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THE EFFECT OF PROSTAGLANDIN E_2 ON POTASSIUM CHANNELS IN RAT NEUROHYPOPHYSEAL NERVE TERMINALS

by

LEI ZHANG (C)

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfilment of the requirements for the degree of

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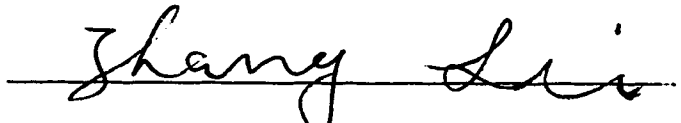

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Effect of Prostaglandin E_2 on Potassium Channels in Rat Neurohypophyseal Nerve Terminals" submitted by LEI ZHANG in partial fulfillment of the requirements for the degree of Master of Science.

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Dedicated with love to Thomas, James, Mom and Dad.

ABSTRACT

Three distinct voltage-dependent outward K^+ currents were characterized in the acutely dissociated nerve terminals from the rat posterior pituitary. They were the fast-inactivating K^+ current, which was 4-AP sensitive and α-dendrotoxin resistant, the non-inactivating current, which could be blocked by TEA and 4-AP, but not by α- or β-dendrotoxin, and the Ca^{2+}-activated K^+ current.

A high concentration of prostaglandin E_2 (PGE_2) has been found to be active in the neurohypophysis. These studies show that PGE_2 modulates the voltage-dependent K^+ channels in neurohypophyseal nerve terminals, which may contribute to regulation of neuropeptide secretion. These regulatory effects were via a receptor-mediated process. G proteins were found to be responsible for the postreceptor activation of signal transduction mechanisms. PGE_2 increased the non-inactivating component, and this increase was concentration dependent. The mechanism(s) underlying this effect remain as yet unknown. Following longer incubations with PGE_2, the fast-inactivating component increased, while the non-inactivating component declined to control levels. The ability of cAMP analogues to mimic, and the ability of a cAMP antagonist to abolish the long-term actions induced by PGE_2 indicated that cAMP accumulation and the subsequent activation of PKA were involved in these processes. PGE_2 did not affect the Ca^{2+}-activated K^+ channel.

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AA	arachidonic acid
AC	adenylate cyclase
ACh	acetylcholine
ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone (vasopressin)
4-AP	4-aminopyridine
ATP	adenosine 5'-triphosphate
Ba^{2+}	barium
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BK_{Ca}	large-conductance Ca^{2+}-activated K^+ channels
8-Br-cAMP	8-bromoadenosine 3',5'-cyclic monophosphate
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine 3',5'-monophosphate
Cd^{2+}	cadmium
cGMP	cyclic guanosine 3',5'-monophosphate
Cl^-	chloride
CRH	corticotropin-releasing hormone
Cs^+	cesium
Db-cAMP	dibutyl adenosine 3',5'-cyclic monophosphate
DG	diacylglycerol
DMSO	dimethyl sulfoxide
DTX	dendrotoxin
E_K	K^+ equilibrium potential
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EP(s)	PGE receptor(s)

F	Faraday constant
FSH	follicle-stimulating hormone
G protein(s)	guanine nucleotide binding protein(s)
G_i	inhibitory G proteins
G_o	other (unidentified) G proteins
G_s	stimulatory G proteins
GDP	guanosine 5'-diphosphate
GDPβS	guanosine-5'-O-(2-thiodiphosphate)
GH	growth hormone
GHRH	growth hormone-releasing hormone
GnRH	gonadotropin-releasing hormone
GppNHp	5'-guanylylimidodiphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine-5'-O-(3-thiotriphosphate)
HEPES	N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid
HNC	hypothalamo-neurohypophyseal complex
HPLC	high performance liquid chromatography
5-HT	serotonin
I_A	transient outward K^+ currents ("A" currents)
I_{Af}	subtype of "A" currents which activates and inactivates rapidly
I_{As}	subtype of "A" currents which activates and inactivates more slowly
I_f	fast-inactivating K^+ current
I_K	delayed rectifiers
I_M	M-channels
I_n	non-inactivating K^+ current
IC_{50}	median inhibitory concentration
IK_{Ca}	Intermediate-conductance Ca^{2+}-activated K^+ channels
IM	indomethacin
im	intramuscular
ip	intraperitoneal

IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
I-V	current-voltage
iv	intravenous
ivt	intraventricular
K aspartate	L-aspartic acid monopotassium salt
K^+	potassium
K_{Ca}	Ca^{2+}-activated K^+ channels
$[K]_i$	intracellular K^+ concentration
K_{Na}	Na^+-activated K^+ channels
$[K]_o$	extracellular K^+ concentration
La^{3+}	lanthanum
LH	luteinizing hormone
Li^+	lithium
Meclo	sodium meclofenamate
Mg^{2+}	magnesium
MSH	melanocyte-stimulating hormone
Na^+	sodium
OXT	oxytocin
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PI	phosphatidyl inositol
PIH	prolactin-inhibiting hormone
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	cAMP-dependent protein kinase (protein kinase A)
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C

PLD	phospholipase D
PRL	prolactin
PTX	pertussis toxin
R	gas constant
R_S	seal resistance
Rp-cAMPs	adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer
S-A node	cardiac sinoatrial node
S-K^+ channel	the serotonin-sensitive K^+ channel
S1-S6	postulated transmembrane α-helical segments (1-6) of K^+ channel protein
sc	subcutaneous
SD	Sprague-Dawley
S.E.	standard error
SITS	4-acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene
SK_{Ca}	small-conductance Ca^{2+}-activated K^+ channels
Sp-cAMPs	adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer
SR	sarcoplasmic reticulum
Sr^{2+}	strontium
T	absolute temperature
TEA	tetraethyl ammonium chloride
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TTX	tetrodotoxin
TXB_2	thromboxane B_2
$V_{1/2}$	membrane potential at which 50% of the channels are inactivated

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1. NEUROHYPOPHYSIS

Neurohypophyseal research began 100 years ago when Oliver first described the vasopressive action of extracts of the pituitary body (Oliver and Schafer, 1895). Now, the pituitary gland is one of the most extensively studied of all endocrine glands.

1.1.1 Anatomy of the Pituitary and its Relation to the Hypothalamus

The pituitary gland, or hypophysis, is located on the diencephalon of the brain immediately inferior to the hypothalamus. It is linked with the hypothalamus by the pituitary stalk. The pituitary gland is structurally and functionally divided into three separate lobes: the anterior lobe (adenohypophysis), which receives signals or information via the blood, and in turn releases its own hormones directly into the blood; the intermediate lobe, which is rudimentary and is not a distinct lobe *per se*; and the posterior lobe (neurohypophysis), which is an extension of the nervous system and functions like a typical neuroendocrine tissue. The anterior lobe and the intermediate lobe are derived from a pouch of epithelial tissue (Rathke's pouch) that migrates upwards from the embryonic mouth, whereas the posterior lobe is formed as a down-growth of the hypothalamus. Neural connections between the

hypothalamus and neurohypophysis are maintained during development. The posterior pituitary contains the axons and terminals of neurons that have their cell bodies within two distinct areas of the hypothalamus, the supraoptic nucleus and the paraventricular nucleus. Therefore, hormone secretion of neurohypophysis is modulated directly by the innervation from the hypothalamus. In contrast to the posterior pituitary, the function of the anterior and intermediate pituitary is regulated by vascular connections, the hypothalamic-pituitary portal system which consists of two capillary beds in the anterior lobe, with the hypothalamus. Factors from the hypothalamus travel via these capillary beds to the anterior and intermediate pituitary and serve to regulate hormone release. The hormones secreted by the pituitary gland and their regulating factors are listed in Tab. 1-1 (Rhoades and Pflanzer, 1992).

1.1.2 Regulation of Posterior Pituitary Hormone Secretion by the Hypothalamus

The posterior pituitary secretes two hormones: oxytocin (OXT) and antidiuretic hormone (ADH, or vasopressin). These two hormones are actually synthesized within the cell bodies of the neurons in the supraoptic and paraventricular nuclei of the hypothalamus. Akaishi and Negoro (1979) classified the neurosecretory neurons in the paraventricular nucleus into oxytocinergic and vasopressinergic neurons, which synthesize OXT and ADH, respectively. OXT and ADH are then transported to the posterior pituitary through the axons of the same neurons that produce them. Generation of action potentials in these nerves results in the release of OXT or ADH from the nerve endings in the posterior pituitary (Rhoades and

Pflanzer, 1992). Therefore, a variety of stimuli, which can modulate the electrical activity of the neuroendocrine cells in these two nuclei, are capable of regulating the secretion of OXT and ADH.

1.1.3 Stimulus-Secretion Coupling

The mechanisms by which neurohormone or neurotransmitter release occurs have been widely studied during the last three decades. It was the pioneering work of Douglas and co-workers on hormone release from the neurohypophysis that first enunciated the concept of stimulus-secretion coupling -- the basic hypothesis for the mechanism by which a molecule, enclosed in a granule (or vesicle), is released into the circulation (Douglas and Poisner, 1964*a*,*b*). The process of depolarization-secretion coupling has been demonstrated in the neurohypophysis (Nordmann, 1983). The arrival of action potentials induces the depolarization of these nerve endings (Salzberg *et al.*, 1983). The electrically induced depolarization promotes the entry of calcium into the nerve terminals (Brethes *et al.*, 1987). Not only the absolute increase, but, also, periodic changes of the free Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_i$) trigger the release of neurosecretory vesicular contents via exocytosis (Nordmann *et al.*, 1987; Dayanithi *et al.*, 1988; Stuenkel and Nordmann, 1993*a*). In the presence of Ca^{2+}, the amount of evoked hormone release was dependent on the external K^+ concentration (Cazalis *et al.*, 1987*a*). Furthermore, a maintained high $[Ca^{2+}]_i$ is not enough to sustain a high rate of secretion from the neurohypophysis. It is suggested that basal secretion is directly dependent on external Na^+

concentrations, which indicates that intracellular Na^+ is an important factor in the control of secretory mechanisms (Toescu and Nordmann, 1991). An increase in internal Na^+ *per se* can promote, in the absence of a rise in $[Ca^{2+}]_i$, an increase in neuropeptide secretion (Nordmann and Stuenkel, 1991; Stuenkel and Nordmann, 1993*b*). Three groups of K^+ channels (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992), one Na^+ channel (Nordmann and Dyball, 1978) and two types ("N_t", where the $_t$ denotes terminal, and "L") of high-threshold, voltage-gated Ca^{2+} channels (Lemos and Nowycky, 1989; Wang X. *et al.*, 1992, 1993*a*) have been identified in isolated rat neurohypophyseal terminals. Other ions such as Mg^{2+} and Cl^- are also crucial components for exocytosis to occur (Lemos and Nordmann, 1986; Cazalis *et al.*, 1987*b*; Dayanithi and Nordmann, 1989). In accordance with the Ca^{2+} hypothesis, the entry of Ca^{2+} into neurosecretory cells activates, by yet unknown mechanisms, a Ca^{2+}-activated channel (most probably be the Ca^{2+}-activated K^+ channel) in the neurosecretory vesicle membrane and elevates cations and anions inside the vesicle (Stanley and Ehrenstein, 1985; Lemos *et al.*, 1989; Lee *et al.*, 1992). The resulting increase in osmolarity would then lead to swelling of the neurosecretory vesicle and thus promote fusion with the plasma membrane and the release of its contents (Nordmann *et al.*, 1974; Cohen *et al.*, 1982; Stanley and Ehrenstein, 1985; Lindau *et al.*, 1992; Nordmann and Artault, 1992).

1.2. POTASSIUM (K^+) CHANNELS

All cells maintain a K^+ ion concentration gradient across the cell membrane. Even though the entry of Ca^{2+} is the most important event coupling stimulus to secretion in the neurohypophysis, the movement of K^+ ions down their electrochemical gradient, through K^+ channels in the plasma membrane, is of fundamental importance for nerve ending excitability. Generally speaking, the opening of K^+ channels sets the cell's resting potential, keeps fast action potentials short, terminates periods of intense activity, slows the rate of repetitive firing, and lowers the excitability of the cell. By controlling action potential repolarization and the threshold for action potential generation, K^+ channels can regulate the amount of transmitter secreted, and, thus, control synaptic efficacy.

Bernstein (1902) first postulated that selective K^+ permeability is a component of an excitable cell membrane. Since Hodgkin and Huxley (1952) described the role of K^+ currents in action potentials, K^+ channels have been demonstrated to be the most ubiquitous of all the ion channels, and they are found in almost all eukaryotic cells of the animal and plant kingdom (Latorre *et al.*, 1984; Cook, 1988; Rudy, 1988; Hille, 1992*a*; Jan and Jan, 1992*b*). This section contains a broad review of K^+ channels, and then, describes the K^+ channels specific to neurohypophyseal nerve terminals.

