BIOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE HEPATIC ALPHA$_1$-ADRENERGIC RECEPTOR

by

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ABSTRACT

The structure and the regulation of the hepatic α_1-adrenergic receptors have been studied in the rat. The *in vitro* incubation of isolated liver cells in a serum-free buffer for 4 hr leads to the conversion of the adrenergic activation of glycogen phosphorylase from an α_1- to a β-adrenoceptor-mediated event. This change is associated with no change in the glycogenolytic response to vasopressin and a reduction of the glycogenolytic response to glucagon. The time-dependent shift in the adrenergic control of glycogenolysis does not influence the density or the affinity of [^{3}H]prazosin-labeled α_1-receptors and [^{3}H]CGP-12177-labeled β-receptors. The change in the adrenergic control of glycogenolysis is reversed by a 30-min incubation with 50 nM lipomodulin, whereas in freshly isolated cells lipomodulin doesn't affect the predominant α-receptor response. Conversely, exposure of freshly isolated cells to a monoclonal antibody to lipomodulin in the presence of 10 μM phenylephrine, or to 2 μg/ml mellitin, results in a shift in the adrenergic control of glycogenolysis from α_1- to β-type within 30 min. It is proposed that coupling of hepatic α_1- and β-adrenoceptors to postreceptor pathways is regulated in an inverse reciprocal manner by changes in membrane phospholipase A_2 activity.

The mechanism of activation of the Ca^{2+}-linked receptors for vasopressin and adrenaline was studied in isolated liver cells. Sequential treatment of cells with 10^{-7}M vasopressin and 1 mM of bifunctional cross-linker disuccinimidyl suberate (DSS), followed by washout of the drugs, doesn't influence the dissociation of vasopressin from its receptor, but results in permanent activation of glycogen phosphorylase. Similarly, when the cells are stimulated with 10^{-5}M adrenaline and treated with DSS, glycogen phosphorylase is permanently activated. It is proposed that agonist activation of vasopressin or α_1-adrenergic receptors involves the microaggregation of receptors or their coupling to other membrane proteins.

We have attempted to isolate the fragment of the active center of the α_1-adrenergic receptor, which binds [^{3}H]phenoxybenzamine ([^{3}H]POB).

The α_1-adrenergic receptor was labeled with [^{3}H]POB in rat liver plasmamembranes, solubilized and partially purified by gel filtration and wheat germ agglutinin chromatography. The partially purified α_1-receptor was digested with trypsin and the [^{3}H]POB-labeled proteolytic fragment purified by RP-HPLC and GP-HPLC. We were unable to isolate, by this approach, a fragment that could be sequenced.

A novel irreversible antagonist for the α_1-adrenergic receptors, I-phenoxybenzamine (I-POB) have been synthesized and pharmacologically characterized. We have shown that I-POB binds to the α_1-adrenergic receptor in rat liver plasma membranes with high affinity (K_i = 2 nM), inhibits the activation of glycogen phosphorylase in isolated hepatocytes (IC_{50} = 1 nM) and causes prolonged and selective blockade of the rat vascular α_1-adrenergic receptors.

Polyclonal antibodies against the rat liver α_1-adrenergic receptor have been developed in rabbits. The antibodies interfered with the α_1-adrenergic but not the vasopressin-induced activation of glycogen phosphorylase in isolated hepatocytes and bound to the 80,000-dalton form of the SDS-PAGE-purified rat liver α_1-adrenergic receptor.

RESUME

Lors de ce travail de thèse nous nous sommes intéressés à l'étude de la structure et de la régulation du récepteur α_1-adrénergique en utilisant comme modèle d'étude des hépatocytes isolés du foie.

Sur ce type de préparation, un changement de l'activation α_1-adrénergique de la glycogène phosphorylase en une activation β-adrénergique a été observé, suite à l'incubation de ces cellules durant 4 heures dans un milieu Krebs-Henseleit ne contenant pas de sérum. Ce changement α_1 ——> β est accompagné d'une réduction de la réponse glycogénolytique au glucagon, tandis que la réponse à la vaso-pressine reste inchangée et que la densité et l'affinité des récepteurs α_1- (marqués à la [^{3}H]prazosine) et β-adrénergiques (marqués à la [^{3}H]CGP-12177) ne sont pas affectées non plus. Par ailleurs ce changement α_1- ——> β est inversé après incubation durant 30 min avec de la lipomoduline (50 nM). Bien que ce composé n'affecte pas la réponse due à l'activation des récepteurs α_1-adrénergiques, normalement prédominante dans les hépatocytes fraîchement isolés, l'incubation de ce type de cellules avec un anticorps monoclonal dirigé contre la lipomoduline et en présence de phénylephrine (10 µM) ou de mellitine (2 µg/ml) produit, au bout de 30 min, le changement α_1- ——> β. Ces résultats suggèrent que des changements d'activité de la phospholipase A_2 membranaire influencent, de façon réciproque, le couplage des récepteurs hépatiques α_1- et β à leurs effecteurs correspondants.

L'étude du mécanisme d'activation de deux récepteurs couplés au Ca^{2+} - les récepteurs à la vaso-pressine et à l'adrénaline - a également été réalisé. Le traitement d'hépatocytes avec de la vaso-pressine (10^{-7}M) et d'un agent de couplage bifonctionnel, le disuccinimidyl subérate (DSS, 1mM), n'affecte pas la dissociation de la vaso-pressine avec son récepteur, mais produit néamoins une activation permanente de la glycogène phosphorylase. De même, l'incubation d'hépatocytes avec de l'adrénaline (10^{-5}M) et du DSS résulte en une activation permanente de la glycogène phosphorylase. Ces résultats suggèrent que l'activation de chacun des récepteurs par leur hormone respective conduit à une microaggrégation de ces récepteurs ou à leur couplage à d'autres protéines membranaires.

Nous avons également tenté d'isoler, à partir de membranes plasmiques de foies de rats, le fragment du centre actif du récepteur α_1-adrénergique auquel se lie la [^{3}H]phénoxybenzamine (POB). Le récepteur à été marqué à la [^{3}H]POB, solubilisé et partiellement purifié à l'aide de techniques de filtration sur gel et de chromatographie à la lectine de germe de blé. Après une digestion à la trypsine, le fragment du récepteur, marqué par la [^{3}H]POB, a été purifié par chromatographie en phase inversée et chromatographie de perméation sur gel. Toutefois cette approche ne nous a pas permis d'isoler un fragment qui aurait pu être séquencé.

Nos travaux nous ont également conduit à synthétiser et à caractériser pharmacologiquement un nouvau marqueur irréversible des récepteurs α_1-adrénergiques, l'iodo-phénoxybenzamine (I-POB). Nous avons montré que l'I-POB se lie par un mécanisme à haute affinité (K_i = 2 nM) au récepteur α_1-adrénergique des membranes plasmiques isolées à partir de foies de rats, qu'il inhibe l'activation α_1-adrénergique de la glycogène phosphorylase dans des hépatocytes isolés (ID_{50} = 1 nM) et qu'il bloque sélectivement et à long terme les récepteurs α_1 vasculaires du rat.

Nous avons également développé, chez le lapin, des anticorps polyclonaux contre le récepteur α_1-adrénergique de foies de rats. L'utilisation de ces anticorps nous ont montré qu'ils interfèrent avec l'activation α_1-adrénergique, mais non avec celle due à la vaso-pressine, et qu'ils se lient au récepteur α_1-adrénergique (P.M. 80 000 daltons) purifié par électrophorèse sur gel polyacrylamide.

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ABBREVIATIONS

AVP	arginine^{8}vasopressin
cAMP	adenosine 3',5'-monophosphate, cyclic
cGMP	guanosine 3',5'-monophosphate, cyclic
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DSS	disuccinimidyl suberate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-(3-*O*-thio)triphosphate
I-POB	3-iodobenzyl phenoxybenzamine
IP	inositol 1-phosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
NAGA	β-N-acetlyl-D-glucosamine
PhE	*l*-phenylephrine
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PMSF	phenylmethylsulfonyl fluoride
POB	phenoxybenzamine
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
STI	soybean trypsin inhibitor
TFA	trifluoroacetic acid
TPCK	tosyl phenylalanyl chloromethyl ketone
WGA	wheat germ (Triticum vulgaris) agglutinin
B_{max}	maximal binding capacity
EC_{50}	concentration of agonist producing half maximal response
IC_{50}	concentration of unlabeled ligand producing half-maximal suppression of specific labeled ligand binding
K_D	equilibrium dissociation constant

SECTION 1: INTRODUCTION

STRUCTURE AND FUNCTION OF THE Ca^{2+}-LINKED HORMONES IN THE LIVER

1.1 METABOLIC EFFECTS OF Ca^{2+}-LINKED HORMONES ON THE LIVER

The liver is the place where the main stocks of glycogen are kept, and by this fact it plays a key role in the organism's carbohydrate metabolism. It is the target organ for the action of the hormones insulin and glucagon, which are involved in the maintenance of glucose levels in the blood. Under stress, adrenaline, secreted by the adrenal medulla, and noradrenaline, released by the sympathetic nerves innervating the liver take over the control and adapt the liver metabolism to the anticipated "fight or flight" situation. They increase the rate of glycogenolysis and gluconeogenesis, and decrease the synthesis of glycogen, which results in an increased release of glucose in the blood. This, together with the increased levels of free fatty acids in the blood (resulting from increased lipolysis in adipose tissue), provide the organism with readily available energy substrates. The hypertensive peptide hormones vasopressin (secreted by the posterior hypophysis), and angiotensin II (formed through the action of renin), have similar effects on liver carbohydrate metabolism (Guyton, 1986). All these hormones produce their effects through their respective membrane receptors on the surface of the hepatocyte.

The hepatic effects of catecholamines are produced through two different types of receptors (Ahlquist, 1948) - α and β. The net result of their activation is the same - stimulation of the liver glycogen phosphorylase, the rate-limiting step in the degradation of glycogen, but the mechanisms they activate are very different. The β-adrenergic receptor,

similarly to the glucagon receptor, activates the cell membrane adenylate cyclase, which results in an increase in the intracellular levels of cAMP (Sutherland & Rall, 1960; Rall, 1972), whereas activation of the α-adrenergic receptor (α_1 subtype in the liver; Hoffman *et al.*, 1980), the vasopressin receptor (V_1-subtype in the liver; Michell *et al.*, 1979) and the angiotensin II receptor are not linked to such a mechanism. It was first proposed in 1977 that the activation of these receptors triggers a different second messenger system that involves a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$). The hypothesis was based on three lines of evidence: 1. It was found that the enzyme phosphorylase kinase (responsible for the activation of glycogen phosphorylase), is stimulated *in vitro* by $[Ca^{2+}]_i$ concentrations similar to those existing *in vivo*. 2. This group of hormones was producing a change in calcium fluxes and a redistribution of $[Ca^{2+}]_i$. 3. The action of these hormones was mimicked by A23187, a Ca^{2+}-ionophore (Assimacopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Van de Werve *et al.*, 1977). Subsequent studies, by demonstrating directly an increase in $[Ca^{2+}]_i$ in the seconds following the administration of α-adrenergic agonists, vasopressin and angiotensin II, provided further support for this hypothesis (Charest *et al.*, 1983; Berthon *et al.*, 1984).

The increase in free cytosolic Ca^{2+} leads to a saturation of the four Ca^{2+}-binding sites of calmodulin. The Ca^{2+}-calmodulin complex is an important modulator of the activity of glycogen phosphorylase kinase and is able to activate, independently of cAMP levels, the glycogen phosphorylase (Van den Heede *et al.*, 1979). The same Ca^{2+}-calmodulin complex inhibits, by a similar mechanism, the activity of glycogen synthase (Payne & Soderling, 1980). The Ca^{2+}-linked hormones also inhibit glycolysis at two

different levels: by decreasing the activity of phosphofructokinase (Richards & Ueda, 1982) as well as the activity of pyruvate kinase (Hers & Hue, 1983). The increase in hepatic gluconeogenesis by the Ca^{2+}-linked hormones is due to stimulation of fructose 1,6-diphosphatase and mitochondrial pyruvate carboxylase, and to inhibition of the pyruvate kinase (Hers & Hue, 1983).

α_1-Adrenergic stimulation in the liver produces many other effects, namely alteration of K^+ fluxes, increase in respiration, amino acid transport and ureagenesis, and inhibition of lipogenesis (Exton, 1981).

1.2 MECHANISM OF Ca^{2+} MOBILIZATION IN THE HEPATOCYTE

From a basic level of 160 - 200 nM, the adrenergic agonists and the hypertensive peptide hormones increase the cytosolic Ca^{2+} to about 800 nM. The rise in $[Ca^{2+}]_i$ is rapid (< 1 sec, maximum at 5 sec for noradrenaline), and sustained (>20 min) (Berthon *et al.*, 1984; Joseph *et al.*, 1985). The response is composed of two phases. There is an initial large transient phase, followed by a much smaller sustained phase. Removal of extracellular Ca^{2+} doesn't alter the first phase, but abolishes the second (DeWitt & Putney, 1983, 1984; Joseph *et al.*, 1985). This means that the Ca^{2+}-linked hormones alter the intracellular Ca^{2+} homeostasis by a dual mechanism: rapid release of Ca^{2+} from a hormone-sensitive intracellular pool, and increase in Ca^{2+} influx through the plasma membrane.

The origin and regulation of the hormone-sensitive intracellular Ca^{2+} pool have been subjects of debate. There are two organelles, that could be possible sources of calcium: the mitochondria and the rough endoplasmic reticulum. The size of their Ca^{2+} content is similar. Estimates

of the Ca^{2+} content of the endoplasmic reticulum range from 0.9 nmol/mg protein (Murphy *et al.*, 1980) to 5 nmol/mg protein (Somlyo *et al.*, 1985), i.e. 5 to 30% of the total cell Ca^{2+} in the hepatocyte. The mitochondrial Ca^{2+} content has been estimated to be 5 nmol/mg protein (Murphy *et al.*, 1980), although a more recent study suggests a lower level, accounting for for only 5% of the total cellular Ca^{2+} (Somlyo, 1985). Both organelles possess energy-dependent Ca^{2+}-uptake systems but, importantly, it has been shown that the Ca^{2+}-uptake system of the endoplasmic reticulum has a much lower K_D (0.1 - 0.3 uM) for Ca^{2+} (Famulski & Carafoli, 1982), than the mitochondrial K_D (1 - 10 μM) (Akerman & Nichols, 1983). Several studies have produced evidence that hormone-induced Ca^{2+} release occurs from a microsomal fraction of the rough endoplasmic reticulum, rather than from the mitochondria. In experiments with saponin-permeabilized hepatocytes, Joseph *et al.* (1984) have shown that, at micromolar $[Ca^{2+}]_i$ concentration and in conditions when only the mitochondrial pool was saturated with Ca^{2+}, stimulation of permeabilized hepatocytes with inositol (1,4,5)trisphosphate (IP_3) was not able to release Ca^{2+}. On the other hand, it was found that IP_3 releases Ca^{2+}, accumulated in the presence of ATP, by a subcellular fraction, enriched with enzyme markers for the endoplasmic reticulum, and the amount of released Ca^{2+} was not affected by mitochondrial inhibitors (Dawson & Irvine, 1984). Experiments with saponin-permeabilized hepatocytes have also indicated that at physiological levels of $[Ca^{2+}]_i$, the endoplasmic reticulum acts as the main hormone-sensitive Ca^{2+} pool (Burgess *et al.*, 1983). One of the most convincing arguments in favor of the endoplasmic reticulum comes from the studies of Spät *et al.* (1986), which have demonstrated that IP_3 releases Ca^{2+} from the endo-

plasmic reticulum, but not from the mitochondria, and the existence of a saturable receptor for IP_3 on the endoplasmic reticulum of hepatocytes. The microsomal fraction of the endoplasmic reticulum, implicated in the first phase of the $[Ca^{2+}]_i$ rise, is not yet well characterized, and it is still unclear how many Ca^{2+} pools are affected by IP_3. There are two binding sites for IP_3 in the endoplasmic reticulum (with K_D's of 8 nM and 0.16 µM; Spät *et al.*, 1986), and it is not known which one is the physiological receptor, since their affinity doesn't correspond to the EC_{50} of IP_3 for Ca^{2+} release (100 - 200 nM in permeabilized hepatocytes; Joseph *et al.*, 1984). On the other hand, Taylor & Putney (1985) have shown that IP_3 is able to release only one third of the non-mitochondrial Ca^{2+} pool, whereas Muellem *et al.* (1985) have demonstrated that after a first stimulation with IP_3, which results in rapid uptake of the released Ca^{2+} back in the pool, a second stimulation with IP_3 was not able to release it again. All these findings have led to speculations about the existence of IP_3-sensitive and IP_3-insensitive Ca^{2+} pools in the endoplasmic reticulum (Muellem *et al.*, 1985). The process by which IP_3 releases Ca^{2+} is also unclear. The release of Ca^{2+} by IP_3 requires GTP, and the nucleotide has to be hydrolyzed, because the effect of GTP (which is able to release by itself a small amount of Ca^{2+}), is antagonized by GDP, and is not mimicked by non-hydrolysable analogs of GTP (Dawson, 1985; Dawson *et al.* 1986). The same investigators have found a GTP-dependent phosphorylation of two proteins with *M* 38,000 and 17,000 daltons from the microsomal fraction. Since the effect of GTP on the IP_3-mediated Ca^{2+} release was also dependent on the presence of the fusogen polyethylene glycol, which enhances the binding of the 38,000-dalton protein to the microsomal membrane, they have

proposed that the binding of this protein, after a GTP-dependent phosphorylation, is necessary for the process.

The second, more sustained phase of the $[Ca^{2+}]_i$ increase seems to be maintained through an opening of Ca^{2+} channels in the plasma membrane by the Ca^{2+}-linked hormones (Mauger *et al.*, 1984), and inhibition of the plasma membrane Ca^{2+}/Mg^{2+}-ATPase (Prpic *et al.*, 1984).

In summary, the presently accepted mechanism of hormonal Ca^{2+} mobilization in the hepatocyte is the following: activation of hormone receptors leads to a release from the plasma membrane of IP_3, which liberates Ca^{2+} from the endoplasmic reticulum, responsible for the first, rapid rise in the $[Ca^{2+}]_i$. The sustained second phase of the response is maintained by an increase in Ca^{2+} influx through the plasma membrane. The $[Ca^{2+}]_i$ increase is limited by mitochondrial uptake, where the increase in the concentration of Ca^{2+} in the matrix is thought to play a role in the increase in respiration and the decrease in glycolysis, produced by the Ca^{2+}-dependent hormones (Burgess, 1987).

1.3 ELEMENTS OF THE MECHANISM OF TRANSMEMBRANE SIGNALING BY Ca^{2+}-LINKED HORMONES

1.3.1 Membrane receptors. None of the Ca^{2+}-linked receptors in the mammalian liver has been cloned and sequenced yet, and little is known about their structure and membrane topography. The presently available data about the structure of the α_1-adrenergic receptor, which is in the main line of interest of this thesis, will be reviewed in the next section. The vasopressin V_1 receptor and the angiotensin II receptor structure will be mentioned briefly.

1.3.1.1 α_1-Adrenergic receptor. The molecular weight (M_r) of the rat liver α_1-adrenergic receptor has been investigated by three approaches: 1) hydrodynamic methods (gel filtration and sucrose gradient centrifugation), 2) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and 3) radiation inactivation (target size) analysis. The hydrodynamic methods, although applied under non-reducing conditions, require the solubilization of the receptor with detergents and have yielded controversial results. Using gel filtration and sedimentation analysis, Graham *et al.* (1982) have found that the digitonin-solubilized receptor has a Stokes radius of 49.4 Å (corresponding to a M_r in the range 137,000 - 153,000 daltons), in contrast with the data of Meier *et al.*, (1984) who found, also for digitonin-solubilized receptors, a much bigger Stokes radius - 68 Å (M_r = 251,000 daltons). Data for the Stokes radius of the detergent-receptor complex, obtained after solubilization with non-ionic detergents, also indicate the presence of a high m.w. complex (Guellaen *et al.* 1979, 57 Å; Kunos *et al.* 1983, 60 Å). Results obtained by this approach are not very accurate, because it is difficult to estimate the homogeneity of the solubilized protein and the exact amount of detergent participating in the complex. Such high m.w. complexes might also include other membrane proteins (Bojanic & Fain, 1986; Fitzgerald *et al.*, 1986). The SDS-PAGE analysis usually yields more reliable results, due to the use of standardized detergent and electrophoretic conditions. The interpretation of the results from the early studies (1979 - 1982) has been complicated by the sensitivity of the receptor protein to proteolysis during purification (Kunos, 1984; Homcy and Graham, 1985). Guellaen *et al.*, (1982), in a study where no protease inhibitors were used, reported a

subunit m.w. of 45,000 daltons; whereas Graham et al. (1982) found a m.w. of 59,000 daltons. Later on, Kunos *et al.* (1983) using several protease inhibitors throughout the purification procedure, identified a major 80,000-dalton band, as well as a minor 58,000-dalton band, and demonstrated that the presence of the higher m.w. peak depends on the use of the inhibitors. Later studies (Venter *et al.*, 1984; Leeb-Lundberg *et al.*, 1984; Seidman *et al.*, 1984) corroborated these findings. Interestingly, SDS-PAGE of the rat liver α_1-adrenergic receptor yields consistently more than one specifically labeled peptides, despite the rigorous purification procedures. Therefore, it was a matter of debate whether the lower molecular weight peptide, which also has ligand-binding capacity, is a product of proteolysis or whether it is a native form of the liver α_1-receptor (Kunos, 1984; Homcy & Graham, 1985). The first possibility was investigated by Leeb-Lundberg *et al.* (1984), who studied the effect of different protease inhibitors (soybean trypsin inhibitor, bacitracin, leupeptin, phenylmethylsulfonyl fluoride and ethylenediamine tetracetate (EDTA) on the labeling pattern of the α_1-adrenergic receptor from different tissues. They found that EDTA was the most efficient protease inhibitor, whose presence decreased the proportion of the lower m.w. peptides, and concluded that an unidentified metalloprotease was responsible for this effect. A similar conclusion was also reached by Venter *et al.* (1984). Endogenous proteolysis has also been implicated in the heterogeneity of the labeling pattern of the liver β-adrenergic receptor (Benovic *et al.*, 1983). SDS-PAGE analysis is indicative mainly of the M_r of the receptor subunits, due to the fact that the disulfide bonds in the protein are reduced. The unique advantage of the third approach, radiation inactiva-

tion analysis, is that it studies the receptor in its natural environment, it is not dependent on the purity of the sample, and it makes it possible to determine the size of the functional unit for several receptor associated functions (Harmon *et al.*, 1980). The method has been used by several groups for the determination of the M_r of the native α_1-adrenergic receptor. For the rat liver, the data obtained by Venter *et al.* (1984) indicate an M_r of 160,000 daltons, and since the amount of irradiation paralleled the loss of the 85,000-dalton subunit of the receptor, they proposed that the native receptor is a dimer of two 85,000-dalton peptides. The α_1-receptor in the rat mesenteric artery also seems to be a dimer (M_r = 123,000 daltons; Agrawal *et al.*, 1986), whereas in the rat cortex the receptor is probably a monomer (M_r = 85,000 or less; Lübbecke *et al.*, 1983; Rank *et al.*, 1985; Mogilnicka & Nielsen, 1986). So far, the only functional characteristic assessed after radiation inactivation of the receptor has been ligand binding, and there have been no attempts to study the functional size of the agonist-activated form of the receptor. Despite the progress in the development of affinity chromatographic methods for the purification of the α_1-adrenergic receptor, and the purification of the hormone-binding subunit of the receptor (Graham *et al.*, 1982; Lomasney *et al.*, 1986), there is still insufficient data about the structure of the receptor and its topography in the plasma membrane. A study using immobilized adrenaline have found that the ligand-binding site of the receptor is exposed to the extracellular space (Dehaye *et al.*, 1980). In a study of the peptides, generated by limited proteolysis of the α_1-receptor from rat liver membranes, Venter *et al.* (1984) have found that digestion with trypsin, chymotrypsin and papain produce peptides of

62,000, 45,000, 40,000, 27,000, 23,000 and 18,000 daltons. The digestion of membrane-bound receptor with chymotrypsin yields water-soluble peptides with M_r 45,000, 40,000, 27,000, 23,000 and 18,000 daltons, all of which contain the ligand-binding site and protrude from the the plasma membrane into the extracellular space. It was proposed that the α_1-adrenergic receptor has two main parts: an extracellular domain, 45,000 daltons, and a 40,000-daltons domain, part membrane (22,000 daltons), part cytoplasmic (16,000 daltons). The ligand-binding site is contained in a 18,000-dalton part of the extracellular 45,000 dalton domain. The α_1-adrenergic receptor is a sialyated glycoprotein (Meier *et al.*, 1984).

1.3.1.2 Vasopressin V_1 receptor. The M_r of the hormone-binding subunit of the vasopressin V_1 receptor from rat liver was found to be 68,000 daltons by SDS-PAGE (Fishman *et al.*, 1987), but radiation inactivation analysis indicates a higher value - 77,000 daltons (Crause *et al.*, 1984). Hydrodynamic measurements of the m.w. of the detergent-solubilized receptor indicate a complex with M_r = 258,000 daltons (Bojanic & Fain, 1986), which also contains tightly bound GTP-binding protein (Fitzgerald *et al.*, 1986).

1.3.1.3 Angiotensin II receptor. The M_r of the hormone-binding subunit of rat liver angiotensin II receptor has been estimated at 64 - 68,000 daltons by SDS-PAGE, following cross-linking with radiolabeled angiotensin II (Sen *et al.*, 1983; Guillemette *et al.*, 1984; 1986). Detergent solubilization of the plasma membrane-bound receptor has also shown the existence of high m.w. complexes (115,000 and 200,000 daltons), and it has been proposed that the native receptor is a dimer of two disulfide-linked, 65,000 dalton subunits (Caponi & Catt, 1980). Similarly to the

nicotinic acetylcholine receptor and the TSH receptor, the liver angiotensin II receptor has disulfide bonds essential for ligand binding (Sen *et al.*, 1984; Sen, 1985). There are two receptor subtypes in rat liver: high-affinity binding sites (K_d = 35 nM, B_{max} = 372 fmol/mg protein) and low-affinity binding sites (K_d = 3.1 nM; B_{max} = 658 fmol/mg protein). The high-affinity receptors are sensitive to dithiothreitol (DTT) treatment and appear to be linked to glycogen phosphorylase stimulation, whereas the low-affinity receptors are linked to adenylate cyclase inhibition. The apparent lack of interconversion by DTT treatment or guanine nucleotides suggests they represent distinct receptor subtypes (Gunther, 1984).

1.3.2 Transducer protein(s). It is now well established that at least four types of GTP-binding transducer proteins - G_s, G_i, G_o and G_t (transducin) participate in the transmission of receptor-generated signals. Their structure has been recently reviewed by Gilman (1987). G_s (stimulatory) and G_i (inhibitory) transmit stimulatory and inhibitory signals to the plasma membrane adenylate cyclase in various tissues, G_o (abundant in the brain and the heart) is inhibitory for the adenylate cyclase, whereas G_t is confined to the retina and is linked to another second messenger - cGMP. Besides the regulation of the activity of adenylate cyclase, the G-proteins participate in the stimulation of retinal cGMP phosphodiesterase (G_t), the regulation of ion channels (G_i and G_o), and in the stimulation of phosphoinositide hydrolysis (G_i and G_o).

Gilman (1987) lists four criteria for involvement of a G protein in the transduction of the signal from a receptor: 1. Both an agonist and GTP are required to initiate the physiologic response. 2. The response can be provoked, independently of the receptor, by stimulators of the G-protein

(non-hydrolysable analogs of GTP, or F^- plus Al^{3+}). 3. The existence of negative heterotropic interaction between the binding of GTP to the G protein, and the binding of agonist to the receptor. 4. Identification, by toxin ADP-ribosylation, immunologic methods, or G-protein-lacking mutant cell lines, of the G-protein. Purification and reconstitution, in a cell-free system, of the components of the receptor pathway. The following text will review, with respect to these criteria, the experimental evidence for the participation of G-proteins in the signal transduction of the Ca^{2+}-linked hormones in the liver.

The first argument in favor of a possible involvement of G protein(s) in the signal transduction of the Ca^{2+}-linked receptors has come from studies of the effect of non-hydrolysable GTP analogs on the agonist binding to Ca^{2+}-linked receptors. Goodhardt *et al.* (1982) has shown that that they produce a three-fold decrease in the affinity of the liver α_1-adrenergic receptor for adrenaline and noradrenaline. In the absence of guanyl nucleotides, the α_1-receptor existed in two affinity states, a high-affinity state with K_D 70 nM (~ 20% of the binding sites), and a low affinity state with K_D 3,920 nM. Guanyl nucleotides converted all the receptors into the low affinity form. A similar proportion of high-affinity binding sites was reported later by Lynch *et al.* (1985). If GTP-modulation of agonist binding was found consistently for vasopressin and angiotensin II, the data for the α_1-adrenergic receptor are less clear, since several groups have reported lack of effect of the guanyl nucleotides (e.g. Hoffman *et al.*, 1980; Preiksaitis *et al.*, 1982; Wikberg *et al.*, 1983). Also, the extent of the coupling of the α_1-receptor to its GTP-binding protein is not constant: it seems to be dependent on the re-

moval of the Ca^{2+} ion from the medium, and to change with the age of the animal (Lynch *et al.*, 1985).

As it will be discussed more below, the activation of the Ca^{2+}-linked receptors results in activation of plasma membrane phospholipase C and phosphoinositide breakdown (Berridge & Irvine, 1984). Vasopressin and α_1-adrenergic agonists have been shown to cause a loss of IP_3 from the rat liver plasma membrane (Wallace *et al.*, 1982; Harrington & Eichberg, 1983), and several groups were able to demonstrate that the plasma membrane phospholipase C is modulated by guanine nucleotides. Wallace & Fain (1985) have found that GTPγS (a non-hydrolysable GTP-analogue) was stimulating phospholipase C in liver plasma membranes. At concentrations from 10 to 100 μM, GTPγS induced a concentration-dependent loss of up to 20% of the plasma membrane phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2), and corresponding increase in IP_3 and inositol bisphosphate (IP_2). Two other studies, by Uhing *et al.* (1985) and Melin *et al.* (1986) demonstrated that rat liver phospholipase C is regulated by guanine nucleotides and Ca^{2+}. The guanine nucleotides decreased the Ca^{2+} requirements of the enzyme and increased its maximal activity.

Another line of evidence has come from the study of Blackmore *et al.* (1985), showing that activators of the G-proteins are able to mimic the effects of Ca^{2+}-linked hormones on hepatocytes. These investigators have shown that the addition of Al^{3+} plus F^-, known to cause dissociation of the subunits of G_s and G_i (Katada *et al.*, 1984), to rat hepatocytes, also produced activation of glycogen phosphorylase, a rise in $[Ca^{2+}]_i$, increase in the cytosolic concentrations of IP_3 and diacylglycerol (DAG), and a decrease in the membrane content of PIP_2.

