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PATCH-CLAMP ANALYSIS OF PEPTIDERGIC EXCITATION IN RAT SUPRAOPTIC NEURONS

by

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of

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RÉSUMÉ

Les effets de l'angiotensine II (AII), de la cholécystokinine (CCK) et de la neurotensine (NT) furent étudié en patch-clamp chez des neurones isolés du noyau supraoptique du rat adulte. Suite à une application locale, chacun de ces peptides produisit une dépolarisation réversible du neurone. Cette réponse était généralement accompagnée d'une augmentation de la fréquence de décharge des potentiels d'action, indiquant la nature excitatrice de ces trois peptides. L'analyse macroscopique en voltage-clamp révéla que les effets excitateurs de l'AII, du CCK et de la NT résultent de l'activation d'une conductance cationique non-sélective. De plus, les rapports de perméabilité ionique des conductances modulées par les trois peptides semblent identiques. Les résultats obtenus lors d'enregistrements membranaires en cellule-attachée permirent de conclure que l'activation d'une conductance cationique non-sélective par l'AII, la CCK et la NT, résulte de la modulation convergente d'un unique type de canal ionique dont la conductance unitaire est de 37 pS. Ces canaux pourraient jouer un rôle important dans le contrôle de la sécrétion d'hormones neurohypophysaires.

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LIST OF ABBREVIATIONS AND SYMBOLS

AII:	angiotensin II
ANP:	atrial natriuretic peptide
ATP:	adenosine triphosphate
AV3V:	anteroventral third ventricle
cAMP:	cyclic-adenosine monophosphate
CCK:	cholecystokinin
CNS:	central nervous system
cNTS:	caudal nucleus tractus solitarii
cVLM:	caudal ventrolateral medulla
EGTA:	ethyleneglycol-bis-(ß-aminoethyl ether) N,N'-tetraacetic acid
EPSP:	excitatory postsynaptic potential
E_{rev}:	reversal potential
GABA:	gamma amino butyric acid
GAD:	glutamic acid decarboxylase
GΩ:	giga-ohm
GTP:	guanosine triphosphate
HEPES:	N-[2-hydroxyethyl]piperazine-N'-[2-ethansulfonic acid]
Hz:	hertz
i.c.v.:	intracerebroventricle
i.d.:	inside diameter
I-V:	current-voltage
IP_3:	inositol 1,4,5-trisphosphate
kHz:	kilohertz
M:	molar
ME:	milk ejection
MCT:	mean closed time
min:	minute
µl:	microlietr
µM:	micromolar
µm:	micrometer
ml:	milliliter
MΩ:	mega-ohm
mOsm:	milliosmole
MOT:	mean open time
mM:	millimolar
mm:	millimeter
MNC:	magnocellular neurosecretory cell
mRNA:	messenger ribonucleic acid
ms:	millisecond
mV:	millivolt
NMDA:	*N*-methyl-*D*-aspartate
NT:	neurotensin
o.d.:	outside diameter
OVLT:	organum vasculosum of lamina terminalis
OXT:	oxytocin

pA:	picoampere
PC:	phosphocreatine
PIPES:	piperazine-N,N'-bis[2-ethanesulfonic acid],1,4-piperazinediethanesulfonic acid
pF:	picofarad
P_o:	open probability
pS:	picosiemens
PVN:	paraventricular nucleus
s:	second
SFO:	subfornical organ
SON:	supraoptic nucleus
SS:	somatostatin
TEA:	tetraethylammonium
TMN:	tubermammillar nucleus
TTX:	tetrodotoxin
V_H:	holding potential
V_m:	membrane potential
VP:	vasopressin

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CHAPTER ONE

INTRODUCTION

The magnocellular neurosecretory cells (MNCs) synthesizing and secreting the hormones vasopressin (VP) and oxytocin (OXT) are best known for their direct role in controlling the body fluid homeostasis through changing their firing rates in response to changes in the osmolarity of the extracellular fluid, and subsequent neurohypophysial hormone release. In response to dehydration, MNCs fire clusters of action potentials to maintain sustained high levels of VP to overcome the circulatory failure (Poulain et al., 1988). Changes in the osmotic pressure have been shown to be a potent regulator of MNC activity and neurohypophysial hormone release. In addition, recent electrophysiological studies have shown that different substances released in the central nervous system (CNS) such as catecholamines and neuropeptides can modulate the excitability of MNCs and play a role in their hormone release into the circulation.

1.1 THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

The hypothalamo-neurohypophysial system comprises the magnocellular neurosecretory cells synthesizing the hormones VP and OXT. The somata of these neurons are located in the paired supraoptic (SON) and paraventricular (PVN) nuclei, as well as in small accessory cell groups of the hypothalamus (see Castel et al., 1984). These cells project their axons through the neural

stalk into the neurohypophysis where each axon branches into thousands of secretory terminals (Nordmann, 1977; Silvermann & Zimmermann, 1983). VP- and OXT-synthesizing MNCs can be distinguished from one another by immunocytochemical techniques in which antibodies against VP or OXT are used (Vandersande & Dierickx, 1975; Sokol et al, 1976; Sofroniew et al., 1979; Swanson & Sawchenko, 1983). While the paraventricular nucleus contains a mixture of both magnocellular and parvocellular neurons (Armstrong et al., 1980), the supraoptic nucleus appears to contain only MNCs. This homogeneity makes SON a popular model for electrophysiological studies on MNCs.

1.2 FIRING PATTERNS OF MNCs

In rats, electrophysiological studies have shown that VP- and OXT-secreting neurons have different electrical response patterns to physiological stimulation.

1.2.1 OXT-secreting neurons

In the lactating female rat, continuous suckling by pups is associated with episodes of milk ejection (ME) occurring every 3-15 min (Wakerley and Lincoln, 1971). In vivo extracellular recordings obtained from the PVN (Wakerley & Lincoln, 1973) and SON (Lincoln and Wakerley, 1974) of anaesthetized female rats during lactation revealed that each episode of ME is immediately (10-20 s) preceded by a *brief* (2-4 secs) *high frequency* (40-80 Hz) burst of action potentials arising from a population of MNCs showing slow irregular (<3 Hz) or fast continuous (3-15 Hz) spontaneous background firing. Similar results

were obtained later from unanaesthetized rats (Summerlee & Lincoln, 1981). Because of the tight temporal correlation between high frequency bursts and episodes of ME, such cells are presumed to be OXT-secreting (Wakerley and Lincoln, 1973). Paired recordings in SON and PVN have revealed that all such OXT-secreting neurons display synchronicity in their ME-related activity, indicating that MNCs in all magnocellular neurosecretory nuclei contribute to each pulse of oxytocin release into the blood (Belin et al., 1984; Belin & Moos, 1986). Similar high frequency bursts of action potentials have also been recorded at parturition (Summerlee, 1981), when they presumably contribute to the high levels of plasma oxytocin associated with uterine contraction.

In contrast to the high frequency bursts fired at lactation and parturition, dehydration (Wakerley et al., 1978), haemorrhage (Harris et al., 1975; Wakerley et al, 1975) and hypertonic stimuli (Brimble and Dyball, 1977) have been found to cause a progressive increase in *continuous* firing in OXT-secreting cells. In agreement, these conditions are associated with continuous (rather than pulsatile) release of oxytocin (Poulain & Wakerley, 1977).

1.2.2 VP-secreting neurons

Since all OXT-releasing MNCs in the hypothalamus contribute to each episode of ME in the lactating female rat, putative VP-secreting neurons can be recognized electrophysiologically by a lack of activity that can be correlated with ME (Brimble and Dyball, 1977; Wakerley et al., 1978). During electrophysiological recordings from MNCs thus identified as VP-secreting, the *phasic* pattern of bursting activity was observed both spontaneously, and during

physiological conditions associated with enhanced VP release, such as haemorrhage (Wakerley et al., 1975), dehydration (Wakerley et al., 1978) or elevation in plasma osmolality (Wakerley et al., 1978). The phasic firing pattern, which was not observed in OXT cells, consisted of consecutive periods of electrical discharge (7-12 Hz) and silence, each lasting tens of seconds, and occurring in a regular manner (Poulain and Wakerley, 1982). Because of its association with VP release and its absence in OXT-releasing MNCs, cells showing phasic firing were hence believed to be VP-secreting (Brimble and Dyball, 1977; Wakerley et al., 1978). Paired recordings from VP neurons indicated that phasic bursts recorded from different MNCs are asynchronous, a feature which contributes to the maintenance of high levels of plasma VP as required during prolonged stimulation (Poulain & Wakerley, 1982). In agreement with the above data, a majority of dye-injected phasically firing MNCs in SON and PVN in vitro were found to display VP-immunoreactivity (Yamashita et al., 1983; Cobbett et al., 1986) but not OXT-immunoreactivity (Armstrong et al., 1991), supporting the concept that phasic firing is a selective feature of VP-secreting MNCs.

1.3 NEUROHYPOPHYSIAL HORMONES

OXT and VP are cyclic nonapeptides with seven common amino acids and with disulphide bridges between the cysteine residues. They are synthesized from prohormones that are structurally similar, known as prooxyphysin and propressophysin, respectively. The main difference between these prohormones is that propressophysin has a longer C-terminal portion

consisting of a glycoprotein (Gainer et al., 1988). The structures of VP and OXT are illustrated here:

arginine-vasopressin H-Cys-Tyr-**Phe**-Gln-Asn-Cys-Pro-**Arg**-Gly-NH_2

|-------S------S-----------|

oxytocin H-Cys-Tyr-**Ile**-Gln-Asn-Cys-Pro-**Leu**-Gly-NH_2

|-------S------S---------|

Vasopressin: VP, also known as antidiuretic hormone, contributes to the maintenance of body fluid balance by acting at renal V_2 receptors. Activation of these receptors increases the permeability of the late distal tubule and collecting duct to water, through the activation of a cyclic AMP-dependent mechanism (see Jard, 1983). VP is also considered to be a potent vasoconstrictor (see Altura and Altura, 1977). By binding to V_1 receptors at the arteriolar smooth muscle, VP causes vasoconstriction through the activation of an inositol 1,4,5-trisphosphate-dependent mechanism (Aiyar et al., 1986; Wu et al., 1995a).

Oxytocin: The major roles of circulating OXT are in the regulation and maintenance of lactation and parturition. During lactation, oxytocin is released by a neuroendocrine reflex activated in response to suckling (see section 1.2.1). Indeed, impulses carried from the nipple to the spinal cord by afferent sensory fibers are necessary to activate the high frequency bursts of OXT neurons

(Goodman and Grosvenor, 1983). OXT released from the neurohypophysis then acts on the myoepithelial cells of the mammary glands causing ME (Wakerley & Lincoln, 1973; Cunningham & Sawchenko, 1991). During pregnancy, the number of oxytocin receptors in the uterus increases as parturition approaches (Kimura et al., 1992). Although OXT is released during parturition, in response to vaginal distention, its role in the initiation of normal labour is still uncertain (reviewed in Jenkin, 1992). It is nonetheless assumed that OXT acts on endometrial smooth muscle to increase the frequency and the intensity of uterine contraction at birth (Garfield and Beier, 1989; Chimura, 1991).

Since the brains of male and female rats have comparable number of OXT immunoreactive-MNCs (Rhodes et al., 1981), circulating OXT might have other physiological functions not related to lactation and parturition. Indeed, recent studies have shown that OXT possesses important natriuretic properties at physiological concentrations in the rat (Balment et al., 1980; Verbalis et al., 1991). Since, in this species, OXT is released during hypertonic stimulation (Brimble et al., 1978), the release of this hormone may also play a role in osmoregulation (Bourque et al., 1994).

1.4 NEUROSECRETION OF NEUROHYPOPHYSIAL HORMONES

As discussed above, stimuli that enhance VP and OXT release have been shown to accelerate the electrical activity of VP- and OXT-secreting MNCs, suggesting that hormone secretion is regulated by the electrical activity of these neurons. Indeed, the secretion of neurohypophysial hormone into the circulation by the axon terminals of MNCs occurs in proportion to the firing

frequency of action potentials generated at the level of MNC somata (Dreifuss et al., 1971; Dutton and Dyball, 1979; Bicknell, 1988; Poulain & Theodosis, 1988). The amount of hormone released per action potential appears to depend on both the *frequency* and the *pattern* of neural lobe stimulation in vitro. By increasing the rate of stimulation, the amount of OXT (Dreifuss et al., 1971) and VP (Dutton & Dyball, 1979; Wolfe & Gainer, 1986; Hobbach et al., 1988) secreted per pulse increases, indicating the existence of a frequency-dependent facilitation of excitation-secretion coupling in the axon terminals of MNCs. Sustained repetitive stimulation, however, leads to a progressive decline in neurohypophysial hormone release, a process known as secretory fatigue (Bicknell et al., 1984). Asynchronous phasic bursting in VP-secreting MNCs, therefore, may facilitate the maintenance of high VP levels required to maintain antidiuresis by preventing the secretory fatigue at the nerve terminals. Similarly, during dehydration, both VP- and OXT-releasing MNCs adopt a spike clustering pattern composed of brief (<2 sec) clusters of action potentials alternating with periods of quiescence of similar duration (Poulain et al., 1988). Such clustering of action potentials has also been shown to potentiate hormone release from the posterior pituitary (Cazalis et al., 1985). Recent in vitro studies on supraoptic neurons have demonstrated that spike clustering can be induced by activating post-synaptic NMDA receptors (Hu & Bourque, 1992), suggesting the involvement of glutamatergic inputs in the expression of spike clustering in MNCs.

1.5 CHEMICAL REGULATION OF ELECTRICAL ACTIVITY IN MNCs

As discussed above, the electrical activity of magnocellular neurosecretory neurons is regulated according to systemic requirements for varying levels of OXT and VP. In addition to the physiological conditions discussed in sections 1.2 and 1.3, plasma levels of VP and OXT are controlled by a wide range of extrinsic factors such as chemoreceptor and baroreceptor activity (Harris, 1979), gastric (Renaud et al., 1987) and vaginal (Moos and Richard, 1975) distension, stimulation of the nipple (Lincoln and Wakerley, 1974), nausea and emetic agents (Verbalis et al., 1986; McCann et al., 1989), as well as a variety of other conditions (Cunningham and Sawchenko, 1991). This control is presumably mediated by afferents to the magnocellular neurosecretory system originating from a variety of regions in the CNS (for review, Bourque and Renaud, 1990). Therefore, synaptic inputs are likely to regulate MNC activity and hormone release through the effects of secreted neurotransmitter substances (Renaud and Bourque, 1991). A concise summary of putative transmitters (in alphabetical order) for which available evidence suggests an important role in the regulation of MNC firing and hormone release is given below.

