACh-induced phosphorylation of chromaffin cell proteins, a series of experiments were carried out to determine the effects of this calmodulin antagonist on catecholamine release and calcium movements in the same cultured cell system.

D.1 Effect of Trifluoperazine on Acetylcholine-Evoked Release of [^{3}H]noradrenaline

Seven-day-old cultured chromaffin cells (10^6 cells/dish) were incubated in Locke's solution for 20 min in the absence or presence of a wide range of concentrations of TFP. Then, the cultured cells were stimulated with 10^{-4}M ACh for 3 successive 3 min periods. In the absence of TFP, the cells responded to ACh with a typical pattern of catecholamine release as reported previously (Kenigsberg & Trifaró, 1980; Trifaró & Lee, 1980). In the presence of TFP, the ACh-evoked response was inhibited although the [^{3}H]NA release pattern was not altered under these conditions (see Fig. 35C). TFP inhibited in a dose-dependent fashion the ACh-evoked catecholamine release

with an IC_{50} of 2×10^{-7}M TFP (Fig. 35A).

D.2 Effect of Trifluoperazine on the Output of [^{3}H]noradrenaline Elicited by a Depolarizing K^+ Concentration

In these experiments, cultured chromaffin cells were stimulated with 56 mM K^+. The [^{3}H]NA release pattern obtained upon stimulation with K^+ was similar to that observed during ACh stimulation. Similarly to its effect on the cholinergic stimulation of chromaffin cells, TFP inhibited the secretory response to 56 mM K^+ without modifying the release pattern. The dose-dependent inhibition of the K^+-evoked amine release produced by TFP is shown in Fig. 35B. In this case, the IC_{50} for the TFP effect was 2.2×10^{-6}M.

D.3 Effect of Different Extracellular Ca^{2+} Concentrations on the Inhibition of Acetylcholine-Evoked [^{3}H]noradrenaline Release by Trifluoperazine

The secretory response of the chromaffin cell to ACh augments with an increase in the extracellular Ca^{2+} concentration. This phenomenon reaches a plateau at Ca^{2+} concentrations in the range of 3-4 mM (Fenwick et al., 1978). The effect of external Ca^{2+} concentrations on ACh-evoked amine release is shown in Table 7. Similar experiments were carried out in the presence of 10^{-7}M TFP, a concentration which produces 40-45% inhibition of the ACh-stimulated secretory response in the presence of 2.2 mM Ca^{2+}, and the results obtained are presented in Table 7. As shown in this table, the

Fig. 35 Inhibition of ACh-evoked and K^+-induced release of [^{3}H]NA from cultured chromaffin cells by TFP. Seven-day-old cultures were labelled with 10^{-7}M [^{3}H]NA for 5 min then subsequently washed for 60 min (see Chap. 2 section D.1). [^{3}H]NA output is expressed as % of total cell [^{3}H]NA content. The ACh-evoked or K^+-induced [^{3}H]NA output is determined by subtracting basal or spontaneous release values from those obtained during three consecutive 3 min periods of stimulation.

A) ACh-evoked [^{3}H]NA output in the presence of TFP (▲) is expressed as a percentage of the output determined in the absence of TFP (△).

B) 56 mM K^+-induced [^{3}H]NA output in the presence of TFP (■) is expressed as a percentage of the output determined in the absence of TFP (□).

C) Effect of TFP on the ACh-evoked release of [^{3}H]NA. [^{3}H]NA output is expressed as percentage of total tissues [^{3}H]NA content.

Each value represents the mean ± S.E.M. of 4 different culture dishes.

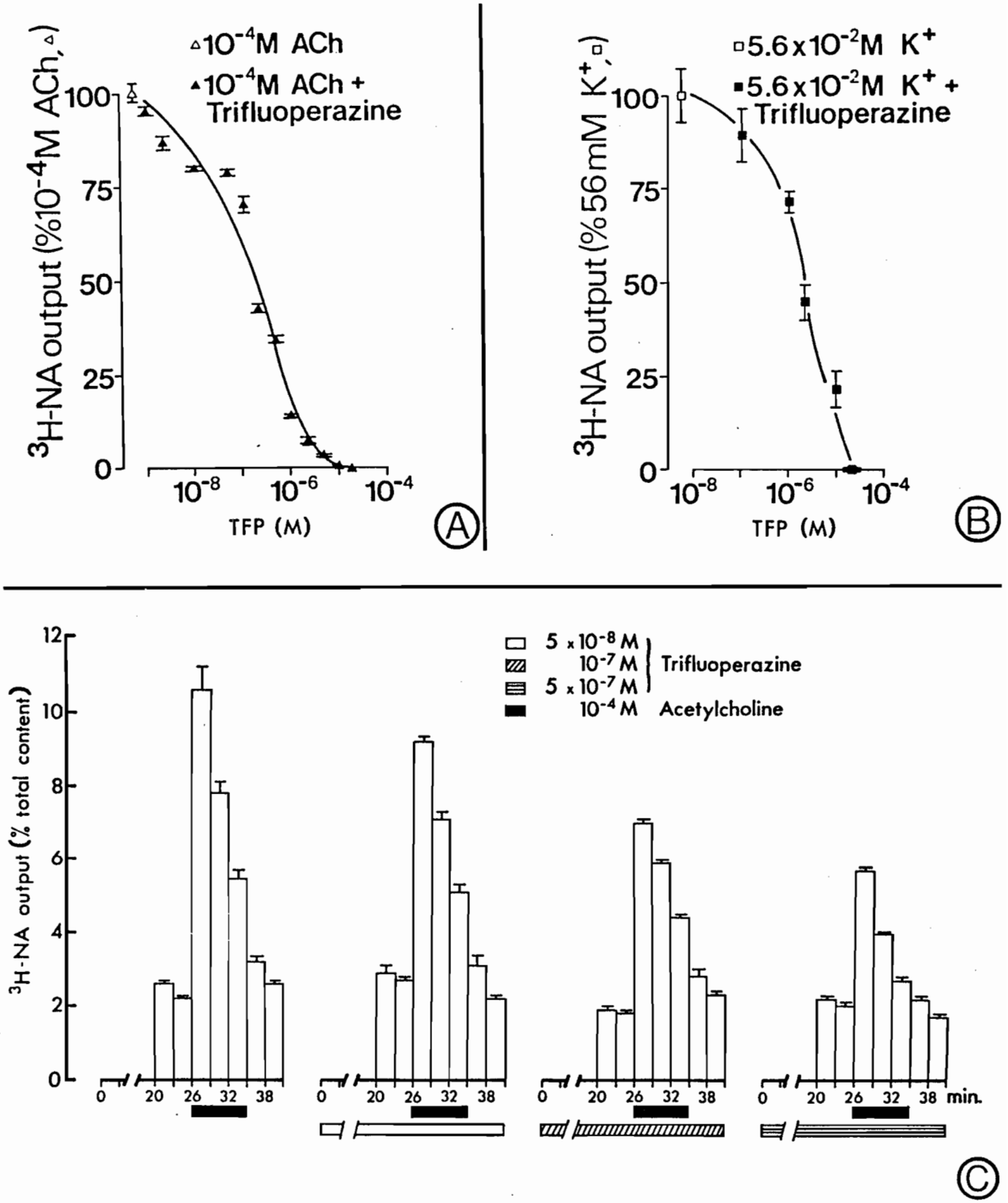

△10⁻⁴M ACh
▲10⁻⁴M ACh + Trifluoperazine
³H-NA output (%10⁻⁴M ACh,△)
100
75
50
25
0
10⁻⁸
10⁻⁶
10⁻⁴
TFP (M)
A
□5.6x10⁻²M K⁺
■5.6x10⁻²M K⁺ + Trifluoperazine
³H-NA output (%56mM K⁺,□)
100
75
50
25
0
10⁻⁸
10⁻⁶
10⁻⁴
TFP (M)
B
5 x 10⁻⁸ M
10⁻⁷ M
5 x 10⁻⁷ M
Trifluoperazine
10⁻⁴ M Acetylcholine
³H-NA output (% total content)
12
10
8
6
4
2
0
0
20
26
32
38
min.
C

Table 7

Effect of TFP on the ACh-evoked [^{3}H]noradrenaline release from cultured chromaffin cells in the presence of various concentrations of Ca^{2+}.

Extracellular	[^{3}H]NA output, % of total stores		Inhibition
[Ca^{2+}](mM)	10^{-4}M ACh	10^{-4}M ACh + 10^{-7}M TFP	by TFP (%)
0.275	5.6 ± 0.4	0.2 ± 0.002	96.5
0.55	8.8 ± 0.5	1.3 ± 0.2	85.2
1.1	10.9 ± 0.1	4.1 ± 0.2	62.4
2.2	13.9 ± 0.3	8.0 ± 0.3	42.4
4.4	16.9 ± 0.03	13.4 ± 0.5	20.7

ACh-induced [^{3}H]NA output in the absence or presence of TFP was determined by subtracting basal or spontaneous release values from those obtained during the three consecutive 3 min stimulation periods with 10^{-4}M ACh. Basal and ACh-induced release of [^{3}H]NA was monitored in the presence of various extracellular concentrations of Ca^{2+} as described in Chap. 2 section D. Each value represents the mean ± S.E. of 6 different culture dishes.

percentage of inhibition produced by TFP increased as the extracellular Ca^{2+} concentration decreased and reached 96.5% when the Ca^{2+} concentration was 0.275 mM. However, it should be noted that with the exception of the responses obtained in the presence of 4.4 mM Ca^{2+}, TFP inhibited the ACh-evoked [^{3}H]NA output by a similar magnitude for all Ca^{2+} concentrations tested. These values correspond to a decrease in the total [^{3}H]NA stores of 5.4, 7.5, 6.8 and 5.9% for the four lower extracellular Ca^{2+} concentrations (0.275-2.2 mM) tested.

D.4 Effect of Trifluoperazine on Depolarization-Induced Ca^{2+} Uptake and on Ca^{2+} Efflux from Cultured Chromaffin Cells

It has been demonstrated previously that a depolarizing K^+ concentration (56 mM) produces an increase in ^{45}Ca influx into the perfused adrenal medulla or the cultured chromaffin cell (Aguirre et al., 1977; Bourne & Trifaró, 1982). In these experiments, when 2.5 mM Ni^{2+} was present in the incubation medium during the 5 min exposure to 56 mM K^+ in the presence of ^{45}Ca, almost total inhibition (98 ± 3%) of the ^{45}Ca uptake was observed and resulted in very low levels of ^{45}Ca as shown in Fig. 36. When TFP was present in the incubation medium during K^+-induced depolarization, the following results were obtained: TFP at concentrations of 10^{-6}-10^{-5}M did not modify either the K^+-induced ^{45}Ca uptake (Fig. 37) or the ^{45}Ca efflux. TFP at a concentration of 2.5×10^{-5}M produced a small decrease in the ^{45}Ca efflux curve (Fig. 36) and a decrease in the K^+-evoked ^{45}Ca uptake of 30 ± 14% (Fig. 37). However, it should be noted that this concentration of TFP produced a total inhibi-

Fig. 36 Effect of TFP and Ni^{2+} on the ^{45}Ca uptake and efflux from cultured chromaffin cells during K^+ stimulation. Five groups of five dishes each (10^6 cells/dish) were incubated for two 5 min intervals with Ca^{2+}-free Locke's solution in the absence of (○) or in the presence of 2.5×10^{-6}M (△), 10^{-5}M, 2.5×10^{-5}M TFP (▲) or 2.4×10^{-3}M Ni^{2+} (□). Radiolabelled calcium (5 μCi ^{45}Ca/dish) in 2.2 mM Ca^{2+}-Locke's solution containing 56 mM K^+ with or without the aforementioned concentrations of TFP or Ni^{2+} was applied to the cells for 5 min. Subsequent to ^{45}Ca labelling, the cells were washed with Ca^{2+}-free (2 mM EDTA) Locke's solution for 65 min. The washes were collected and ^{45}Ca content present determined as described in Chap. 2 section D.2. With the values obtained, desaturation curves were plotted (---), and the uptake of ^{45}Ca into chromaffin cells was calculated as described in Chap. 2 section D.2 from the values of these curves (·····).

Each point and bar represents the mean ± S.E.M. from 5 different culture dishes.