1.2.1 K^+ Channel Structure

Our knowledge of the molecular structure of ion channels has expanded

rapidly as cloning techniques have enabled the complete amino acid sequences of large membrane proteins to be determined. Initially, two approaches have facilitated determination of the molecular structure of A-type potassium channels (I_A): (1) the use of dendrotoxin (DTX) -- a peptide toxin which binds to K^+ channels; and (2) the genetic, cloning approach, isolating the defective gene (an A-type K^+ channel) from the *Drosophila* mutant, *Shaker* (Strong, 1990). Since the cloning of the *Shaker* locus in *Drosophila* (Papazian *et al.*, 1987; Tempel *et al.*, 1987; Kamb *et al.*, 1988; Pongs *et al.*, 1988; Schwarz *et al.*, 1988), a growing number of related K^+ channel genes in various vertebrates and invertebrates have been cloned (Frech *et al.*, 1989; Swanson *et al.*, 1990; Wei *et al.*, 1990; Pak *et al.*, 1991; Pfaffinger *et al.*, 1991; Roberds and Tamkun, 1991; Schroter *et al.*, 1991; Kubo *et al.*, 1993*a,b*; Sheng *et al.*, 1993,1994; Reuveny *et al.*, 1994).

In contrast to the structures of the Na^+ and Ca^{2+} channel proteins, which have four internal repeats, with each repeat possessing the familiar six transmembrane-spanning regions, K^+ channels have a single set of six transmembrane segments as shown in Fig. 1-1 (Hille, 1992*b*; Jan and Jan, 1992*a,b*, 1994; Sigworth, 1994), and four sets of such polypeptide assemble to form a functional K^+ channel (Parcej *et al.*, 1992). These subunits could be encoded by different genes, or because of alternate splicing, different proteins could be encoded by the same genes (Schwarz *et al.*, 1988; Chandy *et al.*, 1990; Christie *et al.*, 1990). This provides for both the possibility of great diversity among K^+ channels and similarities within this diversity; thus, various types of K^+ channels may be expressed in different cells, or even in

different regions of the same cell.

According to the postulated structural model of the K^+ channel (Fig. 1-1) (Miller, 1990; Stuhmer, 1991; Lester, 1991; MacKinnon, 1991; Baldwin *et al.*, 1992; Jan and Jan, 1992*a,b*, 1994; Isacoff *et al.*, 1993; Li *et al.*, 1994; Sigworth, 1994), the whole channel protein contains three domains: the N-terminal and the C-terminal are hydrophilic, with both on the cytoplasmic side of the membrane, whereas the middle, core region contains multiple stretches of hydrophobic residues that can potentially span the membrane. The amino-terminal region contains a few basic residues that are involved in fast inactivation (the fast inactivation gate) (Zagotta *et al.*, 1990), which interacts with the S4-S5 loop and occludes the pore (Isacoff *et al.*, 1991). For the predicted hydrophobic core region, K^+ channels have six transmembrane α-helical segments (S1-S6). This model also speculates that segments S1-S3 and S5 form the outer cylinder while segments S4 and S6, as well as the connection segment of S4 and S5, form the inner cylinder (Pongs, 1992; Lopez *et al.*, 1994). The highly conserved N-terminal hydrophilic domain as well as S1 contribute to the coassembly of homo- and heterotetrameric K^+ channels (Li *et al.*, 1992; Babila *et al.*, 1994). The transmembrane segment S4, which is postulated to be the voltage sensor conferring voltage-dependency to the ion channel proteins, contains a characteristic sequence of positive charges (six Lysine or Arginine residues every third residue), and the movements of charges intrinsic to the channel molecule is involved in the channel activation (Lopez *et al.*, 1991; Papazian *et al.*, 1991; Jan and Jan, 1992*a*; Logothetis *et al.*, 1992; Tytgat and Hess, 1992). The sequence

between the S4 and the S5 carries an acidic residue (Glutamate) and probably functions as one of the many K^+ binding sites for the fast inactivation gate (Yellen *et al.*, 1991; Yool and Schwarz, 1991; Slesinger *et al.*, 1993). The H5 sequence, which follows the S5 sequence, constitutes part of the pore (Guy and Conti, 1990). The H5 as well as the S6 determine the single channel conductance and the sensitivity to internal and external tetraethyl ammonium (TEA), and influence charybdotoxin binding affinity (Hartmann *et al.*, 1991; Goldstein *et al.*, 1994; Lopez *et al.*, 1994). A hydrophobic residue in the S6 is crucial for the rate of slow inactivation (Hoshi *et al.*, 1991; Zagotta *et al.*, 1991), although the mechanism underlying slow inactivation is not yet known. This model of K^+ channel placement in the membrane is supported by three predicted N-glycosylation sites being located on the extracellular face of the protein (Hubbard and Ivatt, 1981), and two possible cyclic adenosine 3',5'-monophosphate (cAMP)-dependent phosphorylation sites being located on the cytoplasmic face of the protein (Krebs and Beavo, 1979). Even though there is an amino acid sequence that bears a loose resemblance to the Ca^{2+}-binding domain seen in other Ca^{2+}-binding proteins (Atkinson *et al.*, 1991), the precise location of the Ca^{2+}-binding site in Ca^{2+}-activated K^+ channels is unknown. It is assumed that the Ca^{2+}-binding domain is located on the cytoplasmic face of the channel, since only internal Ca^{2+} will activate the Ca^{2+}-activated K^+ channels.

1.2.2 K^+ Channel Classifications

The great diversity and prevalence of K^+ channels contributes to their versatile

physiological and pathological functions. Distinctions between channel types are based on kinetic behaviour, voltage-dependence, ion-dependence, permeation specificity, ligand sensitivity, as well as pharmacological properties, and these aspects will be discussed. Although there are a number of distinct K^+ channel types, it is not always possible to assign every K^+ channel into a particular class because some K^+ channels possess more than one type of characteristic. Thus, caution is stressed when identifying a K^+ channel type on the basis of only a few non-specific properties.

1.2.2.1 Voltage-dependent K^+ channels

After being first described in the membranes of skeletal muscle and nerve axons, the voltage-dependent K^+ channels have been demonstrated to be the largest and most diversified class of ion channels. They are expressed ubiquitously in excitable as well as non-excitable cells. The voltage-dependent K^+ conductances are classified in terms of three major families, distinguished by their responses to changes in membrane potential and the pattern of activation as well as inactivation (Adams and Nonner, 1990), and confirmed by their sensitivity to various pharmacological agents and toxins. Each family of the voltage-dependent K^+ channels could be further subdivided into channel subtypes by the differences in voltage dependence and gating kinetics (Jan and Jan, 1989, 1992*a*, 1994; Adams and Nonner, 1990; Sokabe *et al.*, 1991; Pallotta and Wagoner, 1992; Pongs, 1992).

1.2.2.1.1 Delayed rectifiers (I_K)

This type of K^+ channel was first recognized in the squid giant axon (Hodgkin and Huxley, 1952), and is responsible for the repolarization of the action potential. The function of I_K is to keep the action potential short or terminate the agonist-induced depolarization.

I_K is activated, with a delay, upon membrane depolarization, and shows very slow or no inactivation while the depolarization is maintained. These characteristics distinguish I_K from a typical "A" current. The $t_{1/2}$ of inactivation is in seconds, and the single-channel conductance is 3-15 pS. A rise in the internal Ca^{2+} concentration is not necessary to activate I_K, and the lack of Ca^{2+} dependence distinguishes I_K from Ca^{2+}-activated K^+ channel. Some ions, such as cesium (Cs^+) and barium (Ba^{2+}), and tetraethyl ammonium chloride (TEA) can block this channel. Some investigators have reported that 4-aminopyridine (4-AP) is also effective (Rudy, 1988; Adams and Nonner, 1990). Several toxins from the snake such as β- and γ-dendrotoxin (DTX) (Benishin *et al.*, 1988; Ren *et al.*, 1994), and β-bungarotoxin (Petersen *et al.*, 1986), have been found to selectively block this channel. In addition to their primary effects on other types of K^+ channels, toxin from snake such as α-dendrotoxin (Schauf, 1987), and toxin from scorpion such as charybdotoxin (Lewis and Cahalan, 1988) have occasionally been documented to block the delayed rectifier K^+ channel.

Many cells, and most neurons, contain at least one type of I_K. Some cells may contain more than one type of I_K, eg. there are at least three independent types of I_K in the frog node of Ranvier. They are distinguished by the activation threshold

and kinetics, the rate of inactivation, as well as pharmacological profiles. It is possible to alter the properties of one without affecting the others, which would not be expected for a multi-state single channel type (Dubois, 1981, 1982, 1983).

1.2.2.1.2 Transient K^+ channels (I_A)

The transient outward K^+ channels were originally described in molluscan neurons (Hagiwara *et al.*, 1961), which produce transient outward currents, called an "A" current (I_A) (Rogawski, 1985). The function of I_A is to lower the rate of repetitive responses, in order to slow down the return of the membrane potential toward action potential threshold when neurons fire repetitively in response to tonic depolarization.

I_A is also activated by membrane depolarization similar to, except faster than, I_K, but decays within tens of milliseconds even with the maintained depolarization. The steady-state inactivation is complete near resting membrane potentials (-40 mV), and the threshold for activation is lower than that of other K^+ currents. Thus, this current operates in the subthreshold region for action potential generation, opening transiently with a small depolarization which starts from hyperpolarized potentials. It has a typical single-channel conductance of 8-30 pS (Rudy, 1988; Adams and Nonner, 1990). I_A is most sensitive to 4-AP as well as α- and δ-dendrotoxin (Halliwell *et al.*, 1986; Benishin *et al.*, 1988). In patch-clamp studies, the sensitivity to 4-AP is commonly used to identify I_A. Despite their primary effects on I_K and Ca^{2+}-activated K^+ channels, TEA and charybdotoxin, respectivately, have also been

reported to block I_A (MacKinnon and Miller, 1989).

There is some variation among I_A, particularly in terms of voltage-dependence of activation and inactivation rates. For example, two subtypes of I_A have been identified in neonatal rat sensory neurons: one activates and inactivates rapidly (I_{Af}), while the other activates and inactivates more slowly (I_{As}) (McFarlane and Cooper, 1991, 1992). Inactivation of K^+ currents can be very complex, and may depend on the pulse pattern (Aldrich *et al.*, 1978). Therefore, caution should be exercised when using inactivation as a unique criterion to define a channel type.

1.2.2.1.3 Inward rectifiers

Most voltage-dependent K^+ channels are outward rectifying, due to the large K^+ concentration difference between inside and outside the membrane. However, Katz (1949) first reported an anomalous rectifying K^+ channel in frog skeletal muscle, which is activated by membrane hyperpolarization beyond the K^+ equilibrium potential (E_K) and passes K^+ current more easily inward than outward. Inward rectification is an important property of cardiac (Noble, 1984; Reuter, 1984), skeletal and smooth muscles (Adrian, 1969; Benham *et al.*, 1987), neurones (North, 1989), and eggs (Hagiwara, 1979). The function of inward rectifiers is to permit long depolarizing responses.

Inward rectifiers, whose conductance increases under hyperpolarization and decreases under depolarization, have characteristics mirroring those of the delayed rectifier. Anomalous rectification may result from Mg^{2+} block at inner sites of the

channel. The typical single-channel conductance is about 20 pS. External K^+ concentration is capable of regulating the permeability, or the conductance of this type of the voltage-dependent K^+ channel. Inward rectifiers can be blocked by Cs^+, Ba^{2+} and intracellular application of TEA.

There are also some subtypes of inward rectifying channels which have various time or agonist dependence, such as instantaneous (fast activation), H-current (slow activation) and agonist gated channels. For example, in cardiac sinoatrial (S-A) nodal cells there are both muscarine responsive and non-responsive inward rectifiers (Sakmann *et al.*, 1983).

1.2.2.2 Ca^{2+}-activated K^+ channels (K_{Ca})

Gardos (1956) first reported an increase in the K^+ permeability of the cell membrane in response to a rise in cytosolic Ca^{2+} in red blood cells. It has since been shown that Ca^{2+}-activated K^+ channels exist in a wide range of excitable and non-excitable cell membranes. This family of channels opens following an elevation in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$), although in some cases Ca^{2+}-activated K^+ channels may also be voltage-dependent. The function of K_{Ca} is to hyperpolarize the cell membrane. Therefore, K_{Ca} contributes to spike repolarization (Grega and Macdonald, 1987), transient hyperpolarization (Blatz and Magleby, 1986), the resting potential (Bourque, 1988), and control of neuronal excitability (Lang and Ritchie, 1987). Several excellent reviews on K_{Ca} have appeared (Rudy, 1988; Haylett and Jenkinson, 1990; Kolb, 1990; Hille, 1992*a*; Hinrichsen, 1993).

The correlation between macroscopic currents and single K_{Ca} channel currents is inferred on the basis of single channel conductances, voltage, calcium and pharmacological sensitivities.

1.2.2.2.1 Large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca})

The large-conductance Ca^{2+}-activated K^+ channels are found in a variety of excitable and non-excitable cell types. In neurones, BK_{Ca} contributes to the spike repolarization, and thus, controls the frequency of repetitive firing (Meech, 1978). In smooth muscles, the function of such channels may be to terminate the excitatory processes which are initiated or maintained by an increase in $[Ca^{2+}]_i$. In secretory cells, BK_{Ca} has been proposed to regulate the resting potential and plays an important role in controlling secretion (Petersen and Maruyama, 1984).