All the above studies have produced circumstantial evidence for the involvement of a G-protein in the signal transduction of the Ca^{2+}-mobilizing hormones in the liver. For a direct proof from such an interaction, a purification of all the components of the system - the receptor, the transducer and the effector is needed, and after that they could be reconstituted in a cell-free system (e.g. phospholipid vesicles) and their interaction can be studied. So far, only methods for the purification of the α_1-adrenergic receptor (Graham *et al.*, 1982; Lomasney *et al.*, 1986) and plasma membrane phospholipase C (Deckmyn *et al.*, 1986) in active state are available. The attempts to identify the transducer protein(s) involved have been unsuccessful. There are two bacterial toxins, cholera toxin and the pertussis toxin, which ADP-ribosylate G_s and G_i, respectively. Neither of them interferes with the receptor binding characteristics or the agonist activation of glycogen phosphorylase in the liver (Lynch *et al.*, 1986; Uhing *et al.*, 1986), and no mutant cell line, missing some of the components of the Ca^{2+}-linked receptor system is available yet. For these reasons, the molecular characterization of this receptor system is still lagging behind that of the β-adrenergic receptor system.

1.3.3 Effector. As mentioned above, there is a well described correlation between the activation of the Ca^{2+}-linked receptors in the liver and a change in the metabolism of some plasma membrane inositol-containing phospholipids. The stimulatior of these receptors leads to incorporation of ^{32}P into phosphatidylinositol (PI), but not into other major phospholipids (Tolbert *et al.*, 1980). PI is sequentially phosphorylated to form phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), each of which makes up about 1% of the

total inositol phospholipids of hepatocytes. These phosphorylations are continually reversed by specific phosphomonoesterases so that all the lipids are in equilibrium. It was established that the Ca^{2+}-linked hormones induce a rapid breakdown of the plasma membrane PIP_2, and this effect was only partially decreased by removal of the extracellular Ca^{2+}, and relatively independent of the levels of $[Ca^{2+}]_i$. It was noted also that the Ca^{2+}-ionophore A23187 was not able to mimic the effects of the Ca^{2+}-linked hormones on the phosphoinositide metabolism. These results led Michell *et al.* (1981) to propose that that the initial event in the hormonal activation was the increase in the breakdown of the polyphosphoinositides, which was leading to an increase in the $[Ca^{2+}]_i$. The mammalian cell has a variety of phospholipases, which participate in the catabolism of dietary lipids and in the metabolism of structural membrane phospholipids. These enzymes have been divided into four groups with respect to the specificity of their action: phospholipases A_1 and A_2 remove the fatty acid from position 1 and 2 of the diacylglycerophospholipids, respectively. Phospholipases C hydrolyze the glycerophosphate ester bond of phospholipids to yield diacylglycerol and phosphobase moiety, whereas phospholipases D release phosphatidic acid from the phospholipids. Phospholipases A_2 are involved in the control of the synthesis of eikosanoids and platelet-activating factor, whereas mammalian phospholipases C specifically degrade inositol phospholipids (Daniel, 1985). It is now presumed that there exist two types of phospholipases C. The first type are soluble enzymes, which metabolize all three phosphoinositides, while the second type are membrane bound enzyme(s), which are polyphosphoinositide-specific (Deckmyn *et al.*, 1986) and regulated by Ca^{2+} and guanine nucleo-

tides (Uhing *et al.*, 1985; Melin *et al.*, 1986). The phospholipase C-mediated hydrolysis of plasma membrane PIP_2 yields two substances with second messenger functions: IP_3 and DAG. As with other, previously identified second messengers, their concentration is controlled within strict boundaries by specialized systems: IP_3 is sequentially degraded by specific phosphomonoesterases to IP_2, IP and inositol (Storey *et al.*, 1984), and DAG is metabolized by the DAG kinase to form phosphatidic acid. As mentioned above, the main function of IP_3 is to trigger the release of Ca^{2+} from the endoplasmic reticulum. On the other hand, DAG has been shown to induce, concomitantly with the increased $[Ca^{2+}]_i$, an activation of the cytosolic enzyme protein kinase C and its subsequent translocation from the cytosol to the plasma membrane (Hirasawa & Nishizuka, 1985). Thus, in contrast to the cAMP-linked receptors, the propagation of the signal of the Ca^{2+}-linked receptors occurs through a dual messenger system, resulting in parallel activation of two classes of protein kinases: calmodulin-dependent and calcium-phospholipid-dependent enzymes (protein kinase C). In this complex picture, the function of protein kinase C is still less clear than the other aspects of the receptor mechanism of the Ca^{2+}-linked receptors. In contrast to other systems, in hepatic glycogenolysis protein kinase C activation by phorbol esters has no synergistic effect on the hormonal stimulation (Lynch *et al.*, 1985). Interestingly, the enzyme activation has a differential effects on the function of the Ca^{2+}-linked receptors: it inhibits the α_1-receptor responses (PIP_2 breakdown, Ca^{2+} mobilization and phosphorylase activation; Corvera *et al.*, 1986), but only slightly reduces the effects of vasopressin (Cooper *et al.*, 1985), and doesn't influence those of angiotensin II (Corvera *et al.*, 1986). And

since the effect of phorbol esters mimics the vasopressin and angiotensin II-induced block of the α_1-adrenergic effects in hepatocytes (García-Sainz *et al.*, 1986), it is possible that protein kinase C might play a regulatory role in the function of the α_1-adrenergic receptor. This idea is also supported by the implication of protein kinase C activation in the interconversion of the adrenergic control of glycogenolysis in cultured hepatocytes, found recently by Kunos *et al.* (1987). The mechanism of the regulatory change is not clear, but accumulating evidence points to a direct effect on the receptor protein. A recent work of Bouvier *et al.* (1987) have demonstrated that protein kinase C phosphorylates the α_1-adrenergic receptor *in vitro*, and these data, together with the observations that phorbol esters induce desensitization (Corvera *et al.*, 1986) and uncoupling from the inositol phosphate metabolism (Leeb-Lundberg *et al.*, 1985), might indicate the existence, for the α_1-adrenergic receptor, of a regulatory mechanism similar to that described for the β-adrenergic receptor (Sibley *et al.*, 1987).

1.4 STATEMENT OF THE PROBLEM

In the last 8 years, there have been considerable advances in the understanding of the structure and function of the Ca^{2+}-linked hormone receptors in the liver. Similarly to the study of the principles of function of the cAMP-linked hormones, the progress in this area of knowledge comes through the isolation and the study of the cell membrane components for transmission of the hormonal signal, and their mode of regulation. The liver of the male rat is a widely used model for such studies. This organ is rich in α_1-adrenergic receptors, and in most of the studies to date it has been the vehicle for the molecular characterization of the hormone-binding subunit of the receptor. The existence of well established methods for isolation and *in vitro* culture of hepatocytes offers also the possibility to study the function and the mechanisms of regulation of the Ca^{2+}-linked receptor systems. In the mammalian liver, the α- and β-type of adrenergic receptors are present, and, unlike in other systems, they are both coupled in a stimulatory fashion to the same physiological response, liver glycogenolysis. One of these receptor subtypes, the α_1-receptor, is predominant in the male rat, but this predominance is able to shift to the other type of adrenergic receptor under some experimental and pathological conditions. Since the shift appears in cultured liver cells, they represent so far a unique *in vitro* model for the study of the regulatory mechanisms involved in the change.

The work of the present thesis was oriented towards three goals: 1) to study some of the regulatory mechanisms involved in shift of the adrenergic control of the glycogenolysis in the rat liver; 2) continue the study of the hormone-binding subunit of the α_1-adrenergic receptor by

biochemical and immunological methods; 3) to study the mechanism of agonist activation of the α_1-adrenergic and the vasopressin V_1 receptors in the liver.

To achieve these goals, we have 1) studied the effects of modulators of the activity of plasma membrane phospholipase A_2 on the conversion of the adrenergic control of glycogenolysis in isolated hepatocytes; 2) studied the effects of the cross-linker disuccinimidyl suberate on the agonist activation of the α_1-adrenergic and the vasopressin V_1 receptor in isolated hepatocytes; 3) made a partial purification of the hormone-binding subunit of the α_1-adrenergic receptor from rat liver membranes and tried to purify and sequence the [^{3}H]POB-binding site; 4) developed and characterized rabbit polyclonal antibodies against the rat liver α_1-adrenergic receptor; 5) studied the pharmacological characteristics of a novel irreversible α_1-adrenergic antagonist: I-phenoxybenzamine.

SECTION 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 DRUGS. L-arterenol bitartarate, *l*-phenylephrine (PhE), [Arg^8]Vasopressin (AVP), *l*-isoproterenol and mellitin were from Sigma Chemical Co. Phenoxybenzamine HCl (POB) and Prazosin were generous gifts from Smith, Kline and French and from Pfizer respectively. [4-(tert-butyramino-2-hydropropoxy)benzimidazole] was from Amersham. Disuccinimidyl suberate (DSS) was from Pierce Chemical Co.

2.1.2 RADIOLIGANDS. α-D-[^{14}C(U)]-glucose-1-phosphate (322 mCi/mmol), [Furoyl—5-^{3}H]Prazosin (10-30 Ci/mmol), [7-methoxy-^{3}H]Prazosin (70-87 Ci/mmol), [^{3}H]-Phenoxybenzamine (^{3}H-POB, lot 2189-176, 41.57 Ci/-mmol) and 2-[4-(4-azido-3-[^{125}I]iodo-benzyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline ([^{125}I]Arylazidoprazosin, lots N403255, N403070 and N403024, 2200 Ci/mmol) were from NEN. [^{3}H]CGP-12177 (30-50 Ci/mmol) was from Amersham.

2.1.3 OTHER MATERIALS AND EQUIPMENT.

Isolation of hepatocytes and measurement of phosphorylase *a* activity: $NaHCO_3$ and glucose were from Fisher, gelatin was from Difco. Glycogen type III, α-D-glucose-1-phosphate and collagenase (EC 3.4.24.3) were from Sigma. Perfusions were done using a peristaltic pump (mod. 1203, Harvard Apparatus), incubations in a Thermomix 1440 incubator (B. Brann Melsungen AG) and Shaker Water Bath (mod. 406015, American Optical).

SDS-PAGE: SDS, acrylamide, BIS, Coomassie Brilliant Blue R-250, Ammonium persulfate, TEMED and glycine were electrophoresis grade from Bio-Rad. Trisma Base (Tris) was from Sigma. Dithiothreitol (DTT) was from Calbiochem-Behring Co. Molecular weight markers were from Bio-Rad and

Sigma. Electrophoresis was made with Protean dual vertical slab gel electrophoresis cell (Bio-Rad), with Heathkit regulated H.V. power supply (mod. IPW-17, Heath Co).

Transblotting: Genescreen hybridization transfer membrane was from NEN, Nonidet P40 - from Shell, and 4-chloro-1-naphthol - from Aldrich. Peroxydase conjugated-goat anti-rabbit IgG (whole molecule) and bovine serum albumin were from Sigma. Protein transfer was made with Trans-Blot Cell with Model 250/2.5 power supply (Bio-Rad).

Plasma membrane solubilization: Triton X-100 and glycerol were from J.T. Baker Chemical Co. Digitonin (lot B4010) was from Galard Schlesinger. CHAPS, EGTA, 1,10-phenanthroline, bacitracin, trypsin inhibitor type I-S (STI), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Homogenization was made with a Polytron homogenizer (type PT 10/35, Kinematica GmbH). Samples were centrifuged using a L3-40 Beckman ultracentrifuge and a T-40 rotor.

Gel filtration: Sephadex G-200 sf (lot F116421), Sephacryl S-400 sf (lot GB19006), Sepharose CL-4B (lot II32318) and gel filtration calibration kit (lot 1F047A) were from Pharmacia. Columns were from Pharmacia and Bio-Rad. Peristaltic pump (Micropex 2132) and fraction collector (Multirac 2111) were from LKB.

Wheat germ agglutinin (WGA)-affinity chromatography: *A) Analytical chromatography* - WGA-agarose (lot 423608) was from P-L Biochemicals, Inc.; *B) Preparative chromatography* - WGA-agarose (lot 021610 and 030602) was from E-Y Labs, Inc. β-N-acetyl-D-glucosamine (NAGA, lot 64F-0697) was from Sigma.

Plasma membrane isolation: *A) Method of Neville:* Sucrose (Ultra Pure, lot 06879) was from Schwarz/Mann Biotech. Centrifugation was done on L3-40 Beckman centrifuge with rotor SW 28 at 24,000 rpm and on a Sorvall RC-5 centrifuge with rotor SS-34 at 17,000 rpm. *B) Method of Prpic et al.:* Percoll (lot MF 02356) was from Pharmacia.

Concentration: Dialysis tubing (cat. No 8-667A) was from Fisher, Aquacide II-A – from Calbiochem-Behring Corp., YM 10 ultrafiltration membrane and Centriflo membrane cones (type CF25) – from Amicon. Concentration was made using Ultrafiltration Cell (mod. 8200, Amicon), and lyophilization – using Freeze Dryer 8 (Labconco).

Immunization of rabbits: Freund's complete adjuvant was from Gibco Laboratories).

Protein digestion: Trypsin (EC 3.4.21.4), type XIII, TPCK-treated (lot 55F-8065) was from Sigma.

Thin layer chromatography (TLC): Silica Gel G and C18 TLC plates, 250 μ thick, were from Analtech, Inc. Solvents, analytical grade, were from Fisher. POB was visualized by exposure to iodine vapors, and ^{3}H-POB was quantitated by scanning with TLC plate scanner.

Reverse phase (RP) and gel permeation (GP) HPLC: *A) Analytical RP HPLC* was made with a μBondapack C18 column, (0.5 x 30 cm, Waters); *B) Preparative RP HPLC* – with a C18 column (type 201HS10n, 1 x 25 cm, Vydac); *C) GP HPLC* – with column I-60 (No T20961K03, Waters Ass.). Solvents were HPLC grade from Fisher. For HPLC we used a model 6000A Solvent Delivery System, a model M45 pump, model U6K injector and a model 660 solvent mixer, all from Waters. UV absorption was measured with Lambda-Max Model 481 LC spectrophotometer (Waters). ^{3}H was measured on line with

a Ramona flow scintillation detector (Raytest), using a 0.6 ml yttrium silicate cell (Raytest), at 5% efficiency. Data were collected and processed with PC (Raytest, Inc) using Triple Trace standard program (ver. 5.4e, Nuclear Interface GmbH). Fractions were collected with a Helirac 1212 fraction collector (LKB).

Equilibrium receptor labeling: Polyethylenimine (PEI) was from Sigma, Carbowax PEG 8000 (PEG) - from Fisher, Γ-Globulins (Bovine Cohn Fraction II) - from Sigma, and GF/B or GF/C glass fiber filters - from Whatman. Incubations were terminated by vacuum filtration using 1225 Sampling Manifold Filter Holder (Millipore).

Photoaffinity receptor labeling: The UV lamps used were UVS-11 (Mineralight) and 450 W mercury vapor lamp (mod. H400-E1T, General Electric). Photolysis was done in 2-ml quartz cuvettes with 1 cm light path (Pyrocell Mfg. Co.).

Radioactivity counting: A) ^{3}H was measured with RackBeta 1211 Liquid Scintillation Counter (LKB-Wallac) at 40-53% efficiency. The sample was solubilized in Formula 963 or in 4% Protosol in Econofluor (NEN). B) ^{14}C was measured using Formula 965 (NEN) with RackBeta 1211 LSC at 94% efficiency. C) ^{125}I was measured with CG 4000 Gamma Counter (IN Intertechnique), at 70% efficiency.

2.1.4 ANIMALS.

Production of antisera: White New Zealand rabbits, male, 3 - 3.5 kg (Fermes des Chènes Bleus, Québec).

Isolation of hepatocytes: Sprague-Dawley rats, male, 300-350 g (Charles River, Québec).

Isolation of rat liver plasma membranes: Sprague-Dawley rats, male, retired breeders, >400 g (Charles River, Québec).

2.2 METHODS

2.2.1 PREPARATION OF LIVER CELLS

Hepatocytes were isolated from livers of male rats by the method of Berry & Friend (1969) with some modifications (Preiksaitis & Kunos, 1979; Preiksaitis *et al.*, 1982). Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and heparinized (500 I.U./kg i.v.). The abdominal cavity was opened and the liver was perfused through the portal vein with calcium-free Krebs-Henseleit bicarbonate buffer (115 mM NaCl, 3.7 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 20 mM glucose; Krebs & Henseleit, 1932), for 10 min at a flow rate of 30-35 ml/min. During this period the liver was transferred to a perfusion apparatus. After this preperfusion the medium was changed to 100 ml Krebs-Henseleit buffer with Ca^{2+} (2.5 mM) and crude collagenase (0.05%), and the perfusion was continued, with recycling, for another 25-30 min. The pH of the medium was monitored and maintained between 7.0 and 7.4 by the addition of NaOH. Throughout the perfusion period, the medium was bubbled with 5% CO_2 in O_2 and maintained at 37°C. At the end of the collagenase perfusion, the liver was disrupted by gentle combing and the suspension of hepatocytes incubated in a shaking incubator for 10 min, under continuous oxygenation. At the end of the incubation, the cell suspension was filtered through 4 layers of gauze and a nylon mesh and washed from the collagenase by centrifugation (2 min at 50 x g) and resuspension in collagenase-free buffer. The final cell pallet was resuspended in Ca^{2+}-containing Krebs-Henseleit buffer, supplemented with 1.5% gelatin. The cells obtained by this method routinely displayed >95% viability (by the Trypan

Blue exclusion method). Cell density was measured by determinating the wet weight of the cell pellet obtained by centrifugation of a 1 ml aliquot of cell suspension for 2 min in an Eppendorf centrifuge. Cells were preincubated for 30 min before use. *Drug treatment of isolated hepatocytes:* Isolated hepatocytes were exposed to receptor agonists for a standard period of 2 min. Preincubation with other drugs were for times indicated in the feet. *Treatment of isolated hepatocytes with disuccinimidyl suberate (DSS):* Isolated hepatocytes were treated with DSS as described by Pilch & Czech (1979). Hepatocytes, suspended at a concentration of 20 mg/ml, were treated with 1 mM DSS (dissolved in DMSO, delivered as 1% of the total volume of the cell suspension) for 5 min. The reaction was quenched by the addition of 10 mM of ammonium acetate, followed by two washes by centrifugation and resuspension of the hepatocytes in fresh gelatin buffer. Control cells were incubated with 1% DMSO.

2.2.2 GLYCOGEN PHOSPHORYLASE ACTIVITY

Glycogen phosphorylase activity was measured by the method of Stalmans & Hers (1975) and Thomas *et al.* (1968). Ten ml of the hepatocyte suspension (2 mg/ml) were used for determination of the activity of glycogen phosphorylase. The cell suspension was incubated in 25-ml Erlenmeyer flasks, at 37°C and under 5% CO_2/95% O_2 atmosphere. The shaking incubator was set at 90-100 cycles/min. Drugs were added in 10 µl volumes. After 3 min the cell suspension was transferred into a 15-ml test tube and centrifuged for 30 sec in IEC (model CL) table-top centrifuge. The cell pellet was immediately homogenized in 1 ml ice-cold glycogen phosphorylase assay buffer (50 mM morfolinosulfonic acid, 0.5 mM caffeine,

150 mM NaF, 2,5 mM EDTA, 2.0 mM DTT, pH 6.5). The homogenization was done with Polytron (setting 6) for 10 sec. The homogenate was centrifuged for 10 min at 3000 x g and duplicate aliquots of the supernatant were used for measurement of the activity of the enzyme. 50 µl of the supernatant and 50 µl of assay buffer (glycogen phosphorylase assay buffer, containing 1 g% glycogen, 15 mM [^{14}C]-glucose-1-phosphate) were incubated for 30 min at 30°C. The reaction was terminated by spotting 50 µl of the assay mixture on Whatman 41 filter paper discs which were washed 2 x with ice-cold 66% ethanol and once with acetone. ^{14}C was measured by liquid scintillation counting. The activity of glycogen phosphorylase was calculated as pmoles of [^{14}C]glucose incorporated into glycogen per min per mg protein in the supernatant.

2.2.3 LIVER PLASMA MEMBRANE PREPARATION

Method of Neville (1968): Plasma membranes were prepared by the method of Neville (1968), as modified by Wolfe *et al.* (1976). All operations were performed at 4°C. The liver was minced with scissors in 20 volumes of 1 mM $NaHCO_3$ and homogenized. The homogenate was filtered through 4 layers of gauze and centrifuged for 10 min at 4,000 g. The pellet was resuspended in 1 mM $NaHCO_3$ and mixed with 2 vol 69% (w/w) sucrose. After 10 min stirring, the homogenate was transferred to ultracentrifuge tubes and overlaid with 42.3% (w/w) sucrose. The preparation was centrifuged for 2 hr. at 100,000 g. Plasma membranes were collected from the top of the upper layer of sucrose, washed 2 times with 50 mM Tris, 1 mM $MgCl_2$ and stored at -70°C until used. Method of Prpic et al. (1984): Starting material were isolated hepatocytes, washed with

gelatin-free Krebs-Henseleit buffer. The hepatocytes were pelleted by centrifugation and homogenized by Polytron (setting 5, 2 x 5 sec) to give 6% (w/v) homogenate, in ice-cold buffer (250 mM sucrose, 5 mM HEPES-KOH, 1 mM EGTA, pH 7.4). The homogenate was centrifuged at 1,464 g for 10 min and the pellet was resuspended in the same buffer to give 6% suspension. 10.4 ml of the suspension were mixed with 1.4 ml of Percoll (11.9% final concentration) and centrifuged at 34,540 g for 30 min. The plasma membranes were collected from the second layer from the top, washed 2 x with 50 mM Tris, 1 mM $MgCl_2$ and stored at -70°C in the same buffer.

Treatment of rat liver plasma membranes with disuccinimidyl suberate (DSS): Rat liver plasma membranes were diluted to 0.2 mg/ml in 50 mM potassium phosphate buffer, 1 mM $MgCl_2$, pH 7.4 buffer, at 37°C and exposed to 1 mM DSS (delivered in DMSO in 1% of the volume of the sample) for 5 min. The reaction was quenched by 10 mM ammonium acetate, followed by 2 washes by centrifugation and resuspension in fresh buffer.

2.2.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

One-dimensional SDS-PAGE was done using a modified discontinuous buffer system (Laemmli, 1970; Laemmli & Favre, 1973). Samples for electrophoresis were solubilized for 20 min at 80°C, cooled to room t° and supplemented with 0.1 M dithiothreitol. Particulate matter was removed by centrifugation for 3 min in an Eppendorf centrifuge. Up to 40 µg of protein, in a maximal volume of 100 µl, were applied to the well. Molecular weight standards and samples were applied always in equal volume. Separation was made using 3 mm-thick running gels, containing 5, 7.5 or 10% acrylamide and a 4% stacking gel. Electrophoresis was carried out at

constant voltage, with initial currents of 5 mA/well (1 h) for stacking, and 10 mA/well (3.5–4 hr.) for separation. For measurement of radioactivity, the gels were sliced into 3 mm-wide slices. ^{3}H was measured following digestion of the gel slices with Protosol (2 hr., 60°C). The lanes with the molecular weight standards were stained with Coomassie Brilliant Blue R-250.

2.2.5 TRANSBLOTTING

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose paper was made according to Towbin *et al.* (1979). After the SDS-PAGE was completed, the gel was placed in a sandwich of filter paper, with the nitrocellulose sheet layered over one of its sides. The sandwich was placed, with the nitrocellulose sheet facing the anode, in the transblotting apparatus and maintained 0.2 A for 4 hr., with water cooling. At the end of the transfer, the nitrocellulose paper was cut into strips corresponding to the different wells of the SDS-PAGE gel and incubated separately. First, the free binding sites of the nitrocellulose paper were blocked with 5% bovine serum albumin in 10 mM Tris 7.4-saline and incubated with 1:10 dilution of antiserum for 90 min at room temperature. The antiserum was removed by washing with the same buffer with 0.05% Nonidet P-40, followed by a second exposure to 5% BSA, and a 10^{-4} dilution of horseradish peroxydase-linked anti-rabbit IgG. The latter were visualized by treatment with 50 mM Tris pH 7.4, 30% H_2O_2, 0.4 mg/ml 4-chloro-1-naphthol.

2.2.6 GEL FILTRATION

Separation of rat liver plasma membrane components by gel filtration was done as described first by Porath & Flodin (1959). *A) Analytical gel filtration experiments* were performed using Sephadex G-200 sf and Sephacryl S-400 sf. The volume of the sample was kept at 1-2% of the total volume of the separating gel. The flow rate was kept at 2 $ml.cm^{-2}.h^{-1}$. *B) Preparative gel filtration* was made on Sepharose CL-4B, using a flow rate 4 $ml.cm^{-2}.h^{-1}$. All experiments were performed at 4°C with previously degassed buffers. Columns were calibrated in separate runs using appropriate molecular weight standards.

2.2.7 RECEPTOR SOLUBILIZATION

All operations were carried out at 4°C. The plasma membranes were pelleted by centrifugation for 10 min at 35,000 g and homogenized in solubilization buffer containing 50 mM Tris, pH 7.4, with 10 mM $MgCl_2$, 0.1 mM 1,10-phenanthroline, 10 µM PMSF, 10 µg/ml bacitracin, 1 µg/ml STI and 5 mM EDTA. Detergent to protein ratio was 2:1 to 5:1. Triton X-100 was used at a final concentration of 1% (v/v) (Kunos *et al.*, 1983), CHAPS - 10 mM (Hjelmeland, 1980) and digitonin - 1.36% (Graham *et al.*, 1982). The preparation was shaken for 30 min and centrifuged 1 h at 100,000 g. The pellet was extracted with 1/2 volume of the solubilization buffer and the two supernatants combined, concentrated 10-fold and stored at -70°C until used.

2.2.8 BINDING ASSAYS

2.2.8.1 [^{3}H]Phenoxybenzamine binding assay: Labeling of rat liver α_1-adrenergic receptors with ^{3}H-POB was as described by Kunos *et al.* (1983). Plasma membranes were diluted in 50 mM Tris pH 7.4, 10 mM $MgCl_2$ to 0.1 mg/ml. ^{3}H-POB was precyclized for 1 min at 37°C in the same buffer and added to the plasma membrane suspension (precooled to 10°C), to a final concentration of 1 nM. After a 4 min incubation, unbound radioligand was removed 2 cycles of centrifugation (20,000 x g, 5 min) and resuspension in fresh, ice-cold buffer. Specific binding was defined as the difference between the ^{3}H-POB bound in the absence of competitor (total binding) and in the presence of 0.1 µM Prazosin (non-specific binding).

2.2.8.2 [^{3}H]prazosin binding assay: Binding of ^{3}H-prazosin to rat liver α_1-adrenergic receptors was assayed at 31°C, using 50-100 µg of protein/assay, in a buffer of 50 mM Tris 7.4, 10 mM $MgCl_2$. The final concentration of [Furoyl-5-^{3}H]prazosin was 1 nM, and of [7-methoxy-^{3}H]prazosin - 0.4 nM. Incubation was carried out for 30 min, and was terminated by vacuum filtration onto GF/C filters, washed rapidly with three 4-ml aliquots of the same buffer at room t°. Specific binding was calculated from the difference between the binding in the absence and presence of 2 µM phentolamine. Radioactivity was measured by LSC.

2.2.8.3 [^{3}H]CGP-12177 binding assay: Binding of ^{3}H-CGP-12177 to rat liver β-adrenergic receptors was assayed at 31°C, using 1 mg of protein/assay, in 50 mM Tris pH 8.0, 4 mM $MgCl_2$ buffer. Incubation was carried out for 30 min and was terminated by vacuum filtration onto GF/C

filters. Filters were washed rapidly with three 4-ml aliquots of the same buffer. Specific binding was defined as the difference between binding of the ligand in the absence and presence of 300 μM *l*-isoproterenol.

2.2.8.4 ^{125}I-Arylazidoprazosin binding assay: Photoaffinity labeling of rat liver α_1-adrenergic receptors was done according to Graham *et al.* (1984). The composition of the binding buffer was 100 mM Tris pH 7.4, 1 mM $MgCl_2$, 5 mM EDTA, 0.02% NaN_3, 0.1 mM PMSF, 10 μg/ml bacitracin, 1 μg/ml STI and 1 μg/ml leupeptin. Plasma membranes, at a protein concentration of 0.5 mg/ml, were incubated with 0.3 nM of the ligand for 45 min, at 25°C in the dark. Photolysis was done in quartz cuvettes, for 20 s, 10 cm away from a 450 W mercury vapor UV lamp. The unbound ligand was removed by 2 steps of centrifugation and wash.

2.2.9 PREPARATION OF LIPOMODULIN AND MONOCLONAL ANTIBODY TO LIPOMODULIN

Lipomodulin and monoclonal antibody to lipomodulin were prepared in the laboratory of Dr. F. Hirata (Laboratory of Cell Biology, NIMH, Bethesda, MD). Lipomodulin was purified from culture medium of rabbit peritoneal neutrophils (Hirata, 1981). Specific antilipomodulin monoclonal antibody was isolated by screening monoclonal antibodies raised against rat cerebral synaptosomes.

2.2.10 PREPARATION OF ANTISERUM AGAINST RAT LIVER α_1-RECEPTOR

Antisera were raised in rabbits. The antigen was prepared by solubilization of rat liver plasma membranes with 1% Triton X-100 and gel filtration on Sephadex G-200 sf as described in fig.8 and by Kunos *et al.* (1983). The fractions corresponding to the elution point of the ^{3}H-POB-labeled α_1-adrenergic receptor were pooled and concentrated 10-fold. The rabbits were inoculated i.m. with 0.5 mg protein/injection, diluted with an equal volume of complete Freund's adjuvant. Animals were boosted at 2 week-intervals with the same amount of antigen s.c. Blood was collected from an ear vein starting one month after the first inoculation, in 10-ml portions at 2 week intervals. After clotting at room t°, the serum was separated by centrifugation at 3,000 g and was stored at -70°C until use.

2.2.11 WHEAT GERM AGGLUTININ CHROMATOGRAPHY

Analytical WGA-affinity chromatography was done as described by Kunicki *et al.* (1981), using 2 columns of 10 ml of WGA-agarose. The experiment was performed at 4°C. The starting material was rat liver plasma membranes, labeled with ^{3}H-POB and partially purified by gel filtration on Sephadex G-200 sf. Samples labeled in absence ("total") and in presence of competitor ("non-specific") were processed in parallel. The sample (1-2 ml, 20-30 µg protein, in 50 mM Tris/1 mM $MgCl_2$ buffer containing 0.1% Triton X-100, 0.2 M NaCl and protease inhibitors as indicated above) was applied to the column and recycled for 90 min, at a flow rate 5 ml/h. The column was washed with 8 volumes of sample buffer and the glycoproteins were eluted with 0.2 M β-N-acetylglucosamine in sample buffer (80 ml, 5 ml/h). The column was regenerated by washing with 0.5 M

NaCl and 10 volumes of sample buffer. *Preparative batch WGA-affinity chromatography* was made with 2 x 20 ml of WGA-agarose, using as starting material ^{3}H-POB labeled rat liver plasma membranes, partially purified by gel filtration on Sepharose CL-4B. The sample (2-3 ml, 5-10 mg protein) was incubated with the WGA-agarose for 30 min at 37°C in a shaking incubator. The gel was transferred onto a paper filter inside a funnel, washed with 5 vol. sample buffer and incubated with 0.2 M NAGA (30 min, room t°). The gel was regenerated by washing with 0.5 M NaCl and sample buffer. At the end of this step, the samples were concentrated 10 times and stored at -70°C until use.