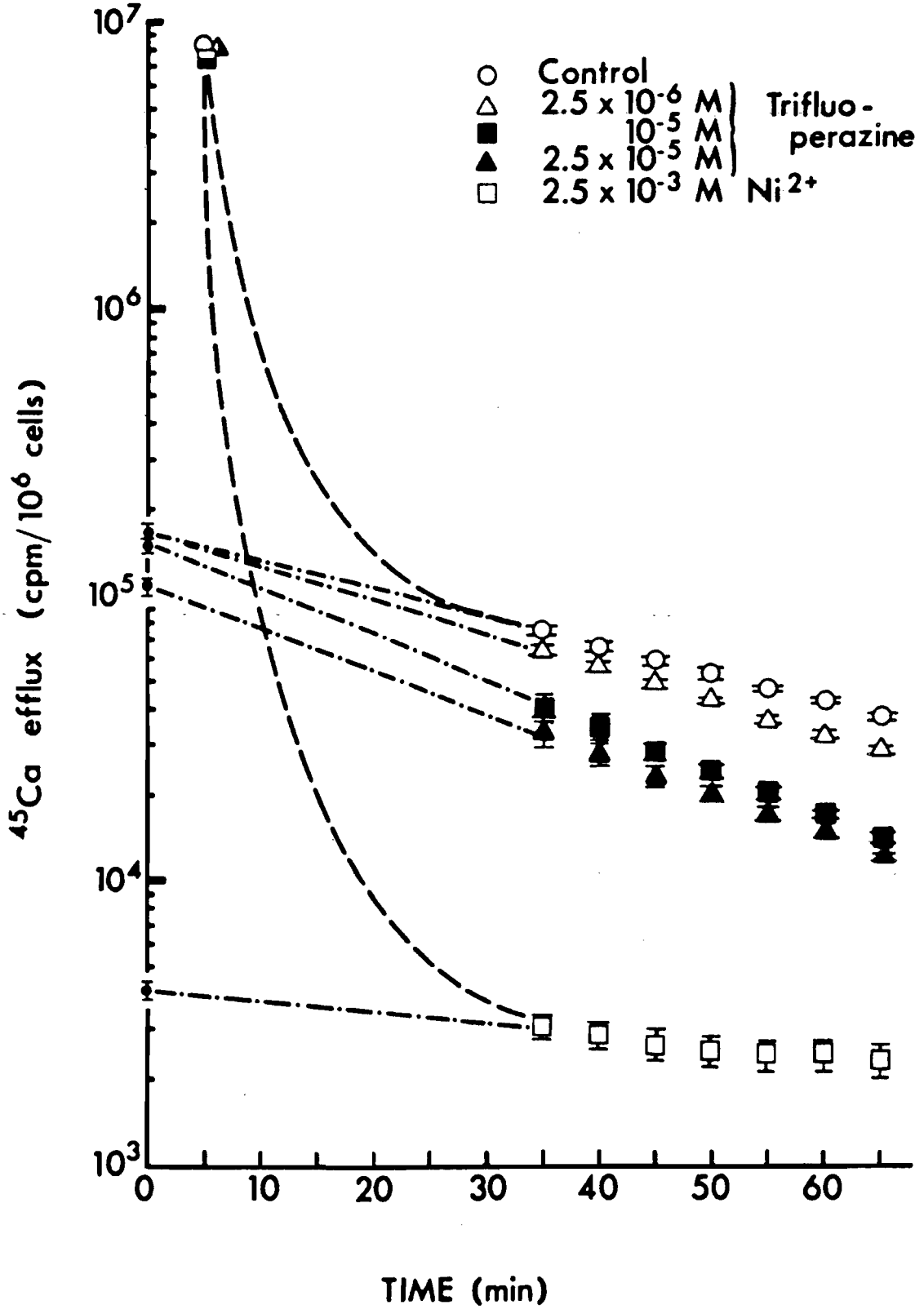
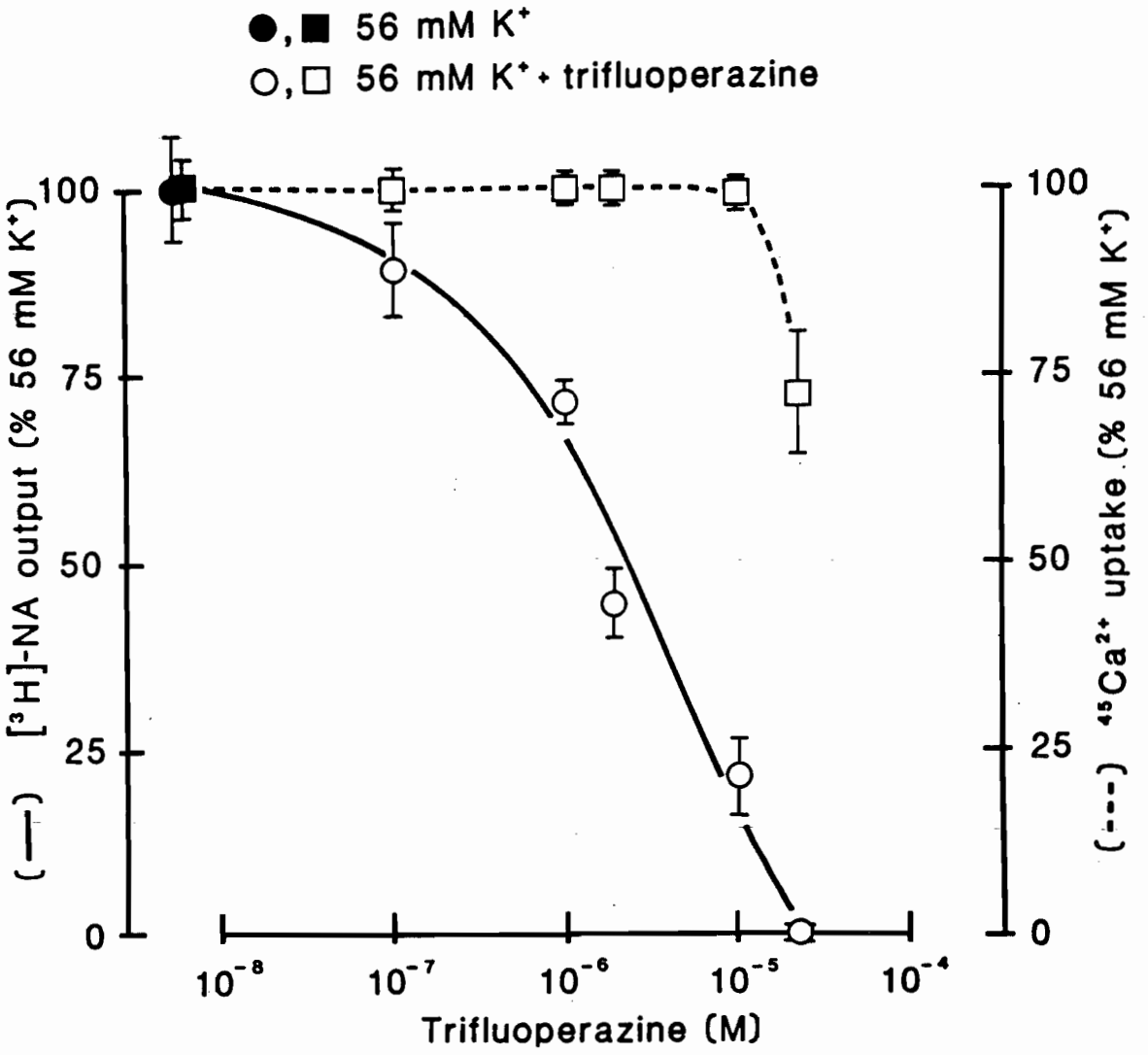

Control
2.5 x 10-6 M
10-5 M
2.5 x 10-5 M
Trifluo-perazine
2.5 x 10-3 M Ni2+
45Ca efflux (cpm/10^6 cells)
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50
60
TIME (min)

Fig. 37 Effect of various concentrations of TFP on 56 mM K^+-induced $^{45}Ca^{2+}$ uptake and [^{3}H]NA output from cultured chromaffin cells. Seven-day-old cultures were labelled with 10^{-7}M [^{3}H]NA for 5 min and subsequently washed as described previously (Chap. 2 section D.1). [^{3}H]NA output was calculated as % of total cell [^{3}H] content. K^+-induced [^{3}H]NA output was determined by subtracting basal or spontaneous release values from those obtained during the stimulation periods with 56 mM K^+.

K^+-induced [^{3}H]NA output in the presence of TFP (○) is expressed as a percentage of the output determined in the absence of TFP (●). $^{45}Ca^{2+}$ uptake was determined from the desaturation curves plotted with the values obtained (for further experimental details see Chap. 2 section D.2). The K^+-induced $^{45}Ca^{2+}$ uptake in the presence of TFP (□) is expressed as a percentage of the uptake determined in the absence of TFP (■).

Each value represents the mean ± S.E.M. of 4 separate culture dishes.


●, ■ 56 mM K+
○, □ 56 mM K+ + trifluoperazine
(—) [3H]-NA output (% 56 mM K+)
(---) 45Ca2+ uptake (% 56 mM K+)
100
75
50
25
0
10-8
10-7
10-6
10-5
10-4
Trifluoperazine (M)

tion of [^{3}H]NA output (Fig. 37). Furthermore, TFP at concentrations lower than 2.5×10^{-5}M did not affect ^{45}Ca uptake but produced significant inhibition of [^{3}H]NA output (Fig. 37).

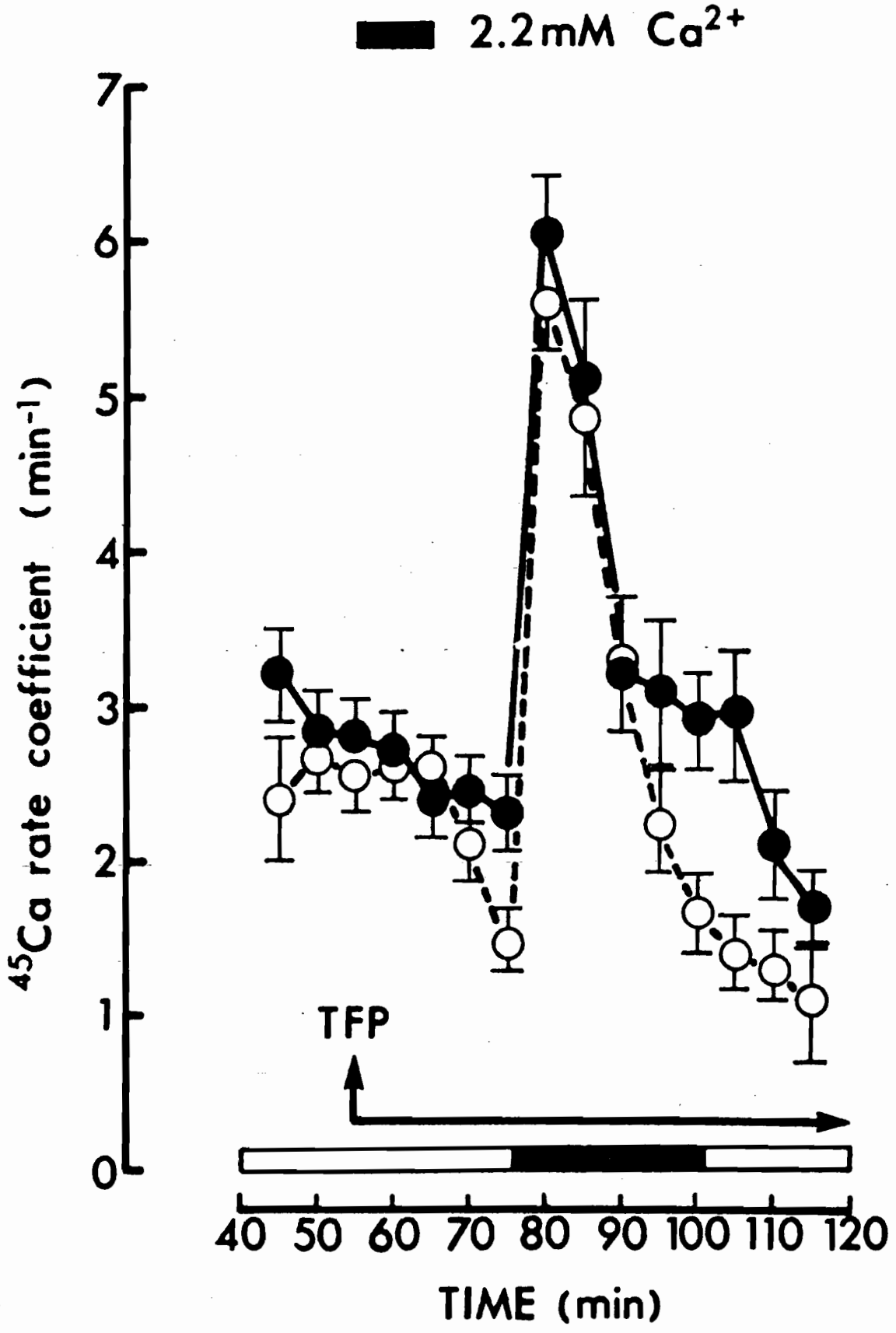
D.5 Effect of Trifluoperazine on the Ca^{2+}-Ca^{2+} Exchange Mechanism

It has been demonstrated previously that reintroduction of Ca^{2+} into Ca^{2+}-free solutions perfusing adrenal glands or into Ca^{2+}-free media incubating cultured chromaffin cells produces catecholamine release and concomitant ^{45}Ca efflux from prelabelled cells as a result of the activation of a Ca^{2+}-Ca^{2+} exchange mechanism (Douglas & Rubin, 1961; Aguirre et al., 1977; Bourne & Trifaró, 1982).