The activation of BK_{Ca} is dependent upon both internal Ca^{2+} binding and membrane depolarization, and unlike I_K, the K^+ current resulting from activation is not delayed. The BK_{Ca} channel exhibits voltage-dependence even at a constant elevated level of Ca^{2+} concentration. The macroscopic current through BK_{Ca} channels is typically a large and long-lasting outward current. The single-channel conductance is 130-300 pS, which is near to the theoretical maximum conductance calculated for a membrane pore. Two toxins, charybdotoxin and noxiustoxin, can block BK_{Ca} (Valdivia *et al.*, 1988). Charybdotoxin is well known as a blocker of BK_{Ca}, and has been used to identify BK_{Ca}. External TEA (Petersen and Marayama, 1984), internal Na^+ (Yellen, 1984), Ba^{2+} (Miller, 1987) and quinine (Glavinovic and

Trifaro, 1988) can also block BK_{Ca}.

1.2.2.2.2 Intermediate-conductance Ca^{2+}-activated K^+ channels (IK_{Ca})

IK_{Ca} is a very diverse group of ion channels, and has been identified in many cell types such as red blood cells (Hamill, 1981), and neurones (Hermann, 1986), *etc.* The channel is active at the resting membrane potential, suggesting a possible role in the maintenance of resting membrane potential. IK_{Ca} has a typical single-channel conductance in the range of 25-135 pS (Hinrichsen, 1993). Some are voltage-independent (Saimi and Martinac, 1989), and some are not (Maue and Dionne, 1987). The channel exhibits intermediate Ca^{2+}-sensitivity; that is, it is in between that of BK_{Ca} and SK_{Ca}. This channel can be blocked by quinine, quinidine (Deitmer and Eckert, 1985), TEA or charybdotoxin (Reinhart *et al.*, 1989). Apamin appears to have little or no effect on any of the IK_{Ca} channels.

1.2.2.2.3 Small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca})

SK_{Ca} was first described in muscle cells (Romey and Lazdunski, 1984), and is defined to be a channel with a single-channel conductance that is less than 20 pS (Hinrichsen, 1993). These channels are known to participate in after-hyperpolarization (Pennefather *et al.*, 1985; Lancaster *et al.*, 1991). SK_{Ca} has less dependence on the membrane potential, and is more sensitive to intracellular Ca^{2+} than BK_{Ca}. Apamin is a specific inhibitor of the SK_{Ca}, and as such has been used to identify SK_{Ca} (Blatz and Magleby, 1986; Cook and Quast, 1990). Leiurotoxin I, a

toxin from scorpion *Leiurus quinquestriatus* venom, also blocks the SK_{Ca} (Castle and Strong, 1986). TEA has little or no effect on SK_{Ca} (Blatz and Magleby, 1986).

1.2.2.3 Na^+-activated K^+ channels (K_{Na})

There are far fewer reports describing Na^+-activated K^+ currents than Ca^{2+}-activated K^+ currents (for review, see Bader *et al.*, 1990). Both intracellular Na^+ accumulation, and membrane depolarization are necessary for the activation of these channels. The time-course of K_{Na} activation is somewhat similar to that of the I_A, except K_{Na} does not inactivate during a steady depolarization (Connor and Stevens, 1971). The single channel conductance is about 50 pS. Tetrodotoxin (TTX), TEA and 4-AP can block these channels (Bader *et al.*, 1985; Dryer *et al.*, 1989). Whenever the intracellular Na^+ increases, K_{Na} promotes a hyperpolarization, and thus, accelerates the repolarizing phase of the action potential.

1.2.2.4 Ligand-gated K^+ channels

A number of hormones and neurotransmitters are known to modify cellular excitability through modulation of K^+ channels. Ligand-gated K^+ channels are, as a group of K^+ channels, modulated by the interaction of ligands with their receptors on the plasma membrane. They have been found in many cell types under the control of a variety of receptors (Brown, 1988; Pfaffinger and Siegelbaum, 1990). Two well known ligand-gated K^+ channels are described below.

1.2.2.4.1 M-channels (I_M)

The M current, which was originally identified in frog sympathetic neurons, is suppressed by the cholinergic agonist muscarine (Brown and Adams, 1980). Later the M current was also shown to be inhibited by a variety of other transmitters including LHRH, substance P, and nucleotide triphosphates, all acting through different receptors (Pfaffinger, 1988). In addition to suppression by certain agonists, M currents can also be up-regulated by a variety of other agonists; in smooth muscle cells, for example, treatment with a β-adrenergic agonist causes up-regulation of the M current (Sims *et al.*, 1988). I_M, like I_K, opens slowly when the membrane is depolarized, with no inactivation. The single channel conductance is 3-10 pS. I_M functions as a physiological voltage clamp to oppose the effect of an imposed depolarization in order to limit repeat discharge of action potentials.

1.2.2.4.2 S-K^+ channels

Application of serotonin (5-HT) to *Aplysia* sensory neurones closes a type of K^+ channel, termed the serotonin-sensitive or S-K^+ channel, which is responsible for the observed presynaptic facilitatory effect of 5-HT in increasing both action potential duration and neurotransmitter release (Siegelbaum *et al.*, 1982). In contrast, FMRFamide, a neuropeptide, opens the S-K^+ channel, producing a slow hyperpolarization, a shortening of action potential duration as well as a decrease in the neurotransmitter release. 5-HT and FMRFamide appear to act at different sites on the S-K^+ channel (Volterra and Siegelbaum, 1990). Therefore, the S-K^+ channels

contribute to both the resting conductance of the membrane and action potential repolarization.

1.2.2.5 Others

There have been numerous reports describing the existence of other classes of K^+ channels that are not listed above. They could be voltage-dependent or ligand-gated. Some K^+ channels, for example, can be activated by hormones and neurotransmitters through direct modulation by guanine nucleotide binding proteins (G proteins) (Dunlap *et al.*, 1987), while others can be modulated by a variety of specific intracellular agents and second messengers, including: adenosine 5'-trisphosphate (ATP) (Standen *et al.*, 1989), cyclic adenosine 3',5'-monophosphate (cAMP) (Belardetti and Siegelbaum, 1988), cyclic guanosine 3',5'-monophosphate (cGMP) (Cook and Babcock, 1993), inositol 1,4,5-trisphosphate (IP_3) (Hamada *et al.*, 1993), and inositol 1,3,4,5-tetrakisphosphate (IP_4) (Chadwick *et al.*, 1992). Also, there are reports of ion sensitive K^+ channels (e.g. lithium-sensitive channels) (Hartung, 1985), or cell volume activated (stretch-activated) channels (Richards and Dawson, 1986). Finally, a Ca^{2+}-activated or cyclic nucleotide activated, non-specific cation current which has equal permeability for K^+ and Na^+ has also been characterized (Reuter, 1984; Nakamura and Gold, 1987).

1.2.3 K^+ Channel Modulation by G Proteins and Second Messenger Cascades

The modulation of K^+ channels by different hormones and neurotransmitters,

through changes in current amplitude and/or channel kinetics, can result in the regulation of the electrical properties in various cells (Pfaffinger and Siegelbaum, 1990). The modulatory actions of hormones and neurotransmitters on K^+ channels are generally considered to be mediated by two distinct receptor families. One family includes the directly gated receptors, where the receptors and ion channels are part of one macromolecular protein complex (Miller, 1989). Binding of the ligand to its membrane receptor directly leads to opening or closure of the channel, which produces very short and rapid changes in conductance. The nicotinic acetylcholine (ACh) receptor is an excellent example of such a channel (Karlin, 1980; Noda *et al.*, 1983). In contrast to the directly gated receptors, the second family of receptor is characterized that the receptor and the channel are two distinct structures. Binding of ligand to its receptor does not directly lead to either an opening or a closure of the channel. Rather, this ligand-receptor interaction is able to trigger the activation of certain G protein(s), which, in turn, interacts with the channel protein directly or trigger the second messenger system(s), leading to changes in the channel conductance. The resultant alteration of conductance tends to be much slower and last much longer. The function of this type of receptor is to produce long-lasting modulatory changes in K^+ channels and cellular activity. The α- and β-adrenergic receptors, muscarinic ACh receptor, and prostaglandin receptors are all members of this family (Gilman, 1987).

1.2.3.1 K^+ channels and G proteins

G proteins are a large family of signal transducing proteins that couple membrane receptors to their effectors, which include ion channel proteins (Gilman, 1984; 1987; Stryer and Bourne, 1986; Brown *et al.*, 1990; Hinrichsen, 1993). Modulation of K^+ channel activity by either hormones or neurotransmitters is believed to occur via G protein activity, which may or may not involve the activation of intracellular second messengers.

The involvement of membrane bound G proteins in the coupling process, either directly or indirectly, has been suggested by the fact that intracellular guanosine 5'-triphosphate (GTP), which binds to G proteins to activate them (Gilman, 1987), modulates the activity of both ACh-gated K^+ channels (Brown *et al.*, 1990; Nakajima *et al.*, 1991) and K_{Ca} (Vaca *et al.*, 1992; Suzuki *et al.*, 1994). Also, the idea that there is coupling of G protein activity with K^+ channel modulation is supported by following observations: pertussis toxin (PTX), which acts on certain G proteins to block the interaction of a receptor with the G protein α-subunit (Ui, 1984), alters the activation of inward rectifier (Wu and Assmann, 1994) as well as the outward rectifier K^+ channels (Boddeke *et al.*, 1993), and cholera toxin, which inhibits the inactivation of certain G proteins by preventing GTP hydrolysis (Abood *et al.*, 1982), affects the activity of K_{Ca} (Fan *et al.*, 1993) as well as the inward rectifier (Wu and Assmann, 1994). Furthermore, G protein involvement is demonstrated by the reports that the non-hydrolysable GTP analogue 5'-guanylylimidodiphosphate (GppNHp) and guanosine-5'-O-(3-thiotriphosphate)

(GTPγS) regulate the activation of K_{Ca} (Vaca *et al.*, 1992; Hamada *et al.*, 1993), inward rectifiers (Wu and Assmann, 1994), outward rectifiers (Benz *et al.*, 1991) and ACh-gated K^+ channels (Breitwieser and Szabo, 1985), and the non-hydrolysable guanosine 5'-diphosphate (GDP) analogue guanosine-5'-O-(2-thiodiphosphate) (GDPβS) modulates the activity of K_{Ca} (Vaca *et al.*, 1992; Fan *et al.*, 1993; Hamada *et al.*, 1993), inward rectifiers (Wu and Assmann, 1994) and ACh-gated K^+ channel (Nakajima *et al.*, 1991).

So far, a number of hormones and neurotransmitters have been reported to either stimulate or inhibit adenylate cyclase (AC) activity through the action of either stimulatory or inhibitory G proteins -- abbreviated as G_s and G_i, respectively. Activation of phospholipase C (PLC) through an unidentified G protein (G_o) has also been confirmed. However, in some cases, the receptor-effector coupling may not involve second messenger cascades, and, instead, the G protein interacts directly with the K^+ channel protein. Examples include the coupling of the atrial muscarinic receptor with the ACh-gated K^+ channel (Pfaffinger *et al.*, 1985, Yatani *et al.*, 1987; Brown *et al.*, 1990), as well as the molluscan neuron dopamine and muscarinic receptors with a common K^+ channel (Bolshakov *et al.*, 1993).

1.2.3.2 K^+ channels and second messengers

In addition to direct interaction with K^+ channels, G proteins can also modulate K^+ channel activity through the activation of various intracellular second messengers, such as cAMP, cGMP, Ca^{2+}, IP_3, diacylglycerol (DG) and arachidonic

acid (AA) (Kaczmarek and Levitan, 1987; Levitzki, 1988; Walsh and Kass, 1988; Pfaffinger and Siegelbaum, 1990; Chen and Yu, 1994). The receptor and channel need not necessarily be intimately associated in a single macromolecular complex, but may communicate via an intracellular second messenger which is produced upon occupancy of the receptor by agonist. The long-lasting modulation of ion channel activity, and, hence, of the electrical activity of the cell, by various neurotransmitters or hormones, is not only dependent on the continued occupation of a receptor, but, rather, is a result of a long-lasting metabolic modification of an ion channel. Protein phosphorylation by various kinases is one of the most important mechanisms for the functional regulation of ion channels by second messengers (Siegelbaum and Tsien, 1983; Nairn *et al.*, 1985; Lemos *et al.*, 1986*a*; Levitan, 1985; 1988).

1.2.3.2.1 The cyclic AMP cascade

The first step in the cAMP cascade is the activation of the stimulatory G protein, G_s, as a consequence of ligand-receptor binding. The activated α subunit of G_s stimulates adenylate cyclase, an integral membrane protein, to catalyse the conversion of ATP into cAMP. The cAMP then activates cAMP-dependent protein kinase (PKA) (Blackshear *et al.*, 1988), leading to the phosphorylation of a wide variety of substrate proteins, including ion channel proteins, on the serine or threonine residues (Scott and Soderling, 1992).