2.2.12 REVERSE PHASE HPLC

Separation of proteolytic fragments of partially purified α_1-adrenergic receptor, labeled with ^{3}H-POB, was made by reverse phase HPLC as described by Mayes (1984). Starting material was the product of the trypsin digestion, suspended in 0.1% trifluoroacetic acid (TFA) in H_2O. The separation was made by a linear gradient of 0-100% acetonitrile (ACN) in 0.1%TFA/H_2O, for 56 min at a flow rate 1 ml/min. Pilot experiments were done on *analytical* µBondapack C18 column, using 10-20 µg of protein digest per injection. *Preparative RP HPLC* was done on a Vydac C18 column, using up to 300 µg/injection. The fractions containing the radioactive peak were pooled, lyophilized and repurified using the same column and gradient.

2.2.13 GEL PERMEATION HPLC

The ^{3}H-POB-labeled proteolytic fragment of the α_1-adrenergic receptor, purified by RP-HPLC, was further studied by GP-HPLC, using a Waters I-60 column. The mobile phase was 0.1% TFA in acetonitrile, and elution was at a flow rate of 0.5 ml/min. Relevant fractions were collected and concentrated by lyophilization.

2.2.14 α_1-ADRENERGIC RECEPTOR TRYPSINIZATION

The partially purified ^{3}H-POB-labeled α_1-adrenergic receptor was incubated with 2 x 5% trypsin (TPCK-treated) for 24 h, at 37°C and pH 7.6, as described by Mayes (1984). At the end, the preparations were centrifuged at 100,000 x g and the supernatants were frozen at -70°C and lyophilized.

2.2.15 RADIOCHEMICAL PURITY CHECK OF [^{3}H]PHENOXYBENZAMINE

^{3}H-POB was stored in ethanol at -20°C. The radiochemical purity of the ligand was checked by thin layer chromatography (TLC) using two systems. The first TLC system was heptane:chlorophorm:methanol (50:35:15) using silica gel G plates as described by Kan *et al.* (1979). The R_f value of POB in this system is 0.71. The second TLC system was methanol:acetonitrile (9:1), using C_{18} reverse phase plates. The R_f value of POB was 0.78. Purity checks were made also by RP HPLC on a μBondapack C18 reverse phase column with the second solvent system.

2.2.16 SYNTHESIS OF I-PHENOXYBENZAMINE (I-POB)

I-POB was synthesized by Dr. T. Mansour of the Department of Chemistry, McGill University, by the following procedure: A mixture of N-phenoxyisopropyl-ethanolamine, triethylamine, and anhydrous sodium iodide, in anhydrous methylene chloride, containing DMF, was stirred under reflux for 18 h. N-(phenoxyisopropyl)-N-(3-iodobenzyl)-ethanolamine was purified from the reaction products by silica gel flash chromatography. On the next step, the residue was dissolved in trichloromethane and dry hydrogen chloride was bubbled into the solution until pH 2 was reached. Thionyl chloride was added, and the mixture was heated for 3 h at 60°C in water bath. After several washes with trichloromethane, the N-(phenoxyisopropyl)-N-(3-iodobenzyl)-beta-chloroethylamine hydrochloride (I-POB) was purified by recrystallization from ethanol. I-POB is a white powder, readily soluble in ethanol (Fig. 1).

2.2.17 CONCENTRATION OF PROTEIN SOLUTIONS

Protein solutions were concentrated by three techniques: *A) Dehydratation:* the sample was transferred in dialysis tubing, which was covered with Aquacide II-A for 4-8 hr, at 4°C; *B) Ultrafiltration:* centrifugation-ultrafiltration in Centriflo membrane cones at 800-900×g (for small volumes) and ultrafiltration under positive pressure (50-60 psi N_2) in Amicon ultrafiltration cell for larger volumes; *C) Lyophilization:* for protein solutions in volatile solvents.

2.2.18 MEASUREMENT OF PROTEIN CONCENTRATION

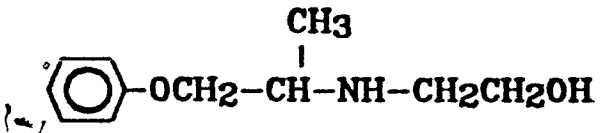
In the absence of interfering substances, protein was measured by the method of Lowry *et al.*, (1951). In the presence of SDS, the modification of Lees & Paxman (1972) was used. In other cases, protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as standard.

2.3. STATISTICAL ANALYSIS

For the assessment of statistical differences between means determined from two independent samples, the Student's t-test for unpaired data was used. Probability (p) of less than 0.05 was considered significant.

Figure 1. Synthesis of I–POB

For further explanations, see section 2.2.16

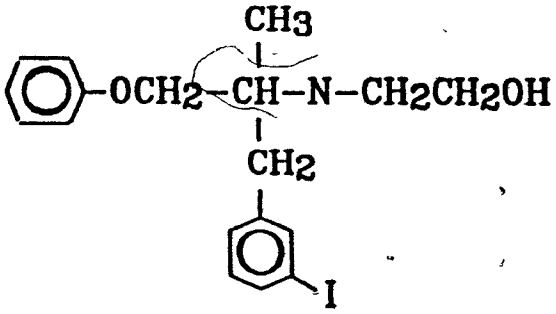


N–(phenoxyisopropyl)–ethanolamine

3–iodo–benzylbromide

$CH_2Cl_2/DMF/\Delta$

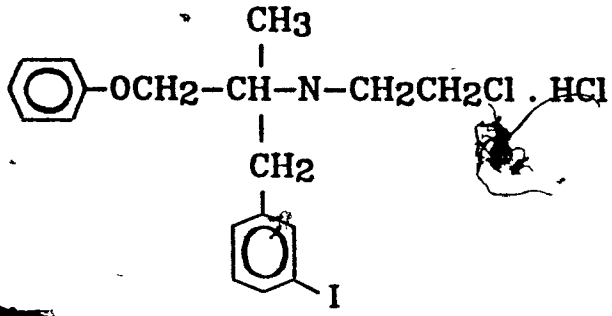
Et_3N, NaI



N–(phenoxyisopropyl)–N–(3–iodobenzyl)–ethanolamine

HCl

$SOCl_2/\Delta$



N–(phenoxyisopropyl)–N–(3–iodobenzyl)–
–β–chloroethylamine hydrochloride (I–POB)

SECTION 3: RESULTS

3.1 TIME-DEPENDENT CONVERSION OF α_1- TO β-ADRENOCEPTOR - MEDIATED GLYCOGENOLYSIS IN ISOLATED RAT LIVER CELLS.

The adrenergic activation of hepatic glycogenolysis in the adult male rat is an α_1-adrenergic receptor-controlled event. β-Adrenergic receptors are also present (approx. 5% of all adrenoreceptors in the hepatocyte; Kunos, 1984), but they don't contribute appreciably to the regulation of glycogenolysis (Hutson *et al.*, 1976). This unique feature makes the liver of the adult male rat a valuable model for the study of the molecular properties and the regulation of the α_1-adrenergic receptor, since in female and young rats, as well as in most other mammalian species, the β-receptor is predominant. It has been observed that the predominance of a given adrenergic receptor subtype over the control of rat liver glycogenolysis may change, within the same animal, after an alteration of the local hepatic condition, such as cholestasis (Schmelck *et al.*, 1979; Aggerbeck *et al.*, 1983; Okajima & Ui, 1984), regeneration following partial hepatectomy (Aggerbeck *et al.*, 1983), and malignant transformation (Boyd & Martin, 1975; Christoffersen & Berg, 1975; Hornbrook, 1979), or after an alteration of the general hormonal homeostasis (Chan *et al.*, 1979; Preiksaitis & Kunos, 1979; Malbon, 1980; Preiksaitis *et al.*, 1982). As first reported by Okajima & Ui (1982) primary culturing of isolated rat hepatocytes may also induce a conversion of the adrenergic control of glycogenolysis. A common denominator in all these processes is the induction of a lower level of differentiation or "fetalization", as suggested by Kunos (1980) and Okajima & Ui (1982). Thus, it appears that the receptor control of liver glycogen metabolism is not fixed, but sub-

ject to adaptation to the level of cell differentiation and to the pathophysiological state of the organism. This would also imply that the conversion of the adrenergic receptor response under the various conditions mentioned above may have a common underlying mechanism.

To study this mechanism, we used isolated rat hepatocytes, maintained in suspension (in serum-free medium), because in this *in vitro* system the change in receptor response develops within a few hours.

In agreement with earlier observations from our laboratory (Preiksaitis *et al.*, 1982), and from others (Hutson *et al.*, 1976), in freshly isolated hepatocytes glycogen phosphorylase is activated predominantly through the α_1-adrenergic receptor: phenylephrine has a pD_2 value ($pD_2 = -\log EC_{50}$) of 6.58 ± 0.21, whereas isoproterenol is ineffective (Fig 2, *Upper*). After incubation of the cells for 4 hours, phenylephrine loses both potency and efficacy ($pD_2 = 5.64 \pm 0.30$, $P < 0.005$), and isoproterenol becomes a potent activator ($pD_2 = 8.10 \pm 0.22$). For comparison, fig. 2 *(Lower)* shows the changes for two other hormones: vasopressin and glucagon. 4-Hour incubation produces no change in the response to vasopressin, but the potency of glucagon is markedly reduced: in freshly isolated hepatocytes $pD_2 = 10.82 \pm 0.15$, in 4-hour cells $pD_2 = 9.57 \pm 0.25$, $P < 0.001$. Since glucagon activates glycogen phosphorylase through the same cAMP cascade mechanism as isoproterenol, and vasopressin uses the same cAMP-independent second messenger system as phenylephrine (Chen *et al.*, 1978) these results suggest that the reciprocal changes in the adrenergic response of hepatocytes after 4 hours of incubation probably do not result from parallel changes in the function of the second messenger systems. This is also indicated by the fact that

the Ca^{2+}-dependent vasopressin retains its potency (in contrast to phenylephrine), whereas the cAMP-dependent glucagon becomes much less potent (the opposite to isoproterenol).

In order to find out if the shift in the adrenergic activation of glycogen phosphorylase is correlated with changes in the number and/or affinity of the corresponding receptors, we studied their density and affinity. These data are summarized in Table 1. α_1-Adrenergic receptors were identified by ^{3}H-prazosin binding, and β-adrenergic receptors - by ^{3}H-CGP-12177 binding. Assays were done using both crude homogenate and purified plasma membranes. For the α_1-adrenergic receptors, despite the significant loss of receptor function, we found no change in the density of receptors or their affinity for ^{3}H-prazosin. Similarly, the emergence of a potent β-adrenergic response in hepatocytes after 4 hours of incubation was not associated with a change in the density of receptors or their affinity for the radioligand. These results do not exclude other possible modifications of receptors, or alteration of their interaction with the transducer mechanism. Recent data from our laboratory indicate that 4 hour incubation of isolated hepatocytes decreases the number of high agonist affinity form of α_1-adrenergic receptors and increases the high agonist affinity form of β-adrenergic receptors (Kunos & Ishac, 1987). Taken together, these data indicate that prolonged incubation of isolated hepatocytes decreases the coupling of α- and increases the coupling of β-adrenergic receptors.

Adrenalectomy can trigger an interconversion of the adrenergic control of glycogen phosphorylase from α- to β-type, and this change is reversible by *in vivo* glucocorticoid treatment (Chan *et al.*, 1979).

Recently, it has become evident that most of the biological effects of glucocorticoids, including their effects on cell differentiation, are mediated indirectly by the induction of the synthesis of an endogenous protein inhibitor of membrane phospholipase A_2 (Hirata *et al.*, 1980). The suppression of phospholipase A_2 activity in the plasma membrane leads to a reduction in the formation of arachidonate metabolites (Flower & Blackwell, 1979). Two endogenous inhibitors of phospholipase A_2 have been identified: lipomodulin, (m.w. 40,000 daltons), and macrocortin (Hirata, 1981; Blackwell *et al.*, 1980). It is currently believed that macrocortin is an active fragment of lipomodulin (Hirata *et al.*, 1982). Using purified lipomodulin and a monoclonal antibody against lipomodulin (prepared in the laboratory of Dr. F. Hirata, NIMH), we tried to find out if changes in the activity of membrane phospholipase A_2 are implicated in the interconversion of the adrenoceptor control of glycogenolysis. As illustrated in fig. 3, a 30 min incubation of 4-hour cells with 50 nM lipomodulin reverses the effect of the 4-hour incubation: the potency of phenylephrine is increased to the level seen in 0-hour cells, and isoproterenol loses its effectiveness. Lipomodulin had no effect on 0-hour cells, and its effect on 4-hour cells was prevented by preincubation of the lipomodulin with an excess of antilipomodulin monoclonal antibody. All this suggests that the changes after 4 hours of incubation are related to an activation of membrane phospholipase A_2, possibly caused by the loss of an endogenous inhibitor, such as lipomodulin.

This possibility is further supported by experiments in which 0-hour cells were incubated for 10 min with a 1:50 dilution of an antilipomodulin monoclonal antibody (Fig. 4). While this treatment caused

only a small reduction in the potency of phenylephrine, and isoproterenol remained ineffective, when the cells were preincubated for 10 min with 10 μM phenylephrine, and then treated with the antibody, the phenylephrine potency was markedly decreased and isoproterenol became a potent agonist (indicating a shift from α- to β-predominance).

Mellitin is a polypeptide activator of phospholipase A_2 (Mufson *et al.*, 1979). Incubation of freshly isolated hepatocytes with 2 μg/ml mellitin for 30 min produces a similar suppression of the α- and emergence of the β-adrenergic response (Fig. 5), further supporting the role of phospholipase A_2 in the conversion response.

3.2 MOLECULAR CHARACTERIZATION OF α_1-ADRENERGIC RECEPTOR FROM RAT LIVER

3.2.1 PARTIAL PURIFICATION OF THE HORMONE-BINDING SUBUNIT OF THE α_1-ADRENERGIC RECEPTOR

In the last 8 years, considerable research effort has been directed towards the elucidation of the molecular structure of hormone receptors. Information on their primary amino acid sequence has helped to shed light on their membrane topography, principles of function and mechanisms of regulation, and the pathogeny of some genetic and autoimmune disorders. The molecular characterization of hormone receptors requires their purification. The purification of adrenergic receptors has advanced mainly through the development of affinity chromatography methods, which

use derivatives of high affinity receptor ligands, immobilized on a chromatographic support. The structure of prazosin (Hess, 1974), a high affinity α_1-adrenergic receptor antagonist, was the source of two such derivatives: CP 57,609 (Graham *et al.*, 1982), and A55453 (Leeb-Lundberg *et al.*, 1984). These studies have also provided methods of solubilization of the receptor in an active state. The first study found that the hormone-binding subunit of the receptor is a single peptide with a m.w. of 59,000 daltons. The second study identified a series of specifically labeled peptides with m.w ranging from 80,000 to 16,000 daltons, with the predominance of a 52,000 daltons peptide. A similar controversy characterized earlier studies, using [^{3}H]POB for the specific labeling of the receptor (Guellaen *et al.*, 1979; Kunos *et al.*, 1983). Leeb-Lundberg *et al.* (1984) demonstrated that the variability in the results was probably due to partial proteolytic degradation of the receptor, caused by the action of an unidentified metalloprotease. Despite the recent progress in the purification and the biophysical characterization of α_1-adrenergic receptors from different sources, the receptor gene has not yet been cloned, and the amino acid sequence and membrane topography of the receptor are unknown. Earlier results from our laboratory have shown that [^{3}H]POB could be used as an irreversible label for the molecular characterization of the α_1-adrenergic receptor from rat liver (Kunos *et al.*, 1983). Using this method, we partially purified the receptor protein and tried, after proteolytic digestion with trypsin, to purify the [^{3}H]POB-labeled fragment of the receptor. The following section describes the results from this study.

In a pilot study, we compared the potency of different detergents to solubilize [^{3}H]POB-labeled α_1-adrenergic receptors from rat liver plasma membrane, and characterized the ligand binding capacity of the receptor after solubilization. Four times 10 mg of rat liver plasma membranes (α_1-adrenergic receptor density 0.6 pmols/mg protein) were solubilized with Triton X-100 (Kunos *et al.*, 1983), digitonin (Graham *et al.*, 1982), CHAPS and CHAPS/NaCl (Bruns *et al.*, 1983) accordingly to published protocols. "Active" receptors were defined as soluble α_1-receptors specifically labeled by a saturating concentration of ^{3}H-prazosin (1 nM). Specific binding was the difference between the binding in the absence and the presence of 10 µM phentolamine, assayed after 60 min incubation at 37°C, in triplicate aliquots from the 100,000 x g supernatant of the detergent extract. The assay of soluble receptors was done as described by Graham *et al.* (1982). The data obtained were compared with the amount of receptors recovered after solubilization of equal amounts of ^{3}H-POB-labeled rat liver plasma membranes with the same detergents. (Fig. 6). We found that only 10 mM CHAPS was able to solubilize a significant amount (40%) of the α_1-adrenergic receptors in an "active" state. The discrepancy between our results and those of Graham et al. (1982), which used digitonin-solubilized preparations is probably due to the variability of the properties of digitonin from different sources (Caron, 1986). Based on these findings, we used 10 mM CHAPS for the solubilization of rat liver plasma membrane α_1-adrenergic receptors and resolved the detergent extract by gel filtration. Ten mg plasma membranes were extracted by CHAPS as described by Bruns *et al.* (1983) and the preparation was centrifuged at 100,000 x g for 1 h. The supernatant was loaded on top

of a Sephacryl S-400 column (2.5 x 47 cm) and eluted at a flow rate of 25 ml/h with a buffer containing 0.1% CHAPS. The eluate was assayed for presence of active α_1-adrenergic receptors by two methods: PEG precipitation (Graham *et al.*, 1982) and binding to polyethylenimine-treated glass-fiber filters (Bruns *et al.*, 1983). As illustrated at fig. 7, we found no peak containing specific ^{3}H-Prazosin-binding activity by either method. The most likely explanation of this negative finding is that gel filtration delipidates the receptor, leading to its inactivation. This made us to abandon the idea of detecting the receptor along the purification procedure by ligand binding.

An alternative approach consists in labeling the α_1-adrenergic receptor in rat liver plasma membranes with [^{3}H]POB, followed by solubilization. After a partial purification, the preparation is to be digested with trypsin and the [^{3}H]POB-labeled fragment of the receptor is to be purified and characterized. For solubilization of the labeled receptor, Triton X-100 was chosen as the most potent detergent (Fig. 6). Fig. 8 describes the results of the gel filtration of such a preparation. Rat liver plasma membranes were prepared using the method of Neville (1968) and labeled with 1 nM ^{3}H-POB at 10°C, in the absence ("total binding") and in the presence of 0.1 µM Prazosin ("non-specific binding"). These two preparations were then solubilized with 1% Triton X-100 (30 min at 4°C), and the 100,000 g supernatant subjected to analytical gel filtration. 0.5 ml of the Triton extract, containing 2 mg protein, was loaded on a Sephadex G-200 sf column (1.6 x 32 cm) and eluted with at a flow rate of 5 ml/h. The solubilization and the elution buffers contained protease inhibitors (see Section 2.2.7), in order to

minimize the proteolytic degradation of the α_1-adrenergic receptor. In these conditions, the Stokes radius of the ^{3}H-POB-α_1-adrenergic receptor complex was found to be 63 Å, close to the value found by Kunos *et al.* (1983) (60 Å), and by Meier *et al.* (1984) (68 Å).

Gel filtration provides little purification of the α_1-adrenergic receptor, and for further purification we used lectin affinity chromatography (see Section 2.2.11). The starting material was the product of the Sephadex G-200 sf gel filtration of ^{3}H-POB-labeled α_1-adrenergic receptors from rat liver plasma membranes. The fractions containing the peak of the "specific" binding (suppressed by 10 µM prazosin) were pooled together and concentrated by ultrafiltration over a filter with a cut-off limit of 10,000 daltons. In the same operation, the detergent concentration was decreased to 0.1%. One ml fractions of the preparation were applied to a 10-ml column of agarose, coupled to wheat germ agglutinin (WGA) (fig 9). We found that 86.2% of the radioactive label in the sample was retained by the WGA-agarose, and 46.5% (53.9% of the retained) was eluted biospecifically with β-N-acetyl-D-glucosamine.

In order to isolate larger amounts of the α_1-adrenergic receptor, we isolated 3 g of rat liver plasma membranes from the livers of 150 animals. Each preparation, (≈200 mg, prepared from a batch of 5-6 livers), was separated in two and labeled with 0.5 nM ^{3}H-POB in the presence and in the absence of 0.1 µM prazosin, as described above. Preparations having less than 200 fmols/mg protein specific binding were rejected. The ^{3}H-POB-labeled plasma membranes (~1 g) were solubilized in 1% Triton X-100 (detergent to protein ratio 5:1), and centrifuged at 100,000 x g for 1 h. The 100,000 x g supernatant was concentrated 10-fold

by ultrafiltration and stored at -70°C. For preparative gel filtration, we used Sepharose CL-4B, packed in two columns (3 x 50 cm). This matrix was preferred over Sephadex G-200 for preparative purposes, because its separation range (10^4 to 10^7 daltons) is larger than that of Sephadex G-200 sf (10^4 to 2 x 10^5 daltons), and the elution point of the ^{3}H-POB-receptor complex is closer to its midpoint. Also, the higher rigidity of Sepharose CL-4B allows for much higher flow rates. The Triton extract was applied in 3 ml aliquots on the columns and eluted at a flow rate of 30 ml/h (fig.3). A total of 20 runs were made, and the fractions containing the peak of specific binding were concentrated by ultrafiltration and stored at -70°C. For WGA-affinity chromatography, we used two batches of 20 ml WGA-agarose (see Section 2.2.11). The biospecific elution was made by 0.2M β-N-acetyl-D-glucosamine in sample buffer. The results of this work are summarized in Table 2. The WGA eluate contained 2.7 mg of protein, containing 17.1 pmols/mg of receptor-specific label. Thus, the overall yield was 0.3% and the purification was 81.4-fold.

Next the partially purified, ^{3}H-POB-labeled α_1-adrenergic receptor was digested with TPCK-treated trypsin. The protein was incubated for 24 h with 5% trypsin (added twice at 12 h intervals), at 37°C. The reaction was stopped by cooling to 4°C, and addition of 0.1% trifluoroacetic acid. The treatment with trypsin was able to solubilize only 30% of the radiolabel. The ^{3}H-POB-labeled peptide(s), released by the trypsin treatment, were separated from the undigested protein by 100,000 x g centrifugation.

The 100,000 x g supernatant was concentrated by lyophilization, solubilized in 1 ml 0.1% trifluoroacetic acid in H_2O, and resolved by reverse phase HPLC on a C_{18} matrix, by application of a 0-100% acetonitrile/0.1% TFA in H_2O gradient for 56 min. The fractions containing radioactive peaks were collected, concentrated by lyophilization and repurified under the same conditions.

RP HPLC of the tryptic digest of the ^{3}H-POB-labeled α_1-adrenergic receptor revealed one main radioactive peak, eluted at 93% acetonitrile, and containing 43% of the label. Also, several small radioactive peaks were eluted in the region 65-90% acetonitrile, each containing 4-6% of the total radioactivity of the preparation (fig 10).

In a separate experiment, the main radioactive peak from the trypsin digestion was rechromatographed using a 20-100% gradient for 50 min. Again, a single, symmetrical peak was observed.

Since RP HPLC is separating peptides by hydrophobicity and gives no direct indication of their molecular weight, we injected the material, obtained from the main radioactive peak of the tryptic digestion, into a gel permeation HPLC column with a separating range of 1,000 - 20,000 D for globular and 600 - 8,000 D for random coil proteins (Fig. 11). Two peaks of radioactivity were detected: a major peak, with apparent m.w. of ~600 D, which contained 71% of the radioactive label, and a smaller peak, containing 29% of the radioactive label, with an apparent M_r of 2,600 D.

SDS-PAGE of these two peaks yielded an apparent m.w. of less than 1084 D for both peaks (Fig. 12). The molecular weight of ^{3}H-POB itself being 340.0 daltons, this means that the fragment purified by this method may have contained no more than 2-3 amino acids. The attempt to sequence

it, in collaboration with the Hormone Research Laboratory, UCSF, was unsuccessful. Therefore, one might suggest that the digestion of the ^{3}H-POB labeled protein should be done first by a method known to generate larger fragments, and after the purification of such fragment is successfully completed, a second digestion might be attempted, if necessary, using a different protease other than trypsin. However, at this point we cannot discount the alternative possibility that after a successful covalent attachment of [^{3}H]POB to the α_1-receptor, radiochemical decomposition released a part of the molecule carrying the [^{3}H] label, and this fragment may have represented the bulk of the low molecular weight radioactive peak.

3.2.2 CROSS-LINKING OF ACTIVATED ADRENERGIC α_1- AND VASOPRESSIN V_1 RECEPTORS WITH DSS

The problems encountered with the use of [^{3}H]POB and [^{125}I]Azidoprazosin for the covalent labeling of the α_1-adrenergic receptors (see Section 3.2.3.1 and 4.4.2) prompted us to look for an alternative approach to attach a radioactive label to the receptor. Since [^{3}H]Prazosin, a high affinity reversible ligand for the α_1-adrenergic receptor has an amino group, we investigated the possibility to use disuccinimidyl suberate, an irreversible cross-linker of amino groups (DSS), to attach [^{3}H]Prazosin to the active center of the receptor. This agent has been used to attach adjacent protein molecules, or to link large peptide hormones, such as insulin and EGF, to their receptors in the plasma

membrane (Pilch & Czech, 1979). As described in this section, the treatment with DSS was not able to immobilize [^{3}H]Prazosin to the active center of the receptor, due probably to a lack of free amino group within the span of the cross-linker. Our attempt to cross-link arg^{8}vasopressin to the hepatic V_1 receptor was also unsuccessful. However, when agonist plus cross-linker were added to intact cells, glycogen phosphorylase, the target enzyme for both of these receptors became permanently activated. These preliminary observations appear to be important for the understanding of the mechanism by which activation of the Ca^{2+}-linked receptors lead to a final cellular response (see Introduction). There are presently no data about the structure of the activated form of these receptors. It has been demonstrated for several peptide hormone receptors (insulin, EGF, prolactin, TRH, enkephalin, LH), as well for the muscarinic acetylcholine receptor, that agonist binding induces receptor microaggregation (Blum, 1985). In the case of the GnRh receptor for example, it has been shown that agonist-induced receptor doublets are the form of activated receptor and their presence is sufficient for the triggering of the biological response, in the absence of an agonist (Conn et al., 1982). The following section, we describe the effects of disuccinimidyl suberate (DSS, see fig. 13) on ligand binding to and agonist activation of the vasopressin V_1 and the α_1-adrenergic receptors in rat liver cells.

The attempt to cross-link [^{3}H]Prazosin (illustrated at fig. 13) to the active center of the α_1-adrenergic receptor was made in isolated rat liver plasma membranes (see Section 2.2.3). The plasma membranes, diluted to 0.2 mg/ml protein in 50 mM potassium phosphate buffer, 1 mM $MgCl_2$, pH 7.4, were incubated at 37°C first with 1 nM [^{3}H]Prazosin, for 30 min.

1 mM DSS, dissolved in dimethyl sulfoxide (DMSO, 1% final concentration) was then added, and the incubation was carried on for another 3 min. At the end of this period, the cross-linking reaction was quenched with 10 mM ammonium acetate. The plasma membranes were washed by centrifugation three times, resuspended in drug-free buffer and incubated for the indicated period of time. As shown in the left panel of fig. 13, the dissociation of [^{3}H]Prazosin from DSS-treated plasma membranes is slightly slower, than from the control preparation, but this amount of retained ligand (~10% in excess to the control at the 60th min), is displaced by 2 μM phentolamine (right panel). Similar results were obtained when the cross-linking was made at 4°C. We found, in separate experiments, that the treatment of isolated rat liver plasma membranes with DSS doesn't change the number of binding sites for [^{3}H]Prazosin in the membrane. Similar results were obtained with plasma membranes, isolated from DSS-treated hepatocytes. The negative results from these pilot experiments were interpreted in the sense that there are no free amino groups available in the active center of the α_1-adrenergic receptor, within the the limited span of the cross-linker (11 Å).

In the next step, we tested the possibility to link, using the same approach, the peptide hormone arg^{8}vasopressin to its receptor in the plasma membrane. Arg^{8}vasopressin is a peptide with the amino acid sequence of Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH_2, and molecular weight of 1084 daltons. Beside the terminal amino group, this peptide has 4 additional amino groups on the side chains of the Gln, Asn an Arg residues. For the experiment, the rat liver plasma membranes were suspended, at 0.2 mg/ml protein concentration, in the same buffer as above, and in-

cubated first for 20 min with 10 nM unlabeled arg^{8}vasopressin. The cross-linking with DSS was done as described above, and the preparation was washed and resuspended in fresh buffer. At the end, the binding capacity of the vasopressin V_1 receptors in the preparation was determined. As shown at fig. 14, the [^{3}H]vasopressin binding capacity of the cross-linked plasma membranes remained at the control level. Therefore, unlabeled vasopressin was not cross-linked to its receptor.

The same experiment was repeated with isolated, intact rat hepatocytes. The cells were isolated from the livers of male, SD rats, 300 - 350 g weight, as described in Section 2.2.1. They were suspended at a concentration of 20 mg wet cells/ml and incubated (at 37°C) with 10^{-7}M vasopressin for 2 min. After this, 1 mM DSS was added (dissolved in DMSO, 1% final concentration) and the incubation was carried on for another 3 min. At the end of this period, the cross-linking reaction was quenched with 10 mM ammonium acetate. The cells were washed by centrifugation three times, resuspended in fresh buffer and incubated for a period of 30 - 45 min. For the measurement of the activity of glycogen phosphorylase, 1 ml aliquots were removed. The treatment with DSS and 1% DMSO was found to have no effect on cell viability, as determined by the Trypan Blue exclusion method. As illustrated by fig. 15/C, when the cells were stimulated with vasopressin alone, the activity of glycogen phosphorylase reached, at the 2nd min, the maximal value of 5.29 ± 0.4 U (1 U = 1 pmole [^{14}C]glucose incorporated into glycogen/1 mg wet cells weight. After the washing out of the hormone, the activity of the enzyme decays rapidly to 1.09 U at the 30th min. In contrast, after the treatment with DSS, the enzyme activity reaches a maximum of 3.42 ± 0.5 U at min 5, and remains

high - 2.96 ± 0.4 U at the 30th min (Fig. 15/D). For the observation period, the activity of glycogen phosphorylase in the control cells, incubated in the presence of 1% vehiculum (DMSO) remained at base-line level (Fig. 15/A), but the treatment with DSS alone produced an increase in the base-line activity of the enzyme, (~ 1 U at the 30th min, fig. 15/B).