The effect of TFP was examined on the Ca^{2+}-Ca^{2+} exchange mechanism. In the presence of 2.5×10^{-6}M TFP, a concentration that blocks catecholamine release by 64%, no inhibition of the Ca^{2+}-Ca^{2+} exchange mechanism was observed (Fig. 38). In contrast, when the experiment was carried out in the presence of Ni^{2+}, a known Ca^{2+} channel blocker, as expected the increase in ^{45}Ca efflux resulting from Ca^{2+} reintroduction was inhibited by 84%.

Fig. 38 Effect of TFP on the ^{45}Ca efflux from cultured chromaffin cells in response to Ca^{2+} reintroduction. Cultured cells were divided into two groups of six dishes each and were loaded with ^{45}Ca in the presence of 56 mM K^{+} as described previously (see Chap. 2 section D.2). Subsequent to the loading period, the cells were washed for 55 min with Ca^{2+}-free (2 mM EDTA) Locke's solution. At this time (indicated by an arrow), 2.5×10^{-6}M TFP was added to the washing media of one (●) of the two groups. Twenty minutes later (75 min) 2.2 mM Ca^{2+}-Locke's solution with (●) or without (○) TFP was applied to the cells for another 25 min (75-100 min interval). Washes were then continued with Ca^{2+}-free (2 mM EDTA) Locke's solution for another 15 min. The washes were collected and the ^{45}Ca efflux rate coefficient determined from these washes as described in Chap. 2 section D.2.

Each point represents the mean ± S.E.M. obtained from 6 different culture dishes.


2.2mM Ca2+
45Ca rate coefficient (min-1)
0
1
2
3
4
5
6
7
TFP
40 50 60 70 80 90 100 110 120
TIME (min)

CHAPTER 4

DISCUSSION

A. PHOSPHORYLATION AND DEPHOSPHORYLATION OF CHROMAFFIN CELL PROTEINS IN RESPONSE TO STIMULATION

The process of protein phosphorylation-dephosphorylation is clearly a major general mechanism by which intracellular events respond to external physiological stimuli. Such a mechanism has been demonstrated to regulate numerous cellular events in a wide variety of cell systems. There is considerable interest in the possibility that protein phosphorylation may play a role in the process of stimulus-secretion coupling. Indeed, changes in the state of phosphorylation of specific proteins have been implicated in the stimulus-secretion coupling of several systems such as nervous tissues and several secretory cell systems including the adrenal medullary chromaffin cell.

In the few previous studies on chromaffin cell phosphorylation in response to various secretagogues, the attention was directed mainly, if not exclusively, toward the 56-59kD and/or the 95-99kD polypeptides which were among the most stimulated phosphoproteins (Holz et al., 1980; Amy & Kirshner, 1981; Pocotte et al., 1985). The paucity of data in those studies appeared to be the result of poor solubilizing conditions and/or the use of poor resolution gel electrophoresis systems. Consequently, in these studies powerful and controlled conditions were used to solubilize the ^{32}P-labelled chromaffin cells and, in addition, a high resolution gel electrophoresis system was utilized to detect small as well as large changes resulting from cell activation.

The principal objectives of the experiments conducted in the present

studies were to provide a more comprehensive picture concerning the phosphoproteins present in chromaffin cells and to determine the state of phosphorylation of these polypeptides following cell stimulation.

ACh was the main secretagogue used throughout these studies. This agent was used at a concentration (10^{-4}M) which was shown to produce maximal catecholamine release from bovine adrenal chromaffin cell cultures (Trifaró & Lee, 1980). The results obtained with ACh were also compared to those obtained with a depolarizing concentration of K^+ (56 mM). These studies focus mainly in the description of how many proteins are phosphorylated, what their molecular characteristics (molecular weights and isoelectric points) are and how their state of phosphorylation is affected following cell stimulation by ACh, in normal or low extracellular concentrations of calcium and in the presence or absence of the calmodulin antagonist TFP. The latter experimental conditions were chosen according to previous studies. Catecholamine release from the adrenal medulla in response to stimulation is blocked by Ca^{2+} deprivation and high Mg^{2+} concentration under similar conditions as used in the present studies (Douglas & Rubin, 1961, 1963). In addition, the concentration of TFP (2×10^{-7}M) used in these studies produces 50% inhibition of ACh-induced catecholamine release from chromaffin cells however without affecting the entry of Ca^{2+} through the voltage-dependent Ca^{2+} channel (see Chap. 3 section D; also Clapham & Neher, 1984b).

a) Phosphorylation of Chromaffin Cell Polypeptides under Stimulation

In this section, the two phosphoproteins (56-60kD and 95-99kD) previously described will first be examined in detail (Holz et al., 1980; Amy & Kirshner, 1981). Then, the results obtained concerning the other chromaffin cell phosphoproteins observed in these studies will be discussed in parallel with some results obtained in other systems.

Phosphorylation of the 63kD polypeptide

Previous studies have demonstrated that stimulation of isolated chromaffin cells increases the incorporation of ^{32}P into a polypeptide of molecular mass of 56-60kD (Amy & Kirshner, 1981; Haycock et al., 1982a, 1982b; Meligeni et al., 1982; Niggli et al., 1984; Pocotte et al., 1985). This polypeptide has also been identified as tyrosine hydroxylase (Haycock et al., 1982a, 1982b; Niggli et al., 1984; Pocotte et al., 1985). The 63kD reported here shows a behavior similar to that of the subunit of tyrosine hydroxylase described above. That is to say, the ^{32}P incorporation into the 63kD polypeptide is increased by either cholinergic stimulation or stimulation by a depolarizing concentration of K^+ (Table 4). Moreover, Ca^{2+} deprivation (low Ca^{2+} and high Mg^{2+} concentrations) produced a 75-80% inhibition of the 63kD polypeptide phosphorylation in response to stimulation. On the other hand, incubation of the chromaffin cells with 2×10^{-7}M TFP, a calmodulin antagonist, produced only a 33% inhibition of the 63kD polypeptide phosphorylation in response to the same stimuli.

Tyrosine hydroxylase is a well characterized enzyme that catalyzes the rate-limiting step in the biosynthesis of catecholamines (Weiner, 1970). This enzyme is phosphorylated, at least in vitro, by three different kinases: calmodulin-dependent, cAMP-dependent and phospholipid-activated (C) kinases (Ames et al., 1978; Yamauchi & Fujisawa, 1979, 1980, 1981; Albert et al., 1984a). A recent publication has suggested that the molecular mass of the tyrosine hydroxylase subunit is 62kD and that the 60kD polypeptide also present in tyrosine hydroxylase isolated preparations is a proteolytic product of the 62kD subunit (Albert et al., 1984a). The phosphorylated polypeptide described in these studies with the characteristics of tyrosine hydroxylase has an apparent molecular weight of 63,000 in the electrophoresis system used and in two-dimensional electrophoresis is resolved into three phosphorylated isoforms of pIs in the range of those described for tyrosine hydroxylase (Pocotte et al., 1985).

At present, it is not possible to attribute the Ca^{2+} dependency and the stimulus-induced phosphorylation of tyrosine hydroxylase observed in intact cells to any one of the known protein kinases. However, the results presented here showing inhibition of the stimulation-induced phosphorylation of the 63kD polypeptide by Ca^{2+} deprivation or TFP, together with the observations of Haycock et al. (1982b) and those of Niggli et al. (1984), suggest that stimulation of the chromaffin cell induces the phosphorylation of tyrosine hydroxylase through at least two separate mechanisms.

Phosphorylation of the 94kD polypeptide

Two previous studies have shown that a chromaffin cell polypeptide of molecular mass of 95-99kD incorporated ^{32}P in response to cell stimulation and that this increased phosphorylation was only observed in the presence of extracellular Ca^{2+} (Holz et al., 1980; Amy & Kirshner, 1981). In the electrophoretic system used in the present studies, this polypeptide shows an apparent molecular weight of 94,000 and pI values of 4.2, 4.3 and 4.9 (Fig. 18). The results obtained in the time course study show that the onset of phosphorylation of the 94kD polypeptide is similar to that observed for the 63kD chromaffin cell polypeptide (tyrosine hydroxylase). Nothing is known of this 94kD polypeptide with the exception that stimuli and conditions known to affect catecholamine release also affect its phosphorylation. In addition, the present results show that TFP at a concentration which blocks ACh-induced catecholamine release by 50% produces a 39% inhibition of the ACh-induced phosphorylation of the 94kD polypeptide.

Therefore, it is quite possible that this polypeptide might play an important role in secretion, since polypeptides of similar molecular weights have been found to be phosphorylated in response to stimulation in other secretory tissues (Schubart et al., 1980b; Dartt et al., 1982; Drust & Martin, 1982). The stimulation of hamster insulinoma cells by high K^+ induced insulin release and phosphorylation of a 98kD polypeptide (Schubart et al., 1980b). In the rat lacrimal gland, another secretory tissue, a 94kD protein is phosphorylated by a calmodulin-dependent system (Dartt et al., 1982). Finally, in prolactin secreting GH_3 rat pituitary cells, thyrotropin-releas-

ing hormone (TRH) stimulation induced the phosphorylation of a 98kD polypeptide (Drust & Martin, 1982). In all of the above-mentioned cases including the present studies, the Ca^{2+} dependency of the polypeptide phosphorylation and the inhibition of this phosphorylation by TFP was demonstrated. In cardiomyocytes, the phosphorylation of a 94-96kD protein was shown to be stimulated by the β-adrenergic agonist isoproterenol, and the authors have postulated phosphorylase as the identity of this phosphoprotein (Onorato & Rudolph, 1981; Hayes et al., 1984). However, this enzyme was identified at pI 6.6 by Garrison and Wagner (1982) in their extensive study on the hormone-induced phosphorylation of distinct substrates in intact hepatocytes. According to this last finding, the very acidic 94kD phosphoprotein described here should not correspond to the phosphorylase. Schubart and collaborators (1980b) have also presented evidence that the 98kD phosphoprotein of insulinoma cells is distinct from the phosphorylase. Further studies are required to determine whether or not the above-mentioned proteins belong to the same family and have similar functions.

Phosphorylation of the 80kD and 117.5kD polypeptides

The soluble proteins of the catecholamine-storing organelles of adrenal medulla, i.e. the chromaffin granules, have been collectively named chromogranins (Blaschko et al., 1967). These proteins are secreted with the catecholamines during exocytosis (Smith & Winkler, 1972; Viveros, 1975). The major secretory protein, chromogranin A, comprises about 40% of total granule soluble proteins (Winkler, 1976). The soluble proteins of chromaffin

granules have been investigated in great detail (Winkler & Westhead, 1980; Winkler & Carmichael, 1982); however, no clear function has yet been attributed to those proteins.