1.5.1 Angiotensin II (AII)

AII is the physiologically active form of angiotensin, produced during the cascade activation of the renin-angiotensin-aldosterone system in the periphery in response to hypovolemia. Because AII promotes rehydration and vasoconstriction, this peptide hormone plays an important role in cardiovascular

homeostasis. AII can also be synthesized in, and released from neurons, in the CNS (Unger et al., 1988). Moreover, a substantial body of evidence now supports an important role for AII as a neurotransmitter and/or neuromodulator in different regions in the CNS, particularly so in those involved in the regulation of cardiovascular, autonomic, and neuroendocrine functions (for reviews, Ferguson & Wall, 1992; Culman et al., 1995; Muratani et al., 1996). High densities of AII-secreting neurons are located in the subfornical organ (SFO), one of the circumventricular organs of the lamina terminalis. AII-secreting neurons in the SFO have been found to send projections to the SON based both on immunohistochemistry (Lind et al., 1985), and in vivo studies where electrical stimulation applied in the SFO caused an excitation in both VP and OXT MNCs (Ferguson et al., 1984; Jhamandas et al., 1989). In agreement with this effect, SFO stimulation has been found to provoke neurohypophysial hormone release (Ferguson, 1987). The excitatory effects of SFO stimulation were found to be blocked upon application of AII antagonists (Jhamandas et al., 1989).

In situ hybridization, immunohistochemical and autoradiographic studies have demonstrated the presence of two AII receptor subtypes, AT1 and AT2, distributed throughout the mammalian CNS. While the AT2 subtype is expressed mainly in brainstem nuclei (Lenkei et al., 1996), a high density of AT1 receptors has been found in the nuclei of the lamina terminalis and in hypothalamic PVN and SON (Plunkett et al., 1987; Mendelsohn et al., 1988; Gehlert et al., 1991; Phillips et al., 1993). Microinjections of AII into the hypothalamus have been shown to induce drinking behaviour, a rise in blood

pressure, and an increase in plasma vasopressin level (Phillips, 1987). Electrophysiologically, AII has been shown to produce depolarization and action potential firing in MNCs by acting on AT1 receptors. This effect appears to result from the activation of a non-selective cationic conductance (Yang et al., 1992). Recent studies, however, have also shown that AII can cause a decrease in A-current amplitude (Nagatomo et al., 1995; Li and Ferguson, 1996).

1.5.2 Atrial natriuretic peptide (ANP)

Within the brain, cells and fibers immunoreactive to ANP are distributed in areas involved in the central regulation of blood pressure and fluid balance, including in the SON and PVN (Imura et al.,1992). ANP applied centrally inhibits osmotically-induced release of VP and OXT both in vivo (e.g. Samson, 1985) and in vitro (Crandall and Gregg, 1986). Local application of natriuretic peptides has been shown to reduce the basal electrical activity of MNCs recorded in vivo (Stadaert et al., 1987) and in vitro (Yamamoto et al., 1991). Recent intracellular recordings from explants have shown that ANP reversibly abolishes the synaptically driven excitation of MNCs induced by hypertonic stimulation of the organum vasculosum of the lamina terminalis (OVLT) via presynaptic inhibition of glutamatergic excitatory postsynaptic potentials (EPSPs) (Richard and Bourque, 1996).

1.5.3 ß-Inhibin

ß-Inhibin, which belongs to the family of transforming growth factor-ß-like

proteins, suppresses the release of follicle-stimulating hormone (Massague, 1990). Combined immunohistochemical and retrograde tracer studies have demonstrated that ß-inhibin-containing neurons projecting to the hypothalamic magnocellular neurosecretory nuclei are located in the caudal nucleus tractus solitarii (cNTS) (Sawchenko et al., 1990). Conversely, fibers immunoreactive to ß-inhibin have been found in the vicinity of the SON and PVN, where they appear to synapse onto OXT-releasing neurons (Sawchenko et al., 1988, 1990). In addition, local application of activin-A, an inhibin $ß_A$ homodimer, in the PVN of anaesthetized male (Sawchenko et al., 1988a) and lactating female (Plotsky et al., 1988) rats induced the secretion of OXT. Electrophysiological recordings in vitro have shown recently that activin-A depolarizes and induces action potential firing in isolated supraoptic MNCs, through the activation of a non-selective cationic conductance (Oliet et al., 1995).

1.5.4 Cholecystokinin (CCK)

CCK is mainly known as a gastrointestinal peptide that controls the secretion of pancreatic enzymes and that thereby regulates gastric emptying. Various studies have shown that CCK is an abundant neuropeptide in the CNS and that CCK-immunoreactivity can be found in different areas in the brain (Beinfeld, 1983; Ingram et al., 1989; Morino et al., 1994). Interestingly, a high density of CCK is present in hypothalamic areas involved in regulating body fluid homeostasis (Ingram et al., 1989), including in the MNCs (Vanderhaeghen et al., 1980, 1981). Based on functional studies, at least two CCK-receptor subtypes, CCK-A and CCK-B, have been found in the mammalian brain

(Pinnock et al., 1991), with CCK-B being expressed on MNCs (Jarvis et al., 1992). CCK application within the SON has been shown to induce the release of OXT (Neumann et al., 1994) and VP (Jarvis et al., 1995) from the neurohypophysis. Intracellular recordings from MNCs in hypothalamic explants have demonstrated that CCK evokes depolarization and increased firing through the activation of a non-selective cationic conductance (Jarvis et al., 1992).

1.5.5 Dopamine

Ultrastructural studies have demonstrated the presence of dopamine-immunoreactive fibers in both the SON and PVN (Lindvall et al., 1984). Although, the origin of these fibers is still unclear, it is speculated that they may arise from the A11 or A13 cell groups in the hypothalamus (Buijs et al., 1984). In vitro studies using extracellular (Mason, 1983) and intracellular (Yang et al., 1991) recordings have demonstrated an excitatory effect of dopamine on MNCs. An electrophysiological analysis has suggested that the excitation of MNCs by dopamine, through D_2 receptors, is calcium dependent and results from the activation of a non-selective cationic conductance (Yang et al., 1991).

1.5.6 Galanin

Although, it was first isolated from gut (Tatemoto et al., 1983), galanin is also widely distributed in the CNS, especially in the hypothalamus (Rokaeus et al., 1984). Galanin-immunoreactive fibers have been visualized in the vicinity of the hypothalamic MNCs (Melander et al., 1986), and mRNA encoding human galanin receptor has recently been found in MNC somata (Gustafson et al.,

1996). Intracerebroventricular (i.c.v.) injections of galanin have been found to inhibit the release of both OXT (Bjorkstrand et al., 1993) and VP (Kondo et al., 1991). Recent intracellular recordings from hypothalamic explants have demonstrated that galanin inhibits both continuous and phasic firing in supraoptic MNCs through activation of a K^+ conductance and reduction of the post-spike depolarizing after potential (Papas and Bourque, 1997).

1.5.7 Gamma amino butyric acid (GABA)

Substantial levels of glutamic acid decarboxylase (GAD) are present both in the SON and PVN (Meyer et al., 1980). Moreover, a high density of GABA-immunoreactive fibers is present in the SON, and GABAergic synapses in this nucleus are believed to account for 30-50% of all synaptic connections onto MNCs (Van den Pol et al., 1985; Buijs et al., 1987; Decavel and Van den Pol, 1990; Gies and Theodosis, 1994). These observations suggest an important role for GABA in the moment-to-moment control of MNCs activity. Electrical stimulation in the anteroventral third ventricle (AV3V) (Nissen and Renaud, 1989) and diagonal band of Broca (Jhamandas and Renaud, 1986; Randle et al., 1986) have been shown to produce a prominent $GABA_A$ receptor-mediated inhibition of SON neurons, suggesting that a GABAergic innervation from these regions contributes to the regulation of MNCs. Inhibition of MNCs by exogenously-applied and synaptically-released GABA has been shown to be mediated by the activation of chloride conductance (Randle et al., 1986; Randle and Renaud, 1987).

1.5.8 Glutamate

Glutamate is the most abundant excitatory transmitter involved in neuroendocrine regulation (Van den Pol et al., 1990). The hypothalamus, including the SON and PVN, contains high levels of glutamate (Palkovits et al., 1986). In addition to the presence of a high density of glutamatergic afferents innervating the hypothalamic neuroendocrine system, MNCs, themselves, are indeed a source for glutamate as determined by immunohistochemical studies (Meeker et al., 1989; 1993; Decavel and Van den Pol, 1992). Electrophysiological techniques have been valuable in determining the origin of glutamatergic afferents onto MNCs. Electrical stimulation in the main olfactory bulb has been found to produce a glutamate-mediated excitation in supraoptic neurons when recorded in vitro (Hatton and Yang, 1989). Moreover, glutamatergic projections to the SON have also been demonstrated to originate from the OVLT (Yang et al., 1994) and the lateral hypothalamus (Dudek and Gribkoff, 1987; Gribkoff and Dudek, 1988). Functional studies have shown that both N-methyl D-aspartate (NMDA) and non-NMDA glutamate receptor subtypes are expressed on MNCs (Hu and Bourque, 1991) and synaptic activation of these receptors is believed to mediate the excitation of MNCs induced by hypertonic stimulation of the OVLT (Richard and Bourque, 1995).

1.5.9 Histamine

Modest levels of histamine are present in the SON and PVN (Brownstein et al., 1974). Moreover, high densities of H_1-binding sites are expressed in both nuclei as determined by autoradiography (Palacois et al., 1981). In the CNS,

histamine-immunoreactive neurons are found in the tuberomammillary nucleus (TMN) (Watanabe et al., 1984). Electrical stimulation in TMN has been shown to activate VP neurons and to inhibit OXT neurons (Weiss et al., 1989). The excitation in VP cells caused by histamine has been found to be mediated through effects on H_1-receptors, while the inhibition of OXT neurons occurs through actions on H_2-receptors (Yang and Hatton, 1989). Recent in vitro electrophysiological studies have shown that local administration of histamine increases the excitability of supraoptic neurons through the activation of a non-specific cationic conductance (Smith and Armstrong, 1996) and/or via suppression of K^+ conductance (Li and Hatton, 1996).

1.5.10 Neurotensin (NT)

NT is a tridecapeptide which was first isolated from the bovine hypothalamus (Carraway and Leeman, 1973) and which is now known to be distributed within many regions in the CNS (Hokfelt et al., 1984, Jannes et al., 1982), as well as in the gastrointestinal system (Kitabgi et al., 1976). NT-immunoreactive fibers have been noticed both within and surrounding the SON (Jannes et al., 1982). In agreement with a possible role as a neurotransmitter, NT has been found to excite a variety of neuronal types (reviewed in, Stowe and Nemeroff, 1991) either through the suppression of K^+ conductances (e.g. Alonso et al., 1994), or via the activation of a non-selective cationic conductance (e.g. Wu et al., 1995b). In superfused hypothalamic explants, application of NT onto MNCs has been found to induce reversible depolarization and spike discharge (Kirkpatrick and Bourque, 1995). Although NT also

attenuated the Ca^{2+}-activated K^+ current I_{AHP}, this study revealed that the depolarizing effect of NT was retained in the presence of apamin (to block I_{AHP}), and appeared to be due to the activation of non-selective cationic conductance.

1.5.11 Noradrenaline

Catecholamine fibers arising from noradrenergic neurons located in the caudal ventrolateral medulla (cVLM) innervate MNCs both in the SON and PVN, as visualized by light microscopy (Cunningham and Sawchenko, 1988). Immunohistochemical studies using anterograde labeling (Swanson et al., 1981; Shioda et al., 1992) suggest that the innervation from the A1 cell group may selectively target VP neurons in both nuclei. In agreement with this anatomical data, discrete electrical stimulation in the cVLM selectively excited VP-secreting neurons (Day et al., 1984; 1985; 1990). Moreover, direct application of norepinephrine into the SON was found to induce VP release both in vivo (Willoughby et al., 1987) and in vitro (Armstrong et al., 1986). Noradrenergic projections from cVLM have been proposed to mediate VP release in response to baroreceptor activation (McAllen and Blessing, 1987).

Another source of noradrenergic input to MNCs in PVN and SON arises from the A2 region in the cNTS (Cunningham and Sawchenko, 1988). Electrical stimulation of the A2 region induced excitation in both OXT- and VP-secreting MNCs (Day et al., 1984; Raby and Renaud, 1989). However, stimulation of cNTS after interrupting synaptic transmission in the cVLM excited only OXT neurons (Raby and Renaud, 1989) suggesting that cNTS may excite OXT-secreting neurons through a direct input, but indirectly excite VP neurons via

the ventrolateral medulla. Signals induced by systemic cholecystokinin application (Verbalis et al., 1986) and suckling (Lincoln and Wakerley, 1974) might be relayed through cNTS projections to trigger OXT release.

Intracellular recordings obtained from hypothalamic explants in vitro have shown that α_1-adrenoceptor activation by norepinephrine induces depolarization and spike discharge accompanied with suppression of the voltage-dependent K^+ current I_A (Randle et al., 1985).

1.5.12 Opioid peptides

A variety of opioid peptides are co-localized within MNCs (e.g. Martin and Voigt, 1981; Watson et al., 1982). In addition, MNCs have been shown to receive opioid innervation from the arcuate nucleus and dorsal medulla as determined by immunohisto-chemistry (Bloom et al., 1978). Moderate to high levels of opioid binding sites are also found on MNCs (e.g. Lightman et al., 1983) and exogenous application of opioids have been shown to inhibit the release of neurohypophysial hormones (e.g. Lincoln and Russell, 1986). In agreement with these findings, electrophysiological studies have shown that opioids can cause a naloxone-sensitive reduction in the excitability of MNCs both in vivo (e.g. Clarke et al., 1980) and in vitro (e.g. Pittman et al., 1980), possibly through effects on μ and kappa receptors (Leng and Russell, 1989). A recent study has shown that activation of kappa receptors inhibits supraoptic neurons by decreasing postsynaptic potential amplitude, and by inhibiting the calcium component of the action potential (Inenaga et al., 1994).

1.5.13 Polypeptide 7B2

7B2 is a secretory granule polypeptide which was first isolated from the pituitary gland (Hsi et al., 1982). In the hypothalamus, it is co-localized in the VP and OXT neurons in both the SON and PVN (Marcinkiewicz et al., 1986). In vitro, intracellular recordings from supraoptic MNCs revealed that application of polypeptide 7B2 produces membrane depolarization and spike discharge through the activation of a non-selective cationic conductance (Senatorov et al., 1993).

1.5.14 Purines

A variety of central cholinergic and catecholaminergic vesicles have been shown to co-store and release nucleotides including adenosine triphosphate (ATP) (Zimmerman, 1994). Moreover, electrophysiological studies have suggested that ATP may play a role as a neurotransmitter in the CNS (Edwards et al., 1992). Interestingly, the excitation of MNCs caused by vagal stimulation can be reversibly blocked by P_2 purinoceptor antagonists (Day et al., 1993), suggesting the involvement of ATP in mediating this response. In agreement with this hypothesis, local application of ATP increases the excitability of MNCs in vivo (Day et al., 1993). Moreover, electrophysiological studies in vitro have demonstrated the existence in MNCs of at least two purinoceptor subtypes: the P_{2X} receptor, selectively activated by α,ß-methylene ATP; and the P_{2U}, selectively activated by uridine triphosphate (Hiruma and Bourque, 1995). The excitatory effects of both receptor subtypes have been shown to be mediated via the activation of a non-selective cationic conductance

(Hiruma and Bourque, 1995).