The above experiments were repeated for the α_1-adrenergic receptor. The experimental conditions were the same as above with the only difference that the agonist used was adrenalin, which has no amino groups, and the activity of glycogen phosphorylase was monitored for a longer period of time. As illustrated in fig. 16/C, incubation of the hepatocytes with 10^{-5}M adrenalin (in the presence of 10^{-5}M propranolol) activates the glycogen phosphorylase to a maximal value of 5.12 ± 0.67 U. After the removal of the hormone, the activity of glycogen phosphorylase decays rapidly and returns to base-line value (0.36 U) within the 45 min period of observation. In contrast, after treatment with 1 mM DSS, the glycogen phosphorylase is activated to a maximum of 3.83 ± 1.07 U, which is reached at the 15th min, and stays approximately at the same level for the observation period. At the 45th min, the activity of the enzyme is still 2.51 U (Fig. 16/D). 1 mM DSS alone produces a small, but significant increase in the activity of glycogen phosphorylase, which reaches a maximum of 0.96 U at the 45th min (Fig. 16/B).

3.2.3 PHARMACOLOGICAL ACTIVITY OF I-POB

In the last ten years, a number of high affinity ligands, such as [^{3}H]dihydroergocryptine (Williams & Lefkowitz, 1976), [^{3}H]WB4101 (U'Prichard *et al.*, 1977), [^{3}H]Prazosin (Karliner *et al.*, 1979), and [^{125}I]BE-2254 (Engel & Hoyer, 1981) have been used for the characterization of α_1-adrenergic receptors from different tissues. In the liver, [^{3}H]Prazosin has been preferred for the characterization of the α_1-adrenergic receptor in isolated hepatocytes (Hoffman *et al.*, 1980, Preiksaitis *et al.*, 1982), because liver cells actively take up and metabolize most of the other ligands (Kunos, 1984). All these ligands, which label the α_1-adrenergic receptor reversibly, have been used for the pharmacological characterization of the receptors in tissue slices or in isolated plasma membrane preparations. On the other hand, an irreversible radioligand may offer a distinct advantage for the molecular characterization of the receptor, which involves detergent solubilization followed by a multi-step purification procedure. The first irreversible radioligand for the α_1-adrenergic receptor was [^{3}H]phenoxybenzamine ([^{3}H]POB) (Lewis & Miller, 1966; Young & Nickerson, 1973; Guellaen & Hanoune, 1979; Kan *et al.*, 1979). Using high specific activity [^{3}H]POB, the molecular weight of the hormone-binding subunit of the rat liver α_1-adrenergic receptor has been determined (Kunos *et al.*, 1983). Subsequently, two irreversible photoaffinity probes for the α_1-adrenergic receptor were synthesized: [^{125}I]CP65,526 (Seidman *et al.*, 1984), and [^{125}I]APDQ (Leeb-Lundberg *et al.*, 1984), and used in the study of hepatic α_1-receptors (Dickinson *et al.*, 1984; Leeb-Lundberg *et al.*, 1984; Seidman *et al.*, 1984).

In our work for the partial purification of the hormone-binding subunit of the α_1-adrenergic receptor (described in detail in Section 3.2.1), we used [^{3}H]POB, supplied from NEN. Results obtained with more recent batches of this probe labeled on the phenoxy ring were inconsistent and variable, due to rapid radiochemical decomposition. Probably as a result of this problem, NEN stopped offering [^{3}H]POB and introduced, as an irreversible probe for the α_1-adrenergic receptor, [^{125}I]Arylazidoprazosin ([^{125}I]CP65,526). We tried to use this ligand but, despite a variety of labeling protocols, did not obtain reproducible labeling pattern of the receptor (see Section 4.4.2). Because of these difficulties, we investigated the possibility to develop an analog of POB, iodinated on the benzyl ring, which could be used for irreversible labeling of the α_1-adrenergic receptor, and would also have the high specific activity of radioiodinated ligands. Iodophenoxybenzamine (I-POB) was synthesized by Dr Tarek Mansour, of the department of Chemistry of McGill University (illustrated at fig. 1). Here we report some preliminary data about the pharmacological activity of this compound.

3.2.3.1 PROBLEMS WITH [^{3}H]POB.

The [^{3}H]POB, as synthesized by Kan *et al.* (1979), was tritiated on the benzyl ring and had a specific activity of 5 -18 Ci/mmol (Fig. 17). Starting from 1983, NEN offered higher specific activity ligand (22 - 55 Ci/mmol), tritiated on the phenoxy ring. Using this latter form of the ligand, we found high variability in the labeling efficiency, ranging from 0.2 - 0.3 pmols/mg to as low as 20 - 50 fmols/mg protein (at 0.5 nM final concentration of [^{3}H]POB). Delipidation of the preparation (as

described by Kunos *et al.*, 1983), removed most of the radioactivity in the preparation (>80%), showing covalent incorporation of an unusually low amount (less than 10% of the total retained). The results of SDS-PAGE analysis confirmed this finding. Only trace labeling was found in the range of the high molecular weight proteins, and 99% of the radioactivity migrated with the front. These findings suggested that the high specific activity [^{3}H]POB we were using, had a faster rate of radiochemical decomposition, than the [^{3}H]benzyl-POB, which was known to be stable for years. To confirm this suspicion, we checked the radiochemical purity of the high specific activity [^{3}H]POB, one month upon receipt, using thin layer chromatography (TLC) as described in Section 2.2.15. In the solvent system methanol:acetonitrile (9:1), in which the R_f value of POB is 0.78 (Kan *et al.*, 1979), we found one small radioactive peak with R_f of 0.78, and several, ~ 2 times higher peaks with R_f in the range 0.75 - 0.78, confirming directly the radiochemical decomposition of the ligand. The TLC profile was similar in batches stored at -10°C and -70°C. Taking into account these facts, we used up the freshly synthesized [^{3}H]POB within 10 days upon receipt, and discarded preparations, containing less than 0.2 pmol/mg specific binding.

Starting from mid-1984, [^{3}H]POB was no more offered by NEN, and was replaced with the novel, radioiodinated photoaffinity ligand [^{125}I]Aryl-azidoprazosin. In the dark, this ligand binds with a high affinity to the α_1-adrenergic receptors in isolated rat liver plasma membranes (K_D = 0.3 nM, Seidman *et al.*, 1984), although the non-specific binding is higher, than for [^{3}H]Prazosin (30 - 40% *vs.* 8 - 10% for [^{3}H]Prazosin). The labeling of rat liver plasma membranes was done as described by

Graham *et al.* (1984) (see Section 2.2.8.4). Samples were UV-irradiated for 20 sec, because we found that longer irradiation periods increased unacceptably the non-specific binding (to 80 - 90% of the total). In these conditions, we found that only 0.5 - 1% of the ligand is protein-linked after SDS-PAGE. The level of non-specific binding and the level of ligand incorporation into protein was not improved by addition of low concentration bovine serum albumin (suggested by Leeb-Lundberg *et al.*, 1984), 1 mM DTT, or by delipidation of the preparation after labeling (as used by Kunos *et al.*, 1983). The low efficiency of covalent binding of this ligand was also reported by others (Terman & Insel, 1986). In a few experiments, we found specific labeling of peptides with molecular weight of 79,000, 66,000 and 52 - 54,000 daltons, but the labeling pattern, and the amount of ligand bound to protein varied between experiments. We were unable to find the reason for this problem.

3.2.3.2 PHARMACOLOGICAL ACTIVITY OF I-POB.

3.2.3.2.1 Antagonism of the stimulation of glycogen phosphorylase through the α_1-adrenergic receptor in isolated hepatocytes

(Fig 18). For these experiments, we used isolated hepatocytes from the livers of male, SD rats, 300 - 350 g, as described in "Methods". The hepatocytes, at a concentration of 20 mg wet weight/ml, were incubated for 20 min with the indicated concentrations of I-POB. At the end of this period, the cells were stimulated with 1 μM adrenaline for 2 min, and the activity of glycogen phosphorylase was determined. As seen in Fig. 18, I-POB is a potent antagonist of adrenaline for the stimulation of glycogen phosphorylase, with an IC_{50} of approximately 1 nM.

3.2.3.2.2 Binding affinity for α_1-receptors to isolated rat liver plasma membranes (Fig 19). The binding affinity of I-POB for α_1-receptors in rat liver plasma membranes was determined from its ability to displace [^{3}H]prazosin. Rat liver plasma membranes were isolated as indicated in Section 2.2.3 and suspended at 31°C in 50 mM Tris/1 mM $MgCl_2$ buffer, pH 7.4, at a concentration of 0.2 mg/ml. After a 20 min preincubation with various concentrations of competing drugs, the binding of [^{3}H]prazosin, final concentration 0.4 nM, was determined in 1 ml aliquots as described in Section 2.2.8.2. In fig. 19, the binding of [^{3}H]prazosin in absence of competitor is expressed as 100%. The K_{50} for the displacement of [^{3}H]prazosin binding was found to be 0.13 nM for unlabeled prazosin, 0.65 nM for POB and 2 nM for I-POB. The binding affinity of I-POB is only 3 times lower than that of POB itself.

3.2.3.2.3 Antagonism of vascular α_1-adrenergic receptors in the rat (Fig. 20). In this experiment, the effect of I-POB as an antagonist of the stimulation of α_1-adrenergic or vasopressin-induced vasoconstriction was determined. The experiment was done in collaboration with Dr R. Mosqueda, using male, SD rats, weighing 500 - 560 g. The animals were anesthetized with pentobarbital and the right femoral vein and artery were cannulated with polyethylene cannulas (PE50). The arterial cannula was connected to a pressure transducer (Statham, Hato Rey, Puerto Rico; P23Db) and polygraph (Grass, Quincy, MA; model 7) for continuous monitoring of blood pressure (BP) and heart rate (HR). Heart rate was monitored through a tachograph preamplifier. Drugs, dissolved in saline, were administered through the venous cannula. Fig. 20 shows the original recording from one animal, which is representative of two

similar experiments. The upper part of the figure demonstrates the agonist-induced changes in blood pressure and heart rate in the absence of antagonist, while the lower part shows the response to the same dose of agonist, tested 20 min after the i.v. administration of I-POB, 5 mg/kg. As seen from this graph, the dose of 5 mg/kg I-POB suppresses completely the response to 5 and 10 µg/kg phenylephrine (PhE) i.v., but has no effect on the vasoconstriction produced by 200 ng/kg i.v. arg^{8}vasopressin. One of the animals was retested the next morning, and the results obtained were similar to those illustrated in fig. 20, indicating a long-term effect (irreversible blockade) of the vascular α_1-adrenergic receptors in the experimental animal.

3.2.4 RABBIT POLYCLONAL ANTIBODIES AGAINST THE α_1-ADRENERGIC RECEPTOR

Immunological methods have been widely used in the study of hormone receptor structure and function. Regarding catecholamine receptors, most of the effort to develop immunological methods has been devoted to the study of the β-adrenergic receptor, due to the more rapid progress in the knowledge about the molecular structure of that receptor. Polyclonal antibodies, developed in rabbits and mice against affinity-purified β-adrenergic receptor have been used to study the interaction of the receptor with ligands (Wrenn & Haber, 1979; Caron et *al.*, 1979), and with the adenylate cyclase system (Couraud *et al.*, 1981; Strader *et al.*, 1983), for the immunocytochemical localization of the receptor in brain

(Strader *et al.*, 1983; Trimmer *et al.*, 1984), and as an independent mean of identification of the hormone-binding binding subunit of the β-adrenergic receptor (Couraud *et al.*, 1983). The main limitation in the use of conventional antibodies resides in their heterogeneity. In 1978 Sege and Peterson proposed to develop a new class of more specific antibodies, representing "the internal image" of an antigen (anti-idiotypic antibodies; Sege & Peterson, 1978). The idea was applied to the study of the ligand-recognizing mechanism of the β-adrenergic receptor (Schreiber *et al.*, 1980), the pathogeny of some receptor-related autoimmune disorders (Venter *et al.*, 1980; Homcy *et al.*, 1982; Wasserman *et al.*, 1982; Strosberg, 1984; Farid *et al.*, 1985) and the mechanisms of modulation of the immune response (Sege & Peterson, 1978; Couraud *et al.*, 1983). An attempt was made to use anti-idiotypic antibodies for immunoaffinity purification of the α_1-adrenergic receptor, but the results were inconclusive (Guellaen *et al.* (1982). The difficulties and the limitations in the use of polyclonal antibodies were resolved by the development of a method for the production of monoclonal antibodies (Köhler & Milstein, 1975). The high specificity of interaction of the monoclonal antibodies, combined with their purity and possibility for production in substantial amounts prompted the development of monoclonal antibodies against the catecholamine receptors (Fraser & Venter, 1980; Dausse & Diop, 1983; Venter *et al.*, 1984). Monoclonal antibodies were used for the purification and the molecular characterization of the β-adrenergic receptor (Fraser & Venter, 1980), and for the molecular characterization of the α_1-adrenergic receptor (Venter *et al.*, 1984). In 1983-84, in our laboratory we developed rabbit antibodies against partially purified preparation of α_1-adrenergic

receptor from rat liver. In the following text, we will describe their pharmacological activity and their application for the molecular characterization of the hormone-binding subunit of the receptor.

The antigen was prepared from plasma membranes from the liver of male rats. The native plasma membranes were solubilized with 1% Triton X-100 and the hormone-binding subunit of the α_1-adrenergic receptor was partially purified by Sephadex chromatography as described in Section 3.2.1 of this thesis, and by Kunos *et al.* (1983). The fractions containing the α_1-adrenergic receptor were identified by parallel processing of [^{3}H]POB-labeled preparations (see fig. 8). The relevant fractions from an unlabeled preparation were concentrated and used (0.5 mg protein/-injection) for the immunization of white male New Zealand rabbits. After repeated boosting at 2-week intervals, the animals were bled and the serum separated. Sera were collected starting from the first month and used at the indicated dilution in the experiments. Control experiments were made in the presence of equivalent dilution of serum from non-immunized animals. (See Section 2.2.10).

As seen at fig. 21, the incubation of isolated rat hepatocytes with 1:10 dilution of rabbit antiserum for 30 min at 37°C produces block of ~80% of the activation of glycogen phosphorylase by the α_1-adrenergic agonist phenylephrine. Based on these findings, in our further experiments, we used 1:10 dilution of the rabbit antiserum in appropriate buffer, and incubation for 30 min at 37°C.

Fig. 22 shows the effect of the antiserum on the activation of glycogen phosphorylase in isolated hepatocytes. The activity of the enzyme is expressed as multiples of basal activity in the absence of

agonist. 10^{-5}M phenylephrine, in the presence of pre-immune serum, increases the activity of glycogen phosphorylase 4.7 ± 1.17 times. In the presence of anti-receptor antiserum, the same concentration of phenylephrine produces only 1.17 ± 0.12 -fold increase in the activity of the enzyme. Preincubation of the antiserum with rat liver plasma membranes (4 mg plasma membranes/1 ml antiserum for 30 min at 37°C) removed the inhibitory effect of the antiserum, phenylephrine being able to increase enzyme activity 3.6 ± 0.4 -fold over basal.

The specificity of the interaction of the rabbit antiserum was determined indirectly by comparing the effect of the antiserum on the activation of glycogen phosphorylase in isolated rat hepatocytes by two different agonists: phenylephrine and arg^{8}vasopressin (Fig. 23). The antiserum interfered only with the activation of the enzyme through the α_1-adrenergic receptor, the response to vasopressin remained unchanged.

The presence, in the rabbit antiserum, of antibodies binding to the active center of the α_1-adrenergic receptor from rat liver was confirmed directly by the method of immunoblotting (Fig. 24). For this experiment, rat liver plasma membranes were solubilized with SDS and resolved by SDS-PAGE (10% acrylamide gel). At the end of the electrophoresis, the proteins were transblotted on nitrocellulose paper and incubated with the rabbit antiserum (dil. 1:10). The antigen-antibody reaction was visualized by reaction with horseradish peroxydase-linked anti-rabbit IgG. The experiment was calibrated with a parallel SDS-PAGE of [^{3}H]POB-labeled rat liver plasma membranes (see Section 2.2.4). As shown in the bottom part of fig. 24, the SDS-PAGE profile of [^{3}H]POB-labeled plasma membranes shows two peaks of α_1-adrenergic receptor specific labeling with M_r of

80,000 and 58,000 daltons. This profile is juxtaposed, in the same X-axis scale, to the results of the interaction of the rabbit antiserum with similarly resolved rat liver plasma membranes preparations. The control (strip A), shows the interaction of native rat liver plasma membranes with the rabbit antiserum. It visualizes a great number of antigen-antibody interactions between the various components of the plasma membranes and the antibodies, elaborated by the experimental animal. Strip B shows the interaction of native plasma membranes with rabbit antiserum, which had been preincubated with plasma membranes (4 mg plasma membrane protein/1 ml serum, 30 min at 37°C), treated with 100 nM POB. The preadsorbtion of the antiserum with POB-labeled plasma membranes removes some of the antibodies (reflected in the decrease in number of the lines and the intensity of staining), except for the line corresponding to the 80,000-dalton peak of the α_1-adrenergic receptor. Strip C shows the interaction of the antiserum with plasma membranes, pretreated with 100 nM POB. The comparison with the control shows that the block of the α_1-adrenergic receptors in the plasma membrane decreases the intensity of the staining of a protein with M_r 80,000 daltons.

FIGURE 2. Effects of *in vitro* incubation of isolated rat liver cells on glycogen phosphorylase activation by glycogenolytic hormones.

Cells were isolated and incubated, and the activity of glycogen phosphorylase measured as described in Section 2.2.1 and 2.2.2. Solid lines and filled symbols, freshly isolated hepatocytes (0-hr cells). Broken lines and open symbols, hepatocytes cultured for 4 hr (4-hr cells). Points represent means of eight (isoproterenol and phenylephrine) or four experiments (glucagon, vasopressin). Five-pointed stars and horizontal bars indicate mean EC_{50} ± SEM. Basal glycogen phosphorylase activity was 12.3 ± 1.1 and 11.3 ± 0.9 units (nmol [^{14}]C glucose incorporated into glycogen/min/mg of protein) for 0-hr and 4-hr cells, respectively.

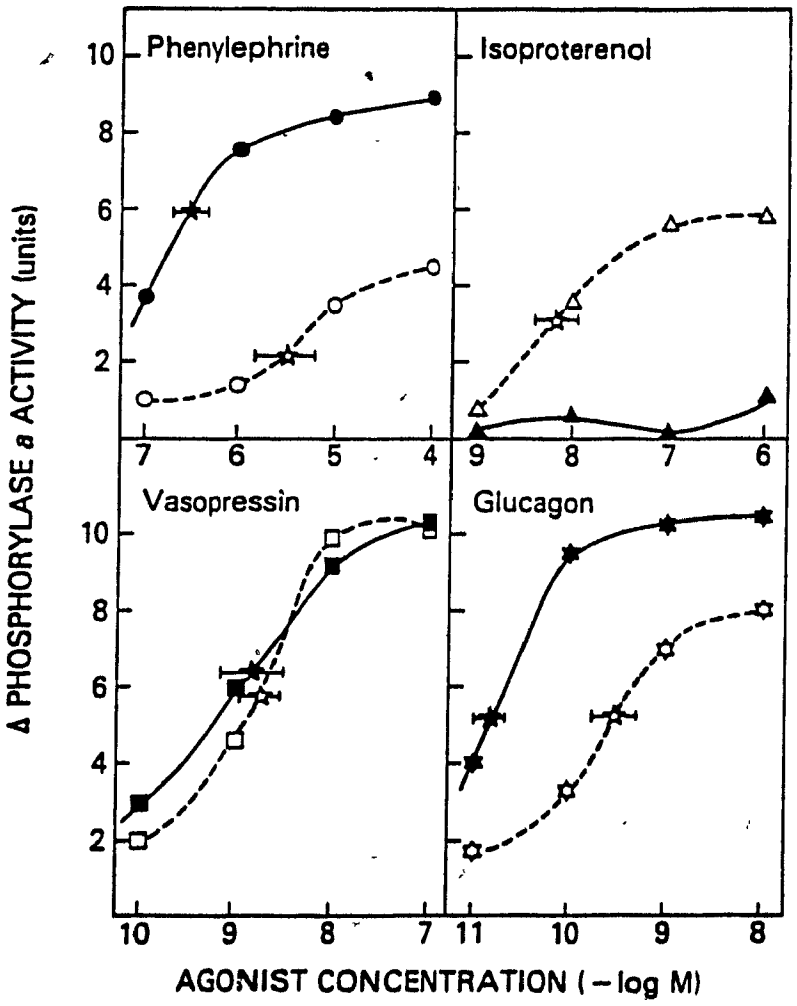

Phenylephrine
Isoproterenol
Vasopressin
Glucagon
Δ PHOSPHORYLASE a ACTIVITY (units)
AGONIST CONCENTRATION (−log M)

FIGURE 3. Reversal by lipomodulin of the time-dependent conversion of the hepatic α_1- to a β-adrenergic response.

The effects of phenylephrine (filled circles, solid line) and isoproterenol (triangles, broken line) were tested in 0-hr cells (*Top*), in 4-hr cells (*Middle*), and in 4-hr cells incubated for a further 30 min with 50 nM purified lipomodulin (*Bottom*). Means of four experiments are shown. Stars and horizontal bars indicate mean EC_{50} ± SEM. Baseline glycogen phosphorylase activity was 13.4 ± 1.3 units (0-hr cells). 11.2 ± 1.1 units (4-hr cells), and 14.4 ± 1.8 units (4-hr cells treated with lipomodulin).

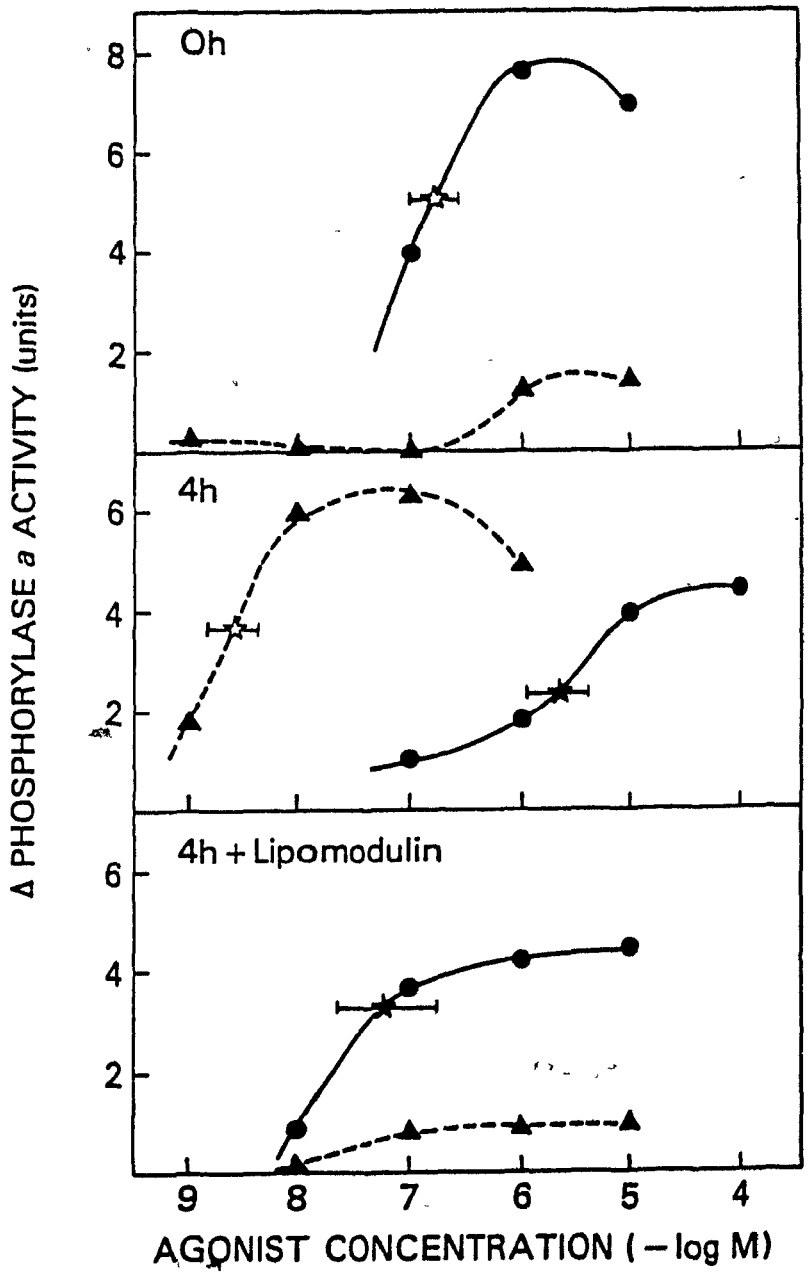

Oh
4h
4h + Lipomodulin
8
6
4
2
9
8
7
6
5
4
Δ PHOSPHORYLASE a ACTIVITY (units)
AGONIST CONCENTRATION (−log M)

FIGURE 4. Effects of preincubation of cells with lipomodulin antibody in the presence of phenylephrine on the adrenergic activation of glycogen phosphorylase.

0-hr rat liver cells were incubated with a 1:50 dilution of a monoclonal antibody to lipomodulin (see Section 2.2.9) in the presence of 10 μM phenylephrine and then washed for 30 min before the effects of phenylephrine and isoproterenol were tested. Filled symbols and solid lines, control cells; open symbols and broken lines, antibody-treated cells. Means of two experiments are shown. Basal phosphorylase activity was 12.2 and 9.4 units in control and antibody + phenylephrine-treated cells, respectively.

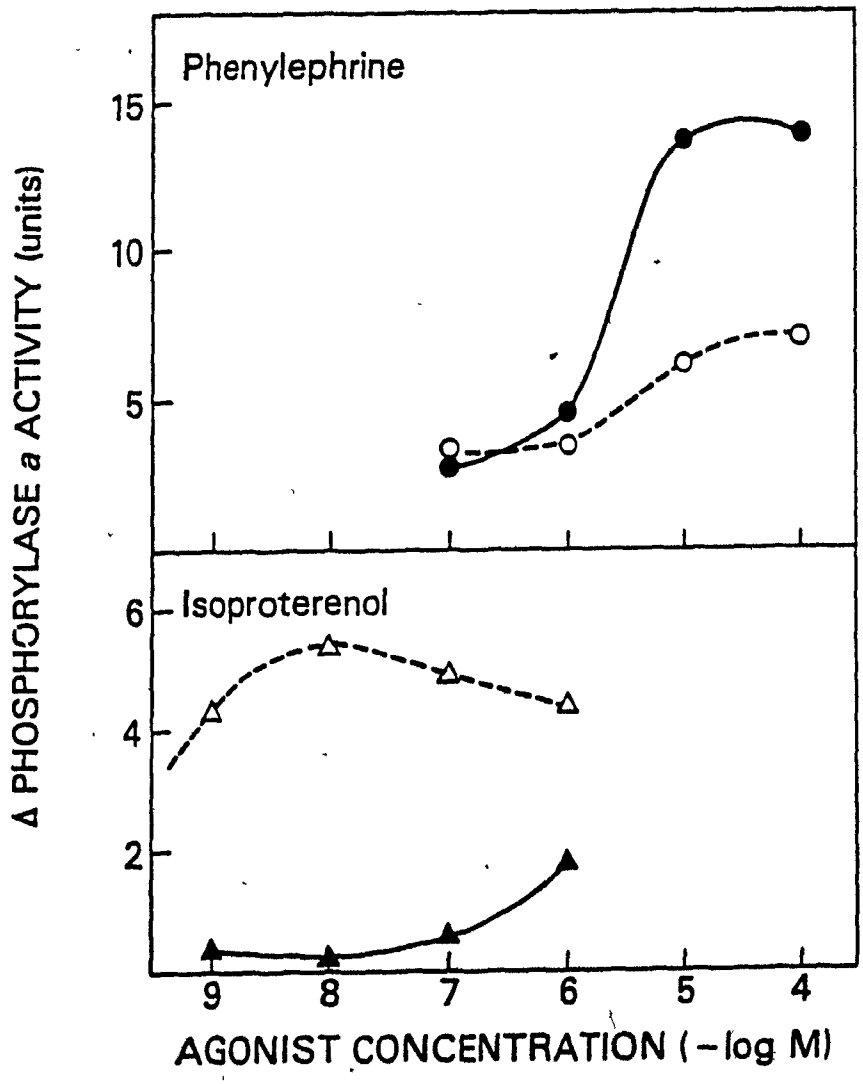

Phenylephrine
15
10
5
Isoproterenol
6
4
2
9
8
7
6
5
4
Δ PHOSPHORYLASE a ACTIVITY (units)
AGONIST CONCENTRATION (−log M)

FIGURE 5. Effect of mellitin on the adrenoceptor-mediated activation of glycogen phosphorylase in freshly isolated liver cells.

0-hr hepatocytes were incubated for 30 min with vehicle only (filled symbols, solid line) or with mellitin at 2 μg/ml (open symbols, broken line). Means of three experiments are shown. Baseline glycogen phosphorylase activity was increased by mellitin from 10.6 ± 2.3 to 18.6 ± 2.1 units.