The 80kD phosphoprotein which focused at pI 4.8 (Fig. 18) corresponds exactly to a major Coomassie blue-stained protein of chromaffin cells (Fig. 17) and furthermore, it reacts with an antiserum directed against chromogranin A (Doucet & Trifaró, unpublished results). The 117.5kD phosphoprotein having a pI of 5.0 (Fig. 18) is also one of the major Coomassie blue-stained proteins of chromaffin cells (Fig. 17) and similarly to the 80kD phosphoprotein, a purified chromaffin granule fraction is enriched in the 117.5kD polypeptide (Doucet & Trifaró, unpublished observations). Because of its molecular properties (molecular weight higher than for chromogranin A and pI slightly more basic) and its localization, the 117.5kD phosphoprotein is likely to correspond to a high molecular form of chromogranin B (Fischer-Colbrie & Frischenschlager, 1985). Chromogranins B have been identified recently as another family of acidic proteins which are immunologically different from chromogranins A (Winkler et al., 1984; Settleman et al., 1985) but also secreted from the adrenal medulla during cholinergic stimulation (Fischer-Colbrie & Frischenschlager, 1985).

It has been demonstrated in a recent study that chromogranin A and two families of very acidic polypeptides, termed secretogranins I and II which appear to be related to chromogranins B, are phosphorylated in cultured chromaffin cells under resting conditions (Rosa et al., 1985). However, the present studies demonstrate for the first time that an increase in phosphorylation of both chromogranins (A and B) occurs following stimulation of cul-

tured chromaffin cells. Under resting conditions (Fig. 16, dotted line and Fig. 18A), the 80kD and the 117.5kD polypeptides are already heavily phosphorylated. Nevertheless, further increases in ^{32}P incorporation are observed in response to either ACh- (30% and 23% for the 80kD and 117.5kD polypeptides, respectively) or high K^+- (16% and 14%) stimulation (Table 4). Again, the receptor-mediated stimulus was more efficient than the direct depolarization with a high concentration of K^+. Furthermore, the ACh-induced increases in phosphorylation show a Ca^{2+} dependency since Ca^{2+} deprivation resulted in an 81% inhibition of phosphorylation for chromogranin A and a 62% inhibition for chromogranin B (Table 5). The presence of TFP greatly decreases the ACh-induced phosphorylation of chromogranin A (57% inhibition) and chromogranin B (38% inhibition) (Table 6).

Recent studies have shown that chromogranin A is not confined only to adrenergic tissues but also occurs in other endocrine organs (Cohn et al., 1982, 1984; O'Connor et al., 1983; Hogue-Angeletti et al., 1985). In addition, chromogranin A has a characteristic distribution in the brain that does not parallel the one of any other known neuroactive substance (Somogyi et al., 1984). Nevertheless, this is the first evidence showing that some of the soluble proteins of chromaffin granules are among the main targets of protein phosphorylation. The physiological significance of chromogranin phosphorylation still remains unclear but the present observations raise many questions about the functional role of chromogranins.

Phosphorylation of the 33kD polypeptide

The 33kD polypeptide (33a) that focused at pI 5.0 (Fig. 18B) has its state of phosphorylation drastically increased upon ACh stimulation, much more in fact, than the change described in Table 4. Indeed, another 33kD polypeptide (pI=5.9) (33b) is present in chromaffin cells. Furthermore, this latter 33kD polypeptide (33b) appears to be phosphorylated under resting conditions (Fig. 18A) but dephosphorylated upon stimulation (Fig. 18B). Because these two phosphoproteins were not resolved in one-dimensional electrophoresis, the inhibition by TFP treatment of the ^{32}P incorporation into the 33kD polypeptides in response to ACh (Table 6) cannot be attributed solely as an effect on the 33kD polypeptide (33a) that focused at pI 5.0. However, the change in phosphorylation of the 33kD phosphoproteins is completely abolished upon Ca^{2+} deprivation (Table 5). The functional significance of the prominent change in phosphorylation of the 33kD (33a) phosphoprotein (pI=5.0) is not known at present but is currently under investigation in our laboratory.

Phosphorylation of the 29kD polypeptides

Another major chromaffin cell phosphoprotein has an apparent molecular weight of 29,000 and corresponds to a well defined doublet with pI values of 5.7 and 5.9 (Fig. 18). Again both stimuli, ACh and high K^+, have a similar effect on the state of phosphorylation of this protein, producing an increase of 43% and 47%, respectively (Table 4). Ca^{2+} deprivation nearly abol-

ished the response to ACh stimulation (Table 5). Moreover, the presence of TFP at a concentration which blocks ACh-induced catecholamine release by 50%, results in a 46% inhibition of the 29kD phosphorylation in response to cholinergic stimulation (Table 6). Treiman and collaborators (1983) have recently reported the in vitro stimulation by Ca^{2+} and calmodulin of a 29.8kD phosphoprotein in a purified membrane preparation of adrenal chromaffin granules. Furthermore, this 29.8kD polypeptide was the only component of the chromaffin granule membranes which specifically responded to exogenous Ca^{2+} and calmodulin (Treiman et al., 1983). A 29kD phosphorylated band is also present in the pattern of phosphoproteins of adrenal chromaffin granule membranes described by Burgoyne and Geisow (1982). According to these investigators, the 29kD chromaffin vesicle membrane phosphoprotein would also be regulated by cAMP.

Recently, a 29kD zymogen granule membrane protein was found to be the only major phosphoprotein labelled in intact pancreatic acinar cells, another secretory tissue (Peiffer et al., 1984). Furthermore, it was shown in these studies that this 29kD phosphoprotein of the zymogen granule membrane was not the 29kD basic ribosomal phosphoprotein S6 described previously by Freedman and Jamieson (1982c). The acid pIs obtained for the 29kD polypeptide of adrenal chromaffin cells in the present studies also rule out this possibility. The functional significance of the state of phosphorylation of the 29kD protein is not known at present, but the results described in these studies together with the studies in the other secretory tissues suggest that this chromaffin cell phosphoprotein may be of functional importance in the secretory processes.

Phosphorylation of the 14.8kD polypeptide

The chromaffin cell phosphoprotein showing one of the largest increases in phosphorylation upon the addition of secretagogue corresponds to a 14.8kD polypeptide as clearly indicated by two-dimensional SDS-PAGE (Fig. 18, see also Table 4). This observation was true for both stimuli, ACh (Δ = 85%) and high K^+ (Δ = 124%), used in the present studies. On two-dimensional slab gels, the 14.8kD chromaffin cell component appears as a doublet of closely related phosphopolypeptides having a pI value of 5.0 (Fig. 18). The 14.8kD phosphorylated protein can probably undergo phosphorylation at multiple sites. Following treatment of the intact cell with ACh, a charge shift was observed (Fig. 18). Indeed, in treated cells, the phosphorylation of the most acidic component of the 14.8kD doublet is markedly increased. The ACh-induced phosphorylation of the 14.8kD chromaffin cell protein is strongly Ca^{2+} dependent considering that Ca^{2+} deprivation completely abolishes the response to ACh stimulation (Table 5). On the other hand, the ACh stimulatory effect on the phosphorylation of the 14.8kD polypeptide is not affected at all by the presence of the calmodulin inhibitor TFP (Table 6).

Interestingly, a very similar polypeptide (15kD; pI=4.77) has also been described among the adrenal medullary chromaffin cell phosphoproteins affected by the protein kinase C activator PMA (Pocotte et al., 1985). Therefore, the present results together with those of Pocotte and collaborators (1985) strongly suggest the participation of protein kinase C in the phosphorylation of the 14.8kD chromaffin cell protein. A 14.8kD protein has been described as a membranous phosphate accepting protein in isolated adrenal

medullary plasma membrane preparations (Konings & De Potter, 1983a). Its in vitro phosphorylation, however, showed a 6-fold increase in the presence of 1 mM EGTA (Konings & De Potter, 1983a). Finally, in human neutrophils, stimulation by PMA also induced the phosphorylation of a 13kD protein (Andrews & Babior, 1983).

Phosphorylation of cytoskeletal proteins

The 57kD phosphoprotein doublet that focused at pI 5.2 (Fig. 18B) corresponds to tubulin subunits which appear as major Coomassie blue-stained polypeptides after two-dimensional electrophoresis (Fig. 17). Under basal conditions, only the more basic component of chromaffin cell tubulin is heavily phosphorylated (Fig. 18A). However, upon stimulation with ACh, the more acidic tubulin component becomes heavily phosphorylated while the other (more basic) appears slightly dephosphorylated (Fig. 18B). Like other phosphoproteins, the 57kD substrate responds better to ACh stimulation (38%) than to the K^+-induced depolarization (22%) (Table 4). Its increase in phosphorylation is highly Ca^{2+} dependent (Table 5) and inhibited by 88% in the presence of TFP (Table 6). By indirect immunofluorescence using an antibody raised against brain tubulin, tubulin has been localized to a fine filamentous network extending throughout the cytoplasm and processes of cultured bovine adrenal chromaffin cells (Bader et al., 1984a). Furthermore, binding sites for tubulin have been found on chromaffin granule membranes (Bernier-Valentin et al., 1983; Bader et al., 1984a), as well as on the secretory granules isolated from the endocrine pancreas and from the anterior pitu-

itary (Sherline et al., 1977; Surprenant & Dentler, 1982). Tubulin has also been found to be a substrate for Ca^{2+}/calmodulin-dependent protein kinase in intact synaptosomes (Burke & DeLorenzo, 1981). Yamamoto and collaborators (1985) have recently reported that tubulin phosphorylation by purified brain Ca^{2+}/calmodulin-dependent protein kinase inhibited microtubule assembly. Furthermore, the phosphorylation of both tubulin and the microtubule-associated protein τ factor had a greater inhibitory effect on microtubule assembly than either of the phosphorylated components alone (Yamamoto et al., 1985). Considering the latter observations and the results described in these studies, it is tempting to postulate that upon stimulation of chromaffin cells, the inhibition of microtubule assembly through the phosphorylation of tubulin and other microtubule-associated proteins might be another important event which, combined with the Ca^{2+}-induced rupture of the actin microfilament network (Trifaró et al., 1985), would allow the free movement of secretory granules towards the plasma membrane-releasing sites.

Myosin has also been isolated from adrenal chromaffin cells and characterized (Trifaró & Ulpian, 1976; Creutz, 1977; Hesketh et al., 1978). Indirect immunofluorescence with anti-myosin antibody showed that chromaffin cells displayed an intense fluorescence localized in the cytosol (Trifaró et al., 1978; Aunis et al., 1980; Bader et al., 1983). Furthermore, when the anti-myosin antibody was added to freshly isolated cells, immunofluorescence showed that antigenic sites also seemed to be localized on or close to the chromaffin cell surface (Trifaró et al., 1978). The estimated molecular weights of the chromaffin cell myosin light chains were 22,000 and 16,500 (Trifaró & Ulpian, 1976) and 23,000 and 20,000 (Hesketh et al., 1978). Con-

sidering those observations and the similar molecular characteristics (MW and pI) reported for nonmuscle cell myosin (Adelstein & Eisenberg, 1980; Trotter et al., 1983; Feuerstein & Cooper, 1984), the 22kD phosphoprotein that focused at pI 4.9 is likely to be the phosphorylated light chain of chromaffin cell myosin. Phosphorylation of myosin light chain by Ca^{2+}/calmodulin-dependent myosin light chain kinase is necessary for its actin-activated ATPase activity and force generation (Harris, 1981). However, this 22kD phosphoprotein is already heavily phosphorylated under basal conditions (Fig. 18A) and does not appear to be significantly affected by stimulation (Fig. 18B). In other systems, similar results (i.e. no change) have been reported for the myosin light chain phosphorylation under activation (Hayes et al., 1984) whereas a prominent increase in phosphorylation was observed in others (Nishikawa et al., 1980; Feinstein & Hadjian, 1982; Naka et al., 1983). Phosphorylation studies followed by myosin light chain immunoprecipitation are needed to determine whether the chromaffin cell 22kD polypeptide corresponds indeed to the light chain of myosin.

Actin is another cytoskeletal protein which has also been isolated from chromaffin cells and characterized (Lee et al., 1979; Trifaró et al., 1985). After two-dimensional electrophoresis, chromaffin cell actin appears as a major protein spot (Fig. 17); its molecular characteristics in this system correspond to a molecular weight of 45,000 and a pI of 5.6. However, no phospholabelled actin is observed in the chromaffin cell under basal or stimulating conditions.