By using such experimental approaches as the intracellular injection of cAMP through microelectrodes, the extracellular application of membrane-permeant

analogues, the use of phosphodiesterase inhibitors, and the use of adenylate cyclase (AC) activators or inhibitors, modulation of K^+ channels by cAMP has been widely documented (Siegelbaum and Tsien, 1983; Kaczmarek and Levitan, 1987; Levitan, 1988; Pfaffinger and Siegelbaum, 1990; Hinrichsen, 1993; Minami *et al.*, 1993; Mori *et al.*, 1993; Chen and Yu, 1994; Harada and Iijima, 1994). cAMP and cAMP-dependent protein phosphorylation could either inhibit or enhance the conductance of certain types of K^+ channels. Intracellular perfusion of cAMP, or the adenylate cyclase activator forskolin was shown to decrease the voltage-dependent K^+ current amplitude in murine B cells and in oligodendrocytes (Choquet *et al.*, 1987; Choquet and Korn, 1988; Soliven *et al.*, 1988), but to increase the delayed rectifier K^+ current in guinea pig ventricular myocytes (Harada and Iijima, 1994). No effect of cAMP on voltage-dependent K^+ channel conductance was observed in human T or B lymphocytes and *Aplysia* neurones (Krause *et al.*, 1988; Zunkler *et al.*, 1988; Kaczmarek and Strumwasser, 1984). In smooth muscle cells, cAMP partially prevented the attenuation of the transient outward K^+ current induced by repetitive stimulations (Ohya *et al.*, 1987). In the case of Ca^{2+}-activated K^+ channels, modulation by cAMP and cAMP-dependent mechanisms has been shown to either potentiate K_{Ca} activity in smooth muscle cells (Sadoshima *et al.*, 1988; Kume *et al.*, 1989; Carl *et al.*, 1991; Minami *et al.*, 1993*a*) and in *Helix* neurons (Lemos *et al.*, 1986*a*), or inhibit K_{Ca} currents in hippocampal pyramidal cells (Madison and Nicoll, 1982). cAMP analogues can increase the current amplitude of an opioid ligand-gated K^+ channel expressed in *Xenopus* oocytes (Chen and Yu, 1994); this increase is

believed to be mediated by PKA phosphorylation, since the PKA catalytic subunit mimicked the effect of cAMP (Chen and Yu, 1994).

Although cAMP-dependent modulation of K^+ channels has been commonly considered to be mediated by protein kinase A (PKA)-dependent phosphorylation of either the ion channel protein itself or a regulatory element intimately associated with the channel (Lemos *et al.*, 1986*a*), it is possible that some cAMP-dependent modulation of K^+ channels is mediated directly by cAMP, independent of protein phosphorylation (Nakamura and Gold, 1987; DiFrancesco and Tortora, 1991; Roy and Sontheimer, 1995). Furthermore, there is some evidence to suggest that PKA activity regulates the expression of a gene coding for a voltage-dependent K^+ channel (Fisch *et al.*, 1989; Grieshammer *et al.*, 1992; Mori *et al.*, 1993). Therefore, cAMP could modulate K^+ channels not only by post-translational processes through phosphorylation of the channel, or associated proteins, but also by pre-translational processes at the level of gene expression.

1.2.3.2.2 The protein kinase C cascade

The interaction of an agonist with its membrane receptor results in the dissociation of G_o pro[illegible] subunits. The uncoupled α subunit of the G_o protein then activates the cytoplasmic effector, phospholipase C (PLC). The activated PLC hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2), causing the release of DG and IP_3 into the cytoplasm. DG directly activates PKC, whereas IP_3 releases Ca^{2+} from the intracellular sarcoplasmic reticulum (SR) stores, which, in turn, activates protein

kinase C (PKC) and calcium/calmodulin dependent kinase (Berridge and Irvine, 1984; Nishizuka, 1984*a,b*).

By using PKC activators and DG analogues, or by intracellular injection of PKC, the PKC cascade has been confirmed to be another important messenger system related to K^+ channel modulation (Shearman *et al.*, 1989; Pfaffinger and Siegelbaum, 1990; Hinrichsen, 1993). Activation of PKC results in a reduction in Ca^{2+}-activated K^+ channel activity in CA1 pyramidal cells (Malenka *et al.*, 1986) and in brain synaptosomes (Ren and Benishin, 1994), a decrease in I_A in *Xenopus* oocytes (Moran *et al.*, 1991) and in rat brain synaptosomes (Colby and Blaustein, 1988), an inhibition of I_K in brain synaptosomes (Colby and Blaustein, 1988), and a block of the opioid receptor-gated K^+ channel in oocytes (Chen and Yu, 1994). Therefore, it is commonly believed that activation of PKC directly inhibits different types of K^+ channels. However, Walsh and Kass (1988) observed the enhancement of I_K in cardiac ventricular myocytes in response to PKC activation.

1.2.4 K^+ Channels in the Neurohypophysis

The release of the neuropeptides vasopressin and oxytocin by the nerve endings of the posterior pituitary is triggered by Ca^{2+} entry during presynaptic action potentials (Douglas and Poisner, 1963*a,b*; Nordmann, 1983). The events leading to the release of neurohormones are essentially the same as those observed at synapses (Llinas *et al.*, 1981). This Ca^{2+}-dependent secretion can be modulated by stimulus frequency (Bondy *et al.*, 1987), complex patterns of activity (Cazalis *et al.*, 1985;

Nordmann and Stuenkel, 1986), and certain peptides (Bondy *et al.*, 1989). Thus, these nerve terminals, which may be affected by many aspects, are an important site for regulation of neuroendocrine function. Although there is considerable evidence that depolarization of nerve terminals leads to the entry of Ca^{2+} and to the secretion of neurohormones, the details of how ionic currents control the release of neuroactive substances from nerve terminals remain largely unknown.

K^+ channels play an important role in modulating secretion from the posterior pituitary. Depolarization of the membrane produces a high density K^+ current. Blockade of K^+ channels or inactivation of K^+ current broadens action potentials (Bondy *et al.*, 1987; Salzberg and Obaid, 1988; Jackson *et al.*, 1991), thus facilitating neuropeptide release (Bondy *et al.*, 1987; Hobbach *et al.*, 1988). Much is known about the K^+ channels in neurons and non-neuronal secretory cells, as previous described. However, the small size and inaccessibility of most nerve terminals have precluded direct analysis of membrane ionic currents and their influence on secretion. It was not possible to apply patch-clamp techniques to study ion channels in mammalian nerve terminals until Lemos and Nordmann established the method of isolating neurosecretory nerve endings from the crab sinus gland (Lemos *et al.*, 1986*b*; Nordmann *et al.*, 1986; Stuenkel *et al.*, 1990) and the rat posterior neurohypophysis (Lemos and Nordmann, 1986; Nordmann *et al.*, 1987). The high density and large size (mean diameter of 2 μm) (Nordmann, 1977) of the posterior pituitary nerve endings (i.e. the release site), which can be easily separated from the transport site (the axons) and from the site of synthesis (the neuronal cell

bodies), make this an excellent preparation for the study of neuronal secretion mechanisms. By using the patch-clamp technique, three types of K^+ channels in the membranes of posterior pituitary nerve endings have been identified on the basis of single channel conductance, kinetic properties, voltage dependence, Ca^{2+} and drug sensitivities (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). They can contribute to action potential repolarization and to the regulation of secretion.

1.2.4.1 Transient K^+ channel

The transient K^+ channel is the first K^+ channel to be characterized in rat neurohypophyseal nerve terminals (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992). With a holding potential -80 mV, membrane depolarization can elicit a fast, transient, outward K^+ current in nerve terminals. The outward current amplitude increases with increasingly depolarized potential steps, while peak current activity occurs at progressively faster times. Hyperpolarizing prepulses enhance this transient K^+ current, while depolarizing prepulses reduce it. The threshold of activation for this current is -60 mV, which is lower than the others described below. The current reaches a peak rapidly (less than 10 ms), and then decays more slowly. The decay is fitted by two exponentials with time constants of 20 and 140 ms, and is not voltage dependent. This transient outward current showed steady-state inactivation with 50% inactivation occurring at -47.9 mV ($V_{1/2}$ = -47.9 mV). The single channel conductance is 32 pS. 4-AP can block this transient outward current. There are,

however, conflicting reports on the TEA sensitivity in this channel. Thorn *et al.* (1991) observed that TEA (100 mM) had no effect on the current in dissociated nerve terminals, while Bielefeldt *et al.* (1992) showed that TEA (5 mM) decreased current amplitude in the thin slice preparation from the rat posterior pituitary. The difference may be due to either differences in the preparation studied, or differences between the vasopressin and oxytocin terminals. The activation of this channel is neither Ca^{2+}-dependent, nor charybdotoxin sensitive. Therefore, it corresponds most closely in its kinetics to I_A. However, it has to be pointed out that this terminal I_A appears to be different from the I_A found in the cell bodies in the hypothalamus which project their axons to the posterior pituitary. There are three major differences between the neurohypophyseal terminal I_A and currents recorded in other cells. First, the terminal I_A half-maximal inactivation occurs at -47.9 mV, and when compared to those reported in other neurons, which ranged from -110 mV in bull-frog sympathetic neurons (Adams *et al.*, 1982) to -60 mV in hippocampal CA3 neurons (Zbicz and Weight, 1985), the inactivation kinetics in neurohypophyseal terminals clearly occurs at more depolarized potentials. Secondly, the inactivation curve for the terminal I_A was much less steep than that reported in other neurons. Thirdly, the rate of recovery from inactivation is slow ($>$ 16 s) in this channel, which would lead to spike broadening, more Ca^{2+} entry, and thus enhanced peptide release from the terminal, when stimulated by a burst of action potentials.

It is believed that this channel is the major current responsible for the neurohypophyseal repolarization after a spike. After prolonged stimulation at high

frequencies, repolarization by I_A would fail because of inactivation and lead to a failure of spike propagation. Thus, terminal I_A tends to act as a brake on spike discharge, retarding the production of successive spikes in a train, thus limiting spike frequency.

1.2.4.2 Ca^{2+}-activated K^+ channel

In physiological solutions with 4-AP (to block I_A), depolarizing voltage-clamp steps from different holding potentials (-90 or -50 mV) elicit a sustained outward K^+ current with slow inactivation, which reaches a peak plateau and then decays slowly (Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). The threshold for activation of this current is -30 mV from holding potential of -80 mV, and the inactivation is fitted by a single exponential with a time constant of 9 s. This channel can be strongly enhanced by hyperpolarization, and virtually eliminated by depolarization. It is activated by intracellular Ca^{2+}, and extracellular Cd^{2+}, a calcium channel blocker, can reversibly abolish this current. Current decay did not show any dependence on voltage but rather on intracellular Ca^{2+}. This channel has a large conductance of 140 pS. 4-AP, dendrotoxin, apamin and charybdotoxin have no effect on this current, while Ba^{2+} and TEA inhibit this current. It corresponds most closely to a large-conductance Ca^{2+}-activated K^+ current. However, this channel is unusual in that it is insensitive to charybdotoxin, which is usually used to identify BK_{Ca}. Additionally, this toxin also fails to affect the compound action potential of the mouse neurohypophysis (Salzburg and Obaid, 1988). Meanwhile, apamin, a blocker of the

SK_{Ca}, also fails to have any effects on this channel. Furthermore, it has properties different from that of the Ca^{2+}-dependent K^+ channel in the magnocellular neurons of the hypothalamus, which are more transient and are sensitive to 4-AP or dendrotoxin, but not to TEA (Bourque, 1988). Therefore, it may be a novel type of K_{Ca} similar to that described by Reinhart *et al.* (1989).

The slow activation/inactivation of the Ca^{2+}-activated K^+ current makes it probable that it may have a role in the termination of a burst of action potentials and in the control of intraburst intervals. Repetitive action potential firing leads to an increase in terminal Ca^{2+} accumulation, which tends to maximally activate K_{Ca} and produce a long-lasting hyperpolarization (Jackson *et al.*, 1991). This would then uncouple terminal excitation from incoming axonal spikes. Thus, maximal peptide release only occurs in response to bursts of electrical activity invading the nerve terminals.

1.2.4.3 Non-inactivating K^+ channel

This K^+ channel can be activated by depolarizing the membrane from a holding potential of -80 mV, which is similar to the normal terminal resting potential, and it responds with slow activation kinetics and little if any inactivation (Bielefeldt *et al.*, 1992). Based on currents obtained from analyzed single channel records, the activation of this current was not sigmoid, but had an exponential onset with a time constant of 65 ms. The threshold for activation is -30 mV, and the single channel conductance is 27 pS. It is not Ca^{2+} sensitive, and both TEA and dendrotoxin can

block this channel. Therefore, this channel may be related to a delayed rectifier family, but its activation is much slower than that found by Cobbett *et al.* (1989) in the hypothalamic neurons from which these nerve ending originated. Likewise, this channel may be a specific axonal or nerve terminal membrane component.