1.5.15 Somatostatin (SS)

SS, which was originally isolated from the hypothalamus as a tetradecapeptide (SS-14) (Brazeau et al., 1973), plays a major role in regulating the secretion of growth hormone from the anterior pituitary. SS-14-immunoreactive material has been visualized in the MNCs (Bugnon et al., 1977), as well as in fibers surrounding MNCs (Kawano et al., 1982). Moreover, a low, but detectable density of SS-14 binding sites, has been reported to be present within the magnocellular nuclei (Leroux et al., 1985). Fibers immunoreactive to SS-28, another prosomatostatin-derived peptide, have also been found in the vicinity of MNCs (Sawchenko et al., 1988b, 1990). Central administration of SS-14 has been reported to inhibit haemorrhage-induced VP release (Wang et al., 1987). In contrast, i.c.v. administration of SS-28 provoked an important rise in plasma levels of VP and OXT (Brown et al., 1988). Surprisingly, in vitro recordings from hypothalamic explants have shown that somatostatin SS-28 hyperpolarizes rat supraoptic neurons (Raby et al., 1989). However, the ionic basis for this action is still unclear.

1.6 HYPOTHESIS AND GOALS

As reviewed in section 1.5, a wide variety of substances can modulate the electrical activity of MNCs by increasing or decreasing their firing rate with concomitant effects on the release of neurohypophysial hormones. While inhibitory substances appear to depress MNC activity through a variety of

different mechanisms (e.g. presynaptic inhibition, activation of K^+ and Cl^- currents, reduction of Ca^{2+} spikes), a common feature of most of the excitatory transmitters studied is the activation of a non-selective cationic conductance. Interestingly, even though often obtained by extrapolation and under various experimental conditions, the reversal potentials associated with the excitatory effects of these drugs appear to be roughly similar (see table 1.1). This observation suggests that a similar ionic mechanism may have been involved in generating each of these responses. It is hereby hypothesized *that a single population of cation permeable channels is modulated by the different excitatory substances contributing to the regulation of MNC activity.* As shown in table 1.1, however, the effects of ionic substitution were not consistently examined in previous studies, making it difficult to establish whether the permeability characteristics of the conductances activated were actually similar in each case. The goal of this thesis is to test whether this hypothesis is correct for three representative excitatory neuromodulators. The substances chosen for this analysis were the neuropeptides AII, CCK and NT.

The experimental approach used consisted of a patch-clamp analysis of the electrophysiological effects of these substances on MNCs acutely dissociated from the SON of the adult rat (see chapter 2 for methods). The specific objectives were: (1) to confirm that the actions of AII, CCK and NT are mediated postsynaptically, (2) to determine whether the macroscopic conductances activated by these peptides share common ionic permeability characteristics, and (3) to investigate whether AII, CCK and NT convergently modulate single non-selective cationic channels.

Substance	E_{rev} (mV)	ΔCl^-	low $[Na+]_o$	ΔK^+	Reference
Angiotensin II	-26.4±2	no effect	-	-	(Yang et al., 1992)
Cholecystokinin	-15±7	no effect	-	-	(Jarvis et al., 1992)
Neurotensin	-34±7	no effect	-	-	(Kirkpatrick & Bourque, 1995)
Dopamine	-25±10	no effect	-70±9.8	-	(Yang et al., 1991)
(7B2)	-27±11	no effect	-	-	(Senatorov et al., 1993)
Purines	-33±6	no effect	-	-	(Hiruma & Bourque, 1995)
Activin-A	-41.6±2.8	no effect	-	-	(Ollet et al., 1995)

TABLE 1.1: Reversal potentials (E_{rev}) of responses to a variety of substances recorded in MNCs, according to the references listed. Also shown are the effects of modifying the chloride (ΔCl^-), sodium (low Na^+), or potassium (ΔK^+) gradients (when tested). Minus (-) signs indicate cases where a specific procedure was not carried out.

CHAPTER TWO

MATERIALS AND METHODS

In order to isolate the effects of AII, CCK and NT on MNCs, and to characterize the conductances underlying the putative excitatory effects of these peptides, recordings were obtained from individual neurons isolated from their natural environment. An enzymatic procedure, first described by Kay and Wong (1986), for the isolation of adult mammalian neurons was recently adapted to magnocellular neurosecretory neurons (Cobbett & Weiss, 1990; Weiss & Cobbett, 1992). Oliet and Bourque (1992) further simplified this procedure for studying the intrinsic osmosensitivity in MNCs. The latter procedure was used in this study to investigate the ionic mechanisms underlying the responses of excitatory neuropeptides in MNCs.

2.1 PREPARATION OF ISOLATED MNCs

Unanaesthetized (140-200 g) adult male Long-Evans rats (Charles River, USA) were killed by decapitation. The skull was removed and optic nerves were cut to release the brain from the cranial vault. Coronal slices of 0.8 to 1 mm thick were obtained from the SON area using a razor blade, and blocks of tissue (≈ 1 mm^3) containing part of SON were dissected using iridectomy scissors. these blocks were incubated at 33° C for 90 min in oxygenated (100% O_2) PIPES solution (see below) containing 0.6-0.7 mg/ml trypsin to digest the connective tissue. After the incubation, blocks were washed with oxygenated

trypsin-free PIPES solution at room temperature for 20-30 min. Individual tissue blocks were triturated with fire-polished pipettes (0.2-0.5 mm i.d.) and about 100 µl of the resulting suspension was plated onto each of 5 to 8 Corning Petri dishes (35 mm). The cells adhered to the plastic within 15 min, after which the cells were perfused with HEPES-buffered solution. Untriturated blocks were kept in trypsin-free, oxygenated PIPES-buffered solution at room temperature for up to 12 hours prior to trituration. Figure 2.1 summarizes the steps used for the isolation of MNCs.

2.2 IDENTIFICATION OF MNCs

Immunocytochemical staining using antibodies directed against VP and OXT has shown that more than 96% of the cells isolated showing a cross sectional area exceeding 160 μm^2 are immunoreactive for either peptide (48% of MNCs being reactive to OXT and 51% being reactive to VP; Oliet & Bourque, 1992). Cells recorded in this study were therefore selected for their large size (diameter >15 µm) and bright appearance under phase contrast. Figure 2.2 shows a phase-contrast photomicrograph of a representative MNC used for patch clamp recording.

2.3 SOLUTIONS

The PIPES-buffered solution used for incubating the blocks in trypsin (pH=6.9-7.0) comprised (mM): NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; piperazine-N,N'-bis[2-ethanesulfonic acid],1,4-piperazinediethanesulfonic acid (PIPES, Sigma), 20; D-glucose, 25. The osmolality of PIPES saline was 290±2 mOsm.

During the recordings, the dishes were perfused with HEPES-buffered solution which comprised (mM): NaCl, 140; KCl, 3; $MgCl_2$, 1; $CaCl_2$, 1; N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES, Sigma), 10; D-glucose, 5-10. The osmolality of HEPES was 290±4 mOsm, and the pH was adjusted to 7.3-7.4 using NaOH. The osmolality of all solutions was measured using a freezing point osmometer (Advanced Instruments Inc.), and the pH using an Accumet pH meter (model 910, Fisher Scientific). The HEPES saline described above was used for perfusing the dishes during all whole-cell current-clamp and cell-attached single-channel recording experiments, and as a control condition in whole-cell voltage-clamp recordings. In ion substitution experiments, the concentration of certain ions in the external perfusing solutions were varied. table 2-1 summarizes the ionic concentrations in the external solutions used in different conditions. Tetrodotoxin (TTX, 0.5 μM) was added to external solutions when performing cell-attached and whole-cell voltage-clamp recordings. The internal solution that was used to fill the patch pipettes in whole-cell experiments (pH= 7.17; 265 mOsm) comprised (mM): K-gluconate, 110; $MgCl_2$, 3; HEPES, 10; ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 1; Na_2-adenosine triphosphate (ATP), 4; Na-guanosine 5'-triphosphate (GTP), 1 ; phosphocreatine (PC), 14. In some experiments, ATP and PC were omitted from the pipette medium, but they had no obvious effect on current and voltage responses.

The pipette solution used in cell-attached single channel recording experiments (pH=7.3-7.4) comprised (mM): Na_2SO_4, 70; KCl, 3; $MgCl_2$, 1; HEPES, 10; tetraethylammonium chloride (TEA), 20; 4-aminopyridine (4-AP), 5;

kynurenic acid, 1; TTX, 0.001. This solution was used as a control condition. For single channel ion substitution experiments, the concentration of certain ions were changed as summarized in table 2.2.

2.4 DRUGS

AII, acetate salt (Sigma), CCK octapeptide 26-33 sulphated (CCK-8S, Bachem, California), and NT fragment 8-13 (NT 8-13, Sigma) were dissolved in distilled water and kept in the freezer as aliquots of 10 µl (AII, 400 µM; CCK, 1 mM; NT, 500 µM). These were diluted to the desired concentration on the day of the experiment using the external medium appropriate for the given condition. Drugs were administered via a puffing micropipette connected to a pressure-driven Picospritzer (General Valve Co.) for 20-40 s, or by hand-held addition of a small bolus (using a Gilson Pipetman) to the entire petri dish. No differences were noted in the delay, or amplitude, of the responses obtained by the two methods. The final concentrations reaching the cells were estimated to be: AII, 0.4-4 µM; CCK, 0.5-1 µM and NT, 0.5-5 µM.

2.5 ELECTROPHYSIOLOGICAL RECORDING

Petri dishes containing isolated cells were placed in a Leiden perfusion chamber (Medical Systems Co.) attached to the stage of an inverted phase contrast microscope (Diaphot; Nikon Co.) which was mounted on top of a vibration isolation table (Technical Manufacturing Co.). When the drug was applied by puffing, constant perfusion of HEPES solution was maintained throughout the recording (2-3 ml/min) using a peristaltic pump (Watson-Marlow).

However, when drugs were added by bolus to the entire petri dish, perfusion was stopped just before the addition, and was resumed 30-40 s later. All recordings were performed at room temperature (20-24°C).

2.5.1 Whole-cell patch-clamp recording

Whole-cell current- and voltage-clamp recordings were obtained from isolated MNCs using an Axopatch-1D amplifier (Axon Instruments Inc.). The patch pipettes (4-5 MΩ) were pulled from single barrel filament capillary glass (A-M Systems Inc.; o.d.= 1mm, i.d.= 0.58 mm) using a Flaming/Browm Micropipette Puller (P-87; Sutter Instrument Co.), and were filled with the internal solution described in section 2.3. A positive pressure of 0.5-1.0 cm H_2O, monitored with a water manometer, was applied by mouth to the inside of the pipette just before placing the patch electrode in the perfusing solution. Under visual control, and with the assistance of an audio monitor, a motorized micro-manipulator (MS 314, Fine Sciences Tools Inc.) was used to advance the electrode toward the plasma membrane of the cell. As soon as the contact between the electrode and the membrane was established, the positive pressure was released and the assembly was left undisturbed for a few minutes to allow sealing of the patch to the glass. In many cases, a little negative pressure (0.2-0.5 cm H_2O) was needed to increase the tightness of the seal (to 1-50 GΩ). The pipette voltage was clamped to -60 mV (near resting membrane potential) just before going whole-cell. The patch was ruptured by applying a little negative pressure and by delivering a 0.2-0.8 ms "zap" using the Axopatch-1D's circuitry. Whole-cell and pipette capacitance was compensated

electronically.

Current signals (filtered at 200 Hz) and voltage signals (filtered at 5 kHz; Axopatch-1D, Axon Instruments Inc.) were stored via a pulse code modulator (Neuro corder DR-384, Neurodata Co.) onto videotape (RCA, Video Cassette Recorder). These data were also displayed either on chart paper (Neo Inkless Series, Graphtec) using a Linearecorder mark VII WR3101 (Graphtec), or displayed and stored using Axotape software (Axon Instruments Inc.). pCLAMP software 5.5.1 (Axon Instruments Inc.) was used to digitize the current and voltage signals, or to generate command voltage ramps.

2.5.2 Cell-attached patch-clamp recordings

To minimize noise, the pipettes used in cell-attached recordings were fire-polished on a microforge (Narashige, Scientific Instrument Lab.), and coated with dental wax up to 0.5 mm of the tip. Single channel currents were amplified and filtered at 5-10 kHz and stored on videotape. Data were digitized at 20 kHz using the Fetchex program (pCLAMP suite), and digitally filtered at 1-2 kHz prior to analysis. Single channel events selected for anlysis were chosen manually to eliminate contamination due to spurious transients. Single channel current amplitude was determined as the difference between baseline and the average current amplitude during the open state (Fetchan, pCLAMP). Single channel open and closed times were determined using pSTAT, from the events lists generated during file analysis in Fetchan.

A major difficulty encountered in cell-attached experiments is estimating the actual membrane potential (V_m) of the cell being recorded. In the present

experiments, V_m was estimated as the average membrane potential (i.e. V_m at I=0) observed during current responses to voltage ramps applied in the presence of each peptide. Since the mean V_m values were similar for each of the peptides tested: -38±1 mV (AII, n=8), -36±1 mV (CCK, n=3) and -37±1 mV (NT, n=6); an average value of -37 mV was used as estimate of V_m in all cell-attached recordings.

2.6 STATISTICAL ANALYSIS

For illustration purposes and in the text, all values are presented as mean ± standard error of the mean (S.E.M.). In comparing data sets for a single condition, statistical significance of any differences was assessed using Student's t-test. Complementary analysis performed using a two way ANOVA, followed by Tukey posthoc test to determine where differences occured was in agreement with that obtained with t-test. The null hypothesis was rejected when $p < 0.05$.

Solution	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NMDG	HEPES	TTX
control	140	3	1	1	147	-	10	0.0005
low $[Cl^-]_o$	140	3	1	1	7	-	10	0.0005
high $[K^+]_o$	140	6	1	1	150	-	10	0.0005
low $[Na^+]_o$	70	3	1	1	147	70	10	0.0005

TABLE 2.1: Concentrations of ions, in mM, present in the various extracellular solutions used in the present experiments.

pipette solution	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NMDG	HEPES	TEA	4-AP	Kynu. acid	TTX
control	140	3	1	1	15	-	10	20	5	1	0.001
High $[K^+]_o$	140	6	1	1	18	-	10	20	5	1	0.001
Low $[Na^+]_o$	70	3	1	1	85	70	10	20	5	1	0.001

TABLE 2.2: Concentrations of ions, in mM, present in the various pipette solutions used in the present cell-attached patch-clamp experiments.