In addition, phosphorylation studies carried out on cytoskeleton preparations obtained from cultured chromaffin cell extracts have provided fur-

ther evidence that several contractile proteins are phosphorylated under both resting and stimulation conditions. Furthermore, for some of these cytoskeletal components, an increase in ^{32}P incorporation and redistribution was observed following cell stimulation (Doucet, Côté & Trifaró, preliminary studies).

The other chromaffin cell phosphoproteins

Depolarization of cultured adrenal chromaffin cells by either cholinergic stimulation or high K^+ in the presence of calcium led to increased incorporation of ^{32}P into a number of other polypeptides (Table 4). Among these other polypeptides, the phosphoproteins of 107, 76, 47, 43, 41.5, 38 and 35.5kD appear to be highly affected by the Ca^{2+}-deprivation treatment (95% to 100% inhibition, Table 5) and by the presence of TFP as well (65% to 100% inhibition, Table 6), again suggesting the involvement of Ca^{2+}/calmodulin-dependent kinase activity in the phosphorylation of those substrates. Interestingly, except for the 38kD and 35.5kD phosphoproteins, the above-mentioned substrates seem to respond better to the receptor-mediated stimulus that the K^+-induced depolarization (Table 4). Therefore, it appears that both type of cell stimulation produce in general a similar response in terms of protein phosphorylation, although some of the components involved in this process might be affected or regulated in a slightly different manner at some intracellular sites.

Bovine chromaffin cells in culture also contain several high molecular weight phosphoprotein bands (147 to ~500kD) which are already very highly

labelled under basal conditions (Figs. 15B and 16). Most of these bands are resolved in many phosphopolypeptides after two-dimensional electrophoresis (Fig. 18). Some of these polypeptides might correspond in fact to well-characterized phosphoproteins such as microtubule-associated proteins and neurofilament subunits, but the great number of phosphopolypeptides resolved by electrophoresis and some other technical limitations in these studies do not permit them to be identified unequivocally. Among these high molecular weight phosphoprotein bands, the 294, 160 and 147kD bands are also affected by Ca^{2+}-deprivation treatment (56 to 92% inhibition, Table 5), but their phosphorylations are not as well inhibited by the calmodulin antagonist (35 to 48% inhibition, Table 6) suggesting the involvement of more than one type of protein kinase. Finally, the state of phosphorylation of the high molecular weight bands 313,000, 249,000, 210,000 and 184,000 are not affected by lower calcium concentration in the external medium (Table 5). However, TFP has a non-negligible inhibitory effect on their state of phosphorylation (Table 6). However, the present studies unfortunately do not provide any clear explanation for this observation.

It should also be noted that a 249.4kD phosphoprotein has been described by Treiman and collaborators (1983) in membranes of bovine chromaffin secretory vesicles. Its <u>in vitro</u> phosphorylation was found to be stimulated by cAMP. Thus, the 249kD polypeptide described in the present studies might be related to this chromaffin granule membrane phosphoprotein.

b) Dephosphorylation of Chromaffin Cell Polypeptides under Stimulation

The state of phosphorylation of a specific substrate protein is of course determined by the relative activities of both a protein kinase and a protein phosphatase. Much more is known about protein kinases than about protein phosphatases. However, several lines of evidence indicate that the activity of some protein phosphatases can be regulated by second messengers and by protein modulators (Albert et al., 1984b; Nestler & Greengard, 1984; Nairn et al., 1985). Furthermore, recent publications have shown that some protein kinases, in addition to directly mediating the phosphorylation of specific substrates, concomitantly inhibit the dephosphorylation of some of these substrates via phosphorylated protein phosphatase inhibitors (Nemenoff et al., 1983; Hemmings et al., 1984a).

The results obtained in the present studies by two-dimensional gel electrophoresis analysis of adrenal chromaffin cell phosphoproteins demonstrate for the first time that a concomitant phosphorylation (about 30) and dephosphorylation (about 40) of many polypeptides occur following ACh stimulation (Fig. 18). Most of the polypeptides which are dephosphorylated upon stimulation correspond to minor phospholabelled components of chromaffin cells. However, the 42kD doublet that focused at pI 5.9 is one of the major chromaffin cell phosphoproteins under basal conditions and is highly dephosphorylated after ACh treatment (Fig. 18). This phosphoprotein corresponds to the α-subunit of pyruvate dehydrogenase and is a well established example of the role of protein phosphorylation-dephosphorylation in the regulation of metabolic activity (Randle, 1976, 1981). The mitochondrial enzyme is known to be

dephosphorylated by a Ca^{2+}-stimulated protein phosphatase, resulting in increased enzymatic activity which facilitates ATP production (Schaffer & Olsen, 1980). The dephosphorylation of this polypeptide has also been observed in rat cortical synaptosome preparations following depolarization (Robinson & Dunkley, 1985).

Another major phosphoprotein dephosphorylated under stimulating condition was, in this case, easily detected after one-dimensional gel electrophoresis (Figs. 15B and 16). The dephosphorylated protein has a molecular weight of 20,400 and a pI of 6.7 (Fig. 18). Dephosphorylation of the 20.4kD protein is stimulated by either ACh or high K^+, -30.5% and -39.2% respectively (Table 4). This effect is highly reproducible (Table 4) and occurs in a time-dependent manner (Fig. 19). Moreover, the ACh-induced dephosphorylation of the 20.4kD polypeptide appears to be Ca^{2+} independent (Table 5) and not affected by the presence of TFP (Table 6). A protein of similar molecular mass (21kD) has been found to be dephosphorylated in intact hepatocytes in response to stimulation with cAMP- (glucagon) or Ca^{2+}-linked hormones (angiotensin II, noradrenaline and vasopressin) (Garrison & Wagner, 1982). Furthermore, Hayes and collaborators (1984) have shown in cultured heart cells that β-adrenergic stimulation causes substantial increases in the phosphorylation state of several proteins but also markedly attenuates the phosphate content of a 21kD polypeptide. The isoproterenol-induced dephosphorylation of this polypeptide was readily reversed following the addition of the β-antagonist propranolol. In human neutrophils, the protein kinase C activator PMA was shown to cause the dephosphorylation of a 20kD protein (Andrews & Babior, 1983). A taurine-induced dephosphorylation of a 20kD

polypeptide was also observed in rat retinal membrane preparation enriched in photoreceptor cell synaptosomes (Lombardini, 1985).

c) Relationship between Protein Phosphorylation and Secretion

Most of the previous phosphorylation studies performed in chromaffin cells have attempted to establish relationships between the phosphorylation of a single protein and catecholamine secretion. Considering the steady-state effects of ACh, it has been possible from the results obtained in the present studies to correlate the increase in catecholamine secretion with the phosphorylation state of all the proteins examined, including the 20.4kD band which is dephosphorylated following stimulation.

However, only the phosphorylation of the 29, 33, 63, 76, 80, 94, 107, 117.5, 147, 160, 184, 210, 249, 294 and 313kD protein bands and the dephosphorylation of the 20.4kD protein occur rapidly enough to be temporally associated with the onset of secretion (Fig. 20). In the case of tubulin (57kD), the correlation between its state of phosphorylation and catecholamine secretion is not too good (data not shown); however very little can be said about this protein since, as mentioned previously, one subunit is phosphorylated while the other is dephosphorylated upon stimulation. Furthermore, the involvement of the 20.4, 184, 210, 249 and 313kD phosphoproteins in the secretory process is unlikely considering that their phosphorylation appears to be independent of extracellular calcium.

Therefore, the results obtained in these studies provide some indications concerning the phosphoproteins which may be involved in the secretory

process of chromaffin cells and also would allow future studies concerning this issue to focus specifically on some of these polypeptides.

B. ADRENAL MEDULLARY AND CHROMAFFIN CELL TROPOMYOSINS: IDENTIFICATION AND CHARACTERIZATION

The existence of tropomyosin in some types of nonmuscle cells has been known for more than a decade (Cohen & Cohen, 1972). At present, tropomyosin has been found to be present in a wide variety of nonmuscle tissues and cultured cell systems (for review see Côté, 1983). In the past five years, numerous studies have focused on nonmuscle tropomyosin since this protein has been found to possess several intriguing structural and functional characteristics (for review see Payne & Rudnick, 1984, 1985).

Tropomyosin has been isolated from the brain as well as from sympathetic neurones grown in cultures (Fine et al., 1973; Dabrowska et al., 1980; Barylko & Sobieszek, 1983; Kieser & Wegner, 1985). Therefore, the presence of this contractile protein was also expected in adrenal medullary chromaffin cells (Trifaró, 1978). However, no study has yet demonstrated the presence of tropomyosin in this cell system.

A series of experiments were conducted in chromaffin cells to determine if tropomyosin was indeed present in these cells and if so, to characterize this protein and to determine if tropomyosin was phosphorylated in this cell system. However, as for other chromaffin cell contractile proteins, since these components are present in relatively low amounts in these cells, tropomyosin was first isolated and characterized from the adrenal medulla.

a) Adrenal medullary tropomyosins

The resistance of tropomyosin to heat treatment provided the basis for the isolation procedure followed in the preparation of adrenal medullary tropomyosins. The entire purification procedure used was a combination of methods previously described for the isolation of brain and platelet tropomyosins (Barylko & Sobieszek, 1983; Côté & Smillie, 1981b). The isolated adrenal medullary tropomyosin preparation contains two major forms with apparent molecular weights of 32,000 and 38,000 and one minor form with an apparent molecular weight of 35,500. It should be noted that the molecular weights of these multiple forms of tropomyosin are apparent since tropomyosin molecules are known to show different migration behaviors on SDS-polyacrylamide gels even though they may have the same chain length (Mak et al., 1980). By quantitative densitometric measurements, it was determined that the major tropomyosin forms of 38kD and 32kD are present in a molar ratio of 2 to 1. A preponderance of the slower mobility form has also been observed for several other nonmuscle tropomyosins (Côté & Smillie, 1981b; Dabrowska et al., 1980; Fattoum et al., 1983; Fowler & Bennett, 1984). The fact that the purification on a hydroxylapatite column partially separates the two predominant forms of the adrenal medullary tropomyosin indicates that molecules composed of two 38kD and 32kD polypeptide chains must exist (Figs. 22 and 25, lanes d and e). The existence of homodimers of each of the polypeptide chains has been demonstrated for tropomyosins from equine platelets (Côté & Smillie, 1981b), bovine brain (Dabrowska et al., 1980) and erythrocyte membrane (Fowler & Bennett, 1984). However, additional experi-

ments would be necessary to determine whether the population of adrenal medullary tropomyosin molecules might also contain some heterodimers of the 38kD and 32kD polypeptide chains.

It is highly probable that the minor form having a molecular weight of 35,500 corresponds to smooth muscle tropomyosin originating from contaminating blood vessels. Indeed, contaminating smooth muscle tropomyosin was separated from kidney tropomyosin (30kD; Kobayashi et al., 1983b) and from thyroid tropomyosin preparations (30kD; Kobayashi et al., 1982) by chromatography on a hydroxylapatite column under very similar conditions to those described in these studies. However, as seen in Fig. 22B, the elution pattern of the 32kD and 38kD forms from adrenal medulla on a hydroxylapatite column does not allow the removal of the 35.5kD component.

One striking feature of the major forms of adrenal medullary tropomyosins is their high apparent molecular weight (Fig. 23). It is commonly held that nonmuscle tropomyosins are smaller in size than muscle tropomyosins. In fact, molecular weights ranging from 26,000-30,000 have been obtained for nonmuscle tropomyosins isolated from various sources (Côté, 1983). As pointed out by Côté (1983), higher molecular weight forms of tropomyosin are often observed in nonmuscle cells grown in culture possibly as the result of some modification of gene expression. The present studies using bovine adrenal medulla as the source of material appear to be the first description in a nonmuscle tissue of the presence of a significant amount of a larger size of tropomyosin.

The three adrenal medullary tropomyosins possess similar isoelectric points of about 4.7 (Fig. 24). These values are close to the isoelectric

point of muscle tropomyosin and that of macrophage tropomyosin (Bailey, 1948; Fattoum et al., 1983). The fact that the three tropomyosin forms of adrenal medulla have similar isoelectric points might first appear curious, but such a result has also been reported for some tropomyosin isoforms described in other systems (Matsumura et al., 1983b; Matsuda et al., 1983; Talbot & MacLeod, 1983; Lin et al., 1984).