Due to its slow activation kinetics, this channel cannot play a role in action potential repolarization. Activation of this channel could raise the threshold for action potential generation, and then limit secretion towards the end of a long burst. Thus, the activation of this non-inactivating K^+ channel could play a role in the fatigue of stimulus-secretion coupling in the posterior pituitary, which corresponds to the pattern of vasopressin secretion, while oxytocin secretion is relatively uniform for long and short stimuli (Bicknell *et al.*, 1984).

1.3. PROSTAGLANDIN E_2 (PGE_2)

1.3.1 Biosynthesis and Metabolism of Prostaglandins

The biological activity of prostaglandins was first described in the 1930s (Kurzrok and Lieb, 1930; Von-Euler, 1935), and study of this field has advanced rapidly since the 1960s, when Bergstrom *et al.* (1963) published the structure of some members of this naturally occurring group of fatty acids. The term prostaglandin is now used as a generic term for a family of closely related derivatives of prostanoic acid (Fig. 1-2), which are essentially unsaturated fatty acids with a 20-carbon skeleton and consisting of a cyclopentane ring with two side chains (Horton, 1969).

Prostaglandins are biosynthesized from arachidonic acid (Bergstrom *et al.*, 1964; McGiff, 1981; Needleman *et al.*, 1986), which is derived from dietary linoleic acid (Lundberg, 1965). Linoleic acid is an unsaturated 18-carbon fatty acid with two double bonds, while arachidonic acid is a 20-carbon fatty acid with four double bonds. Arachinodic acid is esterified to membrane phospholipids at the 2-position of the glycerol backbone, and can be released from membranes, either through the direct action of phospholipase A_2 (PLA_2) (Lapetina, 1982), or through the combined action of phospholipase C and diacylglycerol lipase (Billah *et al.*, 1981; Irvine, 1982; Prescott and Majerus, 1983). In some cases, triglycerides may also be able to release phospholipid bound arachidonic acid in tissues such as renal medullary interstitial cells (Bojesen, 1974). Free arachidonic acid can then be converted through the enzyme cyclooxygenase to produce the prostaglandins, prostacyclins and thromboxanes. Cyclooxygenase is universally present in cells, and can transform arachidonic acid into the prostaglandin endoperoxides PGG_2 and PGH_2. These endoperoxides are quite unstable in aqueous solution and isomerize spontaneously or enzymatically, by specific isomerases, into stable prostaglandins, such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane B_2 (TXB_2) (Moncada and Higgs, 1986; Gresele *et al.*, 1991). At present, nine groups of prostaglandins have been classified, and designated alphabetically from A to I. Each group can be further divided into series one, two and three (e.g. PGE_1, PGE_2, PGE_3), based on the degree of unsaturation, i.e. the number of double bonds in the side chains. The additional subscript α or β, e.g. $PGF_{2\alpha}$, is indicative of the stereometric orientation of the -OH group attached

at carbon site 9. The prostaglandins of the two series, such as PGE_2 and $PGF_{2\alpha}$, have been considered to be the most important biological prostanoids (Greeley *et al.*, 1986; Coleman *et al.*, 1994*b*).

Prostaglandins are synthesized and released on demand *de novo*, rather than being stored in the cell, by most tissues. After eliciting their actions, prostaglandins are very rapidly and effectively removed by the lung and metabolized by 15-hydroxyprostaglandin dehydrogenase and Δ13 reductase into inactive metabolites, which are then excreted by the kidney (Terragno and Terragno, 1979). Therefore, the arterial concentration of these substances under normal conditions is very low (Curtis-Prior, 1988).

1.3.2 Prostaglandins and Neurohypophyseal Hormone Release

Prostaglandins were first reported to be widely distributed in the brain 30 years ago (Samuelsson, 1964). They are released either spontaneously or in response to electrical and/or chemical stimulation. Relatively large amounts of prostaglandins are found in the neurointermediate pituitary lobe (Horton, 1969; Narumiya *et al.*, 1982; Ogorochi *et al.*, 1984; Watanabe *et al.*, 1985). High affinity and selectivity binding of ^{3}H-PGE_2 in the rat hypothalamo-neurohypophyseal system is observed and the specificity of PGE_2 binding has the expected characteristics for receptors which are coupled to adenylate cyclase activation (Malet *et al.*, 1982). The fact that the posterior pituitary contains abundant amounts of PGD_2, PGE_2 and $PGF_{2\alpha}$ (Horton, 1969; Narumiya *et al.*, 1982; Ogorochi *et al.*, 1984; Watanabe *et al.*, 1985), their

specific receptor proteins and related enzymes (Shimizu *et al.*, 1979) strongly suggests a physiological role for these prostaglandins in the pituitary neural lobe, where ADH and OXT are stored in vesicles and released into the systemic circulation.

1.3.2.1 Effects of prostaglandins on ADH release from neurohypophysis

Within the past two decades, it has become increasingly apparent that prostaglandins may influence the central nervous system mechanisms that control ADH secretion, although the effect of prostaglandins on neurohypophyseal ADH release is still controversial.

1.3.2.1.1 Prostaglandins increase ADH release

It is well accepted that the major physiological roles of ADH are the regulation of water balance (antidiuretic action) and the contraction of arterioles (vasopressor action). Thus, one of the first indications of the relationship between prostaglandins and ADH release is provided by the work of Vilhardt and Hedqvist (1970), who used changes in urine flow and osmolarity as indicators of neural lobe ADH release and observed that intracarotid infusion of PGE_2 elicited a dose-related antidiuresis in the water loaded, ethanol-anaesthetized dog. Antidiuresis mediated by intravenous (*iv*) injection of PGE_1 is also found to increase the release of ADH in dogs (Berl *et al.*, 1973) and rats (Ruoff *et al.*, 1974). Subsequently, it has been shown that intraventricular (*ivt*) infusion of PGE_1, PGE_2 or PGH_2 facilitates ADH

secretion in the hydrated conscious goat (Andersson and Leksell, 1975; Leksell, 1976, 1978), and in the rat (Hoffman and Schmid, 1979; Fujimoto *et al.*, 1980). In these studies, however, changes in the plasma concentration of ADH are inferred from the amount of water excretion. Measuring the plasma ADH concentration by radioimmunoassay, Yamamoto *et al.* (1976) were able to demonstrate that perfusion of PGE_2 into the ventriculi-cisternal system of anaesthetized dogs resulted in a significant increase in the plasma ADH concentration, and Hashimoto *et al.* (1988 and 1989) showed that the *ivt* administration of PGE_2 increases plasma concentrations of ADH in rats and rabbits, suggesting it enhances ADH release through a central mechanism(s), i.e. via actions on the hypothalamus nuclei. Furthermore, PGE_2 and $PGF_{2\alpha}$ increases ADH release from the explants of male guinea pig hypothalamo-neurohypophyseal complex (HNC) in organ culture, although $PGF_{2\alpha}$ has a relatively weaker effect than PGE_2 (Ishikawa *et al.*, 1981).

It has been found that administration of indomethacin (IM), an inhibitor of cyclooxygenase, can decrease the plasma ADH concentration in dogs (Yamamoto *et al.*, 1976), diminish urinary ADH in humans (Glasson *et al.*, 1979; Glasson and Vallotton, 1980), and suppress the osmotically or angiotensin II-induced ADH release in dogs (Hoffman *et al.*, 1982; Yamamoto *et al.*, 1978). Intravenous administration of the prostaglandin-antagonist meclofenamate results in a consistent fall in plasma ADH levels in conscious dogs (Walker, 1983), which suggests that prostaglandins are important mediators of basal ADH release. Moreover, IM modulates ADH release from the guinea pig HNC in organ culture (Ishikawa *et al.*, 1981). It has been found

that IM significantly enhances the neurohypophyseal ADH content in rats (Bojanowska *et al.*, 1985). An elevation of ADH content in the neurohypophysis may be the effect of an intensified axonal transport rate (and, indirectly, of enhanced hormone synthesis) and/or of diminished release. Although the present data do not allow one to precisely evaluate the participation of these three processes, these results indicate that endogenous prostaglandins may be involved in the mechanism(s) of ADH release.

There is some evidence to the contrary, namely that: (a) the subcutaneous (*sc*) administration of IM does not influence the osmoregulation of ADH in dogs (Kamoi *et al.*, 1983); (b) the alterations in water excretion caused by IM are not due to any direct effect on the central release of ADH (Berl *et al.*, 1977); (c) in anaesthetized dogs, perfusion of cerebral ventricle with IM alone has no effect on the plasma ADH concentration (Yamamoto *et al*, 1978), and (d) IM does not alter the basal release of ADH from the guinea pig HNC in organ culture (Ishikawa *et al.*, 1981). However, more evidence supports the contention that brain prostaglandins may modulate the control of ADH liberation.

The question of whether prostaglandins can act directly upon the neurohypophysis to stimulate ADH release is also controversial. There has been a failure to observe any change in ADH release in *in vitro* studies when the rat neurohypophysis is incubated with PGD_2, PGE_1, PGE_2 or $PGF_{2\alpha}$ (Vilhardt and Hedqvist, 1970; Ruoff, 1974; Hashimoto *et al.*, 1988 and 1989). Neither basal secretion of ADH, nor that evoked by electrical stimulation is affected by superfusing

the neurointermediate lobe of the hypophysis with IM (Knepel *et al.*, 1985). In contrast to those results, Gagnon *et al.* (1973) first demonstrated that PGE_2 (10^{-9} to 10^{-8} M) induced a significant increase in ADH release from rat neural lobes *in vitro*. This is consistent with the subsequent finding that PGE_2 or $PGF_{2\alpha}$ increases ADH release from isolated rat neurointermediate lobes (Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989). The discrepancy may due to the fact that: (a) some results are expressed as a percentage of the total amount of ADH contained in the incubated gland, while others are expressed as the absolute amount which may vary greatly from one experiment to another, (b) differences in the potassium concentration of the medium, since high concentrations of potassium induces ADH release, and these conditions may approach maximal release, and (c) differences in the time of incubation and the measurement of ADH. Incubation of neurointermediate lobes in a solution containing IM results in the inhibition of ADH release under both resting and depolarized (i.e. in solutions with excessive potassium) conditions (Bojanowska and Guzek, 1991).

It may, therefore, be assumed that some prostaglandin-dependent processes, which are under the stimulatory control of prostaglandins, involved in the mechanism of ADH release are, at least in part, localized in the neurohypophysis. Polyphloretin phosphate, an antagonist of prostaglandins, competitively inhibits PGE_2-elicited ADH release when rat neural lobe is incubated in the presence of both PGE_2 and poluphloretin phosphate (Gagnon *et al.*, 1973), indicating a direct role for PGE_2 in ADH release. Also, the peripheral and neurohypophyseal prostaglandin receptors

may be similar since polyphloretin phosphate antagonizes various prostaglandin-induced activities in the periphery (Gagnon *et al.*, 1973).

1.3.2.1.2 Prostaglandins decrease ADH release

IM can enhance ADH release in unanesthetized rats (Kasting *et al.*, 1985) and reverse the impairment of ADH release in hypokalemic dogs (Rutecki *et al.*, 1982). It would, therefore, appear that prostaglandins in some way exert an inhibitory influence, at least via osmoreceptors, on the release of ADH. By superfusing various prostaglandins on the neurointermediate lobe of the hypophysis: (a) PGE_1 is shown to suppress hypersecretion of ADH induced by surgical stress in humans (Enzan *et al.*, 1994), (b) PGE_2 does not influence resting release of ADH but remarkably inhibits electrically induced release of ADH, (c) $PGF_{2\alpha}$ also inhibits the evoked release of ADH; (d) PGD_2 has no effect on ADH release (Knepel *et al.*, 1985). These studies demonstrate that prostaglandins (especially PGE_2) can inhibit the stimulated release of ADH when acting upon ADH-containing nerve terminals of the neurohypophysis. The actions of PGE_2 and $PGF_{2\alpha}$ on the release of ADH are specific. These findings suggest that prostaglandin receptors may exist on the terminals of ADH neurones in the pars nervosa as well as in the zona externa of the median eminence.

1.3.2.2 Prostaglandins on OXT release from neurohypophysis

PGE_1, PGE_2 and $PGF_{2\alpha}$ have been reported to inhibit or facilitate posterior

pituitary OXT release in lactating animals and women.

1.3.2.2.1 Prostaglandins facilitate OXT release

The major physiological roles of OXT are the contraction of the uterus (oxytocic action) and the stimulation of milk ejection in the lactating mammary gland. Using milk ejection as an indicator, PGE_2 and/or $PGF_{2\alpha}$ is shown to increase OXT release in lactating women (Cobo *et al.*, 1974) and cows (Vorherr, 1979). This is consistent with the results from measuring plasma and/or urine OXT levels by radioimmunoassay. It has been reported that *iv* administration of $PGF_{2\alpha}$ and/or PGE_2 can lead to OXT release in both women and men (Gillespie, 1972), as well as in rabbits of either sex (Desai and Raghavan, 1982); *ivt* injection of PGE_2 and/or PGD_2 increased plasma concentration of OXT in rats (Hashimoto *et al.*, 1988) and rabbits (Hashimoto *et al.*, 1989); *sc* injection of the $PGF_{2\alpha}$ analogue cloprostenol causes an increase in plasma OXT concentration in cows (Aurich *et al.*, 1993), and intramuscular (*im*) injection of $PGF_{2\alpha}$ increases the circulating concentration of OXT in pigs (Ellendorff *et al.*, 1978). This data suggests prostaglandins are central to OXT release.