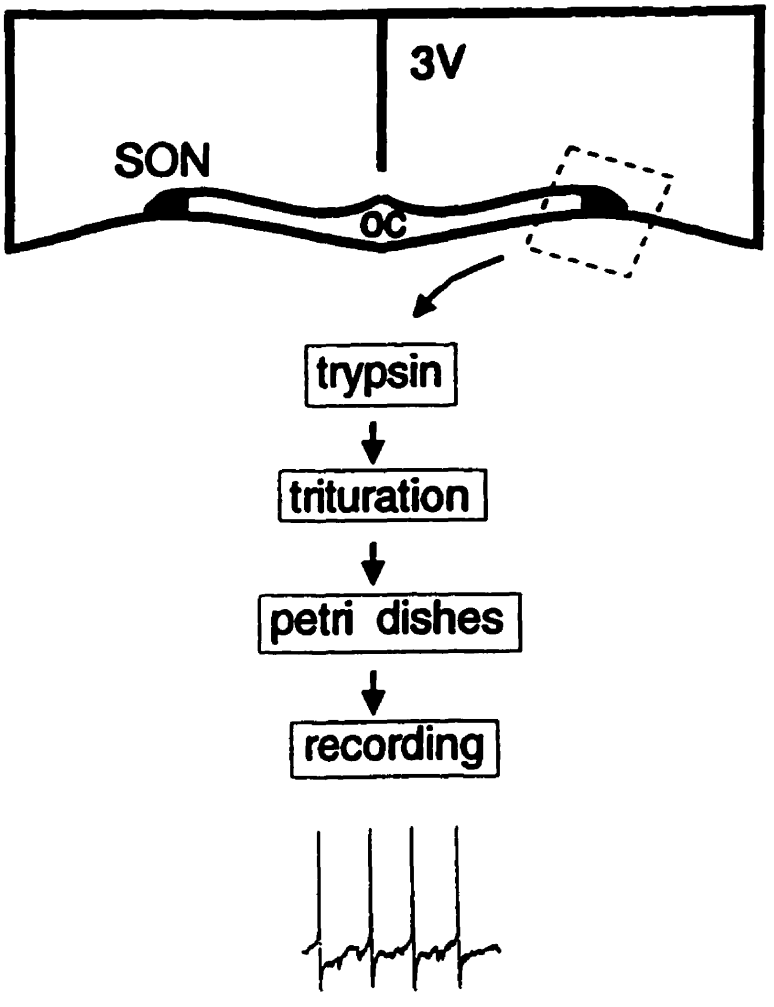

FIGURE 2.1: Scheme of the experimental procedure used for the acute isolation MNCs from coronal hypothalamic tissue slices obtained from the adult rat (top). 3V, third ventricle; SON, supraoptic nucleus; OC, optic chiasm.

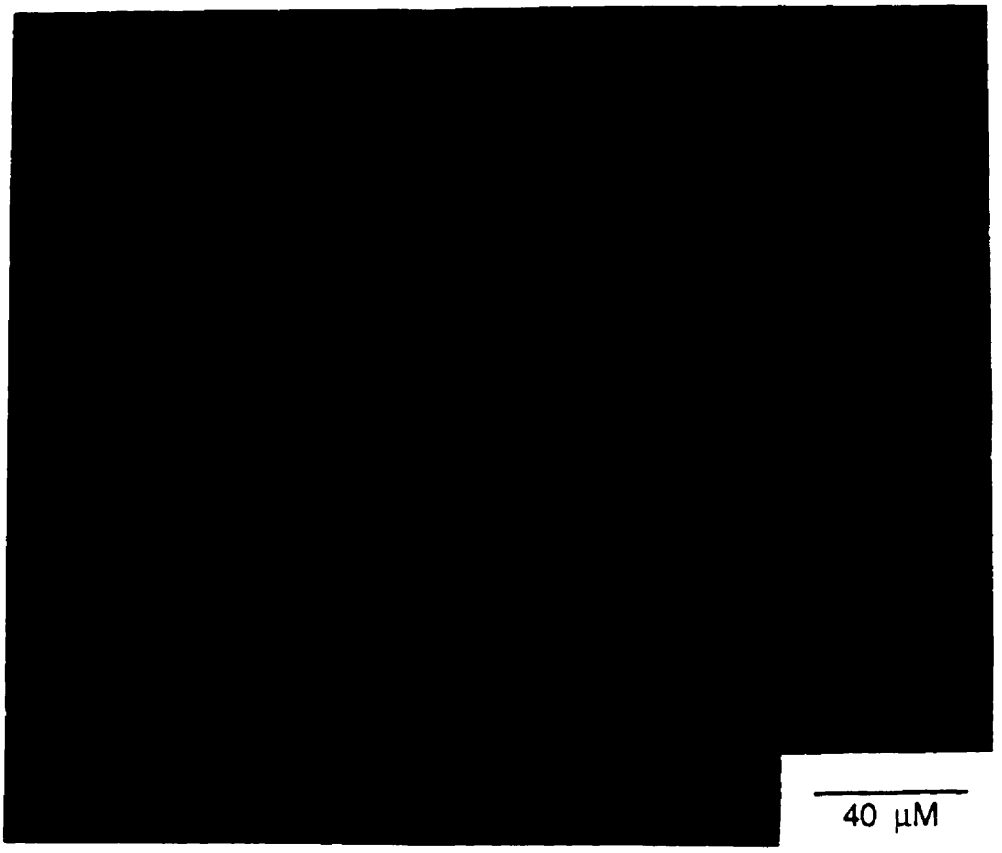

FIGURE 2.2: Photomicrograph of an acutely dissociated MNC (centre) as observed under phase-contrast microscopy. The shadow above the cell is due to the presence of a patch electrode.

CHAPTER THREE

PEPTIDE-ACTIVATED CONDUCTANCE IN RAT SUPRAOPTIC NEURONS

3.1 INTRODUCTION

As described in sections 1.5.1, 1.5.4 and 1.5.10, previous experiments on hypothalamic explants in vitro have shown that AII, CCK and NT may depolarize MNCs by increasing a non-selective cationic conductance. The hypothesis presented in section 1.6 suggests that a number of excitatory transmitters modulate a single group of non-selective cationic channels in MNCs. If this hypothesis is correct, the macroscopic conductances activated by these drugs would be expected to share similar characteristics. In the present experiments, the effects of AII, CCK and NT on isolated MNCs were first examined using whole-cell current-clamp to establish whether the excitatory effects of these drugs are mediated postsynaptically. Second, quantitatively accurate measurements of the reversal potentials of the currents evoked by these peptides were obtained under voltage-clamp. The effects of ion substitution on the reversal potentials were then examined to compare the ionic permeability characteristics of the conductances gated by these representative excitatory neurotransmitters.

3.2 RESULTS

The whole-cell patch-clamp technique was used to study the macroscopic peptide-activated conductances in isolated supraoptic MNCs. This technique allows precise control over the intracellular and extracellular concentration of different solutes. Moreover, the low resistance of the electrodes used maximizes the signal to noise ratio of the recordings and provides the resolution required for precise detection of reversal potentials. Finally, space-clamp problems were not likely to occur with this approach, since the cells used did not retain long processes following dissociation. While more than 150 whole-cell recordings were performed, only data obtained from cells showing reversible responses are reported below. Also excluded from the analysis were data obtained from cells in which responses were observed instantly upon peptide application, and which therefore possibly resulted from a mechanical artifact.

3.2.1 Peptide-evoked responses under current-clamp

To examine the effects of peptides on membrane excitability, whole-cell current-clamp recordings were obtained from 36 MNCs isolated from the SON. Application of either AII, CCK or NT to cells maintained (by 10-20 pA DC current injection) at membrane potentials 7-15 mV below the threshold for action potential discharge (≈-50 mV) induced a reversible depolarization and firing in most of the cells tested: AII (16/20); CCK (4/5); NT (8/11). As shown in figure 3.1, these responses generally lasted between 2 and 4 minutes before complete recovery. The amount of depolarization at the peak of the responses ranged between 9 and 17 mV. When tested with consecutive applications of

different peptides, 5 of the 6 cells examined showed positive responses to each of the drugs. In one cell, however, application of CCK evoked a depolarizing response, but a subsequent application of AII was without effect (data not shown). These findings indicate that AII, CCK and NT activate postsynaptic receptors on MNCs, and that activation of receptors for these peptides produces excitatory responses of similar amplitude and duration.

3.2.2 Effects of peptides on membrane current and input conductance

To investigate the properties of the membrane current underlying the depolarizing effects of AII, CCK and NT, current-voltage analysis was performed under whole-cell voltage-clamp. Although the percentage of cells excited by these peptides was similar to that observed under current-clamp (> 80%), the results described below were obtained from 71 responsive MNCs.

When held near the resting membrane potential (-60 mV), application of AII, CCK and NT resulted in a reversible inward current reaching a peak within 20-40 s (data not shown). To determine the nature of the underlying change in conductance, current responses to slow voltage (17.5 mV/s) ramps were used to approximate steady-state current-voltage (I-V) relations in the absence and presence of drug. Membrane conductance was calculated from the slope of these I-V relations between -90 mV and -60 mV. As shown in figure 3.2, application of the peptides resulted in a reversible increase of membrane conductance. The mean increases in conductance caused by AII (0.37±0.1 nS; n=8), CCK (0.36±0.04 nS; n=3) and NT (0.28±0.06 nS; n=6) were not significantly ($p > 0.05$) different from each other.

3.2.3 Ionic basis of peptide-evoked excitation

To determine the ionic basis of the currents evoked by peptide-mediated increases in conductance, accurate measurements of the reversal potentials were obtained from the intersection point of the I-V curves recorded in the absence and presence of peptides (fig. 3.3). As shown in figure 3.4, the mean reversal potentials of the currents evoked by AII (-37±3 mV; n=8), CCK (-35±3 mV; n=3) and NT (-33±3 mV; n=6) were not significantly ($p > 0.05$) different from each other, suggesting that the permeability characteristics of these currents are similar. If this hypothesis is correct, the reversal potentials of the currents evoked by each peptide should change by a similar amount when evoked under different ionic conditions.

Effects of reducing external Cl

In low $[Cl^-]_o$ solution (see table 2.1), the hypothetical equilibrium potential to chloride (E_{Cl}) estimated using the Nernst equation was -4 mV rather than -77 mV (E_{Cl} in control solution). If the peptide-regulated conductance is partly permeable to Cl^-, therefore, the reversal potentials of peptide-evoked currents would be expected to shift in a positive direction. The mean reversal potentials observed in low $[Cl^-]_o$ solution were: -40±2 mV (AII, n=3), -34±3 mV (CCK, n=3) and -38±5 mV (NT, n=4). These values were not significantly ($p > 0.05$) different from one another and, as shown in figure 3.5, they were not significantly different from those observed in control solution. These observations suggest that the peptide-gated conductances are not permeable to chloride.

Effects of increasing external K^+

In high $[K^+]_o$ solution (see table 2.1), the hypothetical E_K shifted from -86 mV in control solution to -70 mV. If the peptide-activated conductance is permeable to K^+, the reversal potential of the evoked current is therefore expected to shift in a positive direction. In the presence of high $[K^+]_o$, the mean reversal potentials were (in mV): -15±4 (All, n=7), -12±2 (CCK, n=3) and -11±2 (NT, n=7). These values were not significantly ($p>0.05$) different from one another but, as shown in figure 3.6, they were significantly more positive than those obtained in control solution (All, p=0.00003; CCK, p=0.004; NT, p=0.00001). These results indicate that the peptide-induced conductance is partly permeable to K^+.

Effects of reducing external Na^+

In low $[Na^+]_o$ solution (see table 2.1), the hypothetical equilibrium potential for Na^+ was reduced from +69 to +52 mV. If the peptide-regulated conductance is permeable to Na^+, the reversal potentials of the peptide-evoked currents are expected to shift in the negative direction. The mean reversal potentials observed in low $[Na^+]_o$ solution were (in mV): -56±3 (All, n=3), -56±6 (CCK, n=3) and -54±1 (NT, n=3). These values were not different from one another ($p>0.05$) but, as shown in figure 3.7, they were significantly more negative than those obtained in control solution (All, p=0.0028; CCK, p=0.029; NT, p=0.00076). These results suggest that the conductances modulated by All, CCK and NT are permeable to Na^+.

3.2.4 Voltage dependency

The results described in sections 3.2.1, 3.2.2 and 3.2.3 indicate that all of the aspects of the responses to AII, CCK and NT that were studied were quantitatively similar. It is presumed, therefore, that all of these peptides activate a macroscopic current with similar properties. Digital substraction of I-V curves recorded before and after peptidergic stimulation was used to examine the voltage dependency of the peptide-regulated conductance. Figure 3.8 illustrates examples of the I-V relations of currents evoked in control, low $[Na^+]_o$ and high $[K^+]_o$ solutions. As can be seen in this figure, the relations were linear over the voltages tested, suggesting that the peptide-activated conductance is not voltage-sensitive.

3.2.5 Relative permeability

The relative permeability of the peptide-regulated conductance was estimated using the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$E_{rev} = RT/F \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o}{P_K[K^+]_i + P_{Na}[Na^+]_i} \qquad (1)$$

where E_{rev} is the reversal potential of the evoked current, R the universal gas constant (1.987 cal K^{-1} mol^{-1}), T the absolute temperature (295 ^oK), F Faraday's constant (9.648 X 10^4 C mol^{-1}), and P_K and P_{Na} the relative permeability of K^+ and Na^+, respectively. To estimate the permeability of Na^+ relative to K^+, P_K was set to a value of 1, and the value of P_{Na} yielding the appropriate E_{rev} was

determined. In control solution, the value of P_{Na} required to explain a mean reversal potential of -35 mV (the average value of E_{All}, E_{CCK} and E_{NT}) was 0.16. Similarly, a P_{Na} value of 0.12 was required to explain the mean reversal potential of -55 mV observed in low $[Na^+]_o$. Surprisingly, a P_{Na} value of 0.43 was required to explain the mean reversal potential of -13 mV obtained in high $[K^+]_o$. These findings indicate that the peptide-modulated conductance is about 7 times more permeable to potassium than to sodium in control and low $[Na^+]_o$ solutions, but that this ratio decreases to 2 when the concentration of K^+ in the external solution is doubled (from 3 to 6 mM).

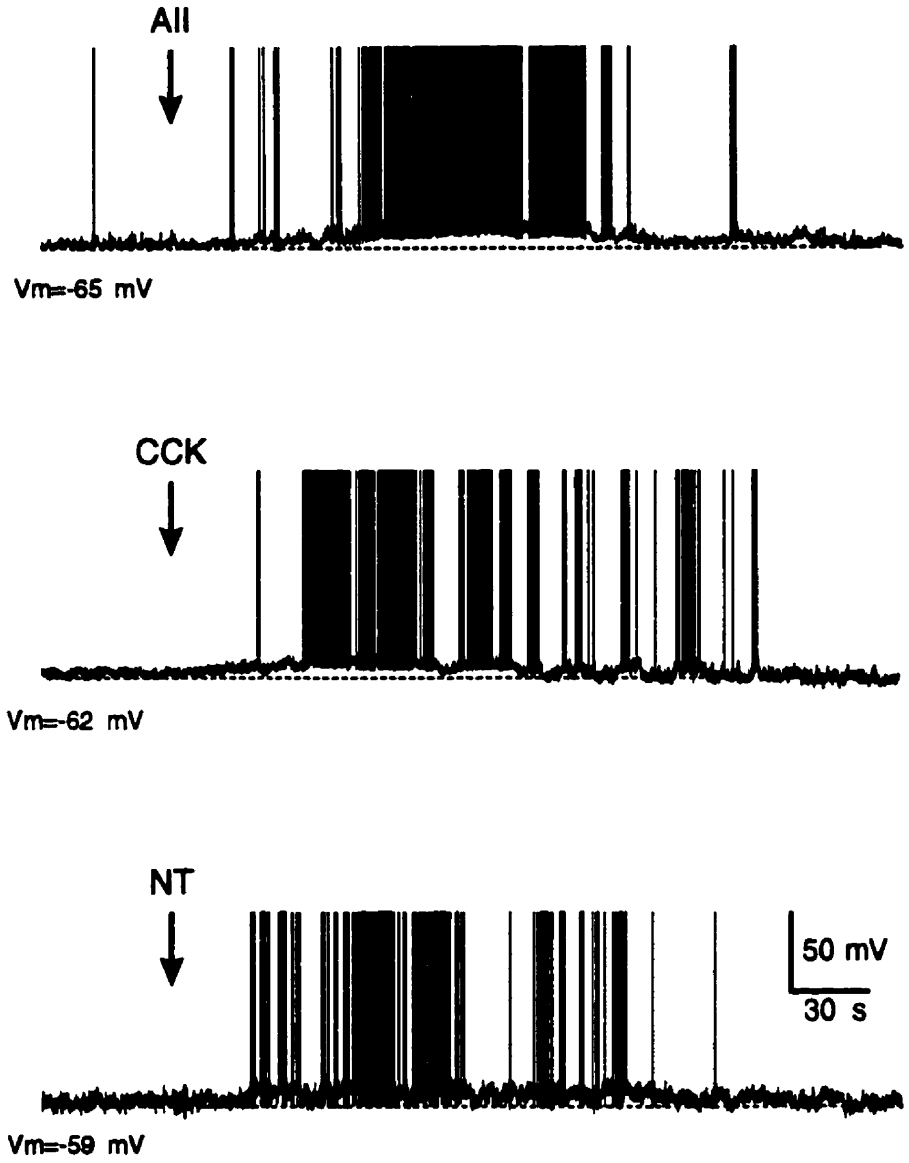

FIGURE 3.1: Whole-cell current-clamp recordings obtained from MNCs isolated from the rat supraoptic nucleus. The traces show depolarizing responses to application (arrows) of AII, CCK and NT. When recorded from initial membrane potentials maintained below the threshold of spike discharge, each peptide induced a reversible depolarization and action potential firing which lasted between 2 and 4 minutes before achieving complete recovery.