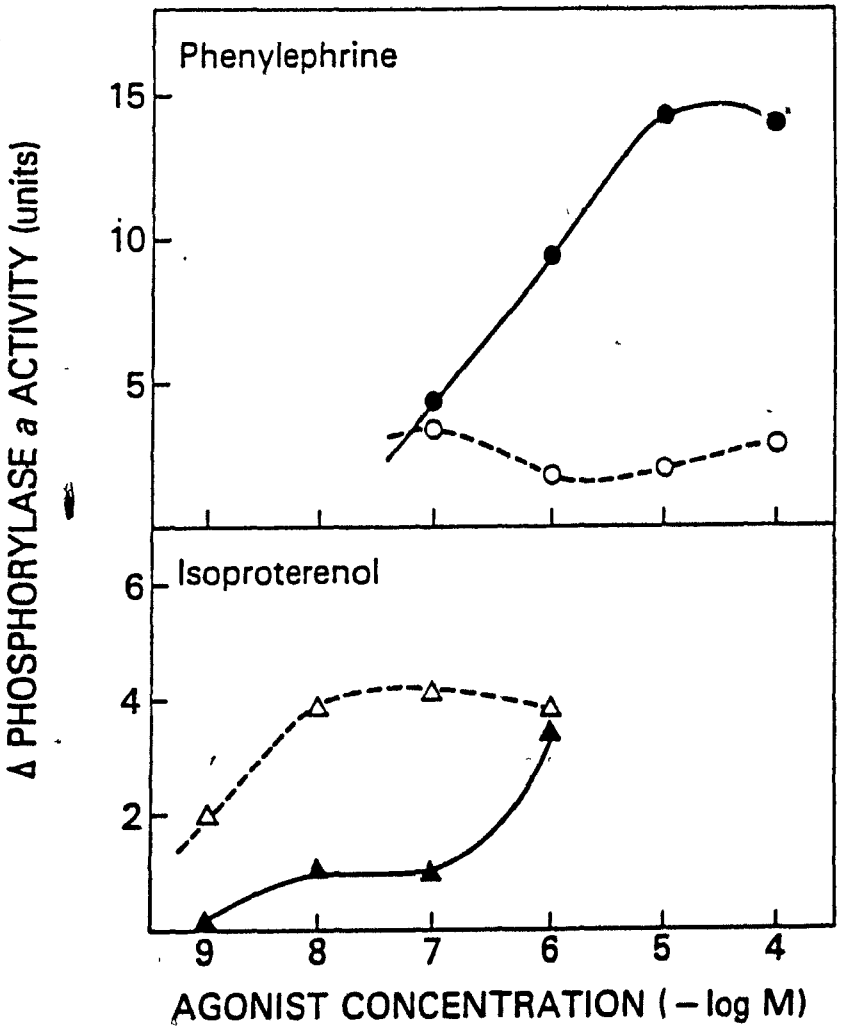

Phenylephrine
15
10
5
Isoproterenol
6
4
2
Δ PHOSPHORYLASE a ACTIVITY (units)
9
8
7
6
5
4
AGONIST CONCENTRATION (−log M)

FIGURE 6. Effect of different detergents on the solubilization of the α_1-adrenergic receptor in an active state.

Plasma membranes were isolated from rat liver as described in Section 2.2.3.1 and resuspended in 50 mM Tris/10 mM $MgCl_2$, pH 7.4 buffer, containing protease inhibitors (see Section 2.2.4) and the indicated concentration of detergents. The preparations were shaken for 30 min on ice and centrifuged for 1 hr at 100,000 g. The pellet was extracted a second time with 1/2 volume solubilization buffer and the two supernatants combined for the experiment. All experiments were done at 4°C. The results are expressed as percentage of the concentration of α1-adrenergic receptor in the plasma membranes, ~0.6 pmol/mg protein. "Active receptor" is defined by the specific binding of 0.4 nM [3H]prazosin. Bars indicate the mean ± SEM of three experiments.

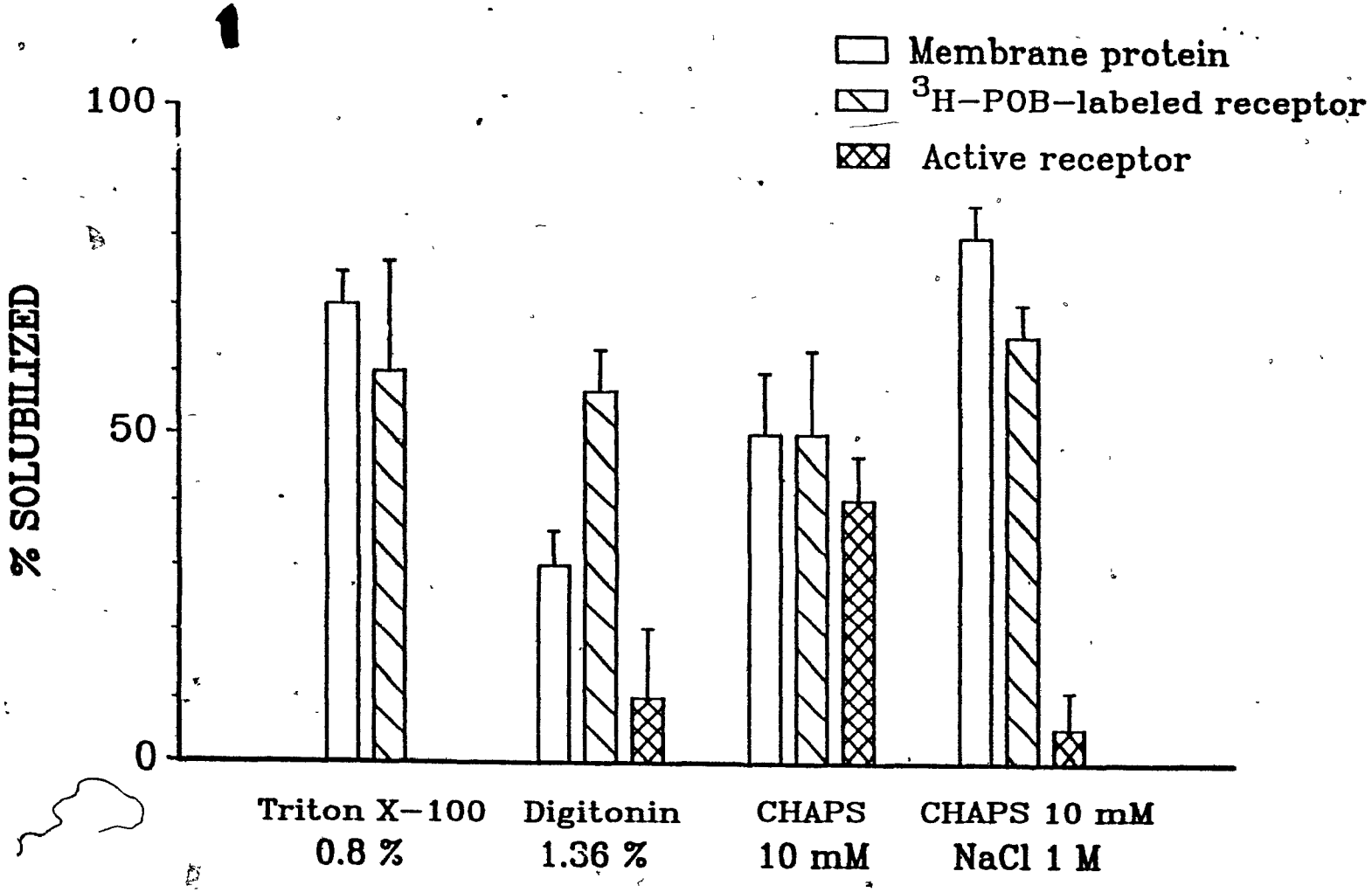

1
Membrane protein
^{3}H–POB–labeled receptor
Active receptor
100
50
0
% SOLUBILIZED
Triton X–100
0.8 %
Digitonin
1.36 %
CHAPS
10 mM
CHAPS 10 mM
NaCl 1 M

FIGURE 7. Sephacryl S-400 gel filtration chromatography of α_1-adrenergic receptor, solubilized with CHAPS.

Ten mg rat liver plasma membranes were solubilized with 10 mM CHAPS as indicated in fig. 6 and in Section 2.2.7. The extract (1 ml) was loaded on top of a Sephacryl S-400 column (2.5 x 40 cm) and eluted with a 50 mM Tris/10 mM $MgCl_2$, pH 7.4, buffer containing 0.1 mM CHAPS and protease inhibitors at a flow rate 25 ml/h. Solid line and filled points indicate the specific binding of [^{3}H]prazosin in the eluate (see Section 3.2.1). Doted line indicates optical density (O.D.) at 280 nm. V_0 indicates the elution point of dextran blue (M_r = 2 x 10^6 daltons). All experiments were done at 4°C. Representative for three experiments.

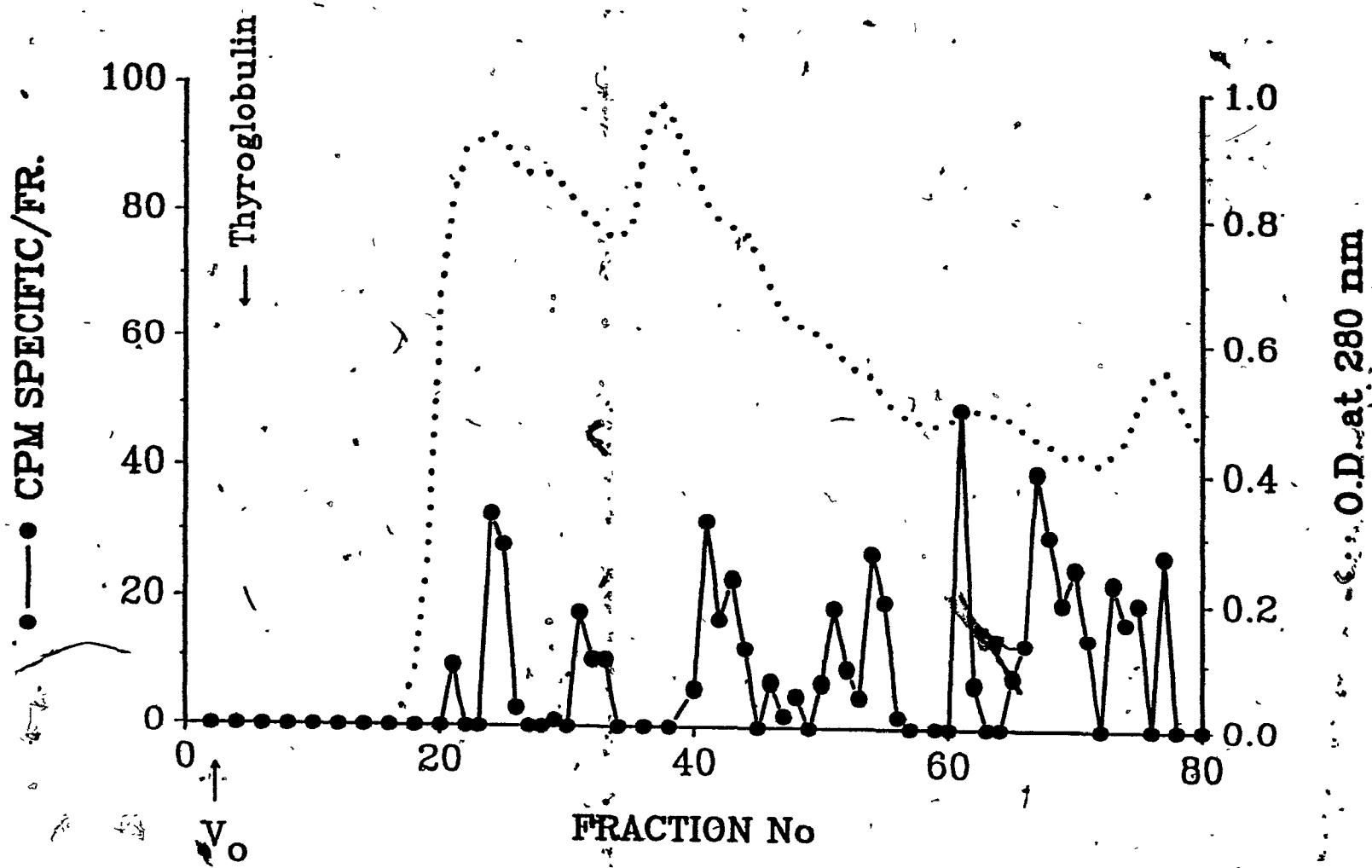

CPM SPECIFIC/FR.
Thyroglobulin
O.D. at 280 nm
100
80
60
40
20
0
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Vo
FRACTION No

FIGURE 8. Sephadex G-200 gel filtration chromatography of [^{3}H]POB-labeled α_1-adrenergic receptor.

Rat liver plasma membranes were labeled with [^{3}H]POB as described (see Section 2.2.8.1) and extracted with 1% Triton. 0.5 ml of the extract was loaded on top of a Sephadex G-200 (fine) column (1.6 x 32 cm) and eluted with 50 mM Tris/10 mM $MgCl_2$ buffer containing 0.8% Triton and protease inhibitors at a flow rate of 5 ml/h. The solid line with filled symbols denotes the difference in the radioactivity in each fraction between parallel runs of preparations labeled with 1 nM [^{3}H]POB in the absence and in the presence of 0.1 µM Prazosin (see Section 2.2.8). The recovery of total radioactivity was 80%. *Inset*, estimation of the M_r of the [^{3}H]POB-labeled α_1-adrenergic receptor (*arrow*). Open circles are molecular weight standards (dextran blue, ferritin, catalase, aldolase, BSA, ovalbumin, chymotrypsinogen A). $K_{av} = (V_T - V_o)/(V_E - V_o)$, where V_T, V_o and V_E are total bed volume, void volume and elution volume of a given protein, respectively. All experiments were done at 4°C. Protein was determined by the method of Bradford (1976). The graph is representative of ten experiments.

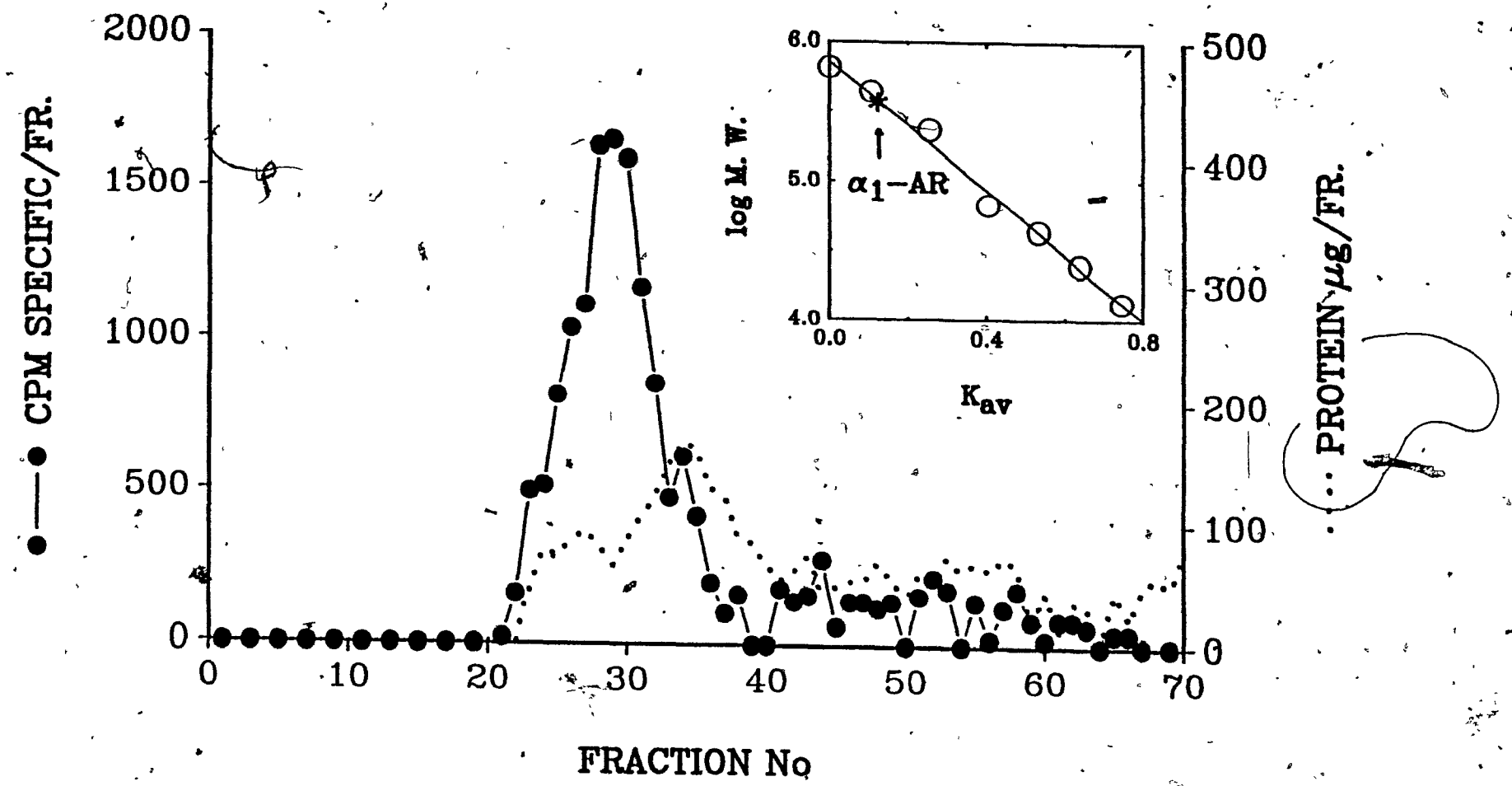

2000
1500
1000
500
0
CPM SPECIFIC/FR.
500
400
300
200
100
0
PROTEIN μg/FR.
0
10
20
30
40
50
60
70
FRACTION No
6.0
5.0
4.0
log M.W.
α1–AR
0.0
0.4
0.8
Kav

FIGURE 9. Wheat germ agglutinin chromatography of partially purified α_1-adrenergic receptor.

Rat liver α_1-adrenergic receptors were solubilized from plasma membranes and partially purified by gel filtration chromatography as described in fig. 8. The receptor-containing fractions were pooled together and applied to a 10 ml column of WGA coupled agarose (see Section 2.2.11). The column was washed with sample buffer (50 mM Tris/10 mM $MgCl_2$, 0.1% Triton, pH 7.4), eluted with sample buffer, containing 0.2 M β-N-acetyl-D-glucosamine, and washed with sample buffer containing 0.5 M NaCl. The preparations labeled with 1 nM [^{3}H]POB (dpm T), and with 1 nM [^{3}H]POB + 0.1 μM prazosin (dpm NS) were processed in parallel. The recovery of total radioactivity was 90%. Experiments were done at 4°C. The graph is representative of three separate experiments.

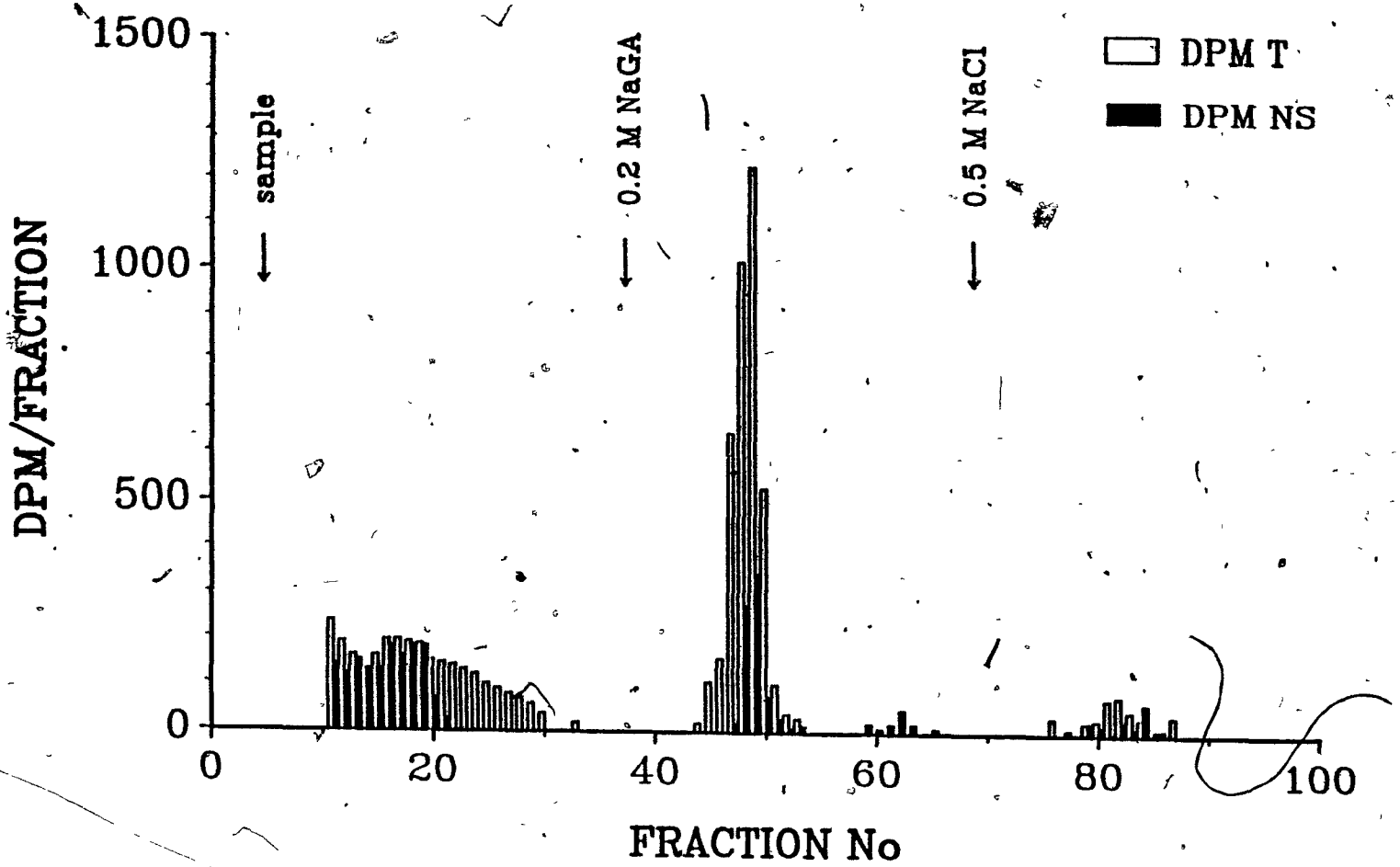

1500
1000
500
0
DPM/FRACTION
sample
0.2 M NaGA
0.5 M NaCl
DPM T
DPM NS
0
20
40
60
80
100
FRACTION No

FIGURE 10. Reverse phase HPLC chromatography of tryptic digest of [^{3}H]POB-labeled α_1-adrenergic receptor.

[^{3}H]POB-labeled α_1-adrenergic receptor, partially purified by gel filtration and WGA-affinity chromatography (see fig. 8 and 9) were digested with TPCK-treated trypsin (see Section 2.2.14). 0.5 ml of the digest, dissolved in 0.1% TFA/H_2O (0.3 mg protein) was injected in a Vydac C18 column (1 x 25 cm) and eluted with a linear gradient 0 - 100% acetonitrile in 0.1%TFA/H_2O for 56 min. The flow rate was 1 ml/min. The graph represents two parallel experiments of preparations labeled with 1 nM [^{3}H]POB in the absence (T) and in the presence of 0.1 µM Prazosin (NS). The recovery of total radioactivity was 85-90%. Experiments were done at room t°. The graph is representative of 40 experiments.

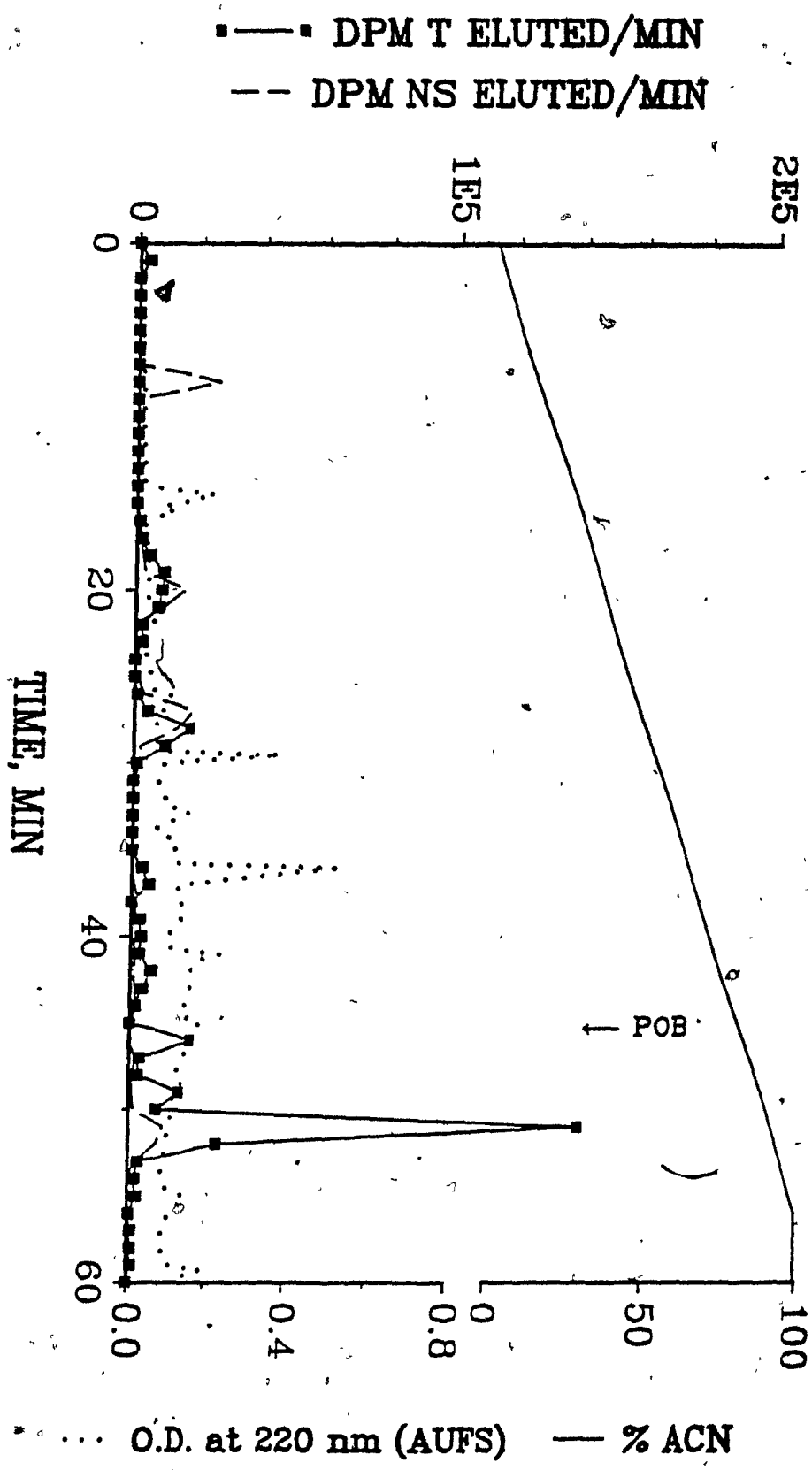

DPM T ELUTED/MIN
DPM NS ELUTED/MIN
0
1E5
2E5
TIME, MIN
0
20
40
60
POB
0.0
0.4
0.8
0
50
100
O.D. at 220 nm (AUFS)
% ACN

FIGURE 11. Gel permeation HPLC chromatography of [^{3}H]POB-labeled proteolytic fragments of the α_1-adrenergic receptor.

The main peak of radioactivity of the tryptic digest of the partially purified α_1-adrenergic receptor (Fig. 10) was collected, lyophilized and dissolved in 0.5 ml 0.1% TFA/acetonitrile. The sample was injected in a Waters I-60 column and eluted with 0.1%TFA/acetonitrile at a flow rate 0.5 ml/min. *Inset*: estimation of M_r of the proteolytic fragments of the α_1-adrenergic receptor (*arrow*). Open circles are molecular weight standards (cytochrome C, β-endorphin, actinomycin D, enkephalinamide and POB). K_{av} as in fig. 8. The recovery of radioactivity was 95%. Experiments were done at room t°. The graph is representative of two separate experiments.

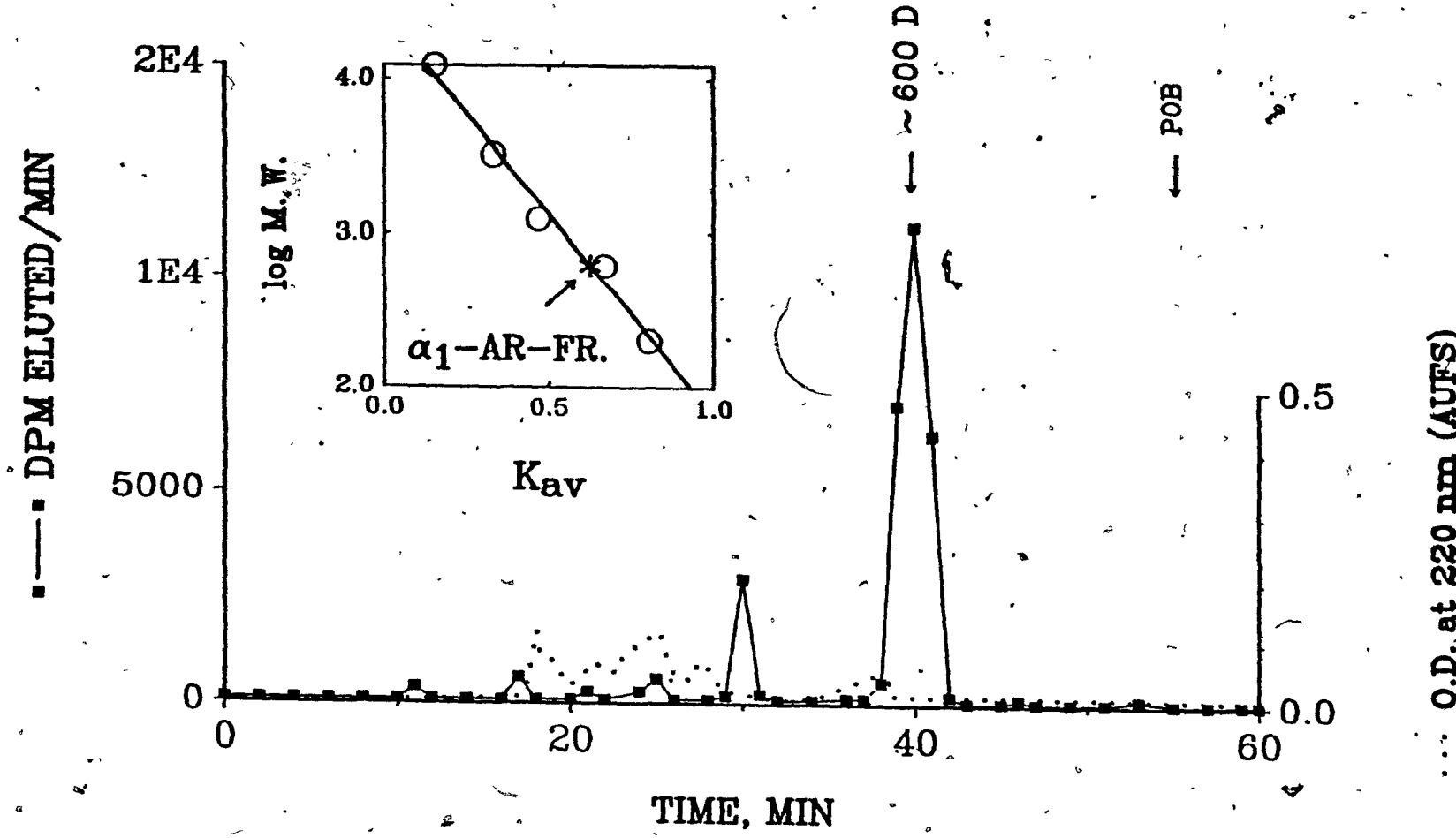

2E4
1E4
5000
0
0
20
40
60
TIME, MIN
DPM ELUTED/MIN
O.D. at 220 nm (AUFS)
0.5
0.0
~ 600 D
POB
4.0
3.0
2.0
0.0
0.5
1.0
log M.W.
Kav
α1–AR–FR.