To decide whether this effect is pharmacological or physiological, IM is once again useful to determine the role of prostaglandins. IM can suppress OXT release in rats (Guzek *et al.*, 1986) and in goats (Cooke and Homeida, 1984). Therefore, these results indicate that endogenous prostaglandins are indeed involved in the stimulation of OXT release.

Where do the prostaglandins act? Gillespie *et al.* (1972) suggested a direct action of prostaglandins on the posterior pituitary, while Desai and Raghavan suspected that the site of prostaglandin action was within the hypothalamus (Desai and Raghavan, 1982). It has been reported that perfusion of the rat posterior pituitary with either PGE_2 or PGD_2 does not significantly affect the release of OXT (Hashimoto *et al.*, 1988 and 1989). However, addition of PGE_2 or $PGF_{2\alpha}$ to incubated fragments of neural lobe of the rat pituitary stimulates secretion of OXT *in vitro* (Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989). Incubation of neurointermediate lobes in a solution containing IM results in a reduction of OXT release during both resting and high potassium-induced depolarization conditions (Bojanowska and Guzek, 1991). This is consistent with the result that previous treatment of rats with IM is followed by a distinct decrease *in vitro* of basal and K^+-evoked release of OXT from incubated neurointermediate lobes (Bojanowska and Guzek, 1991). Therefore, prostaglandins may be supposed to act on the posterior pituitary serving as a modulatory factor for OXT release.

It may then be assumed that some prostaglandin-dependent processes, which are under the stimulatory control of prostaglandins, involved in mechanisms of OXT release are, at least in part, localized in the neurohypophysis.

1.3.2.2.2 Prostaglandins inhibit OXT release

Intraperitoneal (*ip*) injection of PGE_1 and/or $PGF_{2\alpha}$ inhibits milk ejection in lactating rats (Batta *et al.*, 1974; Prilusky and Deis, 1976), which is proposed as the

results of central block of OXT release from the neurohypophysis. *Ivt* injection into the lateral ventricle of rat brain, $PGF_{2\alpha}$ and PGE_2 may act on the CNS to inhibit OXT release (Fujimoto *et al.*, 1978).

These studies, on the whole, present a conflicting picture of the role prostaglandins may play in OXT release. It is also noteworthy that in pregnant rats, $PGF_{2\alpha}$ is capable of inducing lactogenesis but preventing OXT release and milk ejection after delivery (Deis, 1971; Vermouth and Deis, 1972). IM greatly attenuates endotoxin-induced and enhances haemorrhage-induced OXT release in male rats (Kasting, 1986). More interestingly, $PGF_{2\alpha}$ administered *ivt* activates 83% of oxytocinergic neurones and inhibits 75% of vasopressinergic neurones, i.e. $PGF_{2\alpha}$ stimulates OXT release and suppresses ADH release (Akaishi and Negoro, 1979). Therefore, it is possible that in the brain $PGF_{2\alpha}$ acts as a transmitter or modulator of hormone release.

To summarize, these studies have indicated that prostaglandins are indeed involved in the release of hormone from posterior pituitary; there is, however, some conflict as to whether their role is excitatory or inhibitory. Thus, prostaglandins may have a role in modulating the activity of the supraoptic nucleus and the paraventricular nucleus (this includes both the cell bodies in the hypothalamus, the axons and the nerve terminals in the neurohypophysis), depending upon which stimulus is evoking the release. It is also possible that separate populations of OXT

and ADH neurons mediate hormone release evoked by different stimuli and that prostaglandins affect these populations differently. The actions of prostaglandins may determined by the metabolic state of the cells, too. Within the posterior pituitary, the effects of prostaglandins may depend on many factors, such as the stimulus for the OXT and ADH release, the source of prostaglandins (neural tissue or blood-derived), the duration of prostaglandin action, or perhaps the sex of the experimental animals. The physiological roles and the mechanisms of action of prostaglandins in the posterior pituitary still remain to be elucidated.

1.3.3 PGE_2 and K^+ Channel Modulation

PGE_2 exerts an interesting effect on the neurohypophysis; the cellular basis of its action, however, especially with respect to membrane electrical activity, has not yet been documented. As a local hormone, PGE_2 is a very suitable candidate for regulating membrane ion channels. So far, the effect of PGE_2 on K^+ channels has been sparsely reported. PGE_2 inhibits K^+ channel activity in human T lymphocytes, and this effect is due to an increase in cAMP followed by subsequent phosphorylation of the K^+ channel protein by PKA (Bastin *et al.*, 1990). PGE_2 can also block the Ca^{2+}-activated K^+ channel in rabbit visceral neurons (Fowler *et al.*, 1985) and in rat brain synaptosomes, and the latter of which may involve PKC activation (Ren and Benishin, 1994). A delayed rectifier channel is inhibited by PGE_2 in smooth muscle cells (Ren, 1994), which is mediated by activation of PKA and possibly PKC (Ren *et al.*, 1995*a*). It is reported, however, that PGE_2 promotes the opening of the

ligand- (e.g. purinergic, muscarinic and opioid) gated K^+ channels in cortical synaptosomes (Zoltay and Cooper, 1994), and activates cAMP-mediated K^+ channels (Mori *et al.*, 1989) as well as ATP-sensitive K^+ channels (Guillemare *et al.*, 1994) in *Xenopus* oocytes. Additionally, there are arachidonic acid active K^+ channels in smooth muscle cells (Ordway *et al.*, 1989).

1.3.4 PGE_2 and Signal Transduction

Prostaglandins are considered to be paracrine hormones that effect their biological actions through their binding to specific receptors on the plasma membrane (Haluska *et al.*, 1989; Smith, 1989). The first report on PGE receptors (EP) came out over 20 years ago (Kuehl and Humes, 1972; Oien *et al.*, 1975), and subsequent work has indicated that the EP may be subdivided into EP_1, EP_2, EP_3 and EP_4 subtypes (Coleman *et al.*, 1987, 1994*a*). The diverse and sometimes opposite effects of PGE on cell function have been attributed to their interaction via G proteins with two major signal transduction systems, namely, the cAMP cascade and PKC cascade (Haluska *et al.*, 1989; Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990; Goureau *et al.*, 1992). In addition to interaction with these two signal transduction pathways, PGE_2 has also been reported to activate phospholipase D (PLD) (Wu *et al.*, 1992).

As previously described, K^+ channels are subject to modulation by second messengers. Therefore, elucidation of the effect of PGE_2 on the signal transduction mechanisms may give us a clue as to how PGE_2 regulates K^+ channel and produces its cellular functions.

1.3.4.1 PGE_2 and the cAMP pathway

Many of the biological actions of PGE_2 involve the cAMP-PKA second messenger cascade. The interaction of PGE_2 with EP activates at least two regulatory G proteins, G_s and G_i, and, hence, regulates adenylate cyclase, which, in turn, catalyses the conversion of ATP to cAMP. PGE_2 activity has been well documented to result in the accumulation of cAMP via stimulation of adenylate cyclase in neuroblastoma cells (Gilman and Nirenberg, 1971; Yu *et al.*, 1988), smooth muscle cells (Molnar *et al.*, 1987; Bernal *et al.*, 1991; Dixon *et al.*, 1990; Ren *et al.*, 1995*a*), heart and skeletal muscle (Bocek *et al.*, 1976), bone cells (Hagel-Bradway *et al.*, 1991), ovarian follicles (Weiss *et al.*, 1976), epidermal cells (Iizuka *et al.*, 1976; Adachi *et al.*, 1977), epithelial cells (Taylor *et al.*, 1979), spleen cells (Almawi *et al.*, 1987), pancreatic cells (Metz *et al.*, 1982), parietal cells (Zucker *et al.*, 1987), lymphocytes (Goto *et al.*, 1983; Lanefelt and Martinsson, 1985; Bastin *et al.*, 1990; Ploeg *et al.*, 1991), monocytes (Dore-Duffy and Donovan, 1991), mast cells (Saito *et al.*, 1985), and erythroleukaemia cells (Kunapuli *et al.*, 1994). Whereas PGE_2 has been commonly believed to increase cAMP generation in various types of cells (Samuelsson *et al.*, 1978; Coleman *et al.*, 1994*b*), PGE_2 can also attenuate hormone-stimulated cAMP formation in other cells, namely hepatocytes (Melien *et al.*, 1988), kidney cells (Nakao *et al.*, 1989) and adrenal chromaffin cells (Negishi *et al.*, 1989). More interestingly, a high concentration of PGE_2 stimulates cAMP accumulation, while a low concentration inhibits ADH-induced cAMP accumulation in kidney cells (Sonnenburg and Smith, 1988).

K^+ channels can be regulated by cAMP and cAMP-dependent protein phosphorylation, as described previously. Therefore, it has been suggested that the modulation of K^+ channel conductance by PGE_2 can be attributed to the accumulation of cytosolic cAMP and a subsequent phosphorylation of K^+ channels by PKA (Bastin *et al.*, 1990; Dixon *et al.*, 1990).

1.3.4.2 PGE_2 and the PKC pathway

Similar to the cAMP-PKA system, the PLC-PKC cascade is another major signalling system which is involved in K^+ channel modulation. PGE_2 can stimulate PLC by interaction with its specific receptors (Scherer and Breitwieser, 1990), which are either coupled to PTX-sensitive G proteins (Gilman, 1987) or PTX-insensitive G proteins (Negishi *et al.*, 1989; 1990), to hydrolyse phosphoinositides into DG and IP_3 and lead to the activation of PKC and elevation of $[Ca^{2+}]_i$, respectively (Yamaguchi *et al.*, 1988; Yokohama *et al.*, 1988; Aboolian *et al.*, 1989; Hayaishi, 1989). PGE_2 can also affect PKC activity by acting at the level of PKC translocation (El-Fakahany *et al.*, 1988) or at sites distal to DG and IP_3 generation (Piau *et al.*, 1989). Even though the effect of PGE_2 on the PKC cascade is less well documented, PGE_2 has been reported to activate PKC in platelets (Vezza *et al.*, 1993), lymophcytes (Piau *et al.*, 1989), bone cells (Yamaguchi *et al.*, 1988; Hagel-Bradway *et al.*, 1991; Kozawa *et al.*, 1992), adrenal chromaffin cells (Yokohama *et al.*, 1988; Negishi *et al.*, 1989 and 1990), lung cells (Aksamit *et al.*, 1993), kidney cells (Aboolian *et al.*, 1989; Hebert *et al.*, 1990), smooth muscle cells (Heaslip and Sickels, 1989; Goureau

et al., 1992; Ren *et al.*, 1995*a*), and brain synaptosomes (Ren and Benishin, 1994).

1.4. OBJECTIVES

K^+ channels exert an important influence on the control of excitability, and thus, on hormone secretion, in the neurohypophyseal nerve ending. The special structure of the posterior pituitary makes it an excellent preparation for the study of K^+ channels by using the patch-clamp technique. The present study focuses on the following objectives:

a. Identification and classification of the voltage-dependent outward K^+ channel currents in neurohypophyseal nerve terminals;

b. Characterization of the effects of PGE_2 on K^+ currents in neurohypophyseal nerve terminals;

c. Investigation of the relationship between the effect of PGE_2 on K^+ channels and the role of the cAMP cascade in mediating PGE_2 action at the cellular level.

secretion region	hormone	regulating factor(s)
anterior lobe	Luteinizing hormone (LH) Follicle-stimulating hormone (FSH)	(+) Gonadotropin-releasing hormone (GnRH) (-) Sex steroids
	Prolactin (PRL)	(-) Prolactin-inhibiting hormone (PIH)
	Adrenocorticotropic hormone (ACTH)	(+) Corticotropin-releasing hormone (CRH) (-) Glucocorticoids
	Growth hormone (GH)	(+) Growth hormone-releasing hormone (GHRH)
	Thyroid-stimulating hormone (TSH)	(+) Thyrotropin-releasing hormone (TRH) (-) Thyroid hormones
posterior lobe	Oxytocin (OXT) Vasopressin (ADH)	Neuroendocrine reflex
intermediate lobe	Melanocyte-stimulating hormone (MSH)	(+) CRH

Table 1-1. Hormones secreted by pituitary gland. (+): stimulation; (-) inhibition.