An important property of skeletal muscle tropomyosin is its binding to F-actin in a ratio of 0.14, i.e. one tropomyosin molecule to seven actin monomers (Mannherz & Goody, 1976). However, the binding of nonmuscle cell tropomyosin to F-actin is thought to be much weaker than that of skeletal muscle tropomyosin (Fine et al., 1973). Therefore, the three adrenal medullary tropomyosin forms were tested for their capability to interact with F-actin. The 38kD tropomyosin appears to bind to F-actin with considerably higher affinity than the other adrenal medullary tropomyosins (Fig. 26A). Under the conditions used in the present studies, the binding curve reached a plateau at a molar ratio of 0.12 mole of 38kD adrenal medullary tropomyosin per mole of actin monomer (Fig. 26B). This value is similar to those reported for thyroid tropomyosin (Kobayashi et al., 1982), kidney tropomyosin (Kobayashi et al., 1983b), erythrocyte membrane tropomyosin (Fowler & Bennett, 1984) as well as those reported for skeletal muscle tropomyosin (Bernstein & Bamburg, 1982; Dabrowska et al., 1983). The 32kD adrenal medullary tropomyosin having a weaker affinity for F-actin than the 38kD tropomyosin suggests properties which are similar to those of brain tropomyosin (Fine et al., 1973; Dabrowska et al., 1983; Keiser & Wegner, 1985). Indeed, the present results on the tropomyosin binding to F-actin correspond exact-

ly to the observations reported recently for the 30kD, 32kD and 33kD bovine brain tropomyosins isolated by Keiser and Wegner (1985). Using similar conditions as those used in the present studies, the bovine brain 33kD tropomyosin was found to bind to actin filaments, whereas the mixture of 30kD and 32kD brain tropomyosins failed to show binding to F-actin. However, the three brain tropomyosins were shown to decrease the rate of actin polymerization (Keiser & Wegner, 1985). These results obtained from the binding study suggest that the two predominant tropomyosins of adrenal medulla might possibly play different functional roles. Consequently, it would be necessary to purify homogeneous populations of the 38,000 and 32,000 homodimers in order to evaluate this possibility.

Because of the extent to which the 32kD and 38kD polypeptides of adrenal medulla have been characterized and the fact that these polypeptide were recognized specifically by tropomyosin antibodies, it can be concluded with reasonable confidence that these molecules correspond to authentic tropomyosins. These adrenal medullary polypeptides behaved like muscle and nonmuscle tropomyosins in several respects: a) their ability to remain in solution at high temperatures (Bailey, 1948; Côté & Smillie, 1981b); b) their insolubility at or close to the isoelectric point of muscle and nonmuscle tropomyosins (Bailey, 1948; Côté & Smillie, 1981b); c) their elution patterns from anion exchange and hydroxylapatite columns similar to those of skeletal muscle and platelet tropomyosins (Eisenberg & Kielley, 1974; Côté & Smillie, 1981b); d) their different relative mobilities on SDS-PAGE in the presence and absence of urea (Storti et al., 1976; Kobayashi et al., 1982, 1983b; Segura et al., 1982) and e) for the 38kD component, its ability to

bind to F-actin (Fine et al., 1973; Dabrowska et al., 1980; Côté & Smillie, 1981a; Kobayashi et al., 1982, 1983b; Segura et al., 1982).

b) Chromaffin cell tropomyosins

Chromaffin cell tropomyosins were isolated from cultured cells following a procedure very similar to the one utilized for the purification of adrenal medullary tropomyosins. By electrophoresis, it was determined that the partially purified preparation of chromaffin cell tropomyosin contained two polypeptides with apparent molecular weights of 32,000 and 38,000 which comigrated with adrenal medullary tropomyosins. These two polypeptides were shown to have a behavior on DEAE-Sephacel column similar to the one observed for adrenal medullary tropomyosins (Fig. 29). However, the chromaffin cell tropomyosin preparation did not appear to contain a polypeptide which corresponds to the 35.5kD form of adrenal medullary tropomyosin, thus supporting the possibility that this tropomyosin form might indeed originate from contaminating sources (vascular smooth muscles). Therefore, it appears that chromaffin cells possess two forms of tropomyosin with apparent molecular weights of 32,000 and 38,000.

The molar ratio of the 38kD to the 32kD form of tropomyosin obtained for cultured chromaffin cells appears to be different from the one determined for the major forms of adrenal medullary tropomyosins. That is to say, the 32kD form of tropomyosin appears to be present in larger amounts in cultured chromaffin cells than the 38kD tropomyosin component. This observation might be explained by the facts that, as determined previously, the 38kD

tropomyosin component interacts strongly with F-actin and that in cultured chromaffin cells, the actin filament network extends throughout the entire cell. Therefore, it is possible that the 38kD form of tropomyosin is bound tightly to F-actin and not readily available for extraction under the experimental conditions used. In fact, the results obtained by indirect immunofluorescence using an antibody raised against smooth muscle tropomyosin seem to agree with this explanation since at least in bipolar chromaffin cells, a number of fine filaments were arranged in a parallel fashion and some of them extended from a point of cell attachment to the next one (Fig. 32). Therefore, it is possible that in chromaffin cells, tropomyosin may be associated with actin filaments as described previously in fibroblasts (Lazarides, 1976).

Tropomyosin has been shown to be phosphorylated in several systems, both in vivo and in situ (for review see Payne & Rudnick, 1985). At present, little is known concerning the mechanism of tropomyosin phosphorylation. It has been suggested that a separate protein kinase (tropomyosin kinase) may be required to phosphorylate the appropriate site (Ser-283) on tropomyosin. This suggestion arose from the fact that tropomyosin was shown not to be phosphorylated by either phosphorylase kinase or cAMP-dependent protein kinase in cultured cell systems as well as in isolated preparations (Stull & Buss, 1977; Gard & Lazarides, 1982). In this regard, a tropomyosin kinase was recently isolated from chicken embryo, and it was shown that such a kinase can phosphorylate tropomyosin in vitro (Montgomery & Mak, 1984). However, the exact nature and characteristics of this kinase are still unknown.

A phosphoprotein of molecular weight of 38,000 was detected in the partially purified tropomyosin preparation obtained from ^{32}P-labelled chromaffin cells (Fig. 30). This chromaffin cell phosphoprotein was shown by electrophoresis to correspond to the 38kD form of tropomyosin (Fig. 31). However, under the experimental conditions used in these experiments, no phospholabelled polypeptide appears to correspond to the 32kD form of chromaffin cell tropomyosin (Fig. 31). Therefore, it appears that only the 38kD form of tropomyosin is phosphorylated in cultured chromaffin cells. In this regard, it has also been observed in other cell systems that only some of the tropomyosin polypeptides present are phosphorylated in these systems (for review see Payne & Rudnick, 1985). It was also observed in intact cultured chromaffin cells that a polypeptide with molecular characteristics (38kD, pI=4.8) similar to adrenal tropomyosin was phosphorylated (see Chap. 3 section A). The ^{32}P incorporation into this chromaffin cell polypeptide was shown to be increased following cell stimulation by both ACh and a depolarizing concentration of K^+. Furthermore, Ca^{2+} deprivation and TFP exposure markedly decreased the phosphorylation of this 38kD polypeptide. Therefore, these results suggest that the state of phosphorylation of this chromaffin cell polypeptide may be regulated by a Ca^{2+}/calmodulin-dependent protein kinase.

In summary, the presence of tropomyosin in chromaffin cells was demonstrated by both immunofluorescence and electrophoretic techniques. It appears that cultured chromaffin cells contain two forms of tropomyosin with molecular weights of 32,000 and 38,000. These polypeptides correspond to the two major forms of tropomyosin isolated from the adrenal medulla. It was also observed that the 38kD tropomyosin component is phosphorylated in cul-

tured chromaffin cells. In addition, it was demonstrated that the 38kD adrenal tropomyosin component binds to F-actin with higher affinity than the 32kD form. In view of these results concerning tropomyosin phosphorylation and F-actin-tropomyosin interaction, it could be postulated that the 38kD and 32kD form of chromaffin cell tropomyosin may play different functional roles in these cells as suggested previously for other nonmuscle cell systems (for review see Côté, 1983; Payne & Rudnick, 1984, 1985).

During the course of these studies, a short communication was published concerning the presence of tropomyosin in adrenal chromaffin cells (Burgoyne & Norman, 1985b). It was shown by immunoblotting using a tropomyosin antibody that a heat-stable extract of adrenal medulla contained three polypeptides of molecular weights of 39,000, 36,500 and 31,000. Some evidence was also provided concerning the presence of tropomyosin in chromaffin cells as detected by immunoperoxidase staining of cultured cells by an anti-tropomyosin antibody. In addition, it appeared that a 39kD polypeptide was present in chromaffin granule membranes as observed by immunoblotting. However, it should be pointed out that in the case of the immunoperoxidase staining of the cells no control staining was provided in these studies. Furthermore, the evidence concerning the presence of the 39kD form of tropomyosin in chromaffin granule membranes was provided solely on the basis that a 39kD component of a crude chromaffin granule preparation was detected among other components by immunoblotting. Therefore, the evidence provided in this publication appeared to be rather preliminary and additional experiments are required to determine with certainty if tropomyosin is indeed present in chromaffin granule membranes.

C. TRIFLUOPERAZINE: A PHARMACOLOGICAL TOOL FOR STUDYING CALMODULIN-DEPENDENT PROCESSES IN CULTURED CHROMAFFIN CELLS

The phenothiazine antipsychotics were the first pharmacological agents found to antagonize calmodulin-activated processes (Weiss et al., 1974). At present, a wide variety of chemically unrelated substances have been described in the literature as calmodulin antagonists (see Chap. 1 section F.4). These pharmacological agents have been widely used both in vitro and in situ to examine the Ca^{2+}/calmodulin dependency of various cellular events. In particular, the phenothiazine trifluoperazine (TFP) has been utilized extensively to determine if calmodulin is implicated in cellular secretion in a wide variety of cell systems.

However, it has been reported that these agents produce different types of effects depending upon the experiment conditions utilized in different systems. Consequently, prior to studying the effect of TFP on a particular cellular event in chromaffin cells, several experiments were carried out to determine the effects of this calmodulin antagonist on catecholamine release and calcium movements in this cultured cell system.

The results obtained in the present studies concerning the effects of TFP on catecholamine secretion from chromaffin cells demonstrate that this calmodulin antagonist inhibits in a dose-dependent manner both ACh-induced and K^+-evoked catecholamine release in this cell system (see Chap. 3 section D). According to these experiments, the IC_{50} of TFP for the inhibition of ACh-induced and K^+-evoked release of [^{3}H]NA were 2×10^{-7}M and 2.2×10^{-6}M, respectively (Figs. 35, A and B). These IC_{50} values are within the range of

the apparent K_d (1.5×10^{-6}M) of the high affinity binding sites of calmodulin (Weiss & Wallace, 1980). The findings mentioned above suggest that the inhibition produced by TFP on catecholamine secretion takes place at some intracellular sites. However, some other possible sites of action could not be disregarded without further investigation.

In order to trigger catecholamine release, the aforementioned stimuli (i.e. ACh and high K^+) increase Ca^{2+} influx into chromaffin cells. Ca^{2+} enters into these cells via different routes (Aguirre et al., 1977; Rink, 1977). The entry of Ca^{2+} through the "late Ca^{2+} channel" appears to be involved in the secretory process of many cell systems including the chromaffin cell (Aguirre et al., 1977, 1979; Trifaró, 1977). The influx of Ca^{2+} through this channel as well as catecholamine release was shown to be blocked by methoxyverapamil and Gd^{3+} (Pinto & Trifaró, 1976; Aguirre et al., 1977; Bourne & Trifaró, 1982). Consequently, the effects of TFP on Ca^{2+} movements were examined in chromaffin cells.

The results obtained in the Ca^{2+} movement experiments in presence of TFP demonstrate that this agent at concentrations (10^{-6}-10^{-5}M) which produce significant inhibition of [^{3}H]NA released during stimulation did not block Ca^{2+} influx into chromaffin cells (Fig. 37). Only at higher concentrations of TFP ($\geq 2.5 \times 10^{-5}$M) was it observed that this agent produced a small but significant decrease in Ca^{2+} uptake (Fig. 37). TFP at a concentration of 2.5×10^{-5}M produced only a 30% inhibition of the K^+-induced ^{45}Ca uptake while completely blocking the K^+-evoked catecholamine output (Figs. 36 and 37). In addition, when TFP was used at a concentration (2.5×10^{-6}M) that blocks catecholamine release significantly, no inhibition of the Ca^{2+}-Ca^{2+} exchange

mechanism was observed (Fig. 38).