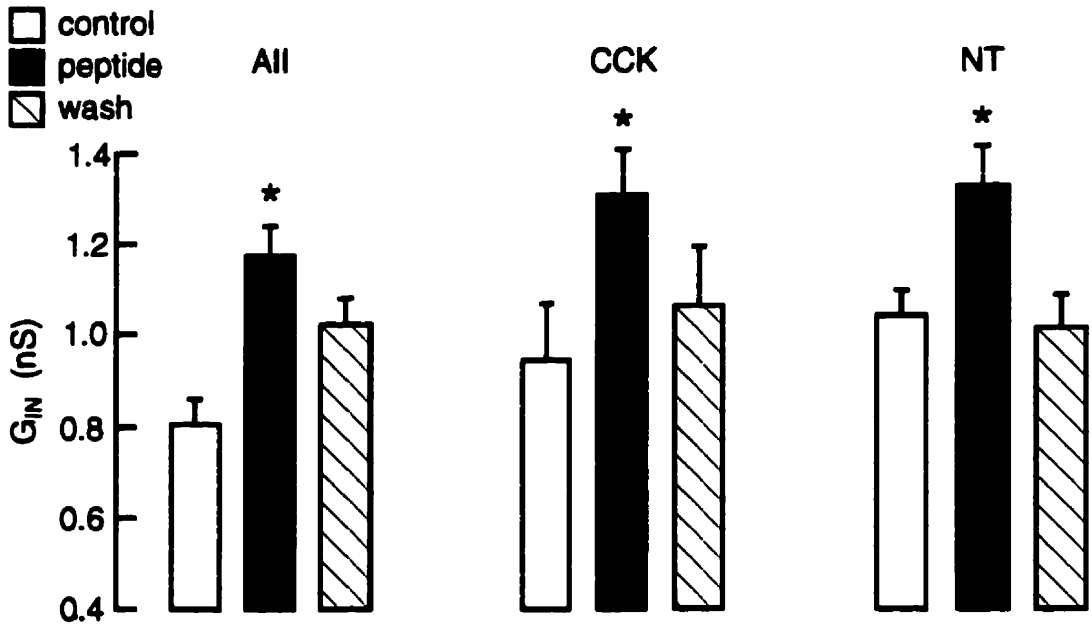

FIGURE 3.2 Bar histograms illustrate the mean (± S.E.M.) changes in input conductance (G_{IN}) recorded from 17 MNCs in response to the application of: AII, (n=8); CCK, (n=3); and NT, (n=6); before, during the peak of the response, and after recovery.

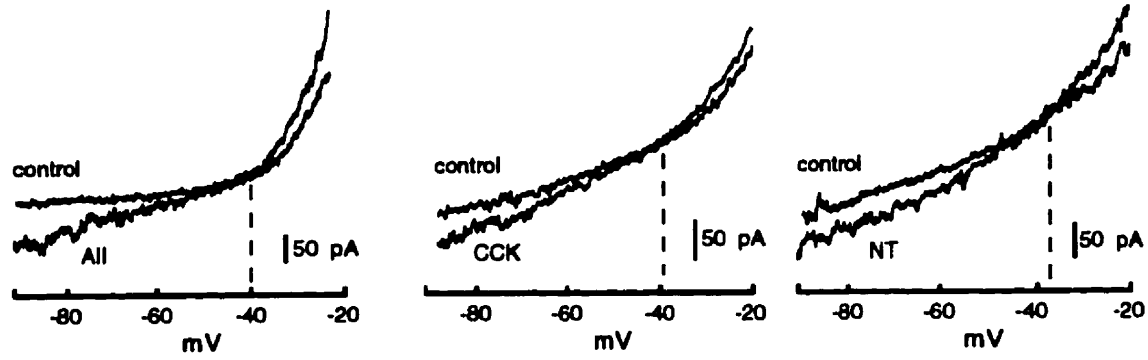

FIGURE 3.3: Current-voltage relations, approximated from membrane current responses to slow voltage ramps (17.5 mV/s), were obtained from MNCs perfused with control solution. Application of AII, CCK and NT induced a reversible membrane current accompanied with a reversible increase in slope conductance.

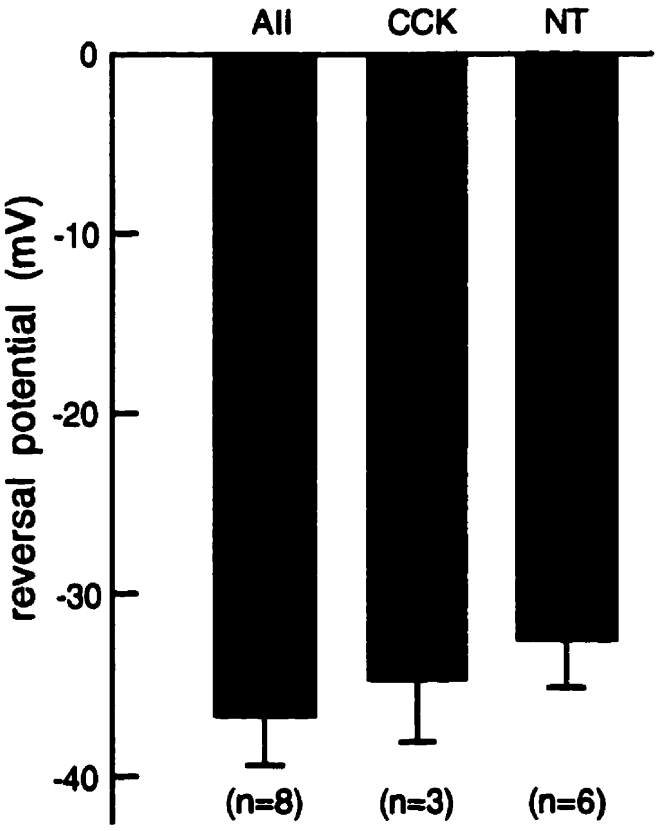

FIGURE 3.4: Bar histograms showing the mean (± S.E.M.) reversal potentials of the currents evoked by AII (n=8), CCK (n=3) and NT (n=6), obtained from the intersection points of I-V relations captured in the presence and absence of drug. The values are not significantly different from each other ($p > 0.05$).

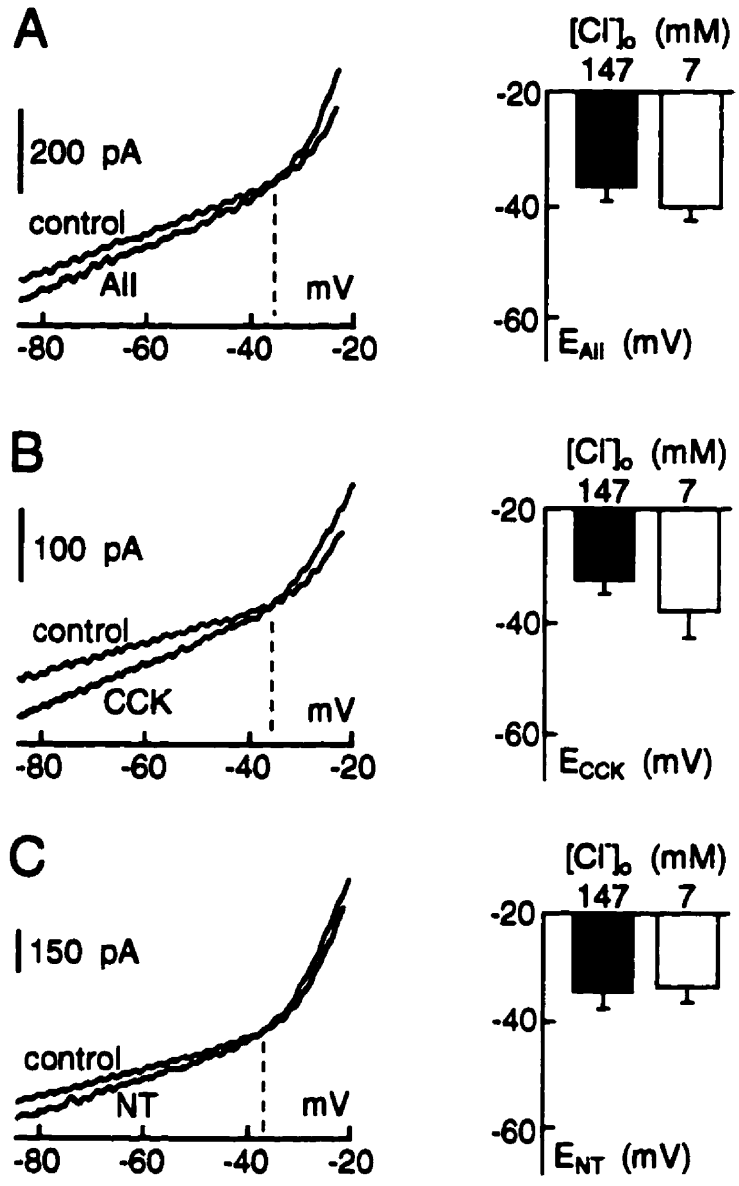

FIGURE 3.5: Effects of reducing the concentration of extracellular chloride ($[Cl^-]_o$) from 147 mM to 7 mM on the reversal potential of the peptide-evoked currents. The left panels show I-V relations recorded in the absence and presence of AII (A), CCK (B) and NT (C). The vertical dashed line shows the current reversal potentials corresponding to each experiment. The right hand panels are bar histograms plotting the mean (± S.E.M) reversal potentials of the currents evoked by AII (E_{AII}), CCK (E_{CCK}) and NT (E_{NT}), in control (147 mM; filled bars) and low (7 mM; open bars) chloride solution. The values are not significantly different from each other ($p > 0.05$), both within and between groups.

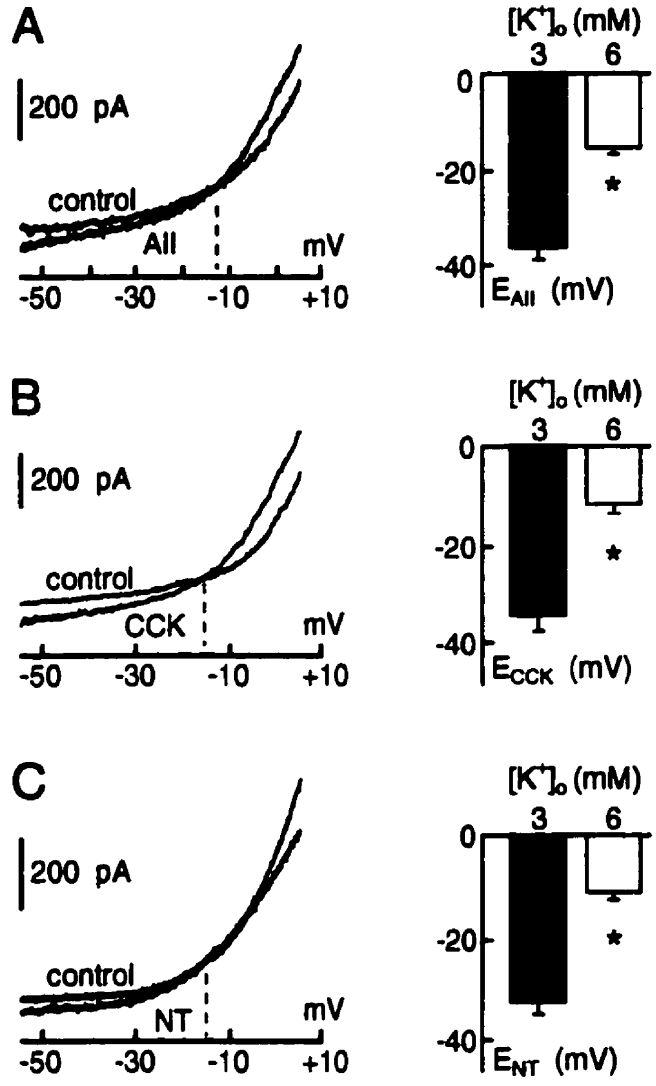

FIGURE 3.6: Effects of increasing the concentration of extracellular potassium ($[K^+]_o$) from 3 mM to 6 mM on the reversal potential of the peptide-evoked currents. The left panels show I-V relations recorded in the absence and presence of AII (A), CCK (B) and NT (C). The vertical dashed line shows the current reversal potentials corresponding to each experiment. The right hand panels are bar histograms plotting the mean (± S.E.M.) reversal potentials of the currents evoked by AII (E_{AII}), CCK (E_{CCK}) and NT (E_{NT}), in normal (3 mM; filled bars) and high (6 mM; open bars) potassium solution. The values are not significantly different from each other ($p > 0.05$) between groups, but they are significantly greater in high $[K^+]_o$ (* indicates $p < 0.05$).