FIGURE 12. SDS-polyacrylamide gel electrophoresis of [^{3}H]POB-labeled proteolytic fragments of the α_1-adrenergic receptor.

The main peak of radioactivity of the tryptic digest of the partially purified α_1-adrenergic receptor (Fig. 11) was collected, lyophilized and dissolved in 1 ml H_2O. 50 µl sample was lyophilized, dissolved in 50 µl SDS-PAGE sample buffer and applied to a 5% polyacrylamide gel. At the end of the electrophoresis, the gel was sliced in 3-mm wide slices and counted by LSC (see Section 2.2.4). *Inset*: estimation of *Mr* of the proteolytic fragments of the α_1-adrenergic receptor (*arrow*). Points are m.w. standards (polypeptide fragments of horse heart myoglobin, MW-SDS-17 Kit, Sigma). AVP indicates the R_f of [^{3}H]Arg^{8}vasopressin (m.w. 1084). *Rf* = distance migrated by the substance/-distance migrated by the tracking dye. The recovery of total radioactivity was 70%.

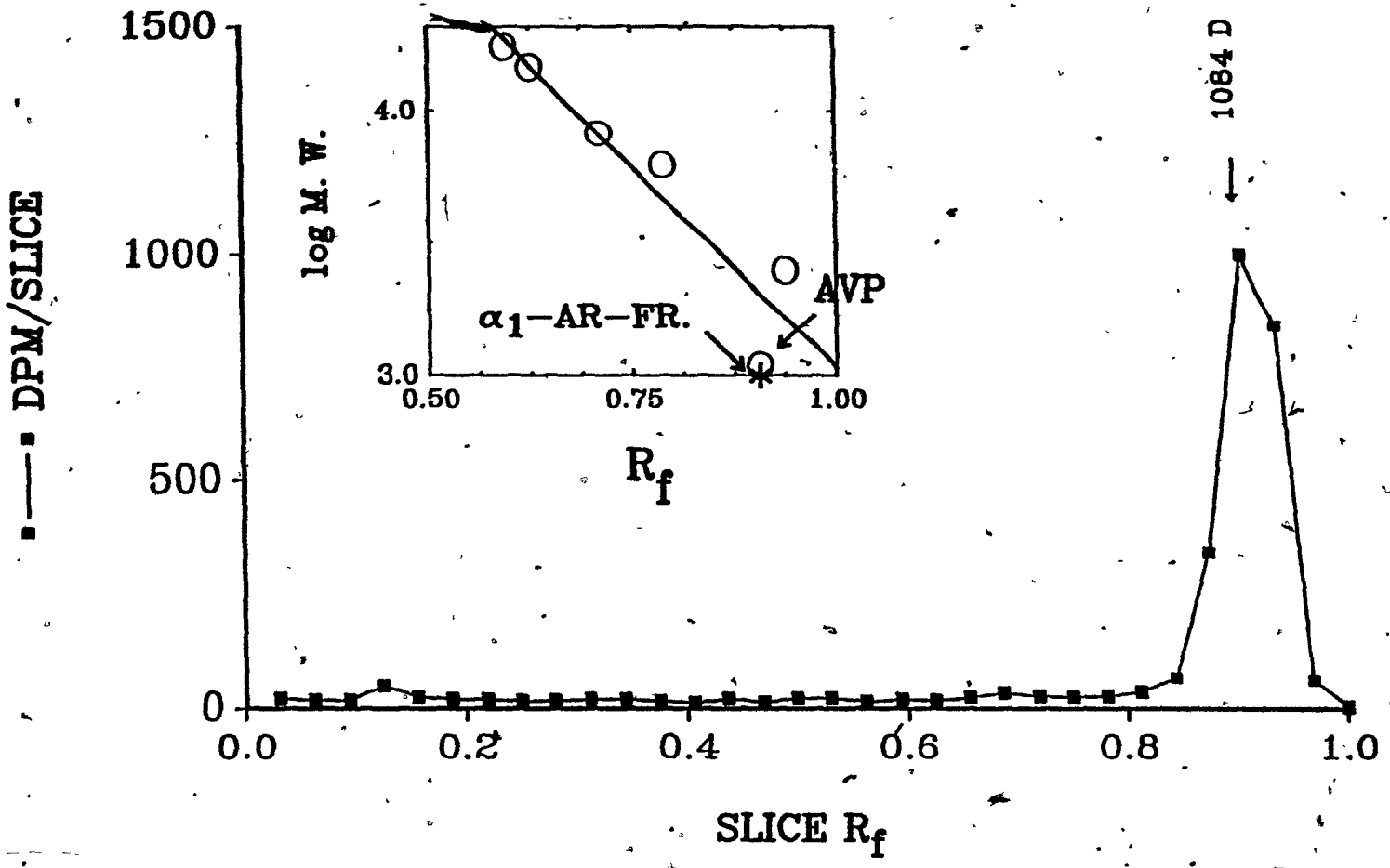
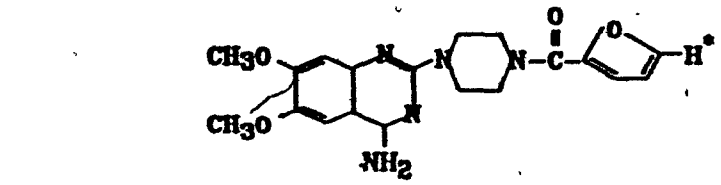
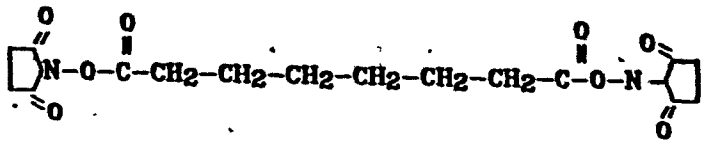

1500
1000
500
0
DPM/SLICE
0.0
0.2
0.4
0.6
0.8
1.0
SLICE R_f
1084 D
4.0
3.0
log M. W.
0.50
0.75
1.00
R_f
AVP
α_1—AR—FR.

FIGURE 13. Effect of disuccinimidyl suberate on the dissociation kinetics of [^{3}H]Prazosin in rat liver plasma membranes.

Rat liver plasma membranes were suspended in 50 mM potassium phosphate/1 mM $MgCl_2$, pH 7.4 at 0.2 mg/ml and labeled with 1 nM [^{3}H]prazosin (see Section 2.2.8.2). The preparation was cross-linked with 1 mM disuccinimidyl suberate for 5 min at 37°C. The cross-linker was dissolved in DMSO (1% of the volume of the sample). The reaction was quenched with 10 mM ammonium acetate, the preparation was washed two times by centrifugation, resuspended in fresh buffer (*Left panel*) or in buffer containing 2 μM phentolamine (*Right panel*) and incubated at 4°C. At the indicated intervals, equal volume samples were removed for determination of the bound radioactivity. The results are expressed as percentage of the binding of 1 nM [^{3}H]prazosin in DMSO-treated (control) membranes (0.6 pmols/mg). The graph is representative for three experiments.



[3H]Prazosin



Disuccinimidyl suberate (DSS)

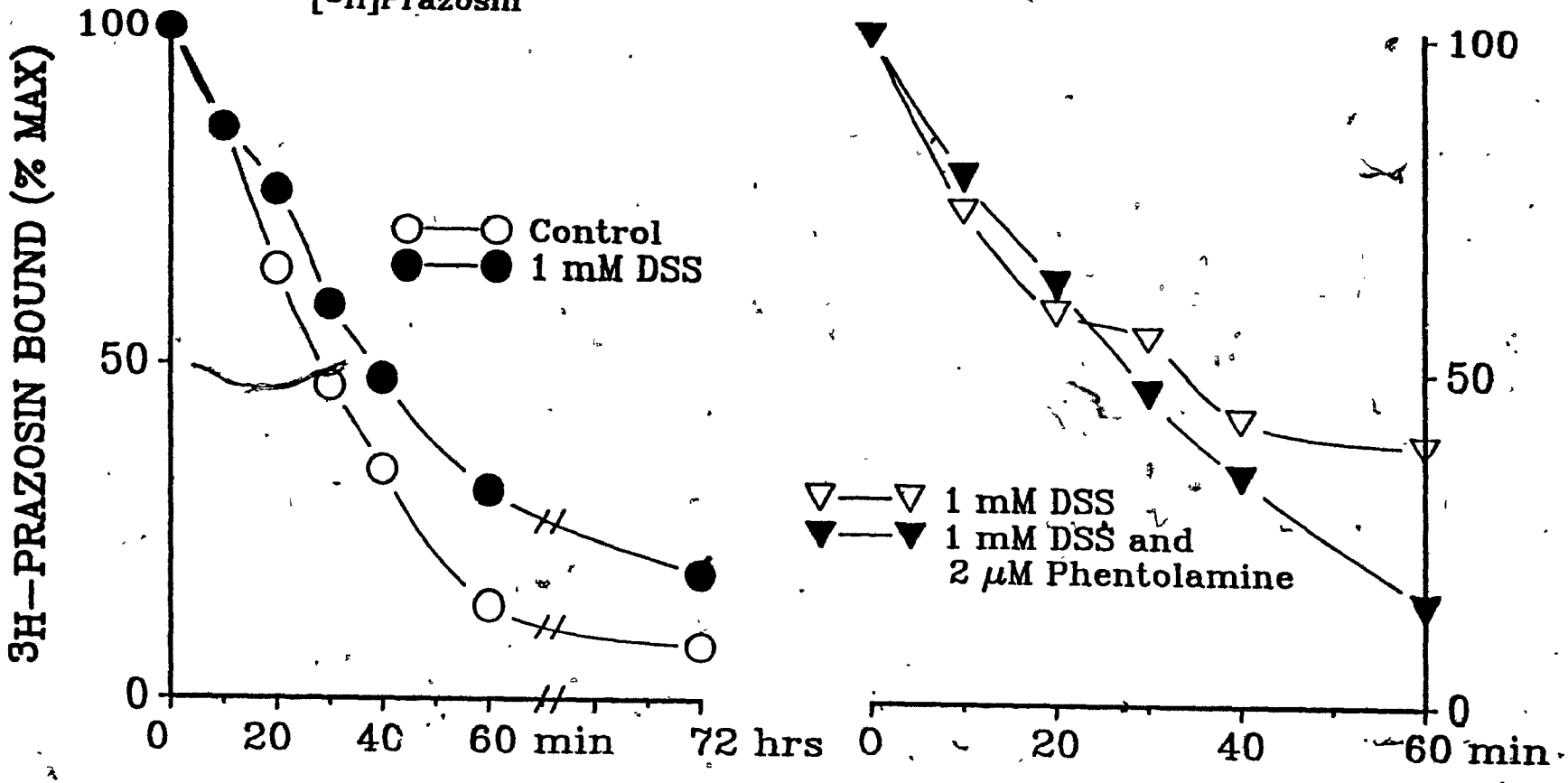


FIGURE 14. Lack of cross-linking of vasopressin to its receptor by disuccinimidyl suberate.

Rat liver plasma membranes were suspended in 50 mM potassium phosphate/1 mM $MgCl_2$, pH 7.4 at 0.2 mg/ml and incubated for 2 min with 10 nM unlabeled arg^8vasopressin, followed by cross-linking with DSS as described in fig. 13, two washes and resuspension in fresh buffer. Following this, the binding of [^{3}H]Arg^8vasopressin was determined (incubation for 20 min at 30°C). The dashed line indicates the maximal [^{3}]$arg8^v$asopressin binding, obtained in DMSO-treated (control) membranes. The graph is the mean of three experiments.

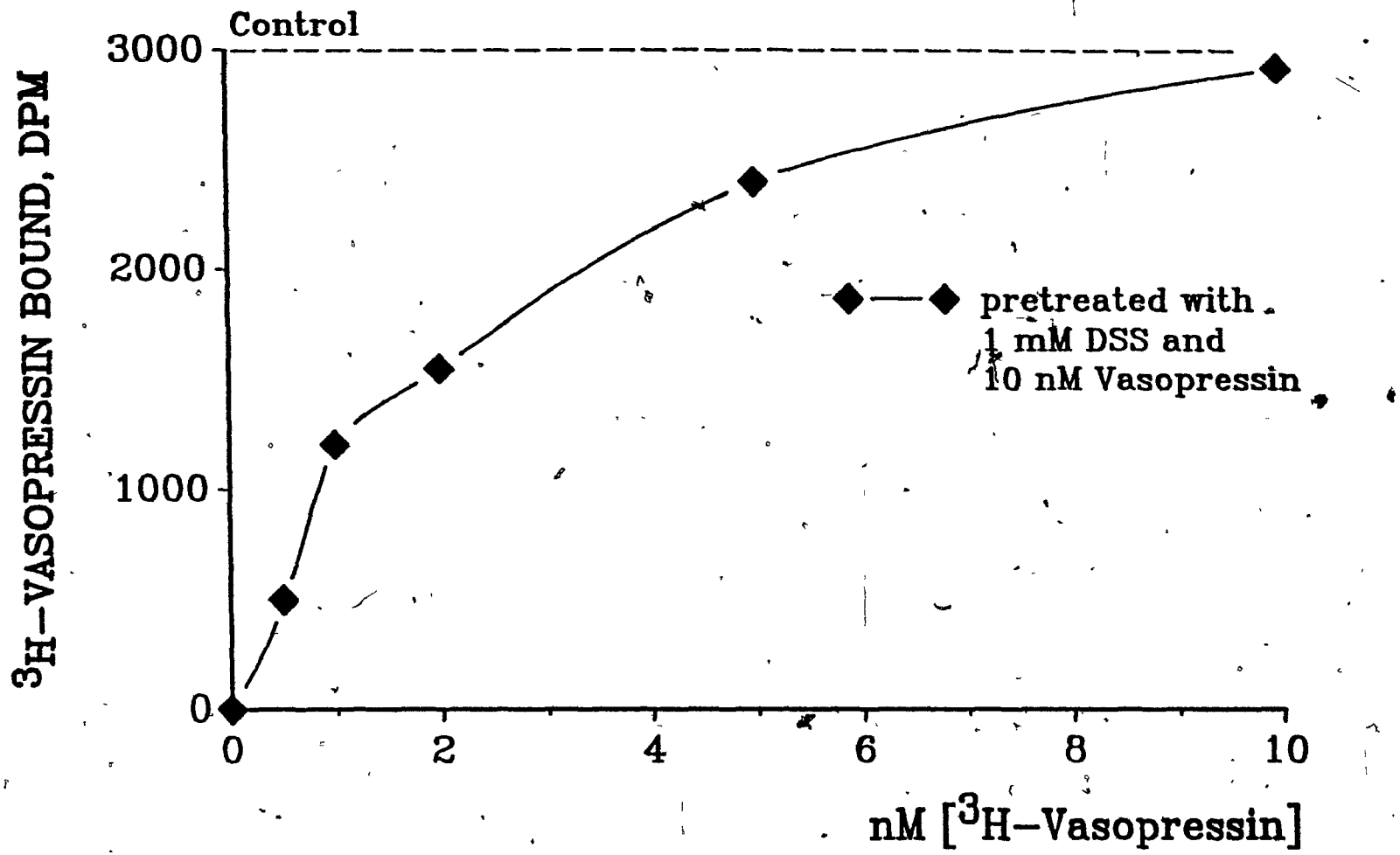

Control
3000
2000
1000
0
0
2
4
6
8
10
^{3}H–VASOPRESSIN BOUND, DPM
pretreated with
1 mM DSS and
10 nM Vasopressin
nM [^{3}H–Vasopressin]

FIGURE 15. Cross-linking of agonist-activated vasopressin V_1 receptors with disuccinimidyl suberate in isolated liver cells.

Rat liver cells were isolated and glycogen phosphorylase activity was determined as described in Section 2.2.1 and 2.2.2. The hepatocytes were suspended at concentration 20 mg wet cells/ml and incubated with 10^{-7}M arg^{8}vasopressin for 2 min at 37°C. Following this, they were treated with DSS for 3 min, the reaction was quenched with 10 mM ammonium acetate and the cell were washed two times and resuspended in fresh medium. At the indicated intervals, samples were removed for the determination of the activity of glycogen phosphorylase. Treatments: *A)* 1% DMSO; *B)* 1 mM DSS; *C)* 10^{-7}M arg^{8}vasopressin + 1%DMSO; *D)* 10^{-7}M arg^{8}vasopressin + 1mM DSS. Basal glycogen phosphorylase activity was 0.5 ÷ 0.2 U (nmol [^{14}]C glucose incorporated into glycogen/min/mg wet cells). Points indicate mean + SEM of experiments made with cells isolated from three different livers.

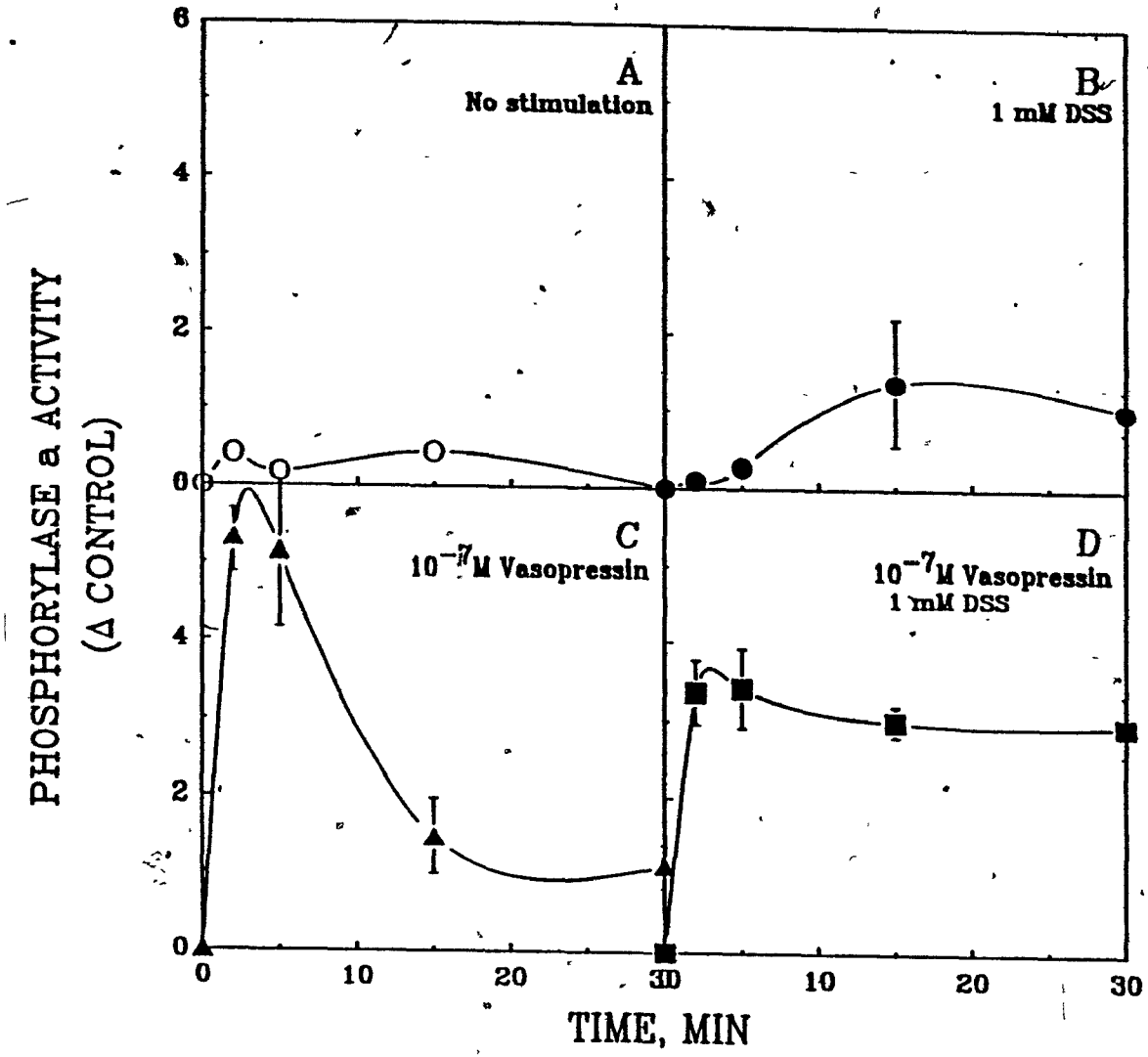

A
No stimulation
B
1 mM DSS
C
10⁻⁷M Vasopressin
D
10⁻⁷M Vasopressin
1 mM DSS
PHOSPHORYLASE a ACTIVITY
(Δ CONTROL)
TIME, MIN
0
2
4
6
0
10
20
30
10
20
30

FIGURE 16. Cross-linking of activated α_1-adrenergic receptors with disuccinimidyl suberate in isolated liver cells.

Rat liver cells were isolated and glycogen phosphorylase activity was determined as described in Section 2.2.1 and 2.2.2. The hepatocytes were suspended at concentration 20 mg wet cells/ml and incubated with 10^{-6}M adrenaline for 2 min at 37°C. Following this, they were treated with DSS for 3 min, the reaction was quenched with 10 mM ammonium acetate and the cells were washed two times and resuspended in fresh medium. At the indicated intervals, samples were removed for the determination of the activity of glycogen phosphorylase. *A)* 1% DMSO. *B)* 1 mM DSS. *C)* 10^{-6}M adrenaline + 1%DMSO. *D)* 10^{-6}M adrenaline + 1mM DSS. Basal glycogen phosphorylase activity was 0.45 ± 0.15 U (nmol [^{14}]C glucose incorporated into glycogen/min/mg wet cells). Points indicate mean ± SEM of three separate experiments.

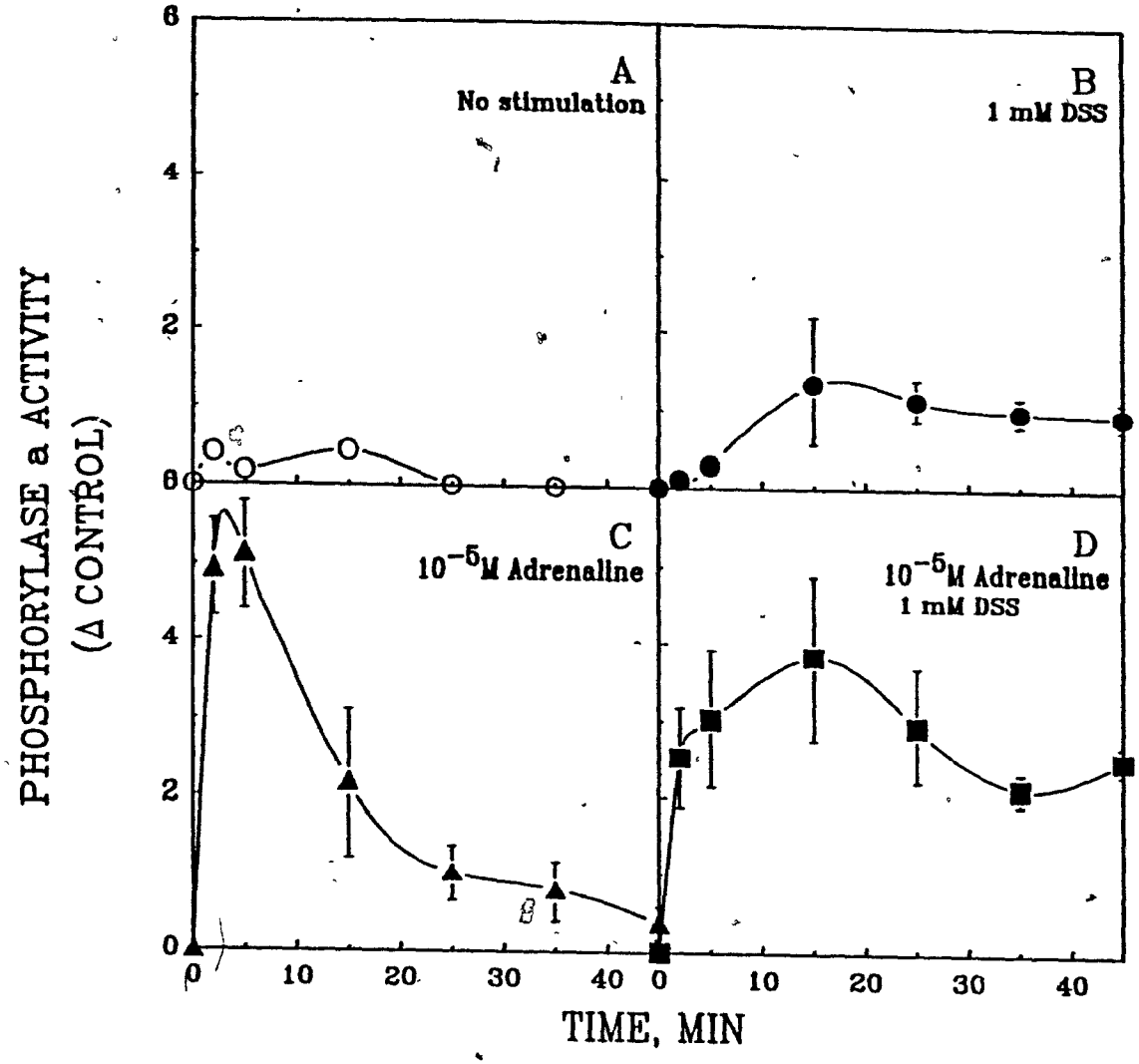
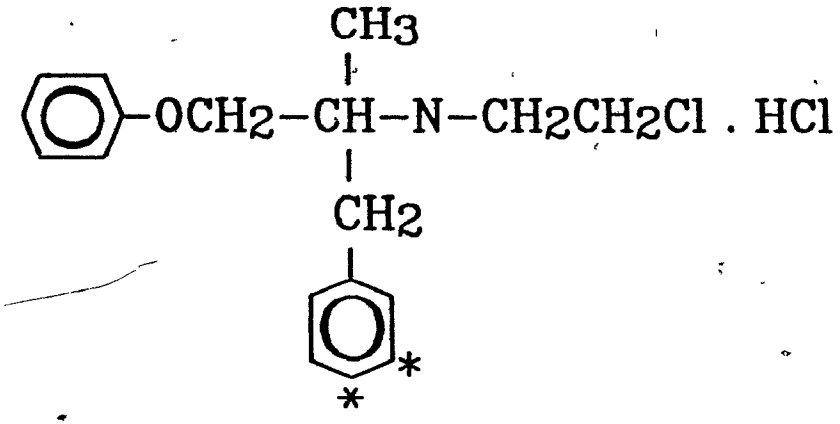
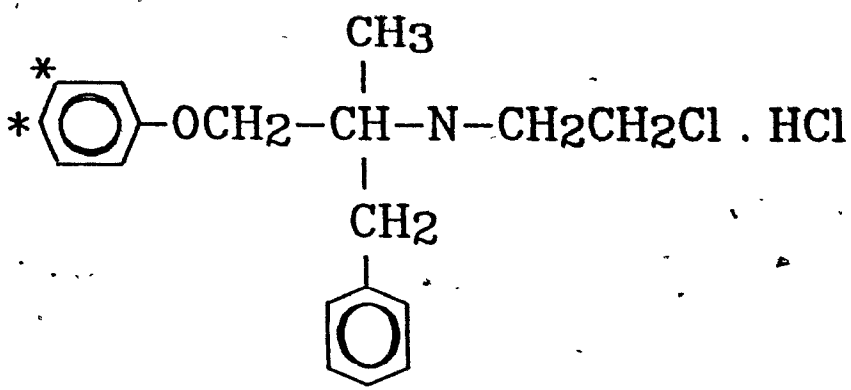

A
No stimulation
B
1 mM DSS
C
10⁻⁵M Adrenaline
D
10⁻⁵M Adrenaline
1 mM DSS
PHOSPHORYLASE a ACTIVITY
(Δ CONTROL)
TIME, MIN
0
2
4
6
0
10
20
30
40

FIGURE 17. Tritiated derivatives of phenoxybenzamine.

For explanations, see Section 3.3.3.1.



[^{3}H]Phenoxybenzamine HCl (Kan et al., 1979)
Sp. activity 5–18 Ci/mmol



[Phenoxy–^{3}H(N)]–Phenoxybenzamine HCl (NEN)
Sp. activity 25–55 Ci/mmol

FIGURE 18. Effect of I-phenoxybenzamine on the α_1-adrenergic stimulation of glycogen phosphorylase in isolated liver cells.

Rat liver cells were isolated and glycogen phosphorylase activity was determined as described in Section 2.2.1 and 2.2.2. The hepatocytes were suspended at a concentration of 20 mg wet cells/ml and incubated for 20 min at 37°C with the indicated concentrations of I-POB. Following this, the cells were washed twice and resuspended in fresh buffer. The cells were incubated for 2 min with 10^{-6}M adrenaline and the activity of glycogen phosphorylase was determined. Data are expressed as percentage of the activation, obtained by 10^{-6}M adrenaline in the absence of I-POB (6.7 ± 0.2 U). Points represent the mean of three experiments.