Therefore, these results demonstrate a dissociation of the effects of TFP on Ca^{2+} movements and catecholamine secretion, thus suggesting that the inhibitory action of TFP on the secretory process in chromaffin cells takes place at a step distal to Ca^{2+} entry. In this regard, a similar conclusion was drawn from experiments carried out in chromaffin cells permeabilized by exposure to electric fields ("leaky" cells). The release of catecholamine from "leaky" cells depends entirely on the concentration of Ca^{2+} present in the extracellular media (Baker & Knight, 1980, 1981). At concentrations in the micromolar range, TFP was shown to inhibit Ca^{2+}-dependent catecholamine release from this cell system, thus indicating that this agent must act at some sites distal to Ca^{2+} entry.

In contrast to these findings, some investigators have observed that the inhibitory action of calmodulin antagonists (TFP and W7) on stimulus-evoked catecholamine release was accompanied by an inhibition of stimulus-induced Ca^{2+} uptake into chromaffin cells. Consequently, it was concluded that these agents produced their inhibitory effects on catecholamine secretion by blocking Ca^{2+} fluxes rather than by interacting with calmodulin and inhibiting calmodulin-dependent events (Slepetis & Kirshner, 1982; Brooks & Treml, 1983b; Sasakawa et al., 1983; Wada et al., 1983).

Although the above-mentioned studies appear to be somewhat controversial with respect to the effect of TFP on Ca^{2+} fluxes in chromaffin cells, this controversy results mainly in the interpretation of the results observed rather than in the experimental findings themselves. In this regard, experiments carried out in cultured chromaffin cells using patch-clamp techniques

have helped to clarify some of these discrepancies (Clapham & Neher, 1984b). Using such techniques, excitation and secretion in chromaffin cells were studied by measurement of unitary inward currents and stimulus-evoked increments in membrane capacitance. These studies have shown that whole cell Ca^{2+} currents were reduced by about 50% in the presence of 10 μM TFP. In addition, the application of 10 μM TFP was found to inhibit capacitance steps which represent the summed result of exocytotic vesicular fusion events. However, the block of capacitance steps by TFP was shown to be independent of the reduction of Ca^{2+} inward ionic currents. Therefore, it was concluded from these studies that although TFP at a high concentration may block stimulus-induced Ca^{2+} uptake in chromaffin cells, this agent also inhibits catecholamine secretion by interacting at some intracellular site distal from Ca^{2+} entry (Clapham & Neher, 1984b).

In summary, the results presented here have demonstrated that trifluoperazine appears to be a suitable pharmacological agent for examining calmodulin-dependent processes in chromaffin cells. However, this agent should be used at relatively low concentrations so as to avoid interference with calcium movements in this cell system.

D. GENERAL DISCUSSION

The adrenal medullary chromaffin cell stores its secretory products in membrane-bound organelles, the chromaffin granules. Upon stimulation, the chromaffin cell depolarizes, Ca^{2+} enters the cell and this results in a transient increase in intracellular Ca^{2+}. Then, a sequence of events is triggered in this cell leading ultimately to the exocytotic release of the chromaffin granule contents into the extracellular space (Smith & Winkler, 1972; Viveros, 1975; Trifaró, 1977). Therefore, it appears that Ca^{2+} plays a predominant role in the secretory process of the chromaffin cell. In fact, Ca^{2+} may be involved at several steps of the secretory cycle which encompasses several cellular events such as synthesis, packaging (storage), transport, fusion of the chromaffin granules with the plasma membrane, release of the chromaffin granule contents via exocytosis and finally membrane retrieval process.

By reason of the similarities between stimulus-secretion coupling and excitation-contraction coupling in muscle, it has been postulated that some type of contractile event might be involved in the secretory process of the chromaffin cell (Poisner & Trifaró, 1967; Douglas, 1968). The presence of contractile proteins, cytoskeletal components as well as regulatory proteins in this cell and the association of some of these components with the chromaffin granules support the idea that some contractile elements may be involved at certain steps of the secretory process of the chromaffin cell (for recent reviews see Trifaró et al., 1984a, 1985).

Protein phosphorylation has also been implicated in the process of stimulus-secretion coupling of several secretory systems including the adrenal chromaffin cells (see Chap. 1 section G.6.1). In this regard, it has been reported that the time course of phosphorylation of some chromaffin cell polypeptides appears to be temporally associated with catecholamine secretion (Holz et al., 1980; Amy & Kirshner, 1981; Baker et al., 1982). Other phosphoproteins have also been identified or detected in subcellular fractions of chromaffin cells, and a protein kinase C activity has been characterized in the adrenal medulla (see Chap. 1 section G.6.2). Thus, these observations support the hypothesis that some phosphoproteins may be involved in the secretory process of chromaffin cells.

In view of the results presented in this thesis and from observations reported previously, several hypotheses can be proposed concerning the role of protein phosphorylation and dephosphorylation in adrenal medullary chromaffin cells.

Protein phosphorylation may be involved in different cellular events in chromaffin cells. Some of these events may be directly related to the secretory cycle of these cells, while others may be associated with the general maintenance of the cell such as intermediary metabolism (e.g. α-subunit of pyruvate dehydrogenase).

Some of the chromaffin cell phosphoproteins could be involved in the synthesis of chromaffin granule constituents, in the packaging of proteins into secretory granules or at some steps related more directly with the exocytotic release of chromaffin granule contents.

For instance, the degree of phosphorylation of tyrosine hydroxylase

(63kD polypeptide) is greatly increased following cell stimulation with either ACh or a depolarizing concentration of K^+. This enzyme has been shown previously to catalyze the rate-limiting step in the synthesis of catecholamines (Weiner, 1970). Therefore, it is possible that an increased phosphorylation of this enzyme corresponds to a signal which would increase the synthesis of catecholamines following cell stimulation. In this regard, *in vitro* experiments have shown that the K_m of the enzyme is decreased when tyrosine hydroxylase is phosphorylated (Haycock *et al.*, 1982b; Vigny & Henry, 1982).

Cell stimulation also increased the phosphorylation of two chromaffin cell components of molecular weights of 80,000 and 117,500. These two phosphoproteins correspond to major components of chromaffin cells as observed by two-dimensional SDS-PAGE. The 80kD polypeptide was found by immunoblotting to be recognized specifically by a chromogranin A antibody (Doucet & Trifaró, unpublished observations). These two phosphoproteins were also also found to be enriched in a purified chromaffin granule fraction (Doucet & Trifaró, unpublished results). Therefore, the molecular characteristics and the observations described above strongly indicate that the 80kD and 117.5kD phosphorylated polypeptides correspond to chromogranin A and chromogranin B, respectively. Recent studies have shown that chromogranin A is not confined to adrenergic tissues but also occurs in other secretory cells where it might play similar roles as in chromaffin cells or paraneurones in general (Cohn *et al.*, 1984; O'Connor *et al.*, 1983; O'Connor & Frigon, 1984; Hogue-Angeletti *et al.*, 1985).

Although the exact function of chromogranins is still unknown, some roles for these secretory components have been proposed recently (Rosa et al., 1985). These authors have postulated that these polypeptides might be involved in the packaging of proteins into the chromaffin granules or may serve as processing enzymes. Therefore, it is possible that the degree of phosphorylation of these polypeptides regulates the rate of protein packaging into the granules or may regulate the activity of these components if they act as processing enzymes. Furthermore, since chromogranins are present in the chromaffin granules and that some form(s) may represent integral membrane protein (Settleman et al., 1985b), it is possible that these phosphoproteins may also play a certain function while inside the granules. For instance, phosphorylation of these polypeptides could change the net charge on these highly acidic components and consequently these changes on the molecules could represent a signal to trigger the exocytotic release of chromaffin granule contents. Another possibility would be that increase in the phosphorylation of chromogranins would result in the binding of these phosphoproteins to the luminal surface of the chromaffin granule membrane which could be required to trigger the membrane retrieval process following exocytosis. In all the proposed roles for chromogranins described above, phosphorylation of these polypeptides would occur only if protein kinase activity is present at the appropriate location in the cell. That is to say, either at the level of the Golgi or inside the chromaffin granule. In fact, it appears that protein phosphorylation occurs on the cytoplasmic side of the Golgi membrane (Lee & Huttner, 1985). In regard to protein kinase activity inside the chromaffin granule, no evidence have been provided yet

concerning such an enzymatic activity. Therefore, further phosphorylation experiments have to be carried out with isolated chromaffin granule preparations in order to determine if indeed this hypothesis is correct.

It was also observed in these present studies, as previously reported by other investigators (Holz et al., 1980; Amy & Kirshner, 1981), that a major increase in the degree of phosphorylation of a 94kD chromaffin cell polypeptides occurred following cell stimulation with either ACh or 56mM K^+. Although the identity of this phosphoprotein is still unknown, preliminary experiments on the subcellular localization of chromaffin cell phosphoproteins have revealed that this polypeptide appears to be a membrane-bound component. Therefore, it would be of great interest to determine if this chromaffin cell protein which is highly phosphorylated upon stimulation is associated with the chromaffin granule.

Phosphorylation or dephosphorylation of contractile proteins and cytoskeletal components may play an important role in cell systems since the state of phosphorylation of some of these components appears to regulate their interaction with other cytoskeletal components. In chromaffin cells, such a process might be of importance since several considerable evidence suggests that these components may be involved in some step of the secretory process of these cells (for review see Trifaró et al., 1985). In this regard, it was determined that some contractile proteins and cytoskeletal components are phosphorylated in this cell system. For instance, it appears that one of the light chains of myosin (22kD polypeptide) is phosphorylated in chromaffin cells. As demonstrated previously in numerous nonmuscle cell systems including PC12 cells (Englert & Perlman, 1984), the phosphorylation

of myosin light chain would activate myosin ATPase and regulate actin-myosin interaction. Therefore, this observation supports the idea that an actomyosin complex may be involved in some chromaffin cell functions. In future studies, it would be of great interest to determine if a Ca^{2+}/calmodulin-dependent system regulate the phosphorylation of chromaffin cell myosin light chain as previously described for other nonmuscle cell systems.

Tubulin (57kD polypeptide), the basic protein subunit of microtubules, has been found to interact with chromaffin granules (Bernier-Valentin et al., 1983; Bader et al., 1984a). In the present studies, it was found that tubulin was phosphorylated in chromaffin cells. It was also observed that tubulin phosphorylation was increased upon stimulation with ACh. Since it has been demonstrated in several systems that tubulin phosphorylation inhibits microtubule assembly, it is possible that upon stimulation of chromaffin cells, microtubule disassembly would occur and consequently would allow the free movement of chromaffin granules towards the plasma membrane. In fact, this mechanism could operate in a coordinated fashion with the Ca^{2+}-induced rupture (gelsolin-like effect?) of the actin microfilament network, a mechanism which has been previously proposed in chromaffin cells (Trifaró et al., 1985). To further support this proposed mechanism, it was reported very recently that a gelsolin-like protein is indeed present in chromaffin cells (Bader et al., 1986).

In addition, it was observed in these studies that numerous high molecular mass polypeptides (150kD - 500kD) have their degree of phosphorylation altered (increased or decreased) following cell stimulation. Although the identity of these phosphoproteins was not revealed in these studies due to

the large number of these polypeptides, there is a high probability that some of these phosphorylated components correspond to neurofilament subunits and microtubule-associated proteins (MAP's). In fact, it has been observed that the three neurofilaments subunits are phosphorylated in cultured chromaffin cells (Georges & Mushynski, personal communication). In this regard, it has been demonstrated in other cell systems that neurofilament phosphorylation and MAP2 phosphorylation may regulate or modulate their association with other cytoskeletal components or may play an important role in the microtubule assembly-disassembly process.