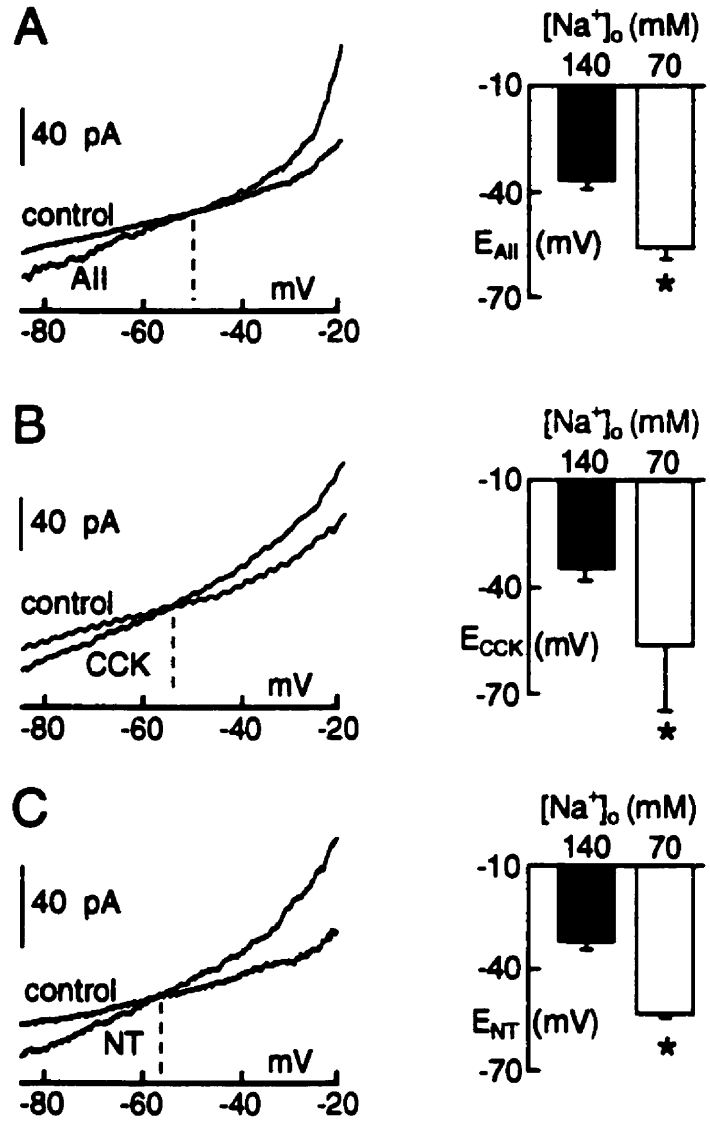

FIGURE 3.7: Effects of reducing the concentration of extracellular sodium ($[Na^+]_o$) from 140 mM to 70 mM on the reversal potential of the peptide-evoked currents. The left panels show I-V relations recorded in the absence and presence of AII (A), CCK (B) and NT (C). The vertical dashed line shows the current reversal potentials corresponding to each experiment. The right hand panels are bar histograms plotting the mean (± S.E.M.) reversal potentials of the currents evoked by AII (E_{AII}), CCK (E_{CCK}) and NT (E_{NT}), in normal (140 mM; filled bars) and low (70 mM; open bars) sodium solution. The values are not significantly different from each other ($p > 0.05$) between groups, but they are significantly lower in low $[Na^+]_o$ (* indicates $p < 0.05$).

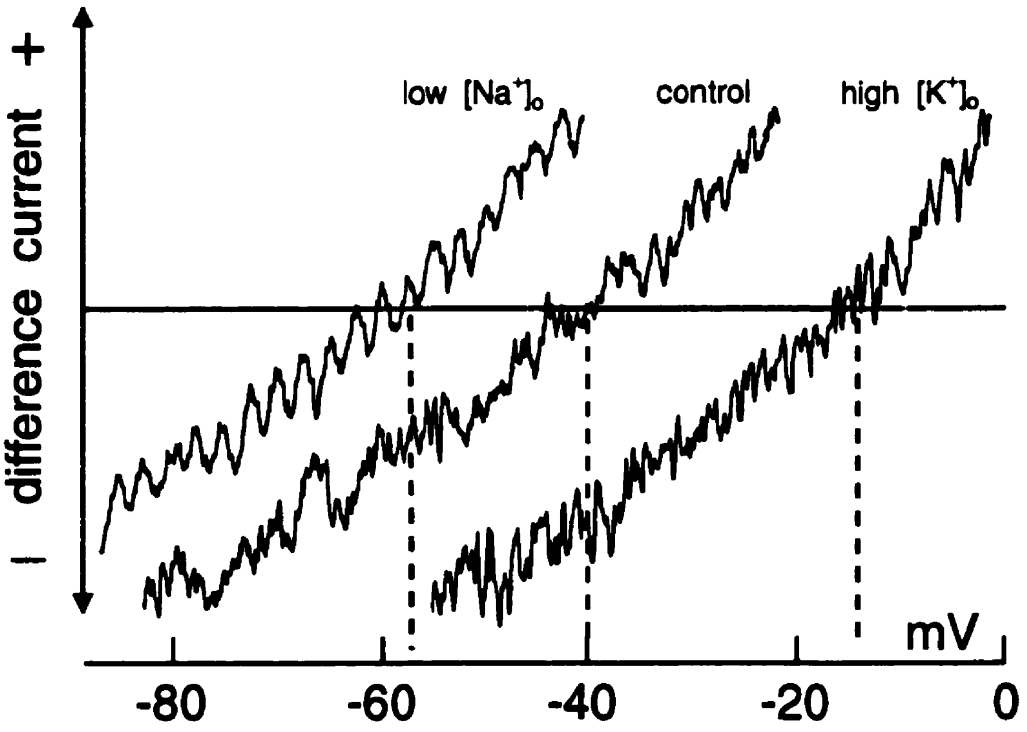

FIGURE 3.8: I-V relations of the peptide-evoked currents obtained by digitally subtracting current responses to voltage ramps obtained in the presence and absence of peptide. The traces shown are representative examples of the currents (scaled to fit the graph) evoked by peptide application under control, high $[K^+]_o$ and low $[Na^+]_o$ conditions. Note that the I-V relationship is linear over the range of potentials tested.

3.3 DISCUSSION

3.3.1 MNCs express postsynaptic receptors to AII, CCK and NT

The data presented in this chapter indicate that AII, CCK and NT depolarize and increase the firing rate of supraoptic MNCs. As these excitatory responses were observed in cells lacking any synaptic connections, it can be concluded that specific receptors for these peptides are expressed directly on MNC somata, as previously suggested from experiments using hypothalamic explants (AII, Yang et al., 1992; CCK, Jarvis et al., 1992; NT, Kirkpatrick and Bourque, 1995). VP- and OXT-secreting MNCs isolated from the SON using the dissociation procedure described in section 2.1 occur in quasi-equal proportions (51% VP and 48% OXT; Oliet and Bourque, 1992; Oliet, 1994). Since excitatory responses to AII, CCK and NT were observed in ≈78% of the cells tested (section 3.2.1), both types of MNCs presumably express receptors to these peptides.

3.3.2 AII, CCK and NT activate macroscopic conductances with common characteristics

The conductance mechanisms underlying the responses of MNCs to AII, CCK and NT seem to be similar, as determined from their quantitatively similar: (1) time course of excitation under current-clamp (fig. 3.1), (2) conductance increase (fig. 3.2), (3) current reversal potentials in control solution (fig. 3.4), (4) lack of sensitivity to changes in external chloride, (5) current reversal potentials in high $[K^+]_o$ solution (fig. 3.6), (6) current reversal potentials in low $[Na^+]_o$ solution (fig. 3.7). It is concluded, therefore, that the macroscopic

conductances activated by AII, CCK and NT share common characteristics and, in fact, that a single conductance may be the target of the three peptides.

3.3.3 Properties of the peptide-gated conductance

Excitatory responses to AII, CCK and NT result from the activation of a conductance that is permeable to Na^+ and K^+, but not to chloride. Moreover, preliminary results indicate that the mean reversal potential of the peptide-evoked current is not significantly afected by increasing $[Ca^{2+}]_o$ from 1 to 2 mM (-32±3 mV; n=3), suggesting that the conductance may not be permeable to this ion as well. Interestingly, the data shown in figure 3.8 suggest that the cationic conductance is not voltage-sensitive, a feature predicted from limited I-V analysis in hypothalamic explants (e.g. Jarvis et al., 1992). Whether any rectification is present at positive potentials remains to be established.

The results obtained from ion substitution experiments indicate that under physiological conditions AII, CCK and NT modulate channels which are seven times more permeable to K^+ than to Na^+. While this permeability ratio was similar in control and low $[Na^+]_o$ solutions, the permeability to Na^+ increased upon raising the concentration of K^+ in the external solution. Although further analysis will be required to establish the basis for this effect, it is possible that this feature indicates the presence of multiple ion binding sites within the pore region of the channels involved (Hille, 1992).

CHAPTER FOUR

PEPTIDE-GATED CHANNELS IN RAT SUPRAOPTIC NEURONS

4.1 INTRODUCTION

The results of chapter three indicate that responses to AII, CCK and NT share numerous common properties and, in fact, that a single conductance may be the target for these peptides. This possibility provides initial support for the hypothesis presented in section 1.6 "*that a single population of cation permeable channels is modulated by the different excitatory substances contributing to the regulation of MNC activity*". If this hypothesis is correct, single channels activated by application of AII, CCK and NT should display common biophysical features. Moreover, it should be possible to demonstrate that individual channels can be activated by consecutive applications of more than one peptide. In this chapter, therefore, cell-attached patch-clamp recordings were obtained from isolated MNCs to investigate the effects of AII, CCK and NT.

4.2 RESULTS

Cell-attached patch-clamp recordings were obtained from 174 MNCs using microelectrodes containing electrolyte concentrations mimicking in situ conditions. In order to isolate the putative cationic channels regulated by peptides, these pipettes also contained blockers of other types of channels that might also be present in membrane patches (see section 2.3 and table 2.2). In response to peptide application, only 24 of the patches recorded showed an increase in ion channel activity. The results presented below were thus analyzed from these particular recordings.

4.2.1 Effects of single peptide application

In most of the responsive patches (n= 20), no channel activity was detected in the background. However, in 4 of the patches, a small amount of basal activity was apparent (opening probability, P_o= 0.0011 ± 0.0002; see section 4.2.4). Bath application of either AII (n=14), CCK (n=3) or NT (n=7) caused a reversible activation, or increase in frequency, of single channel openings (fig. 4.1). Changes in opening frequency associated with peptide application proceeded gradually, and appeared to reach a peak with a delay of 20 to 45 s. This pattern of channel activation was similar from one peptide to the other. In 7 patches, a significant amount of data was obtained before and after peptide application. The data obtained from these recordings were pooled, and figure 4.3 illustrates the resulting mean time course of channel activation following peptide application. The mean maximal P_o determined in 16 patches was 0.013 ± 0.001.

4.2.2 Current-voltage (I-V) analysis of peptide-gated channels

Statistical analysis (see section 2.5.2) revealed that at a holding potential of -77 mV, the mean amplitude of the current flowing during single openings was similar for AII (-1.30 ± 0.02 pA; n= 5), CCK (-1.29 ± 0.05 pA; n= 3) and NT (-1.31 ± 0.03 pA; n= 3) (e.g. fig. 4.3). In the presence of a normal extracellular electrolyte environment, open channel I-V relations were obtained from 5 active membrane patches by changing the holding potential (V_H). As shown in figure 4.4, the open channel I-V was linear. Linear regression analysis through the data points obtained from each of 3 patches activated by application of AII revealed that the mean unitary conductance of the channels was 35.4 pS (R=0.99; n= 12), with a reversal potential (E_{rev}) of the unitary current at -41.2 mV (fig. 4.4). Data points obtained from two other patches containing channels activated by either CCK or NT were found to fall within the 95% confidence interval of the regression fit obtained for AII (fig. 4.4). A lumped regression analysis of data points (n= 21) obtained from all patches in which I-V analysis was performed yielded a mean slope conductance of 36.9 pS (R= 0.99) and a mean E_{rev} of -38.8 mV. It is concluded, therefore, that the channels activated by AII, CCK and NT are similar.

A limited amount of data was also obtained under other ionic conditions. In one patch, the E_{rev} of the current flowing through a single channel activated by AII was found to be -52.9 mV (R= 0.98; n= 5) when determined with a pipette solution containing 70 mM Na^+. In another patch, with 6 mM K^+ in the pipette, the E_{rev} of a NT-activated channel was found to be -15.5 mV (R= 0.99; n=4; fig. 4.5). These combined results suggest that the peptide-activated

channels are permeable to cations, and have permeabilty characteristics similar to the macroscopic conductance described in chapter three.

4.2.3 Single channel kinetics

In order to determine the kinetic basis for the peptidergic activation of cationic channels, data had to be analyzed from patches in which only one channel was present. The analysis was therefore restricted to recordings in which openings with apparent double amplitude were never observed, and in which the probability that 2 channels were nonetheless present was < 5%. The probability of having two channels was approximated using equation 2 (Drapeau, 1990; adapted from Colquhoun and Hawkes, 1983):

$$\text{Probability of 2 channels} \approx [1 / (1 + P_o / 2)]^{\#} \qquad (2)$$

where P_o is the probability of opening, and # the number of openings observed in the recording segment analyzed. Under these conditions, recordings obtained from 19 patches suggested the presence of only one channel in the patch.

In 8 patches, where enough openings were recorded to obtain a good fit, a single exponential was sufficient to fit the dwell time histogram, suggesting the existence of a single open state (fig. 4.6A). The mean time constant of the open state (τ_o) under stimulated conditions was 0.66 ± 0.06 ms. Mean values of τ_o were similar for each peptide (ms): All (0.68 ± 0.10, n=4), CCK (0.63 ± 0.14, n=2), and NT (0.61 ± 0.04, n=3). In contrast, the closed times were distributed more broadly, and the sum of at least two exponentials was needed

to obtain a good fit (fig. 4.6B), implying the presence of at least two closed states. One of these was characterized by a short time constant (τ_{C1} < 10 ms), whereas the other (τ_{C2}) was longer, and ranged between 50 and 1500 ms. Since most of the recordings obtained in these experiments lasted less than 9 minutes, and since channels opened with low probability, it was difficult to obtain an accurate estimation of the closed time distributions under stimulated and non-stimulated conditions.

Because of the constraints described above, the kinetics underlying peptide activation of the cation channels were therefore simply approximated from the changes in mean open time (MOT) and mean closed time (MCT), obtained by dividing the total time spent by the channel in each state (open or closed) by the number of events analyzed (Colquhoun and Sigworth, 1983). Kinetic analysis performed in the 4 patches where it was possible to compare P_o values at different time points during the individual recordings (fig. 4.7) revealed that the reversible increase in channel P_o resulted selectively from a decrease in MCT (slope = 0.83; R=0.99; n=12) without consistent effects on MOT (slope = -16; R= 0.38; n=12).

4.2.4 Effects of consecutive applications of different peptides

The data presented above show that cation channels with similar properties are activated by AII, CCK and NT. To investigate the hypothesis that a single population of channels mediates the effects of different peptides (see section 1.6), the effects of different peptides were examined on patches assumed to contain a single channel, based on criteria described in section

4.2.4 (n=63). In 54 of these patches, none of the peptides tested was able to induce channel activity (fig. 4.8). In 4 patches in which two peptides were applied consecutively, one of the peptides succeeded in inducing channel activity, whereas the other failed. In three of these cases, the failure occured during the second application, while in the other patch a response was observed during the second application, but not the first. Finally, in 5 patches, the activity of a single channel was found to increase in response to separate applications of a different peptide (fig. 4.9).