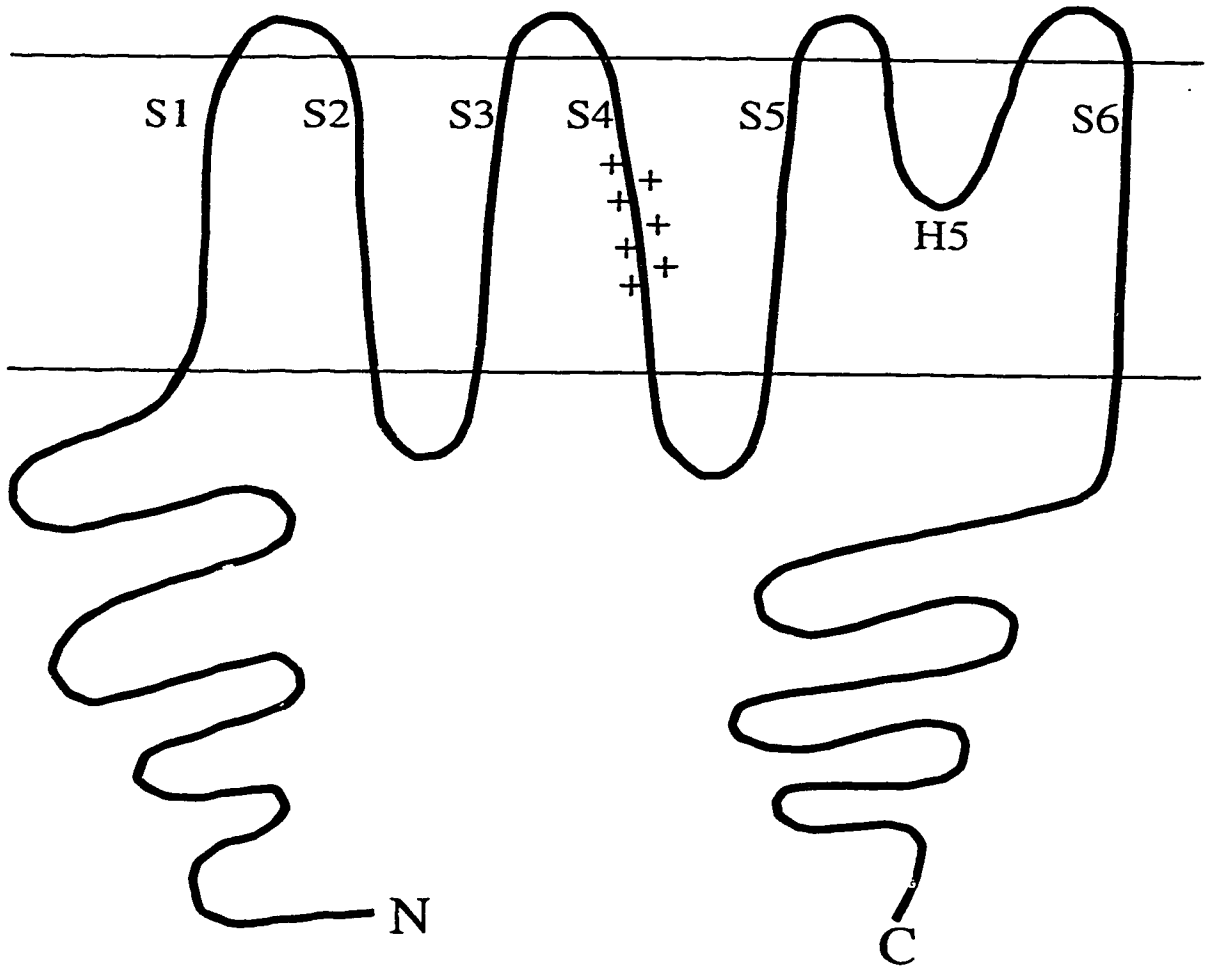

Figure 1-1. Topology of voltage-dependent potassium channels. Both N- and C-terminals are in cytoplasmic side. S1-S6 represent six hydrophobic transmembrane segment, and H5 is the hydrophobic region between S5 and S6, which dips into the membrane as a hairpin loop.

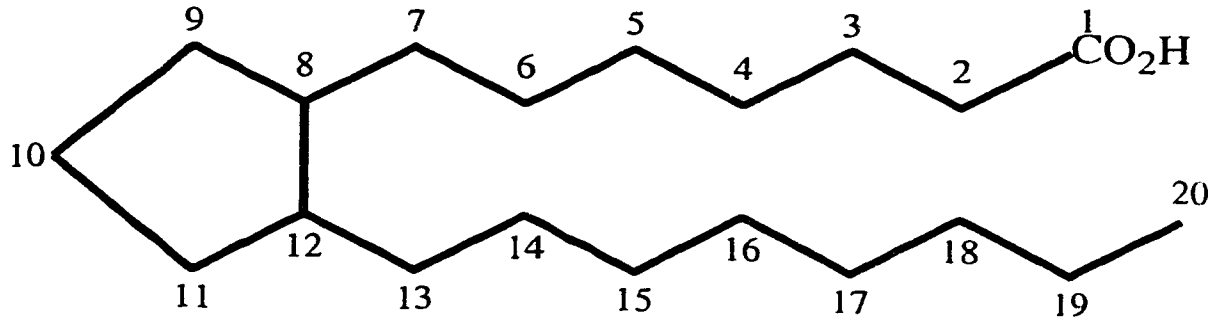

Figure 1-2. Prostanoic acid.

CHAPTER II

MATERIALS AND METHODS

All the methods and materials used in the experiments for the thesis will be described in this chapter. Unless otherwise indicated, this is the protocol used, and it will not be repeated for each chapter.

2.1. PREPARATION OF RAT NEUROHYPOPHYSEAL NERVE TERMINALS

All experiments were performed using isolated peptidergic nerve terminals from Sprague-Dawley (SD) rats according to the method introduced by Nordmann and Lemos (Nordmann *et al.*, 1982, 1987; Wang G. *et al.*, 1992). Briefly, the rats, weighing 350-450 g, were anaesthetized with CO_2 and decapitated. The pituitary was quickly separated from the brain, and was placed in a dish filled with a solution containing 270 mM sucrose, 10 mM N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid (HEPES), and 10 μM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), at a pH of 7.0. The intact posterior lobe was dissected free from the intermediate and anterior lobes of the pituitary. The nerve terminals were dissociated by homogenizing the posterior lobe in the sucrose solution described above. After homogenation, the whole homogenate was transferred to a 0.1% poly-L-lysine coated culture dish. The nerve terminals were allowed to attach to the

bottom of the dish for 10 min. Then they were perfused with reduced-Ca^{2+} Locke solution, containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 15 mM glucose at pH 7.0 and 320 mOsm, for one hour. This preparation has been judged, by immunohistochemistry, to be nearly exclusively nerve terminals (Nordmann *et al.*, 1987; Wang X. *et al.*, 1991*a*, 1993*b*). The isolated neurohypophyseal nerve terminals could be identified under the microscope, since they lacked nuclei, and were of a spherical shape, while other cells exhibited different characteristics, such as the "biconcave" structure associated with a red blood cell. Each brain could generate one culture dish, and there were about 3-5 nerve terminals which qualified for patch-clamp studies. The diameter of the nerve terminals used in these experiments was 6-10 μm. The time during which these nerve terminals could be used to generate voltage-clamp data varied from 15 to 20 min.

2.2. WHOLE-CELL VERSION VOLTAGE-CLAMP

The whole-cell version of the patch-clamp technique (Hamill *et al.*, 1981) was used, and the voltage-clamp method was applied to measure the outward current through voltage-dependent K^+ channels. All the experiments were performed at room temperature (20-22 °C).

2.2.1 Electrodes

The patch pipettes were pulled by using borosilicate capillary tubing with an omega dot fibre (1.2 mm outer diameter and 0.9 mm inner diameter, FHS, Brunswick, ME, USA) on a two-stage electrode puller (Narishige Scientific Instrument, Lab MF-83, Tokyo, Japan). After fire polishing, the microelectrodes used in the experiments had resistances ranging from 6-10 MΩ when filled with the intracellular solution (see below).

2.2.2 Solutions

The contents of both extracellular and intracellular solutions, containing either high or low concentration of Ca^{2+}, are listed in Tab. 2-1. The pH value of all solutions was adjusted to 7.4, using either NaOH (for the extracellular solution) or KOH (for the intracellular solution), and the osmolarity was adjusted to 320 mOsm using sucrose. Intracellular Ca^{2+} was buffered by using BAPTA in the low Ca^{2+} pipette solution or EGTA in the high Ca^{2+} pipette solutions. Both BAPTA and EGTA are Ca^{2+} chelators and either could prevent the rise in intracellular Ca^{2+} concentration which normally follows depolarization due to the entry of extracellular Ca^{2+}. BAPTA, however, is a more effective Ca^{2+} chelator than EGTA.

2.2.3 Experimental Procedures

The isolated rat neurohypophyseal nerve terminals, with their characteristics of a spherical shape and a lack of nuclei, could be identified under an inverted

microscope (Nikon Diaphot, Nikon, Tokyo, Japan). Spherical cells with a clear border and a diameter ranging between 6-10 μm were chosen for study. The cells selected were unattached to other cells, and round or oval in shape, without any processes. The qualified cells were randomly used in each group of experiment, unless indicated otherwise. Dishes with cells attached were mounted onto the stage of an inverted phase-contrast microscope. The liquid junction potential between the bath solution and the pipette solution was compensated by adjusting the pipette current to zero with the tip of the micropipette in the bath solution. Connection and grounding of the pipette and the bath was made via a 1% agar bridge. The pipette tips were manipulated with a hydraulic three dimensional oil driven micromanipulator (Narishige, Tokyo, Japan). Seal resistances (R_S) were always greater than 6 GΩ. The input resistances of the nerve terminals were about 0.5 GΩ. The successful rupture of the membrane was accompanied by an increase in the noise level and a large capacitance-charging current transient in response to a 10 mV command. The currents were recorded using an Axopatch-1C patch-clamp amplifier and filtered with a 4-pole Bessel filter (Axon Instruments, Foster City, CA, USA). The corner frequency was set at 5K Hz.

2.2.4 Leakage Correction and Series Resistance

The leakage subtraction was achieved using the P/N protocol in the pClamp software. The linear leakage conductance was estimated by applying two hyperpolarizing subpulses from the holding potential (-80 mV). These

hyperpolarizing subpulses did not activate any channels, and thus, any currents recorded were solely due to passive leakage conductance. All the current measurements made were corrected by subtracting the linear leakage current on line. In the experimental conditions, the voltage error was 5 mV (series resistance x peak outward current) or less. Hence, the series resistance compensation was not activated.

2.2.5 Membrane Current Measurements

The outward currents were activated by depolarizing the cells from a holding potential of -80 mV to more positive test potentials, at a frequency of 0.1 Hz. The duration of the depolarizing test pulses was 280 msec, unless otherwise indicated. The pulse protocol was generated by using pClamp software and a Zenith personal computer (Zenith 386, Vancouver, Canada). The currents were monitored using a digital oscilloscope (Nicolet 310, Nicolet Instrument Co., Madison, WI, USA), and were recorded on a floppy disc. The membrane current was measured as the peak outward current (leakage corrected) at 5-100 msec and 125-270 msec. For simplification, the current measured at 125-270 msec was considered a non-inactivating current (I_n), while the current differences measured between these two time ranges was considered to be the fast-inactivating component of the current (I_f). Data analysis were carried out by using pClamp software (Axon Instruments, Foster City, CA, USA). The data presented were uncorrected for current rundown. In most cases, the current rundown was less than 1%/min. However, if measured

current rundown exceeded 2%/min, the data was excluded.

2.3. CHEMICALS: THEIR SOURCES AND PREPARATION

Poly-L-lysine hydrobromide, 4-aminopyridine (4-AP), tetraethyl ammonium chloride (TEA), tetrodotoxin (TTX), 4-acetamino-4'-isothiocyano-2,2'-disulfonicacid stilbene (SITS), adenosine 5'-triphosphate (ATP), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), dibutyl adenosine 3',5'-cyclic monophosphate (Db-cAMP), forskolin, nifedipine, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), $CaCl_2$, $CdCl_2$ $LaCl_3$, $MgCl_2$, $SrCl_2$, L-aspartic acid monopotassium salt (K aspartate), sucrose, dimethyl sulfoxide (DMSO), prostaglandin E_2 (PGE_2) and sodium meclofenamate were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPs), and adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPs) were purchased from Biolog (Biolog Life Science Institute, La Jolla, CA, USA). Guanosine-5'-O-(2-thiodiphosphate) (GDPβS) was purchased from Boehringer Mannheim GmbH (Boehringer Mannheim GmbH, Mannheim, Germany). Glucose, KCl and NaCl were purchased from BDH (BDH Inc., Toronto, Canada). N-2-hydroxyethyl piperazine N-2'-ethanesulfonic acid (HEPES) was purchased from Fisher (Fisher Scientific, Fair Lawn, NJ, USA). α- & β-dendrotoxin (DTX) were prepared in the laboratory from *Dendroaspis angusticeps* venom, Latoxan, Rosans, France. Rp- and Sp-cAMPs were

dissolved in distilled water by sonication. Forskolin and BAPTA were dissolved in DMSO. Nifedipine, 4-AP, BAPTA, PGE_2 and sodium meclofenamate were dissolved in 50% ethanol. Other compounds were all dissolved in distilled water. The final concentrations of the drugs were obtained by adding 10 μl of drug stock solution to the 3 ml of extracellular solution. At least 5 minutes was allowed for the stock drug solution to distribute evenly within the dish and produce the response.