The results presented in this dissertation clearly show the presence of two forms of tropomoyosin in chromaffin cells. However, only one of these polypeptides, the 38kD tropomyosin form, was found to be phosphorylated in these cells and to interact strongly with actin filaments (F-actin). Therefore, as suggested previously for other nonmuscle cell systems (for review see Côté, 1983; Payne & Rudnick, 1984, 1985), the fact that the two chromaffin cell tropomyosin polypeptides appear to possess different characteristics may suggest that they play different functional roles in these cells. Although the exact physiological role of tropomyosin in nonmuscle cells is not yet clearly understood, some attractive hypotheses have been recently advanced in this regard (Côté, 1983; Payne & Rudnick, 1984). For instance, tropomyosin forms that exhibit no binding to F-actin would represent a cytoplasmic tropomyosin subpopulation. It has been suggested that such a tropomyosin subpopulation might however regulate the interaction of actin filaments with other cytoskeletal components not directly but via the interaction of tropomyosin-binding proteins. Thus, the 32kD tropomyosin form of

chromaffin cell may indeed constitute such a subpopulation. In contrast, the 38kD form of tropomyosin which can bind strongly to F-actin could modulate the interaction of actin-binding proteins with actin either in a Ca^{2+}-sensitive or in a Ca^{2+}-insensitive manner. Under resting conditions (low intracellular Ca^{2+} concentrations), tropomyosin could bind to F-actin which has a structural role in the cell and then would prevent actin involvement in contractile events. In this case, a Ca^{2+}-insensitive accessory protein would be the only requirement to hold tropomyosin to the inhibitory position on actin (Côté, 1983). In fact, caldesmon which is an actin-binding protein may correspond to a very suitable candidate for this type of regulatory protein. In addition, it has been shown that caldesmon regulates the tropomyosin-enhanced actin-myosin interaction in gizzard smooth muscle (Sobue et al., 1985d). Indeed, caldesmon has been shown to be present in chromaffin cell and could correspond to such an accessory protein (Sobue et al., 1985b, 1985c). Furthermore, it was reported recently that a 70kD form of caldesmon appears to be a chromaffin granule-binding protein (Burgoyne et al., 1986).

It has also been postulated that tropomyosin could bind to actin filaments which are involved in contractile events. However, in this case, a Ca^{2+}-dependent regulatory system such as calmodulin would be required (Côté, 1983). Indeed, calmodulin has been shown to be present in chromaffin cells (Hikita et al., 1984; Trifaró et al., 1984a) and appears to be involved in some cellular events (see Chap. 1 section F.6). Therefore, following cell stimulation (higher intracellular Ca^{2+} concentrations), the inhibitory action of tropomyosin on F-actin could be abolished via the interaction of a Ca^{2+}/calmodulin-dependent mechanism. In this case, it appears that the phos-

phorylation of tropomyosin may regulate its interaction with F-actin. Consequently, actin would be able to interact with other proteins and could be involved in contractile events.

These observations suggest that the phosphorylation of some contractile proteins and cytoskeletal components may provide some mode of regulation for the interaction of these components which may be involved in different contractile events in chromaffin cells.

In addition, other chromaffin cell phosphoproteins might be involved in different cellular events related to the secretory cycle of these cells since their time course of phosphorylation was shown to be temporally associated with catecholamine secretion. Furthermore, the increase in ^{32}P incorporation into some of the polypeptides following ACh stimulation appears to be regulated by Ca^{2+}-dependent systems since experiments performed under Ca^{2+}-deprivation conditions abolished or greatly inhibited the phosphorylation of these polypeptides. The Ca^{2+} regulatory protein calmodulin has been implicated previously in the process of phosphorylation of several secretory systems (see Chap. 1 section G.6.1). Phosphorylation experiments carried out in cultured chromaffin cells in the presence of the calmodulin antagonist trifluoperazine have suggested that the phosphorylation of some polypeptides might be regulated by a Ca^{2+}/calmodulin-dependent system. Analysis of the effects of the calmodulin inhibitor and Ca^{2+} deprivation on individual proteins reveals quantitative differences in the level of protein phosphorylation. These differences allow us to classify the chromaffin cell phosphoproteins into 6 groups (Table 8). The first group which includes the 35.5, 38, 41.5, 43, 47 and 57 (tubulin) kD phosphoproteins, is highly affected by

either Ca^{2+} deprivation or TFP. It is likely that these proteins are major substrates for the Ca^{2+}/calmodulin-dependent kinase(s). Tubulin, for example, has been found to be a substrate for Ca^{2+}/calmodulin-dependent kinase(s) in intact synaptosomes (Burke & DeLorenzo, 1981). The second group corresponding to the 29, 33, 63 (tyrosine hydroxylase), 76, 80 (chromogranin A), 94, 107, 117.5 (chromogranin B) and 147 kD phosphoproteins, is highly affected by Ca^{2+} deprivation but to a lesser extent by TFP than the components of the first group. Ca^{2+}/calmodulin-dependent kinase(s) and other Ca^{2+}-dependent kinase(s) including protein kinase C are likely to be involved in the phosphorylation process of these substrates. The results presented here concerning the phosphorylation of tyrosine hydroxylase (63kD polypeptide) appear to be in agreement with previous studies which suggest that the stimulation of chromaffin cell induces the phosphorylation of the enzyme through at least two separate mechanisms (Haycock et al., 1982; Niggli et al., 1984; Pocotte et al., 1985, 1986). In the third group, the 160 and 294 kD proteins are only partially inhibited by either TFP or Ca^{2+} deprivation. These results suggest that Ca^{2+}-dependent kinase(s) as well as Ca^{2+}-independent kinase(s) possibly including cAMP-dependent kinase(s) might be involved in the regulation of these substrates. The 14.8kD protein is a component apart from all the others. Indeed the phosphorylation of this protein is highly Ca^{2+} dependent but not affected by the presence of TFP. Interestingly, a very similar polypeptide (15kD, pI 4.77) has also been described among the adrenal medullary chromaffin cell phosphoproteins affected by the protein kinase C activator PMA (Pocotte et al., 1985). Therefore, the present results together with those of Pocotte et al. (1985) strongly sug-

gest the participation of protein kinase C in the phosphorylation of the 14.8kD polypeptide. The four phosphoprotein bands at 313, 249, 210 and 184 kD which are listed in the fifth group, are not affected by Ca^{2+}-deprivation conditions. However, the calmodulin antagonist has a substantial inhibitory effect on their state of phosphorylation. We do not have any clear explanation for this observation. However, it possible that either these polypeptides bind very tightly Ca^{2+} or that intracellular pools of Ca^{2+} which are not affected by Ca^{2+}-deprivation conditions regulate their phosphorylation. It should be pointed out that a 249.4kD phosphoprotein has also been described by Treiman and collaborators (1983) in membranes of bovine chromaffin granules. Its in vitro phosphorylation was found to be stimulated by cAMP. Finally, the 20.4kD protein is also a component separate from the others. The ACh-induced dephosphorylation of this protein appears to be Ca^{2+} independent and not affected by the presence of TFP. Therefore, it is possible that its state of phosphorylation is regulated by either cyclic nucleotide-dependent protein kinase(s) or by the so-called independent-protein kinase(s).

In conclusion, protein phosphorylation appears to be a major process involved in several cellular events in chromaffin cells, some of which may be related to the secretory cycle of this cell system.

Table 8

Classification of the adrenal chromaffin cell phosphoproteins.

Groups	Molecular masses	Effects of ACh on phosphorylation	Ca^{2+} dependency	Trifluoperazine sensitivity
1	35.5	↑	+++	+++
	38	↑	+++	+++
	41.5	↑	+++	+++
	43	↑	+++	+++
	47	↑	+++	+++
	57	↑*	+++	+++
2	29	↑*	+++	+
	33	↑*	+++	++
	63	↑*	+++	+
	76	↑	+++	++
	80	↑	+++	++
	94	↑*	+++	+
	107	↑	+++	++
	117.5	↑	+++	++
	147	↑	+++	++
3	160	↑	++	++
	294	↑	+	+
4	14.8	↑*	+++	-
5	184	↑	-	++
	210	↑	-	++
	249	↑	-	++
	313	↑	-	++
6	20.4	↓*	-	-

Chromaffin cell phosphoproteins have been divided into 6 groups according to their response to ACh stimulation in regular Locke's solution, during calcium deprivation or presence of trifluoperazine.
↑ Increase; ↓ Decrease
* The most affected phosphoproteins upon stimulation
Sensitivity: +++ substantial; ++ partial; + slight; - no effect

CHAPTER 5

SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Bovine adrenal medullary chromaffin cells maintained in culture contain numerous polypeptides which can serve as substrates for protein kinases. Some of these substrates correspond to major chromaffin cell proteins while others correspond to minor chromaffin cell components.

2. Stimulation of cultured chromaffin cells affected the state of phosphorylation of several proteins as determined by ^{32}P incorporation.

   a) Enhanced phosphorylation of 22 proteins of apparent molecular weights ranging from 313,000 to 14,800 as well as increased dephosphorylation of a 20.4kD protein was observed when extracts of cultured chromaffin cells stimulated by either ACh ($10^{-4}M$) or 56 mM K^+ were analyzed by one-dimensional SDS-PAGE.

   b) The degree of phosphorylation of 8 proteins was greater in cultured chromaffin cells stimulated by ACh than those challenged by a depolarizing concentration of K^+ (56 mM).

   c) Two-dimensional SDS-PAGE analysis revealed that stimulation of cultured chromaffin cells with ACh produced two types of effect on protein phosphorylation:
      — Activation of protein kinase activities affecting about 30 polypeptides.
      — Activation of protein phosphatase activities resulting in the dephosphorylation of about 40 polypeptides.

d) The time course of protein phosphorylation paralleled or preceded that of [^{3}H]NA release for 15 phosphoproteins. This observation was also true for the 20.4kD polypeptide that was dephosphorylated following ACh stimulation.

e) The largest increase in ^{32}P incorporation was observed in chromaffin cell phosphoproteins with apparent molecular weights of 14,800, 29,000, 33,000, 57,000 (tubulin subunit), 63,000 (tyrosine hydroxylase) and 94,000.

3. The ACh-induced increase in protein phosphorylation was abolished or greatly inhibited (56 to 100%) for 18 proteins during Ca^{2+} deprivation (0.5mM Ca^{2+} plus 15 mM Mg^{2+}).

4. Trifluoperazine, a calmodulin antagonist, when used at a concentration of 2×10^{-7}M was found to produce 50% inhibition of ACh-induced catecholamine release without, however, affecting Ca^{2+} movements in these cells. Under these conditions, trifluoperazine abolished or significantly inhibited (35 to 100%) the stimulation-induced phosphorylation of all the polypeptides previously described with the exception of the 14.8kD and the dephosphorylated 20.4kD components which were not affected.

5. Two major chromaffin cell phosphoproteins of apparent molecular weights of 80,000 and 117,500 were identified as chromogranin A and chromogranin B, respectively. The degree of phosphorylation of both chromogranins (A and B) was increased following chromaffin cell stimulation by two different stimuli (ACh and 56 mM K^+).

6. It was also found that one of the chromaffin cell phosphoproteins (38kD) possesses the physicochemical characteristics of tropomyosin.

7. Bovine adrenal medulla was found to contain tropomyosins. By electrophoretic techniques, it was determined that three forms of tropomyosin are present in this tissue. The three adrenal medullary tropomyosins have apparent molecular weights of 38,000, 35,500 and 32,000 and possess similar isoelectric points of about 4.7. The two major tropomyosin polypeptides (38kD and 32kD) are present in a molar ratio of 2:1.

8. Biochemical characterization of the adrenal medullary tropomyosins has revealed that these polypeptides possess characteristics and properties similar to those described previously for muscle and nonmuscle cell tropomyosins such as heat stability, characteristic molecular weight shift on SDS-PAGE in presence of urea and homologies in peptide profiles after limited proteolytic digestion. It was also shown that the 38kD form of adrenal tropomyosin exhibits a stronger affinity for F-actin than the two other tropomyosin forms.

9. Bovine adrenal medullary chromaffin cells in culture were found to contain two forms of tropomyosin. These tropomyosin polypeptides appear to correspond to the major forms of tropomyosin observed in the adrenal medulla and have apparent molecular weights of 32,000 and 38,000.

10. Tropomyosin distribution in chromaffin cells in monolayer cultures was determined by indirect immunofluorescence techniques using an antibody against smooth muscle tropomyosin. A combined pattern of fluorescence was observed in these cells. The pattern consisted of a certain number of fluorescent granules distributed preferentially in the perinuclear region and of fluorescent filaments distributed over the entire area of the cells. Especially in bipolar chromaffin cells, these fine fluorescent filaments were arranged in a parallel fashion and in some cases, extended from a point of cell attachment to the next.

CHAPTER 6

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