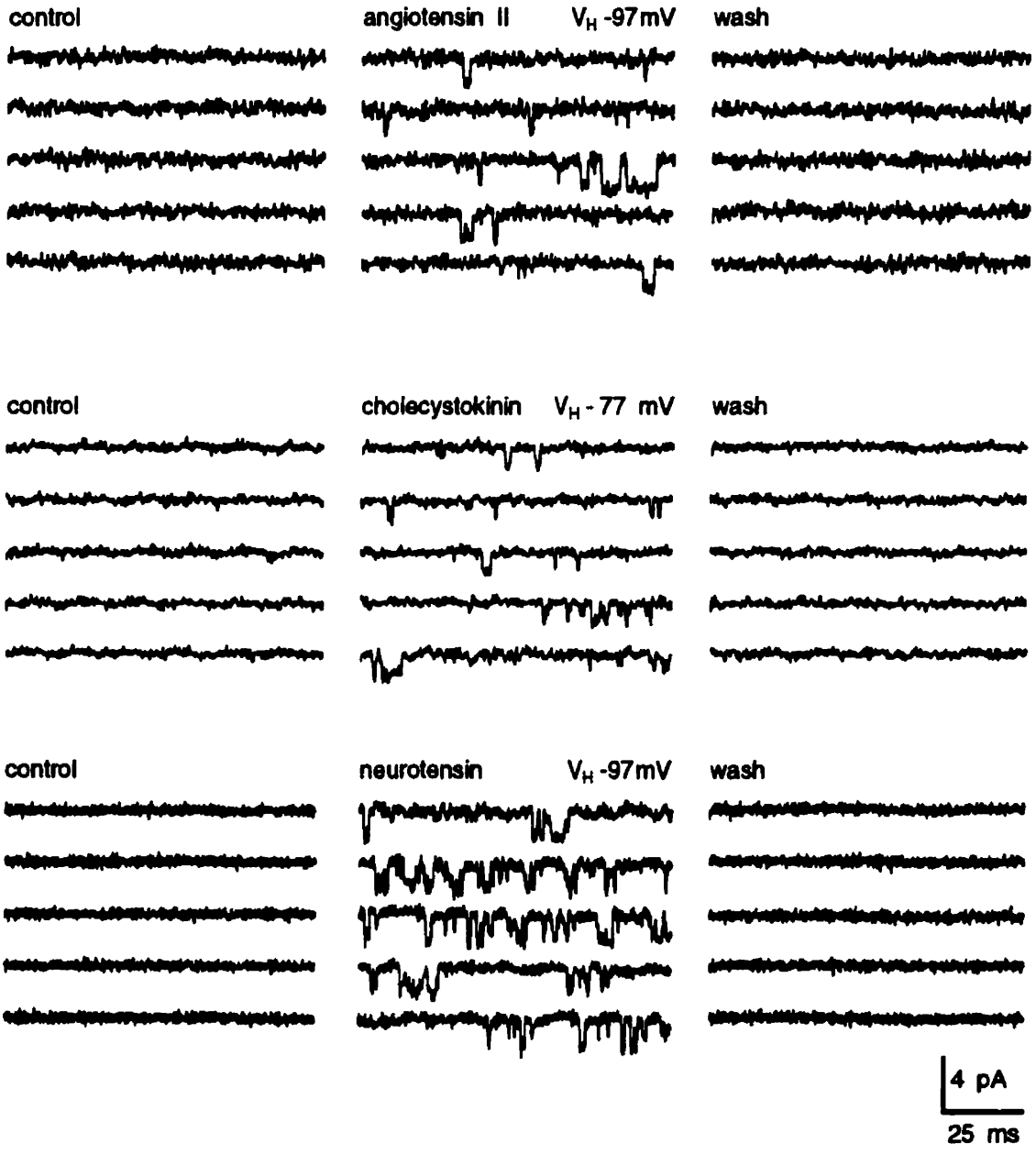

FIGURE 4.1: Excerpts of cell-attached single channel recordings obtained from three patches showing responses to AII (top panels), CCK (middle panels) or NT (lower panels). Note that the single channel currents in response to AII and NT were recorded at more negative potentials than those for CCK.

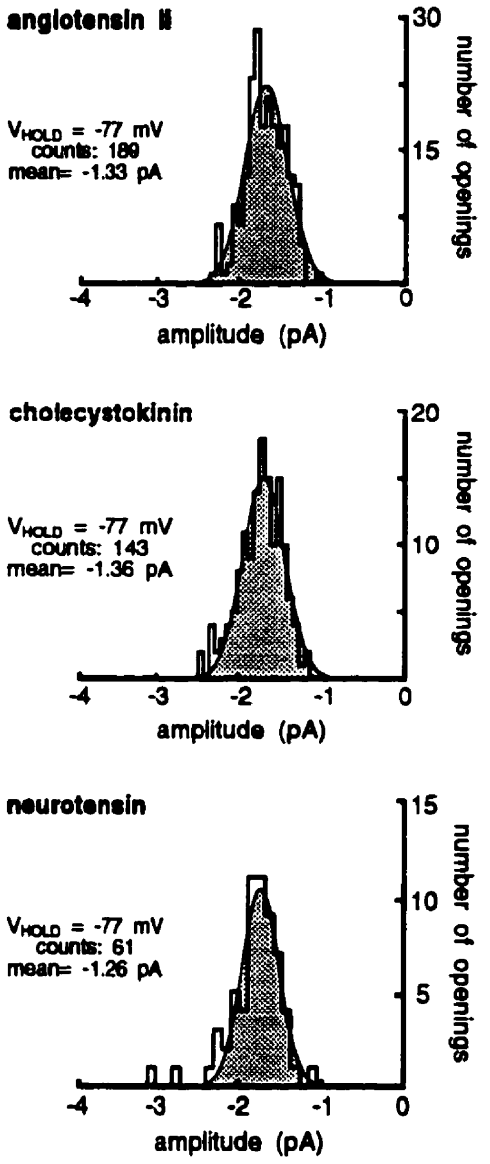

FIGURE 4.2: Current amplitude histograms obtained from three patches in which responses to AII, CCK or NT were recorded at the same holding potential. Note the similar mean current amplitudes observed in the three cases. The shaded areas are the Gaussian fits of the data from which the mean channel current amplitudes were determined.

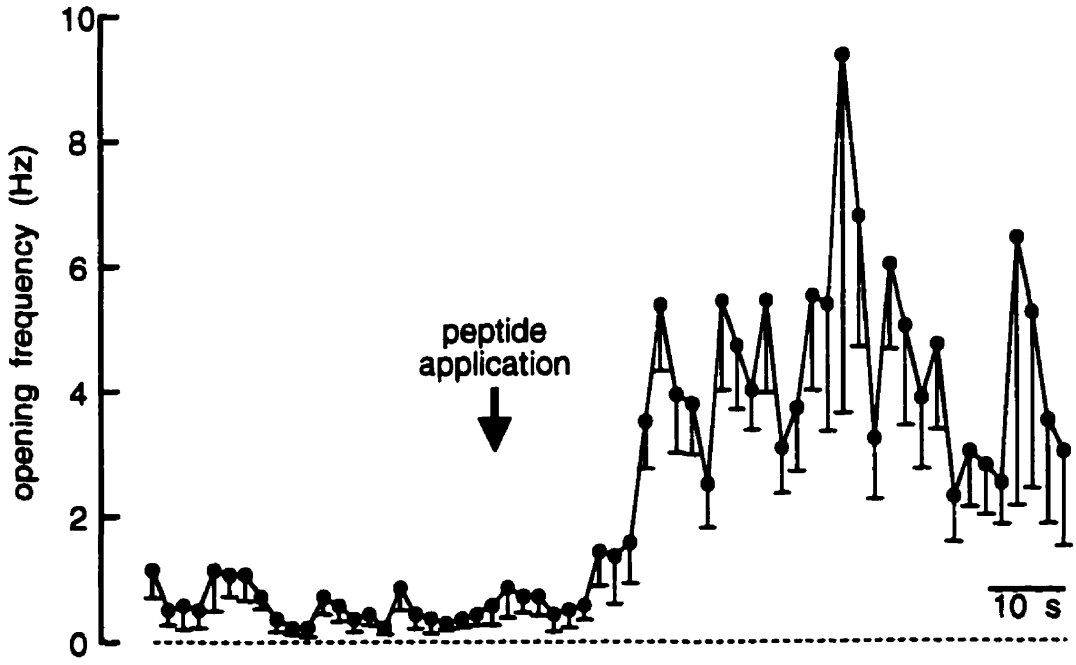

FIGURE 4.3: The plot shows mean (± S.E.M.) single channel opening frequency in response to peptide application (arrow) obtained from 7 patches including responses to AII (n=4), CCK (n=1) and NT (n=2).

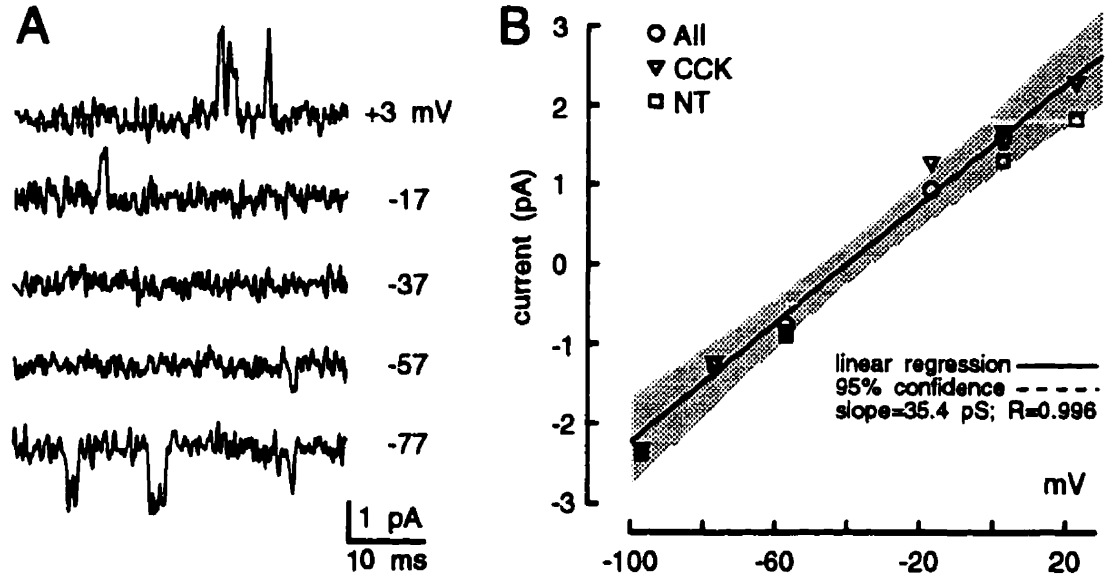

FIGURE 4.4: The traces in A are excerpts of a single channel recording obtained from an AII-stimulated patch held at various holding potentials (as shown). Downward deflections represent inward currents while upward deflections represent outward currents. Panel B shows mean (± S.E.M.) single channel current amplitudes at different voltages obtained from 3 patches stimulated by AII (open circles). The solid line is a linear regression fit through data points (R=0.99; n=12), while the shaded area represents the 95% confidence interval. The slope conductance calculated from the fit is 35.4 pS and the E_{rev} is -41.2 mV. Data points from channels recorded in two other patches stimulated with CCK (open triangles) or NT (open squares) were plotted on the same graph.

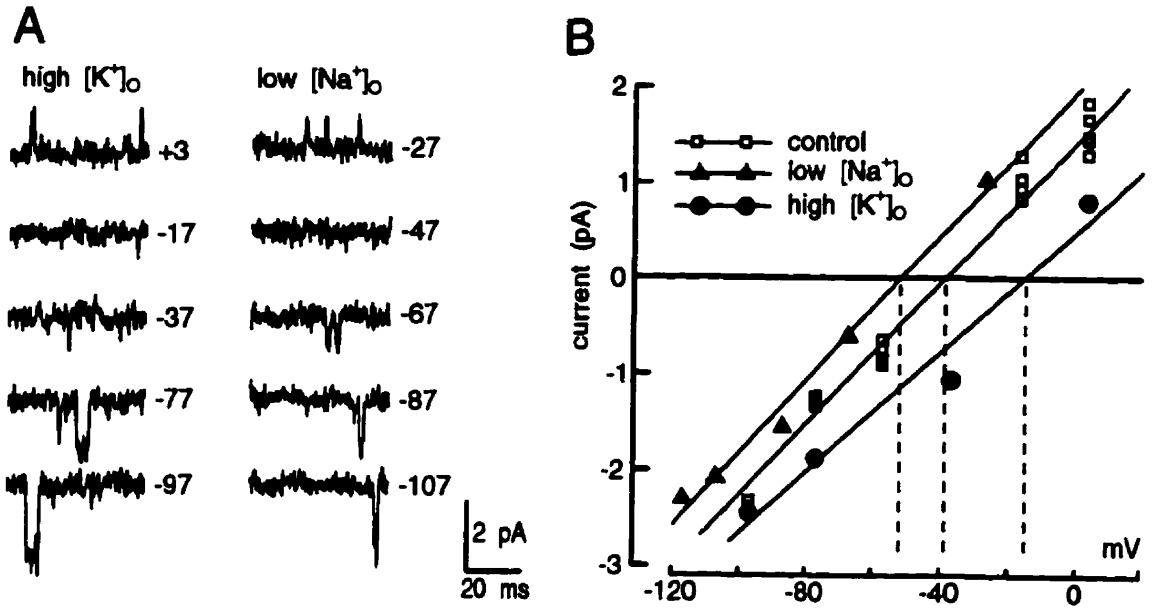

FIGURE 4.5: Traces in A are excerpts of cell-attached recordings from two different peptide-activated patches recorded using pipettes filled with high [K^+] (left) or low [Na^+] (right). Downward deflections are inward currents while upward deflections are outward currents. In panel B, all data points obtained from patches recorded with pipettes filled with control solution are plotted (open squares) along with a regression fit of the data (slope = 36.9 pS; R = 0.99). Data points and corresponding regression fits are also plotted for low [Na^+] (triangles; slope = 37.8 pS; R = 0.99) and high [K^+] (circles; slope = 31.5 pS; R = 0.98). Vertical dashed lines show the positions of the reversal potentials associated with each condition.

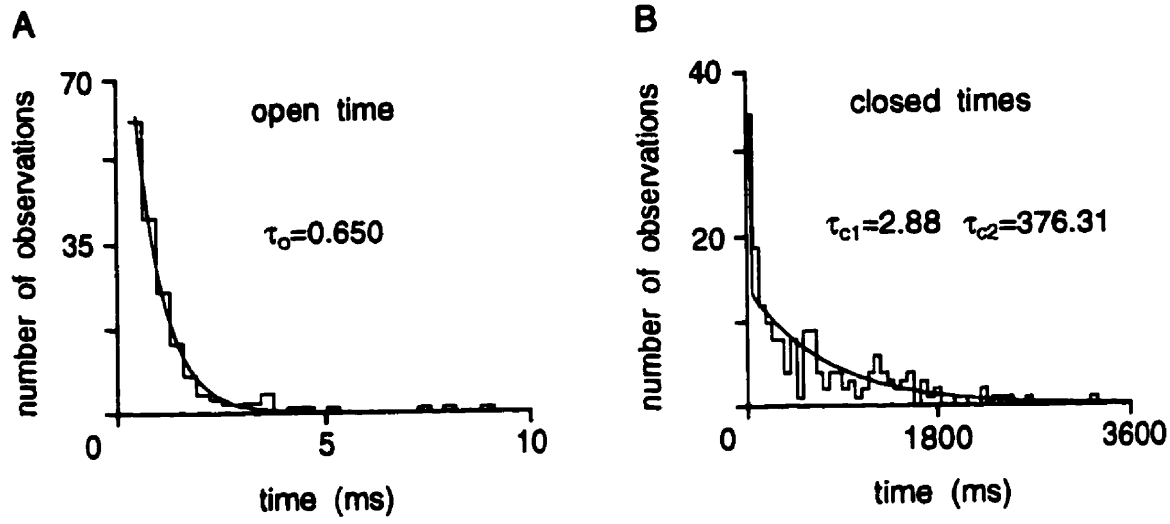

FIGURE 4.6: Distribution of the open (A) and closed times (B) of a peptide-gated cation channel recorded from an isolated MNC. The open time distribution was fitted with one exponential (smooth line) revealing a time constant of τ_o = 0.65 ms. The closed times were fitted with a double exponential function (smooth line) revealing two time constants: τ_{c1} = 2.88 ms and τ_{c2} = 376 ms.