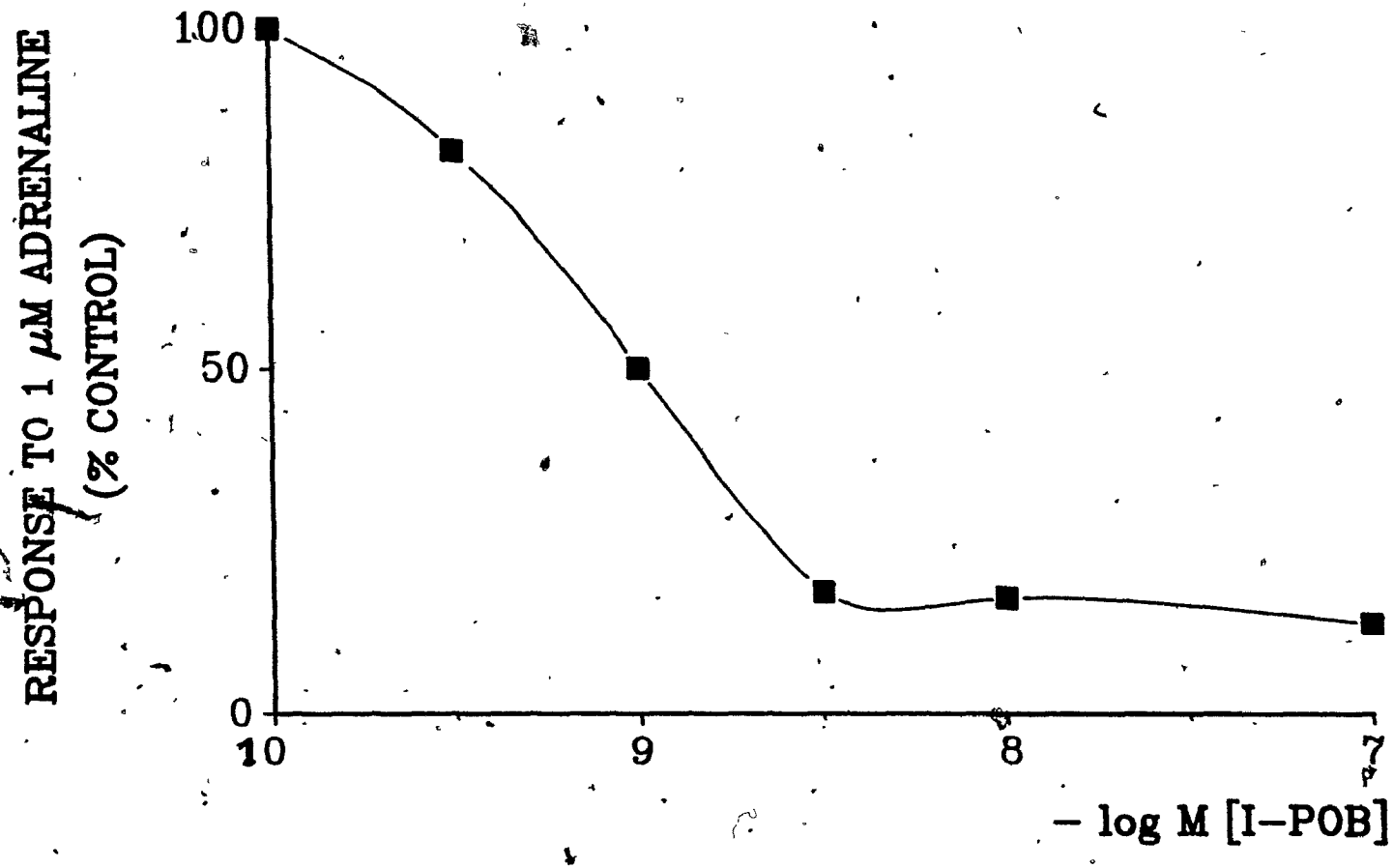

RESPONSE TO 1 μM ADRENALINE
(% CONTROL)
100
50
0
10
9
8
7
– log M [I–POB]

FIGURE 19. Determination of the binding affinity of I-phenoxybenzamine in rat liver plasma membranes.

Rat liver plasma membranes were suspended in 50 mM Tris/1 mM $MgCl_2$, pH 7.4 buffer at 0.2 mg/ml concentration. The preparations were preincubated for 20 min at 37°C with the indicated concentrations of antagonists and the binding of [^{3}H]Prazosin (0.4 nM final concentration) was determined (see Section 2.2.8.2). Dashed line, open circles, prazosin; dashed-dotted line, open triangles, phenoxybenzamine; solid line, filled squares, I-phenoxybenzamine. The results are expressed as percentage of the binding of [^{3}H]Prazosin in the absence of competitor (0.2 pmols/mg). Points represent the mean of three experiments.

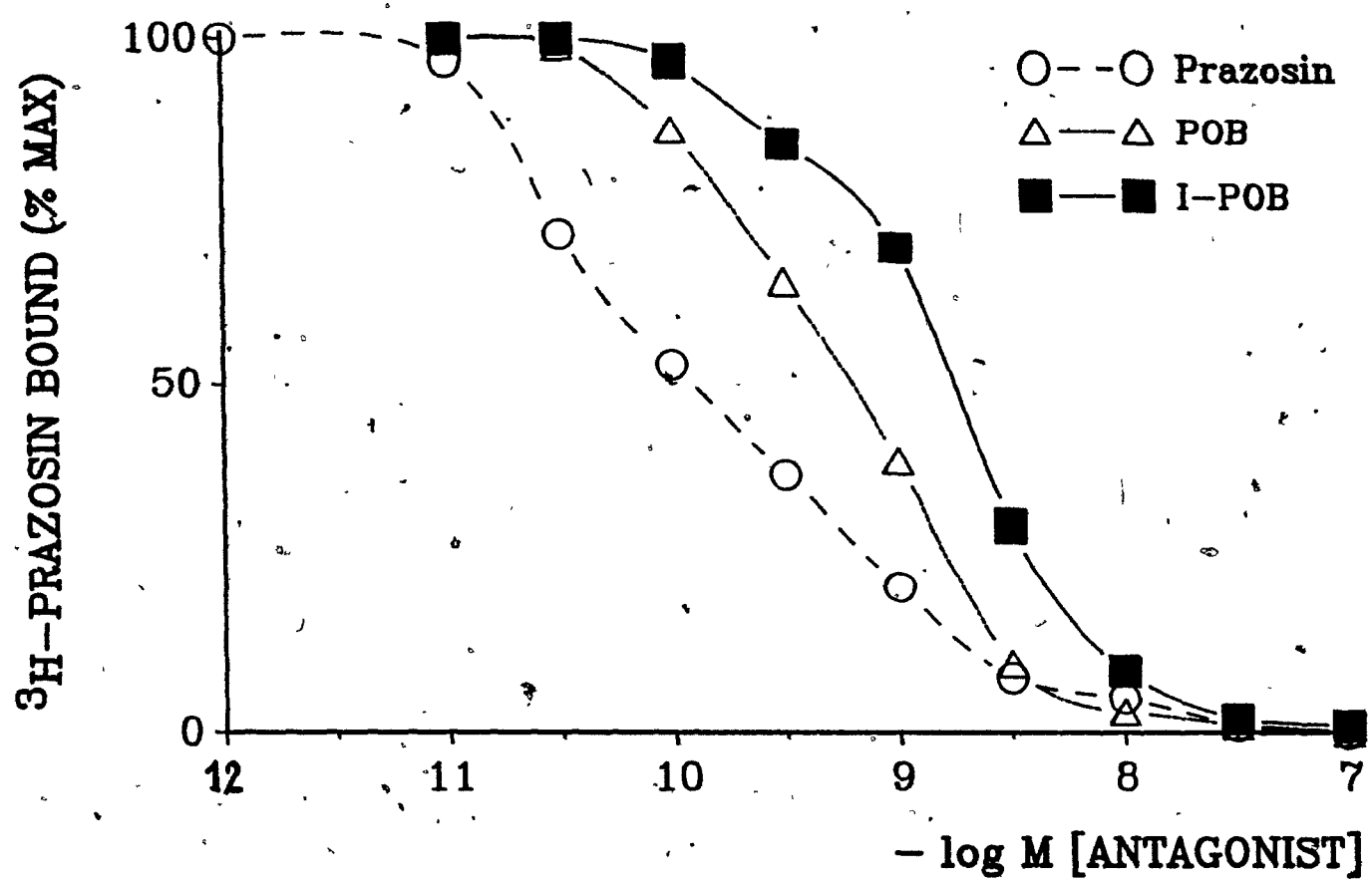

100
50
0
12
11
10
9
8
7
^{3}H–PRAZOSIN BOUND (% MAX)
– log M [ANTAGONIST]
Prazosin
POB
I–POB

FIGURE 20. Antagonism of vascular α_1-adrenergic receptors in the rat by I-phenoxybenzamine.

The experiment was done in male SD rats (weight 500 - 560 g). The animals were anesthetized with pentobarbital (40 mg/kg) and the right femoral vein and artery were cannulated. The arterial cannula was connected to a pressure transducer and the blood pressure (BP) and the heart rate (HR) were monitored. Phenylephrine (PhE), arg^{8}vasopressin (AVP) and I-phenoxybenzamine (I-POB), dissolved in saline, were injected through the venous cannula. The graph shows the actual blood pressure and heart rate recordings and is representative for two experiments. Five divisions of the grid are equal to 1 min.

EFFECT OF I–POB ON THE PRESSOR RESPONSE TO PhE AND AVP

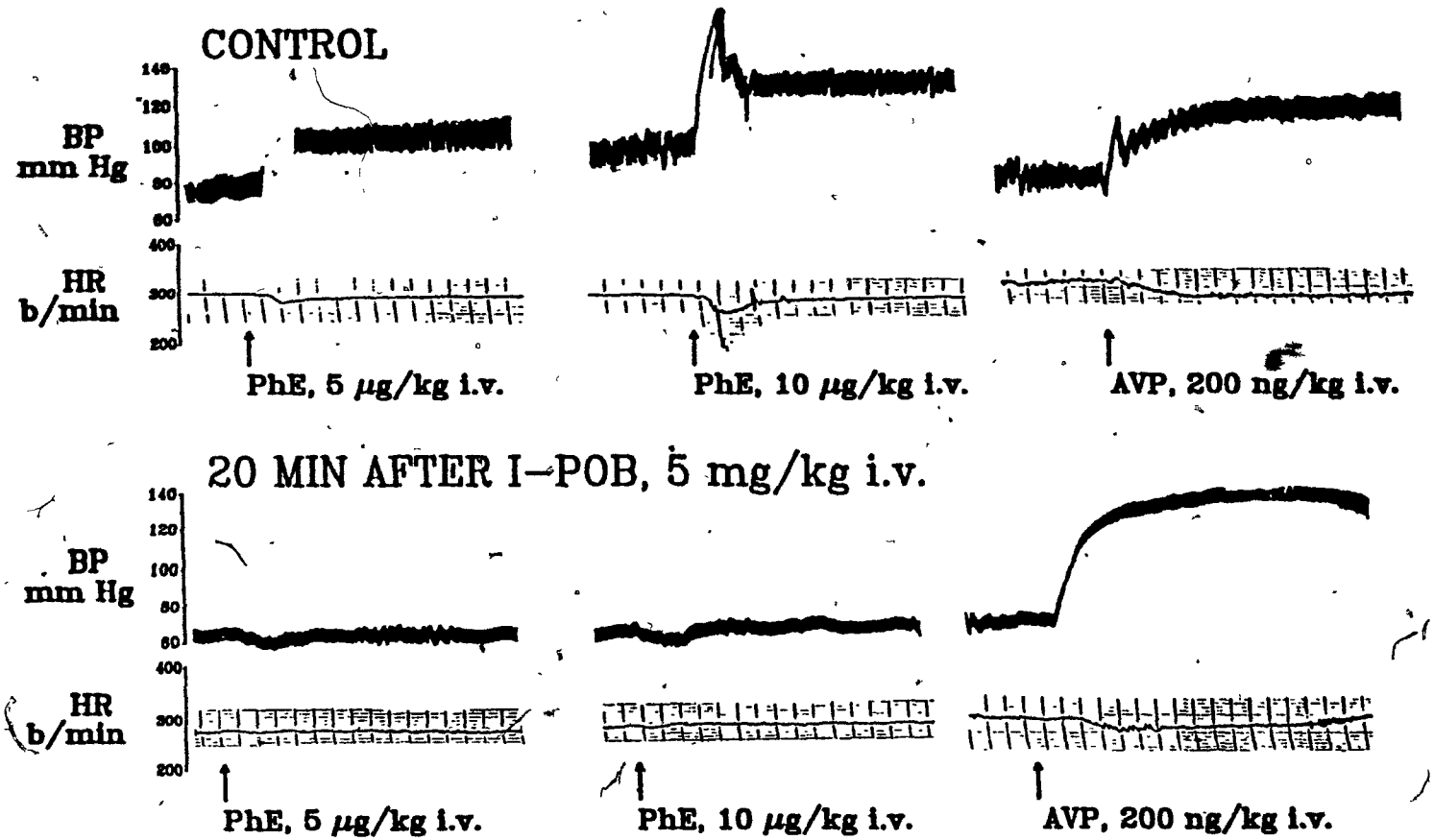


FIGURE 21. Time-course of the block by rabbit antiserum of the activation of glycogen phosphorylase by phenylephrine in isolated hepatocytes.

Rat liver cells were isolated as described in Section 2.2.1. Hepatocytes (10 mg wet weight/ml), were incubated with rabbit antiserum, diluted 1:10 in Krebs-Henseleit buffer. At the indicated time periods, the cells were stimulated with 10^{-5}M phenylephrine and the activity of glycogen phosphorylase was determined (see Section 2.2.2). The results are expressed as percentage of the activation, obtained with 10^{-5}M phenylephrine in cells, incubated with 1:10 dilution of pre-immune rabbit serum (5.8 + 0.2 U). The basal activity of glycogen phosphorylase was 0.8 ± 0.2 U. The graph is the mean of three experiments.

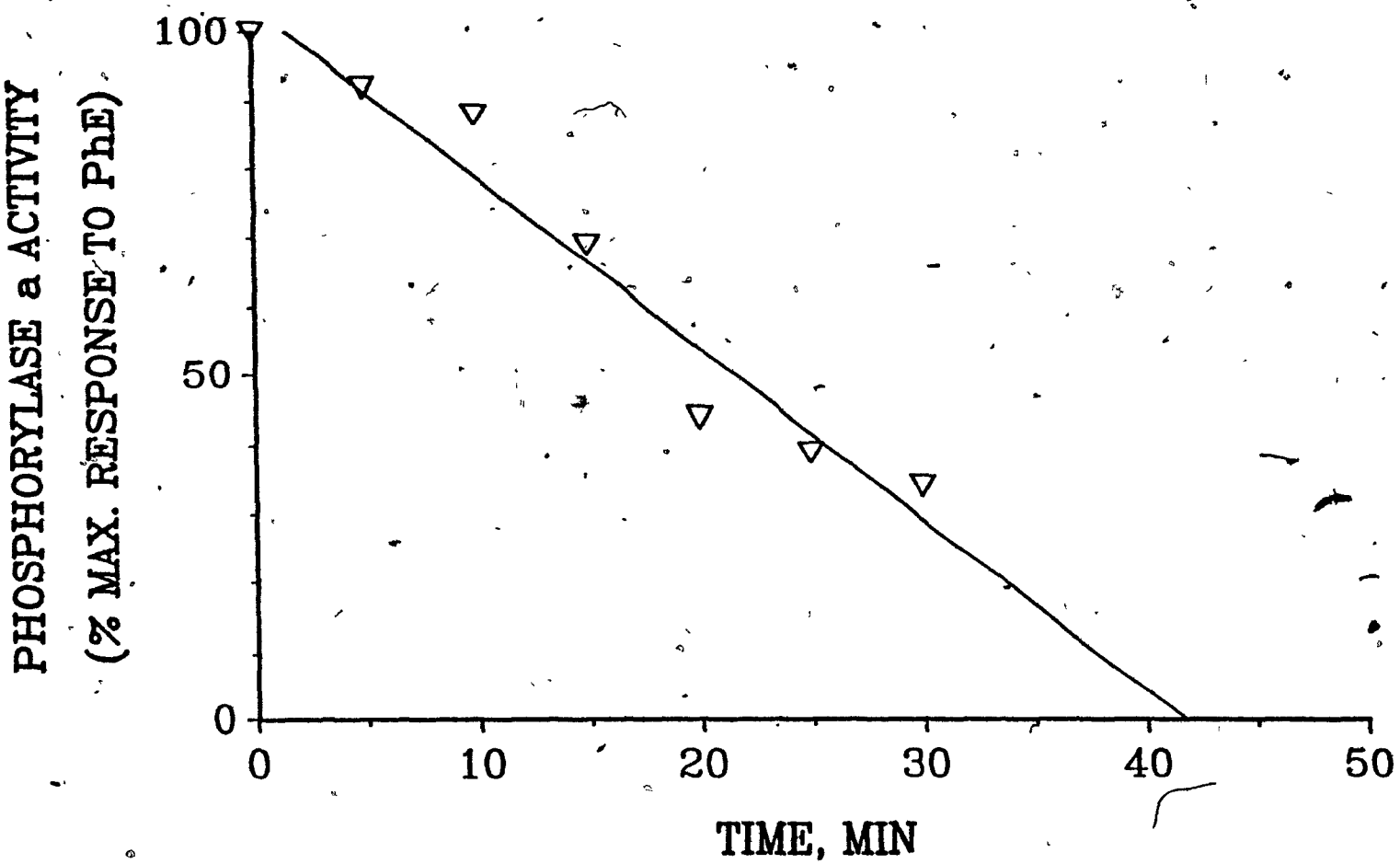

PHOSPHORYLASE a ACTIVITY
(% MAX. RESPONSE TO PhE)
100
50
0
0
10
20
30
40
50
TIME, MIN

FIGURE 22. Effect of rabbit antiserum on the α_1-adrenergic activation of glycogen phosphorylase in isolated hepatocytes.

Hepatocytes were isolated as described in Section 2.2.1. The cells (10 mg wet weight/ml) were incubated for 30 min with 1:10 dilution of untreated (*open columns*) and preadsorbed (*hatched column*) rabbit serum. The preadsorbtion of rabbit serum was done by incubation with 4 mg plasma membranes/1 ml serum for 30 min at 37°C, following which the plasma membranes were pelleted by centrifugation and the serum was used for the treatment of the liver cells. After the treatment with rabbit serum, the cells were stimulated for 2 min with 10^{-5}M phenylephrine and the activity of glycogen phosphorylase was determined (see Section 2.2.2). The results are expressed as multiples of the basal activity of glycogen phosphorylase in the presence of 1:10 dilution of pre-immune rabbit serum (0.8 ± 0.2 U). Vertical bars indicate mean ± SEM of three experiments. * = $p < 0.05$ *versus* pre-immune serum.

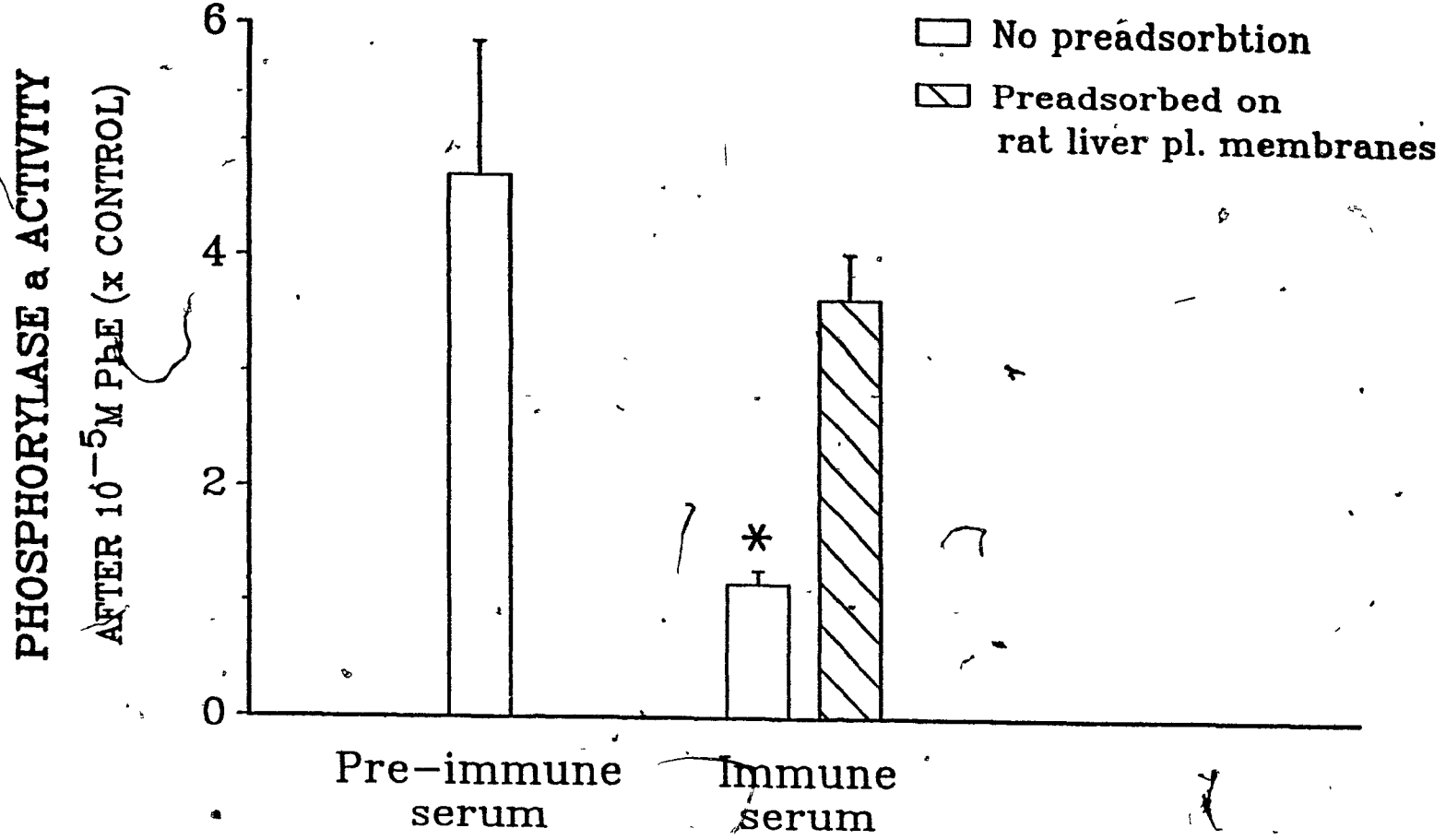

PHOSPHORYLASE a ACTIVITY
AFTER 10^{-5}M PhE (x CONTROL)
6
4
2
0
No preadsorbtion
Preadsorbed on
rat liver pl. membranes
*
Pre-immune
serum
Immune
serum

FIGURE 23. Selective block by rabbit antiserum of the activation of glycogen phosphorylase through the α_1-adrenergic receptor in isolated hepatocytes.

Hepatocytes were isolated and the activity of glycogen phosphorylase was determined as described in Sections 2.2.1 and 2.2.2. The cells were incubated with 1:10 dilution of pre-immune rabbit serum (*broken lines, open symbols*) and with rabbit antiserum (*solid line, filled symbols*) as indicated in fig. 22. At the end of the incubation, the cells were stimulated with the indicated concentrations of phenylephrine (PhE) and arg^{8}vasopressin (AVP) for two min and the activity of glycogen phosphorylase was measured. The results are expressed as multiples of the basal activity of glycogen phosphorylase (0.5 ± 0.1 U). The experiment, shown in the graph, is representative for a group of three experiments.

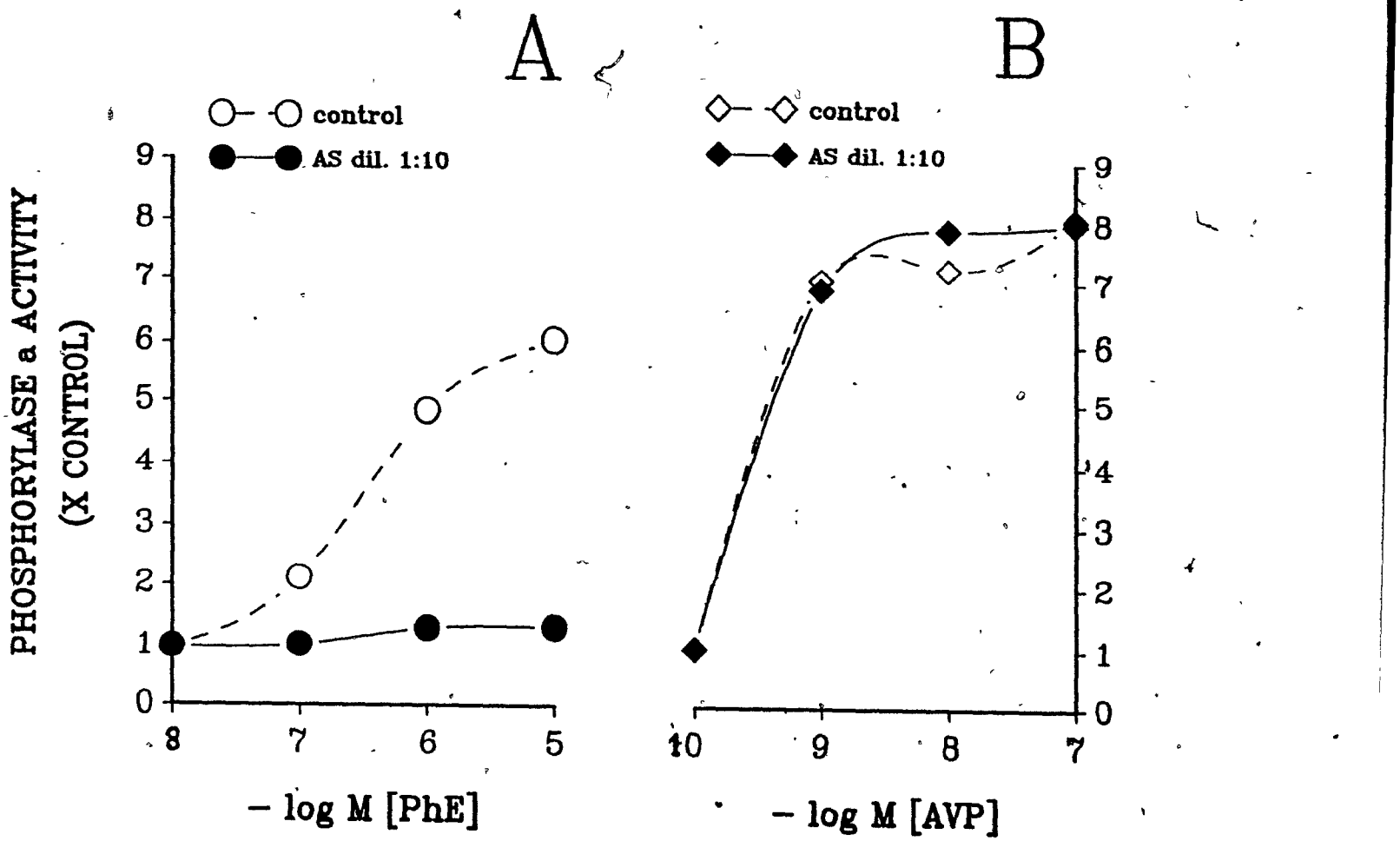

A
B
control
AS dil. 1:10
control
AS dil. 1:10
PHOSPHORYLASE a ACTIVITY
(X CONTROL)
0
1
2
3
4
5
6
7
8
9
8
7
6
5
10
9
8
7
– log M [PhE]
– log M [AVP]

FIGURE 24. Interaction of rabbit antiserum with SDS polyacrylamide gel electrophoresis-purified α_1-adrenergic receptor.

Bottom: Rat liver plasma membranes were labeled with [^{3}H]POB in the absence (T) and in the presence of 0.1 µM Prazosin (NS), solubilized with SDS-PAGE sample buffer and electrophorezed on 10% polyacrylamide gel as described in Section 2.2.4. The gel was sliced and the radioactivity counted (see Section 2.2.4). Specific binding (cpm T - cpm NS) is shown on the x-axis. The determination of the *M* was made by calibration proteins, run on the same gel (not shown). *Top:* SDS-PAGE of unlabeled (A and B) and POB-labeled (100 nM, see Section 2.2.8.1; C) rat liver plasma membranes, made on the same gel. The gel was transblotted onto a nitrocellulose paper and the strips corresponding to A, B, and C were cut, incubated with rabbit antisera and the antigen-antibody interactions were visualized as described in Section 2.2.5. Strips A and C were incubated with untreated rabbit antiserum (dil. 1:10), whereas strip B was incubated with rabbit antiserum, preadsorbed (see fig. 22) on plasma membranes pretreated with 100 nM POB. The graph is representative for two experiments.

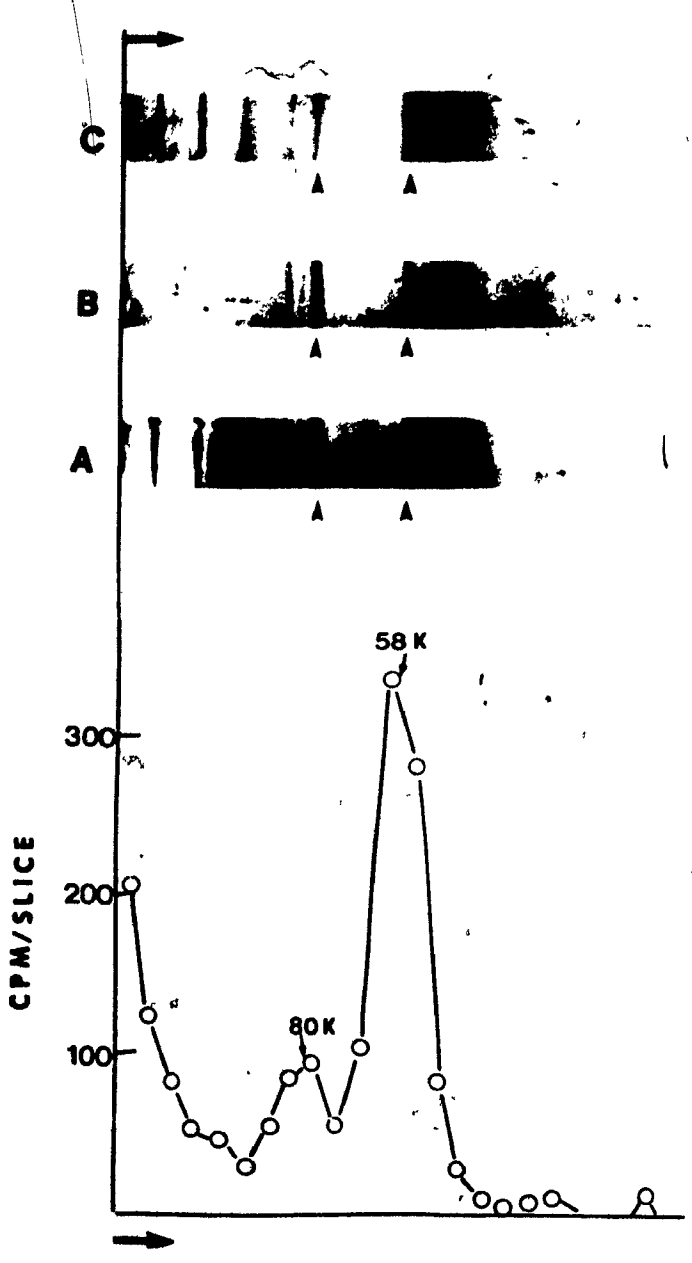

C
B
A
58 K
80 K
300
200
100
CPM/SLICE

FIGURE 25. SDS-polyacrylamide gel electrophoresis of [^{3}H]POB-labeled α_1-adrenergic receptor, partially purified by gel filtration and WGA-affinity chromatography.

Rat liver plasma membranes were labeled with [^{3}H]POB, solubilized with Triton X-100 and the α_1-adrenergic receptor was partially purified by gel filtration and WGA-affinity chromatography (see Section 2.2.8.1, fig. 8 and 9). The SDS-PAGE was made on a 7.5% polyacrylamide gel and the gel was sliced and counted (see Section 2.2.4). *Inset:* estimation of the M_r of the [^{3}H]POB-labeled α_1-adrenergic receptor (*arrow*). Points are standards with known molecular weight (rabbit muscle phosphorylase b, BSA, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, lysozyme). R_f = distance migrated by the substance/distance migrated by the tracking dye. The recovery of total radioactivity was 75%.