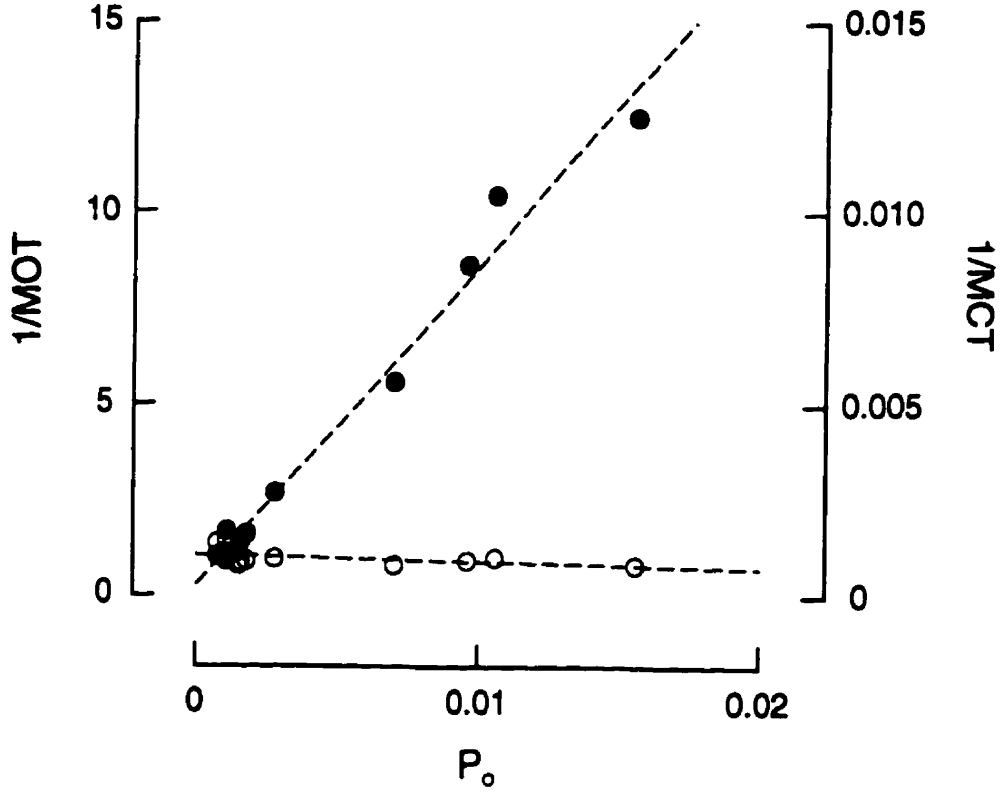

FIGURE 4.7: Effects of peptide stimulation on MOT, MCT and P_o. The data shown were obtained from 4 membrane patches where it was possible to compare P_o values observed during control, peptide stimulation and wash. For each condition, in each patch, MOT and MCT values were plotted as a function of the prevailing P_o. Note that the peptide-induced changes in P_o are achieved via decreasing MCT, with no consistent effect on MOT (see text for details).

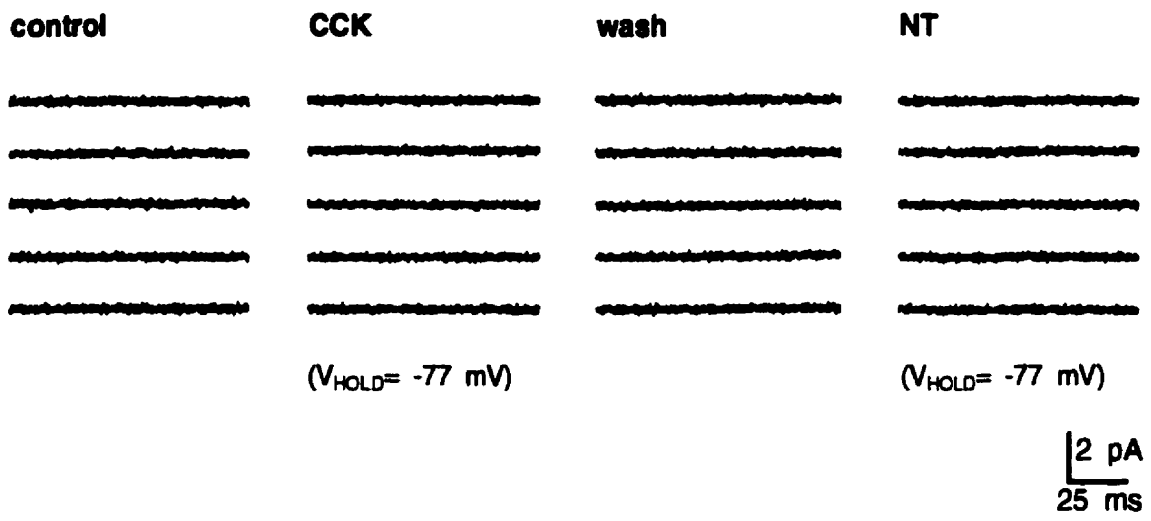

FIGURE 4.8: Excerpts of cell-attached recordings obtained from a single membrane patch before and after separate applications of CCK and NT. None of the peptides succeeded in evoking channel openings in this example.

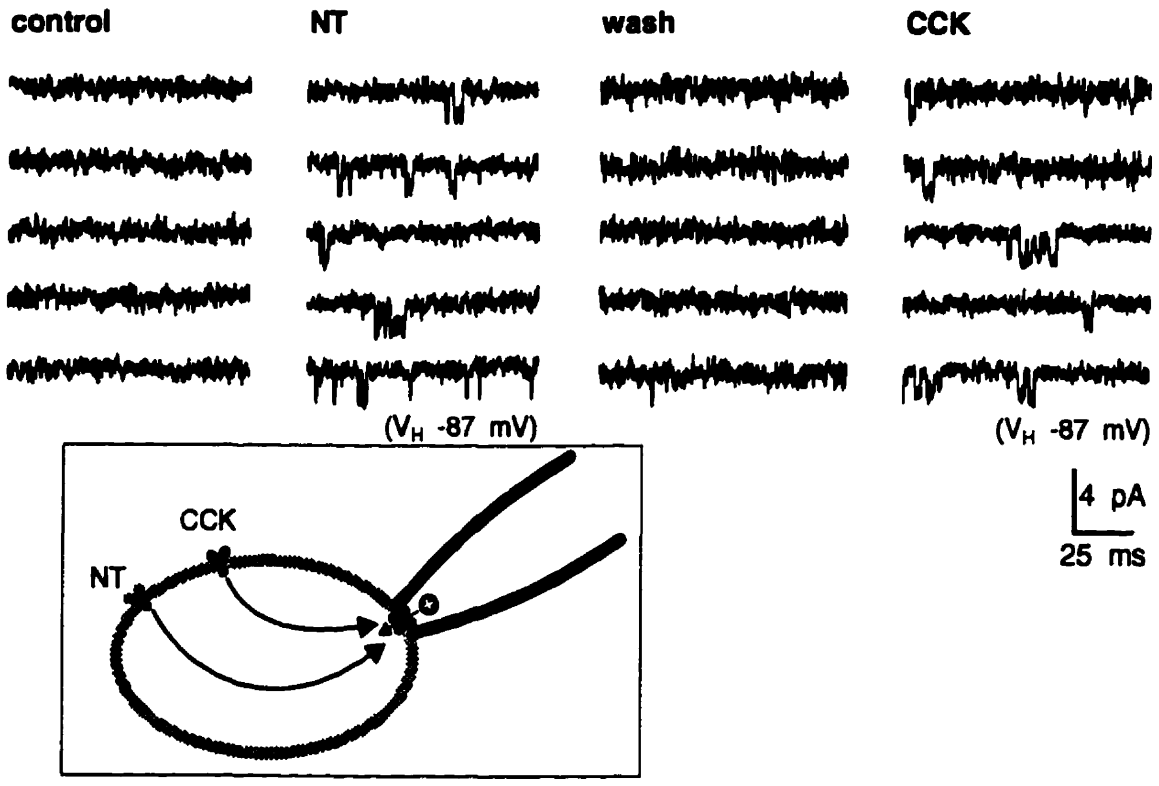

FIGURE 4.9: Exerpts of cell-attached recording obtained from a membrane patch containing a single channel (probability of having two channels <4%). Consecutive applications of NT and CCK induced reversible channel activation. The inset illustrates the hypothetical scheme involved in the convergent activation of a single ion channel.

4.3 DISCUSSION

The results presented in this chapter demonstrate the expression in MNCs of 37 pS cation permeable channels that can be activated by AII, CCK or NT. The mean time course of the enhancement of channel activity upon peptide application (Fig 4.3) was in good agreement with that of the onset of macroscopic voltage and current responses obtained in whole-cell recordings (see fig. 3.1 and section 3.2.2). Moreover, the values of the reversal potentials of the single channel currents observed under various ionic conditions were very similar to those obtained under whole-cell conditions. These channels, therefore, are presumed to be the ones responsible for the changes in peptide-modulated conductance described in chapter three.

4.3.1 Channel density

The minimum number of peptide-regulated cation permeable channels expressed in MNCs can be estimated using the equation:

$$N = I / iP_o \qquad (3)$$

where N is the number of channels, P_o is the mean maximal P_o observed in response to peptide application (0.013), I is the mean maximal macroscopic current amplitude induced by the peptide at -60 mV ($\Delta G[-60 + E_{rev}]$ = 8.5 pA) and i is the unitary current amplitude at the same voltage (0.67 pA). Using these values, the minimum number of peptide-gated channels in single MNCs is estimated to be 976. By dividing this number by the mean surface area (SA)

of isolated MNCs, the minimum channel density can be estimated. The mean SA in MNCs can be calculated by dividing the average input capacitance by the specific capacitance of the cell membrane. MNCs used for patch-clamp recordings in our laboratory have been found to have a mean whole cell input capacitance of 13.3 ± 0.8 pF (n= 52; Fisher et al., 1997). Since specific membrane capacitance is ≈ 1 $\mu F/cm^2$ (Hille, 1992), the average SA of MNCs is ≈ 1330 μm^2. The density of the peptide-regulated channels in MNCs is thus 0.73 μm^{-2}. Assuming that the patch area isolated by the pipette is approximately 1 μm^2, the fraction of patches revealing a single channel might be expected to be 0.73.

Since application of a single peptide succeeded in activating supraoptic MNCs in 78% of the cells tested in whole-cell recordings (see sections 3.2.1 and 3.3.1), it might be expected that in cell-attached recordings, peptide application would evoke channel activity in 57% (0.73 x 78%) of the trials. In practice, however, single peptide applications activated a channel in only 14% of the cell-attached recordings. The low percentage of responses recorded using cell-attached recording, compared to those predicted, suggests that: (1) the channels may be destroyed during the formation of a seal between the cell and the recording pipette, (2) the channels may not be uniformly distributed on the cell membrane, or (3) that the receptors mediating activation need to be intimately associated with the channels to be able to activate them. Interestingly, difficulties in obtaining single channel responses to NT have also been reported during cell-attached recordings from dopaminergic neurons of the rat ventral tegmental area (Chien et al., 1996).

4.3.2 Single cation channel can be modulated by different excitatory peptides

The single channels activated by different peptides shared a number of similar properties, including: (1) unitary current amplitude at a fixed potential (fig. 4.3); (2) onset kinetics (fig. 4.3); (3) slope conductance (fig. 4.4); (4) reversal potential (fig. 4.4); and (5) open time (see section 4.2.4). Moreover, increases in channel P_o selectively resulted from a decrease in MCT during responses to all of the peptides (not shown). While these data suggest that the channels activated by AII, CCK and NT may be the same, direct evidence for this proposal was obtained from observations in five patches, where the activity of a single channel was reversibly enhanced by separate applications of a different peptide.

CHAPTER FIVE

GENERAL DISCUSSION

The results described in this thesis indicate that AII, CCK and NT have profound excitatory effects on both VP- and OXT-releasing MNCs of the rat hypothalamus, and that these effects are mediated through the activation of postsynaptic receptors. As previously suggested from experiments in situ (see section 1.5), the excitation of MNCs evoked by these peptides resulted from the activation of a non-selective cationic conductance. Patch clamp analysis under whole-cell conditions revealed that the properties of the macroscopic responses to AII, CCK and NT were indistinguishable (chapter three), suggesting that a single type of membrane conductance might be regulated by these neuropeptides. Indeed, the cell-attached single channel recording experiments described in chapter four suggest that a single 37 pS, cation permeable, ion channel is modulated by AII, CCK and NT. Previous studies have implied a role for a non-selective cation conductance in mediating responses to other excitatory transmitters (see table 1.1), including: activin-A, ATP (purines), dopamine and polypeptide 7B2. It is possible, therefore, that this 37 pS channel serves as a target for numerous excitatory substances in these hypothalamic neuroendocrine cells. These findings provide the first evidence for convergent regulation of a cationic channel in mammalian neurons.

Little is known about the second messenger mechanisms involved in mediating the effects of peptides on MNCs. However, it may be presumed that

peptide-receptor-mediated ion channel modulation is achieved through an intermediary guanosine nucleotide binding (G) protein. Indeed, all subtypes of AII (Hein et al., 1995), CCK (Zhang et al., 1993; Roettger et al., 1995) and NT (Mitra & Carraway, 1995) receptors have been shown to belong to the superfamily of G-protein-coupled receptors consisting of a single polypeptide chain (350-450 amino acids) comprising seven transmembrane segments, an N-terminal extracellular domain and a C-terminal cytoplasmic domain. Moreover, it has been reported that activation of AII (Tamura & Speth, 1990), CCK (Jensen et al., 1989) and NT (Wu et al., 1995b) receptors stimulates phospholipase C-mediated hydrolysis of phosphatidyl inositol 4,5-bisphosphate, leading to the production of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). In other cells, production of IP_3 following activation of AII (Lynch et al., 1985; Kurtz & Penner, 1989), CCK (Lee et al., 1993) and NT receptors (Wu et al., 1995b) has been found to stimulate the release of Ca^{2+} from internal stores. While further experiments will be required to characterize the biochemical mechanisms involved in regulating the activity of 37 pS cation channels in MNCs, these observations suggest that the intracellular concentration of Ca^{2+} and the activation of protein kinase C may play a role in this process. Indeed, recent studies have suggested that the effects of NT on MNCs require Ca^{2+} mobilization (Kirkpatrick and Bourque, 1993).

Although the physiological conditions under which AII, CCK and NT are released on MNCs in vivo remain to be established, the results presented in this thesis show that activation of receptors for these peptides is likely to play an important role in regulating spike discharge from somata and hormone

release from axon terminals in the neurohypophysis.

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