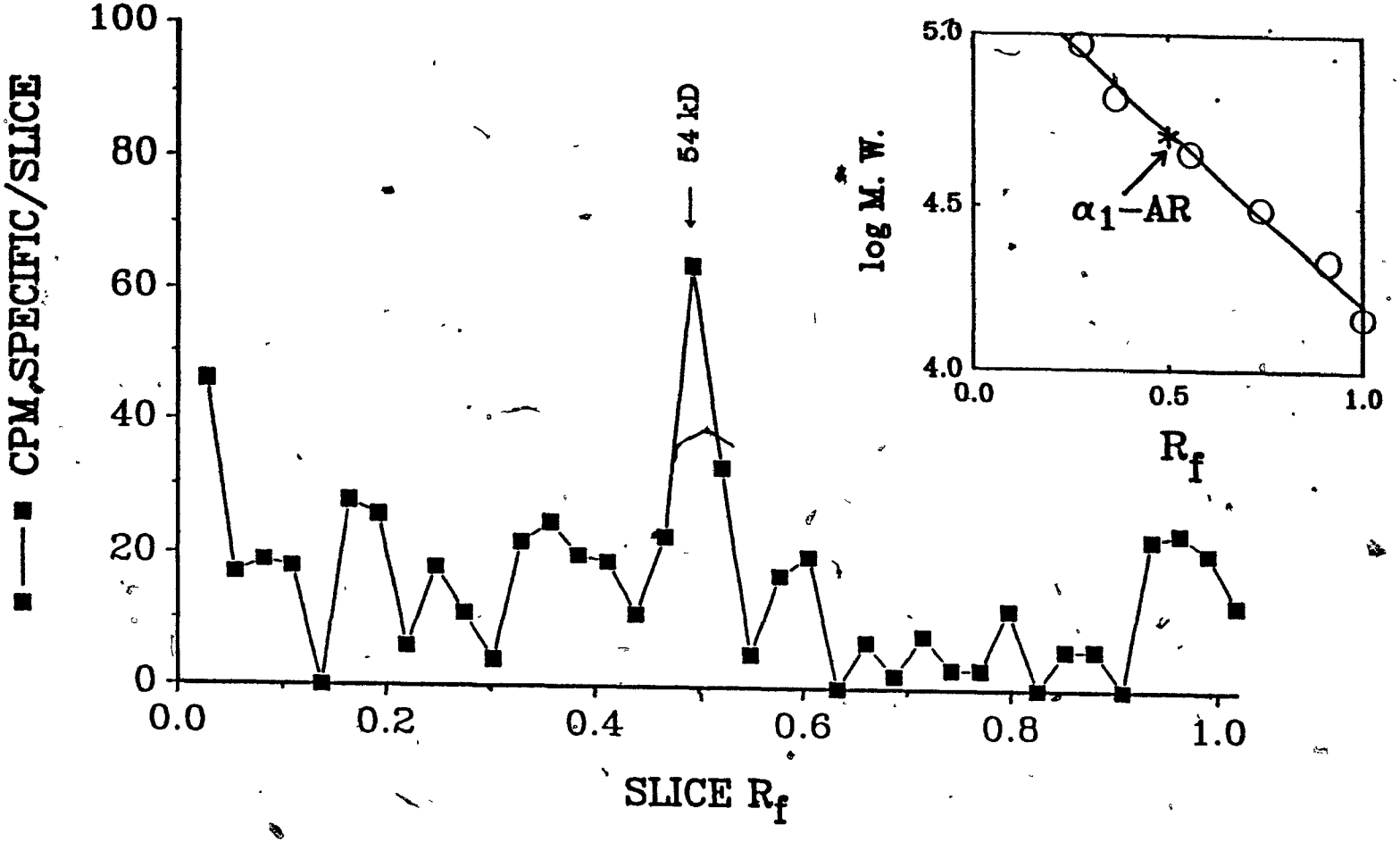

100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
CPM,SPECIFIC/SLICE
54 kD
SLICE R_f
log M. W.
5.0
4.5
4.0
0.0
0.5
1.0
α_1–AR
R_f

TABLE 1. Effect of prolonged in vitro incubation of rat liver calls on the density and affinity of α_1- and β-receptor binding sites.

Rat liver plasma membranes were prepared from isolated hepatocytes by the method of Neville (see Section 2.2.3). Crude cell homogenate was obtained by homogenizing the cells in 1 mM $NaHCO_3$/1 mM EGTA and two times centrifugation at 5000 x g for 10 min. For the binding assay, the crude homogenate and the plasma membranes were suspended in 50 mM Tris/ 4 mM $MgSO_4$ buffer (pH 7.4 for the assay of the α_1- and pH 8.0 for the assay of the β-receptors). α_1-Receptors were assayed with [^{3}H] razosin and β-receptors with [^{3}H]CGP-12177 (see Sections 2.2.8.2 and 2.2.8.3). Specific binding of [^{3}H] razosin (0.05 - 1.0 nM) was 90-75% in crude homogenates and 95-85% in purified membranes. Specific binding of [^{3}H]CGP-12177 (0.25 - 4 nM) was 75-50% in homogenates and 72% at 2 nM in plasma membranes. In each liver, binding was tested in both 0-hr and 4-hr cells. Values are given ± SEM. By the paired *t* test, none of the B_{max} or K_d values were significantly different between the two treatment groups. The total number of livers tested for each receptor was six with crude homogenate and five with plasma membranes.

*Due to the small amount of purified membranes (6 - 8 mg per treatment group per liver), β-receptor binding could be assayed at only a single concentration, 2 nM.

Table 1

Cells	Crude homogenate		Plasma membrane	
	B_{max}, fmol/mg	K_d, pM	B_{max}, fmol/mg	K_d, pM
	Alpha$_1$-adrenoceptors			
0-hr	84.1 ± 17.1	104 ± 22	330.4 ± 96.0	83 ± 24
4-hr	65.4 ± 11.0	99 ± 28	325.8 ± 87.2	102 ± 27
	Beta-Adrenoceptors			
0-hr	8.55 ± 0.98	700 ± 100	11.20 ± 1.65*	
4-hr	7.96 ± 1.42	800 ± 300	10.90 ± 1.90*	

TABLE 2. Summary of partial purification of the α_1-adrenergic receptor from rat liver.

For explanations, see fig. 8 and 9 and Section 3.2.1.

Table 2

Step	Protein	Activity	Specific activity	Yield		x-fold Purification	
				Step	Overall	Step	Overall
	mg	pmol	pmol/mg protein				
Pl. membranes	914.5	192.4	0.21	100	100	1	1
Triton extract	712.1	187.0	0.26	77.8	77.8	1.2	1.2
Gel filtration	282.5	137.9	0.49	39.6	31.0	1.9	2.2
WGA eluate	2.7	46.3	17.1	0.9	0.3	34.9	81.4

SECTION 4: DISCUSSION

4.1 REGULATION OF THE α_1- TO β- CONVERSION OF THE ADRENERGIC CONTROL OF GLYCOGENOLYSIS IN RAT LIVER: A ROLE FOR PHOSPHOLIPASE A_2

The rapid reciprocal change in the coupling of α- and β-adrenergic receptors to the activation of glycogen phosphorylase indicates that they share a common regulatory component, or are affected simultaneously by a regulatory system in the cell membrane, which is triggered by the 4-hour period of culturing. Our data suggest that in freshly isolated hepatocytes the activity of membrane phospholipase A_2 is suppressed by a lipomodulin-like substance, and that 4-hour incubation increases phospholipase A_2 activity. The finding that the antibody against lipomodulin was able to promote the α to β conversions only when α_1-adrenergic receptors were simultaneously stimulated, could suggest a synergistic interaction between these two stimuli. Indeed, plasma membrane phospholipase A_2 was shown to be activated through α_1-adrenergic receptor in certain cell types (Burch *et al.*, 1986), and there are reports that prolonged incubation of isolated hepatocytes with isoproterenol prevents the time-dependent shift from α- to β-controlled glycogenolysis (Okajima & Ui, 1982; Refsnes *et al.*, 1983). This could mean that α_1- and β-adrenergic receptors have opposite regulatory influences on membrane phospholipase A_2 activity. In our experiments, however, the modulation of the activity of phospholipase A_2 is triggered through a different, still unidentified mechanism, since adrenergic agonists are absent from the medium. Some experimental data indicate that a protein factor might be involved in the interconversion phenomenon. Results from several laboratories indicate

that the conversion of the adrenergic response of isolated hepatocytes is prevented by protein synthesis inhibitors (Okajima & Ui, 1982; Nakamura *et al.*, 1983; Ishac & Kunos, 1986). Data, obtained in our laboratory indicate that incubation of freshly isolated hepatocytes with the serine protease inhibitor phenylmethylsulfonylfluoride mimics the interconversion process, and therefore a serine protease, active in freshly isolated hepatocytes, might be responsible for the suppression of membrane phospholipase A_2 activity (Kunos & Ishac, 1987). Our observation (Table 1), that *in vitro* incubation of hepatocytes for 4 hours in a serum-free medium produces no change in receptor densities (later confirmed by Schwarz *et al.*, 1985; Tsujimoto *et al.*, 1986), is in contrast with other studies, reporting reciprocal changes in the density of α_1- and β-adrenergic receptors after primary culturing (Nakamura *et al.*, 1984; Schwarz *et al.*, 1985). However, the time course of the change is much longer (1 to 2 days) than the time course of the altered receptor response and it seems to require both the plating of cells and the presence of serum (Kunos & Ishac, 1987). Thus, it appears that the changes in receptor density are the consequence, rather the cause, of the primary change in receptor coupling.

The mechanism by which phospholipase A_2 activation may influence the coupling of hepatic adrenergic receptors is not yet elucidated. Increased activity of membrane phospholipase A_2 generates lysophosphatidylcholine, a powerful endogenous detergent, and arachidonic acid, which is further metabolized into eikosanoids. The role of an arachidonic acid metabolite in the altered adrenergic response is suggested by the findings of Ishac & Kunos (1986). Incubation of freshly isolated hepatocytes with

arachidonic acid suppressed α_1-adrenergic receptor-controlled glycogenolysis and increased the β-adrenergic response. Conversely, removal of free fatty acids from the incubation medium by addition of fatty acid-free bovine serum albumin throughout the incubation period prevented the interconversion, and a cyclooxygenase inhibitor, ibuprofen, prevented the effects of arachidonic acid (Ishac & Kunos, 1986). This suggests that a cyclooxygenase product is involved in the inverse changes in the coupling of α- and β-receptors, although the mode of action of this product is not yet known.

4.2 BIOCHEMICAL AND IMMUNOLOGICAL APPROACHES IN THE CHARACTERIZATION OF THE RAT LIVER α_1-ADRENERGIC RECEPTOR

4.2.1 PARTIAL PURIFICATION OF THE HORMONE-BINDING SUBUNIT OF THE RECEPTOR

Affinity chromatography is the most efficient method for purification of hormone receptors. However, the method requires solubilization of the receptor in an active state, which we failed to achieve. Therefore, we decided to attempt partial purification of the hormone-binding subunit of the receptor, previously labeled with the irreversible ligand [^{3}H]POB. After a limited tryptic digestion of this material, we planned to purify the [^{3}H]POB-labeled fragment and to sequence it. This would have permitted us to obtain important structural information about a part of the

active center of the receptor. Triton X-100, a potent non-ionic detergent, was chosen for the solubilization of the [^{3}H]POB-labeled α_1-adrenergic receptor (Fig. 6). The first step in the purification was preparative gel filtration, which provides limited purification of the receptor, but separates it from cell membrane lipids and substantial amounts of low-molecular weight proteins. For the next step, we used lectin-affinity chromatography, based on the findings of Meier *et al.*, (1984), who showed that the α_1-adrenergic receptor in MDCK epithelial cell line is a glycoprotein with a carbohydrate specificity of N-acetyl-glucosamine. Our pilot experiments demonstrated that the α_1-adrenergic receptor from rat liver has identical carbohydrate specificity and is retained by WGA-Sepharose columns (Fig. 9). Lectin-affinity chromatography is also compatible with the use of non-ionic type of detergents (Lotan *et al.* (1977). The disadvantages of this type of purification resides in the low efficiency of binding of glycoproteins to lectins, and the similar carbohydrate specificity of different hormone receptors (Hedo, 1984). Indeed, the purification after this step was only 80 fold (Table 2). The SDS-PAGE analysis of the partially purified m.w. of the [^{3}H]POB-labeled α_1-adrenergic receptor yielded a single band of approximately 54,000 daltons (Fig. 25). This results is different from earlier findings of two specifically labeled peptides of M_r 80,000 and 58,000, detected by SDS-PAGE analysis of Sephadex-purified, [^{3}H]POB-labeled preparations (Kunos *et al.*, 1983; also fig. 8). It is generally thought that the lower m.w. peak is a proteolytic product of of the 80,000 m.w. intact receptor (Kunos *et al.*, 1983; Leeb-Lundberg *et al.*, 1984; Venter *et al.*, 1984) and the present results suggest that, despite

the presence of several protease inhibitors, it was not possible to prevent proteolytic degradation of the receptor. This might have occurred at the step of the WGA-affinity chromatography, which was done at room temperature. Therefore, the presence of several protease inhibitors is of limited help, and all the purification steps have to be done at 4°C. Nevertheless, the product of proteolysis still contained the ligand binding region, making further processing possible. The product of the WGA-affinity chromatography was digested by TPCK-treated trypsin, and the proteolytic fragments separated by RP HPLC, as described by Mayes (1984). Trypsin, a serine endopeptidase, cleaves the peptide bonds at the carboxyl side of lysine and arginine. It has the most restricted specificity of action of all known endopeptidases and is well characterized as a tool for the mapping of peptides (Bender & Kezdy, 1965; Blow *et al.*, 1969). Limited proteolysis of membrane-bound α_1-adrenergic receptor with trypsin, chymotrypsin and papain have yielded water-soluble peptides with sizes of 58,000 to 18,000 daltons (Venter *et al.*, 1984). Despite the long digestion period, this method of proteolysis released limited amount (30%) of the radioactive label. This might be due to incomplete unfolding of the protein. The [^{3}H]POB-labeled peptide obtained by these methods had a m.w. of less than 1084 daltons (Fig. 12), and an attempt to determine its amino acid sequence was unsuccessful. The small size of the fragment obtained suggests also that the use of of other proteolytic methods, known to produce bigger fragments, such as CNBr digestion, after carboxymethylation of the preparation, is more suitable for the peptide mapping of the [^{3}H]POB-labeled α_1-adrenergic receptor.

4.2.2 MECHANISM OF ACTIVATION OF THE Ca^{2+}-LINKED RECEPTORS IN LIVER

Our experiments demonstrate that sequential treatment of isolated hepatocytes by an agonist (adrenaline or vasopressin), and the covalent cross-linking agent, DSS, results in a "permanent" activation of glycogen phosphorylase, which is maintained after the removal of the agonist and the cross-linker. We found that the cross-linking does not immobilize a significant amount of either type of agonist on the active center of the receptor, and the latter remains free for binding of receptor-specific ligands.

DSS is a homobifunctional ester of *N*-hydroxysuccinimide, with a span of 11 Å. It is an irreversible cross-linker. Since it is a hydrophobic molecule, it would be expected to react preferentially with amino groups embedded in the membrane, the low dielectric constant of which would favor deprotonation and increased reactivity of the available groups. Cross-linking by a bifunctional reagent may be considered a sequential two-step reaction in which the cross-linker first attaches itself at random to a constituent of the cell by means of one of its functional groups, and then reacts with a neighboring component, producing a covalent link between them. Only the first step of this reaction is dependent on the concentration of free cross-linker. It has been found, in experiments with neutrophils, that cross-linking with 0.4 - 1 mM DSS and other homobifunctional esters of *N*-hydroxysuccinimide has a mild effect and only influences a small number of cellular functions, without interfering with the cell's viability (Aviram *et al.*, 1984). Interestingly, the cross-linking interfered mostly with the functions triggered by the

activation of the fMet-Leu-Phe receptor, but not with the binding of fMet-Leu-Phe to the receptor. Cross-linking had no effect on transmembrane channels such as the glucose transporter, on membrane-bound enzymes such as the Mg^{2+}ATPase and alkaline phosphatase, or on various enzyme functions in neutrophils. In the same study, it was found that DSS and similar cross-linkers interact with only a small number (6.4%) of the available amino groups, from which 22% are located in the plasma membrane. The mildness of the DSS effect was corroborated by the lack of gross changes in the SDS-PAGE pattern of cross-linked cells (Aviram *et al.*, 1984). This is further supported by our observation, that DSS alone has only marginal effect on the glycogen phosphorylase activity in isolated hepatocytes.

Since the treatment of isolated hepatocytes with DSS in the presence of α_1-adrenergic and vasopressin V_1 receptor agonists results in a prolonged activation of at least one of their target enzyme, the glycogen phosphorylase, it is reasonable to suppose that this treatment results in stabilization of an agonist-induced active conformation of these receptors, and that this form of the receptors is able to activate the glycogen phosphorylase after the agonist dissociates from the complex. This hypothesis is further supported by the similarity in the effect of the cross-linking on two different receptor systems: the α_1-adrenergic receptor and the vasopressin V_1 receptor.

In this case, two alternatives have to be considered. One possibility is that the cross-linker exerts mainly an intra-molecular action, which results in the stabilization of an agonist-induced active conformation of the receptor. A second alternative is that the binding of

an agonist to these Ca^{2+}-linked receptors induces, similarly to some peptide receptors, a microaggregation of two or more receptor molecules and, possibly, a transducer protein, in a high molecular weight holoreceptor, which represents the activated form of the receptor, and this complex is stabilized by inter-molecular cross-linking by DSS. This alternative may be tested, first, by radiation-inactivation analysis of agonist-activated receptors. So far, the published measurements have been made in the absence of agonist (Crause *et al.*, 1984; Venter *et al.*, 1984). Another method, often used in such studies, is analytical SDS-PAGE, but for the application of this method it is necessary first to obtain an irreversible labeling of the analyzed receptor. For the α_1-adrenergic receptor, which is in the main line of interest of this thesis, the irreversible labeling of the receptor presents serious technical problems (see Section 3.2.3.1), and our attempts to use the photoactivatable analog of prazosin, [^{125}I]Arylazidoprazosin were unsuccessful. In our hands, this ligand was not able to generate a consistent and reproducible labeling pattern. Our attempt to cross-link [^{3}H]Prazosin to the active site of the receptor with DSS was also unsuccessful, as shown above. For the vasopressin V_1 receptor, a high specific activity [^{3}H]arginine-vasopressin is available, but in the experimental conditions we used it was impossible to attach it irreversibly to its receptor. The reason for this failure is unknown. It is possible that incubation at 37°C favors a receptor conformation in which the amino groups inside or in the vicinity of the active center are shifted away from the range of cross-linking of DSS (11 Å). After the experiments of this thesis were completed (end of June 1987), two methods for irreversible labeling of vasopressin V_1 receptors were

published. The first proposes to use ultraviolet irradiation to cross-link vasopressin to the receptor (Tibonnier, 1987), and the second proposes to perform the cross-linking with 1 mM DSS for 15 min at 4°C (Fishman *et al.*, 1987). No doubt, these two methods, and mainly the last one, developed for the characterization of vasopressin V_1 receptor from rat liver, will be of great help for the study of the molecular weight of the activated form of the vasopressin V_1 receptor.

Another observation in this study was that in both systems studied, the maximal glycogen phosphorylase stimulation following treatment with agonist and cross-linker was smaller than the maximal response to agonist alone, and the rate of activation was slower. This might be due to a smaller than optimal number of receptors stabilized by the cross-linker, or to a slower interaction of cross-linker-modified receptors with the transducer mechanism. It is also possible that, independently from the action on receptors, DSS cross-linking affects the transducer mechanism in the plasma membrane, which leads to a limitation of the maximal physiological response.

We noted also that the treatment with DSS alone produces a small, but significant increase in the activity of glycogen phosphorylase by the end of the period of observation. The mechanism of this effect is unknown, but it might be speculated that it results from the cross-linking of a small number of randomly colliding receptors in the plasma membrane.

In conclusion, the data reported above show that the combined action of agonist and cross-linker on liver α_1-adrenergic and vasopressin V_1 receptors results in a long-term activation of their target enzyme. The cross-linker stabilized form of the receptor seems to be able to ac-

tivate the glycogen phosphorylase in the absence of agonist. Cross-linking with DSS results in a long-term activation of the receptors, but doesn't prevent the dissociation of agonists from their active center.

4.2.3. I-POB - A NOVEL IRREVERSIBLE LIGAND FOR THE α_1-ADRENERGIC RECEPTOR

The persistent difficulties in obtaining a reliable irreversible ligand for the α_1-adrenergic receptor prompted us to develop the iodobenzyl derivative of POB. The preliminary data on the pharmacological activity of I-POB, reported in this thesis, indicate that this compound is an irreversible antagonist of the α_1-adrenergic receptor in rat liver and rat vascular smooth muscle, and it binds with high affinity to α_1-adrenergic receptors in isolated plasma membranes. The chemical structure and the long-term effect of I-POB indicate that its mechanism of action is probably similar to the mechanism of action of POB, its parent compound (for a detailed review of the mechanism of action of POB on rat liver α_1-adrenergic receptor, see Kan, 1983). POB is a β-haloalkylamine, which alkylates proteins through the formation of an azaridinium ion intermediate. This alkylating action is not specific for the α_1-adrenergic receptor, but it has been demonstrated that POB interacts with the α_1-adrenergic receptor by a two step mechanism, the first of which represents specific, high affinity and reversible binding to the receptor. The ligand forms an ion bond with a carboxyl or a phosphate group in the hormone-binding site of the receptor, while its aromatic moiety is at-

tached at a separate site, a short distance away from the first one (Belleau, 1958). The first stage of the binding of POB is a relatively slow process, in which the binding is stereoselective (Portoghese & Riley, 1971), and still reversible - POB may be washed out from the tissue, or displaced by a competing ligand. At the next stage, POB reacts with a nucleophile on the receptor to form a covalent bond, and is no longer in mass-action equilibrium with the receptor (Nickerson, 1957). Used at concentrations in the subnanomolar range (0.1- 0.5 nM), POB has been shown to be selective for α_1-adrenergic receptors in vascular smooth muscle (Cubeddu *et al.*, 1974; Constantine & Lebel, 1980; Constantine *et al.*, 1982), and rat liver (Kan *et al.*, 1979; Kunos *et al.*, 1983), as well as in other tissues (Dubokovich & Langer, 1974; Doxey *et al.*, 1977; Minneman, 1983).

The introduction of an iodine atom on the benzyl ring of POB doesn't interfere with the capacity of the ligand to bind to α_1-adrenergic receptors, but causes a small, approximately 3-fold decrease in its binding affinity. This structural modification also doesn't interfere with the the protein alkylating property, as substantiated by the long-term receptor blockade caused by I-POB. In the future, it will be necessary to determine the exact time-course of the onset and recovery from the α_1-adrenergic blockade by I-POB. Also, it will be important to study in detail the stereoselectivity and the specificity of its binding the the α_1-adrenergic receptor. As it has been demonstrated for POB, higher nanomolar and micromolar concentrations of β-haloalkylamines are able to inactivate several other types of receptors, including the α_2-adrenergic (Regan et al, 1984), the opiate (Spiehler & Randall, 1979), the mus-

carinic acetylcholine (Boyd *et al.*, 1963), the dopamine (McLennan & York, 1967), and histamine receptors (Cook, 1971), as well as some additional cellular processes (Boyd *et al.*, 1960; Iversen *et al.*, 1972; Ross *et al.*, 1968). So far, the high affinity of binding and the ability of I-POB to distinguish between α_1-adrenergic receptors and vasopressin V_1 receptors (Fig. 20), is promising. The eventual use of I-POB as an affinity probe for the isolation and the molecular characterization of α_1-adrenergic receptors will necessitate the development of a radioiodinated form of the compound. For this purpose, an alternative of synthesis has to be developed, in which the iodine-125 atom will be introduced in the ligand molecule as the last synthetic step.

4.2.4 IMMUNOLOGICAL CHARACTERIZATION OF THE α_1-ADRENERGIC RECEPTOR FROM RAT LIVER

The experiments reported above demonstrate that the rat liver α_1-adrenergic receptor solubilized with Triton X-100 and partially purified by gel filtration, is antigenic in rabbits and could be used to elicit the production of antireceptor antibodies. The results of the present study do not permit a more precise analysis of the number of antibodies produced, nor the exact place of binding on the receptor molecule, but are demonstrating that at least some of them bind to a protein with 80,000 D m.w. (Fig. 24 *Top*, strip B & C). These results also indicate the absence of antibodies directed against the vasopressin V_1 receptor, which is even more abundant than the α_1-adrenergic receptor in hepatocytes, and

the hormone-binding subunit of which has a m.w. of in the same range (Fishman *et al.*, 1987). One can also conclude that the antiserum does not contain antibodies interfering with the function of post-receptor mechanisms, common to the Ca^{2+}-linked receptors. Since we found that the binding of [^{3}H]PQB to the active center of the α_1-adrenergic receptor decreases the binding of antibodies to the receptor protein, it might be proposed that the α_1-adrenergic receptor-blocking effect of the antiserum is due to their binding to the active center of the receptor. As evidenced by fig 24, the concentration of these antibodies in the rabbit antiserum was very low, and they were present in the serum of the experimental animals for only 5 months, despite the repetitive boosting. A similar phenomenon has been described by Couraud *et al.* (1983), and has been attributed to a neutralization of the circulating antibody by an anti-idiotypic autoantibody, as proposed by Jerne (1973). Because of such problems, further purification of these antibodies offered little hope to obtain sufficient amounts to be used in immunoaffinity chromatography of the α_1-receptor. Monoclonal antibodies could offer a viable alternative. However, in a collaborative effort with a NIMH group (F. Hirata), we have been unable to identify positive clones with α_1-receptor inhibitory activity, despite screening of >900 clones originating from balb/c mice immunized with a partially purified α_1-adrenergic receptor preparation from rat liver.

In conclusion, the results presented above, describe the development and the characterization of polyclonal antibodies against the rat liver α_1-adrenergic receptor. We have been able to detect the presence of antibodies against the α_1-adrenergic receptor and to show the binding of

such antibodies to the receptor protein. This was used as an independent confirmation of previous determination of the m.w. of the hormone-binding subunit of the receptor (Kunos *et al.*, 1983; Leeb-Lundberg *et al.*, 1984).

SECTION 5: SUMMARY AND CONCLUSIONS

1. The mechanism of the time-dependent shift of the adrenergic control of hepatic glycogenolysis was studied in male rats. Incubation of isolated hepatocytes for 4-hr in a serum-free buffer leads to a reduction of the glycogenolytic effect of phenylephrine and the simultaneous emergence of a glycogenolytic response to isoproterenol within 4 hr. The time-dependent shift in the adrenergic control of glycogenolysis is associated with no change in the glycogenolytic response to the Ca^{2+}-linked activator vasopressin, and a reduction of the glycogenolytic response to the cAMP-linked activator glucagon. *In vitro* incubation of hepatocytes doesn't influence the density or affinity of [^{3}H]prazosin-labeled α_1-receptors and [^{3}H]CGP-12177-labeled β-receptors. In cells preincubated for 4 hr, a further 30-min incubation with 50 nM lipomodulin, an endogenous inhibitor of membrane phospholipase A_2, reverses the adrenergic activation of glycogen phosphorylase from a β- to an α_1-receptor-mediated event, whereas in freshly isolated cells lipomodulin doesn't affect the predominant α-receptor response. Conversely, exposure of freshly isolated cells to a monoclonal antibody to lipomodulin in the presence of 10 μM phenylephrine, or to mellitin, an activator of phospholipase A_2, at 2 μg/ml, results in the suppression of the effect of phenylephrine and the emergence of a response to isoproterenol within 30 min. It is proposed that coupling of hepatic α- and β-adrenergic receptors to postreceptor pathways is regulated in an inverse reciprocal manner by changes in membrane phospholipase A_2 activity.

2. We made an attempt to isolate a the fragment of the hormonal-binding subunit of the α_1-adrenergic receptor to which binds the irreversible antagonist [^{3}H]POB. We found that the rat liver α_1-adrenergic

receptor is a sialyated glycoprotein. The α_1-adrenergic receptor was labeled with [^{3}H]POB in rat liver plasma membranes, solubilized with Triton X-100 and partially purified by gel filtration and wheat germ agglutinin chromatography. The resulting material (2.7 mg protein), was digested with TPCK-treated trypsin, which released ~30% of the protein-bound [^{3}H]POB. The [^{3}H]POB-labeled peptide was purified by reverse phase HPLC and gel permeation HPLC. It was found that trypsin digestion of the [^{3}H]POB-labeled α_1-adrenergic receptor releases a peptide with M_r of <600 daltons, unsuitable for sequencing.

3. The mechanism of the agonist activation of two Ca^{2+}-linked hormones, vasopressin and adrenaline, was studied in isolated rat liver cells. In rat liver plasma membranes, treatment with 1 mM of the homobifunctional cross-linker of amino groups disuccinimidyl suberate, was not able to prevent the dissociation of bound vasopressin from the V_1 receptor or bound [^{3}H]Prazosin from the α_1-receptor. When isolated hepatocytes were stimulated with 10^{-7}M vasopressin, followed by cross-linking with 1 mM disuccinimidyl suberate, we found that glycogen phosphorylase remained activated after the dissociation of the hormone from the receptor. Similarly, when the cells were stimulated with 10^{-6}M adrenaline, and treated with the cross-linker, glycogen phosphorylase was activated for a long time. It is proposed that agonist binding to the Ca^{2+}-linked receptors for vasopressin and adrenaline results in microaggregation, which leads to activation of the postreceptor mechanisms.

4. The pharmacological activity of a novel irreversible α_1-adrenergic receptor antagonist was studied. I-phenoxybenzamine, a derivative of phenoxybenzamine, was found to be a potent inhibitor of the α_1-adre-

nergic activation of glycogen phosphorylase in isolated hepatocytes, with an EC_{50} of 1 nM. The K_{50} for the displacement of [^{3}H]Prazosin binding to the α_1-adrenergic receptor in rat liver plasma membranes was 2 nM. We found that I-phenoxybenzamine prevented the activation of the vascular α_1-adrenergic receptors in the rat by phenylephrine, but didn't interfere with the activation of the vasopressin receptors by vasopressin. We found that the α_1-adrenergic block lasted for at least 24 hr.

5. The pharmacological activity of anti-α_1-adrenergic receptor polyclonal antibodies was investigated. Rabbits were immunized with α_1-adrenergic receptor preparation, obtained by solubilization of rat liver plasma membranes with Triton X-100 and partial purification by gel filtration. The rabbit antisera, used at dilution 1:10, interfered with the α_1-adrenergic activation of glycogen phosphorylase in isolated hepatocytes, but not with the activation of glycogen phosphorylase by vasopressin. When rat liver plasma membranes are solubilized with SDS resolved by SDS-PAGE and transblotted onto a nitrocellulose paper, treatment with rabbit antiserum showed binding of antibodies to a protein with M_r 80,000 daltons, shown to be the hormonal binding subunit of the α_1-adrenergic receptor. Pretreatment of the plasma membranes with 100 nM POB interfered with the antigen-antibody interaction.

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