2.4. ADMINISTRATION OF DRUGS

Drugs were added directly to the bath. The currents were recorded 5 or 10 min later. In order to study the mechanism of PGE_2 on K^+ channels, several inhibitors were included in the intracellular solution, and introduced (intracellularly) to the cell upon penetration of the cell membrane. The solvents, ethanol and DMSO at the concentration used in the studies, had no effect on the K^+ currents themselves.

2.5. STATISTICS

Data were expressed as mean $\pm$ S.E., except for single experiment current recording and current-voltage (I-V) relationships. The currents recorded prior to addition of the drugs were used as control. The Student's t-test (paired or grouped) was used to estimate the statistically significant differences between the cells without and with drug treatment. Analysis of variance in conjunction with the Neuman-

Keul's test was used for multiple group comparisons. P values less than 0.05 were accepted as being significant.

The equilibrium potential of K^+ (E_K) ion was calculated, using Nernst equation: $E_K = (RT/F)ln([K]_o/[K]_i)$. Here R was the gas constant, T was absolute temperature, F was Faraday constant, $[K]_o$ was extracellular K^+ concentration, and $[K]_i$ was intracellular K^+ concentration. A linear regression curve fitted to the instantaneous tail current-voltage relationship data to compare the measured reversal potential to the calculated value. The steady-state inactivation of the fast-inactivating K^+ current was fitted by the Boltzmann equation: $I/I_{max} = [1 + exp(V-V_{1/2}/k)]^{-1}$, where I was the peak current (minus plateau current) measured, I_{max} was the maximum peak current (minus plateau current), V was the holding potential, $V_{1/2}$ was the membrane potential at which 50% of the channels are inactivated, and k was slope factor. $V_{1/2}$ and k were calculated using this equation.

	Extracellular Solution (mM)		Intracellular Solution (mM)	
low Ca^{2+}	NaCl	145	K Aspartate	115
	KCl	5	KCl	25
	$MgCl_2$	2	$MgCl_2$	2
	$CaCl_2$	2	BAPTA	11
	HEPES	10	HEPES	10
	Glucose	10	ATP	2
	TTX	0.005		
		pH 7.4 mOsm 320		pH 7.4 mOsm 320
high Ca^{2+}	NaCl	145	K Aspartate	115
	KCl	5	KCl	25
	$MgCl_2$	2	$MgCl_2$	2
	$CaCl_2$	10	EGTA	1.1
	HEPES	10	HEPES	10
	Glucose	10	ATP	2
	TTX	0.005		
		pH 7.4 mOsm 320		pH 7.4 mOsm 320

Table 2-1. The components of external and internal solutions used for recording K^+ currents.

CHAPTER III

CHARACTERIZATION OF VOLTAGE-DEPENDENT K^+ CHANNELS IN NEUROHYPOPHYSEAL NERVE TERMINALS

3.1. INTRODUCTION

K^+ channels exert an important influence on determining the degree of nerve terminal cellular excitability, and thus, the synaptic efficiency and hormone release. Three kinds of K^+ channels have been identified in the membrane of the posterior pituitary (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). Each of them play a slightly different role in controlling the membrane potential. The transient K^+ current is involved in during neurohypophyseal repolarization after a spike (Thorn *et al.*, 1991); while the activation of Ca^{2+}-activated K^+ channels (K_{Ca}) produces a long-lasting hyperpolarization (Jackson *et al.*, 1991). Both of these channels contribute to action potential repolarization, while the non-inactivating K^+ channel is involved in the fatigue of stimulus-secretion coupling in the neurohypophysis (Bielefeldt *et al.*, 1992). In order to understand the regulation of secretion from the posterior pituitary, our work is focused on how the K^+ channels cooperate with each other in nerve endings, and what the mechanisms are for regulating different types of K^+ channels.

It is more difficult to isolate and purify K^+ channels than any of the other ion channels because of the lack of specific and high-affinity pharmacological ligands for

the channel proteins. Traditional K^+ channel blockers, such as tetraethyl ammonium chloride (TEA), 4-aminopyridine (4-AP), and quinine, cannot be used for isolation studies, since they are non-specific inhibitors of K^+ channel function. Several toxins have been used as specific antagonists for certain types of K^+ channels, such as apamin for the small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) (Blatz and Magleby, 1987; Cook and Quast, 1990), charybdotoxin for the big-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Valdivia *et al.*, 1988), α-dendrotoxin (α-DTX) for the transient outward K^+ channels (I_A), and β-dendrotoxin (β-DTX) for the delayed rectifier K^+ channels (I_K) (Benishin *et al.*, 1988). However, the three types of K^+ channels identified in neurohypophyseal nerve terminals seem to be more specialized. Neither apamin nor charybdotoxin have any effect on K_{Ca}, while dendrotoxin can only block the non-inactivating component (Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). The effects of TEA may be variable. TEA can inhibit all three types of K^+ channels in the thin slice preparation of the rat neurohypophysis (Bielefeldt *et al.*, 1992), while failing to block transient K^+ channels in dissociated nerve terminals from the rat posterior pituitary (Wang G. *et al.*, 1992). Since different strain of male rats are used to isolate neurohypophyseal nerve terminals, the characterization of voltage-dependent K^+ channels is presented and compared with those which were reported in this chapter.

3.2. EXPERIMENTAL DESIGN

The experimental procedures and protocol used are described in chapter 2. The high density and large size of the nerve endings in the posterior pituitary make this an excellent preparation for the study of neuronal secretion mechanisms. The voltage-dependent outward K^+ current present in rat posterior pituitary nerve terminals was recorded, with low and high intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (which could be achieved by using internal high (11 mM) and low (1.1 mM) concentrations of Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) or ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and external low (2 mM) and high (10 mM) concentrations of Ca^{2+}, respectively), in order to distinguish Ca^{2+} dependency. Pharmacological profiles of the outward K^+ channel currents were investigated to establish the identity of these outward K^+ channel currents and to rule out possible contributions from other sources of outward currents. K^+ channel blockers, such as TEA, 4-AP and dendrotoxins were used to identify the K^+ currents. Ca^{2+} channel antagonists, such as nifedipine, La^{3+} and Cd^{2+}, and the Ca^{2+} chelator EGTA, were employed to determine the contribution of the Ca^{2+} dependent component. The chloride channel blocker 4-acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) (Lewis *et al.*, 1993; McEwan *et al.*, 1993) was added to determine whether Cl^- currents contributed to the overall currents which were recorded. The instantaneous tail currents were analyzed to determine the reversal potential (equilibrium potential) and the major permeable ion through the channel. A K^+ selectivity study was also carried out to compare the

relationship between the equilibrium potential and various extracellular K^+ concentrations. Steady-state inactivation was assessed to help distinguish the fast inactivating component (I_f) from the non-inactivating component (I_n).

3.3. RESULTS

3.3.1 The Voltage-Dependent Outward K^+ Current in Neurohypophyseal Nerve Terminals with Low Free $[Ca^{2+}]_i$

3.3.1.1 The outward current recording

In order to rule out the contribution of the Ca^{2+}-activated component to the outward current, 11 mM intracellular BAPTA, a Ca^{2+} chelator, was used to buffer Ca^{2+} intracellularly, while the extracellular Ca^{2+} concentration was set at 2 mM to minimize the activation of the Ca^{2+}-activated K^+ channels. The currents were activated by stepwise depolarization of the cells from a holding potential of -80 mV to more positive potentials. Fig. 3-1 shows two examples of the K^+ currents recorded from isolated nerve terminals of the rat neurohypophysis and the voltage-clamp pulse protocol. Fig. 3-1*A* shows a typical recording exhibiting the fast-inactivating (I_f) and non-inactivating components (I_n). Fig. 3-1*B* shows a typical recording exhibiting an I_n and minimal, or no, I_f. About 1/3 (79 in 246) of the nerve terminals exhibit K^+ currents as shown in Fig. 3-1*A*, and 2/3 (167 in 246) as shown in Fig. 3-1*B*. The I_n was activated at -40 to -30 mV. The current amplitude measured at a test potential of +20 mV at different times after the whole-cell

configuration was established is shown in Fig. 3-2. Using the corresponding current amplitude recorded 3-5 min after penetrating the membrane as control (expressed as 100%, at time 0), the outward K^+ current recorded 5, 10, 15, 20 and 30 min afterwards declined steadily at the rate of 1%/min. The cells, which rundown at the rate over 2%/min, were not be used in further experiments.

3.3.1.2 Identification of the outward current

3.3.1.2.1 Sensitivity to chloride channel blocker

The uneven distribution of Cl^- across the membrane raises the possibility that part of the outward current may be due to the inward movement of Cl^- via Cl^- channels. However, use of the extracellular chloride channel antagonists SITS (1 and 2 mM) had no effect on I_n, as shown in Fig. 3-3, and this result suggested that the current recorded was due to K^+ ions moving outward through K^+ channels.

3.3.1.2.2 Reversal potential and selectivity of the outward current

In order to determine the reversal potential of the outward currents, tail currents were measured by using a two-voltage step protocol. The terminals were depolarized for 140 ms to +20 mV from holding potential of -80 mV to activate the outward current, and, then, repolarized to various membrane potentials, as shown in Fig. 3-4. The amplitude of the tail currents were measured at the peak after the onset of the second step. The reversal potential was obtained by linear regression analysis. The value of the measured reversal potential was -83.65 $\pm$ 1.35 mV

(n = 8), which was similar to the calculated K^+ equilibrium potential of -83.90 mV, according to the Nernst equation. To test the selectivity of I_n for K^+ ions, the subsequent experiments used elevated extracellular K^+ concentrations (as K aspartate) ranging from 5 mM to 15 mM, while gradients for other ions, including Cl^- ion, remained unchanged. The reversal potential shifted towards more positive voltage values depending on the K^+ gradient. The measured reversal potentials with external K^+ concentrations of 10 mM and 15 mM were -66.00 $\pm$ 0.98 mV (n = 5) and -49.64 $\pm$ 0.73 mV (n = 4), respectively, while the calculated K^+ equilibrium potentials were -66.47 mV and -56.25 mV, respectively. These results indicated the outward current (I_n) is highly selective for K^+ ions.

3.3.1.2.3 Sensitivity to Ca^{2+}

To determine the contribution of Ca^{2+}-activated K^+ channels toward generation of the outward currents under conditions of low $[Ca^{2+}]_i$, Ca^{2+} channel antagonists such as nifedipine, lanthanum and cadmium were used to block Ca^{2+} channels, or EGTA was added to the bath to chelate the extracellular calcium. Fig. 3-5 shows that nifedipine (10 μM), La^{3+} (1 mM), Cd^{2+} (300 μM) and EGTA (2 mM) did not have any significant effect on the I_n. This indicates that Ca^{2+}-activated components did not significantly contribute to the generation of the outward K^+ currents recorded under these experimental conditions.

3.3.1.3 Pharmacological profiles of the outward K^+ current

3.3.1.3.1 Sensitivity to the K^+ channel blocker TEA

TEA, a quaternary ammonium ion, blocks most classes of K^+ channels, albeit with varying affinities (Cook and Quast, 1990). At a membrane potential of +20 mV, I_n was measured 5 min after the external application of TEA of 15 mM, 30 mM and 60 mM (Fig. 3-6). This result suggests that the non-inactivating K^+ channel in posterior pituitary nerve endings is inhibited by TEA, and this inhibition is concentration dependent.

3.3.1.3.2 Sensitivity to K^+ channel blocker 4-AP

4-AP, the K^+ blocker traditionally used for block of the transient outward "I_A" current, was used to characterize the outward K^+ currents (Fig. 3-7). Cells, exhibiting both fast- and non-inactivating components, were selected for this group of experiments. At a membrane potential of +10 mV, both I_f and I_n were measured 5 min after extracellular administration of 3 mM and 6 mM 4-AP. The results suggest that both I_f and I_n are sensitive to 4-AP, but 4-AP may have a greater sensitivity for the transient K^+ channel.

3.3.1.3.3 Sensitivity to K^+ channel blocker α-dendrotoxin

α-DTX, a toxin from the venom of the green mamba (*Dendroaspis angusticeps*), has been reported to preferentially block the inactivating K^+ channel in brain synaptosomes (Benishin *et al.*, 1988), and hippocampus neurons (Dolly *et*

al., 1984; Halliwell *et al.*, 1986). It has been shown to also inhibit I_K in axons (Schauf, 1987), motor nerve terminals (Dreyer, 1990; Dreyer and Penner, 1987), and peripheral neurones (Penner *et al.*, 1986; Stansfeld *et al.*, 1987). Cells exhibiting both fast- and non-inactivating components, were chosen in this group of experiments. There was no noticeable change in either the fast- or the non-inactivating current amplitudes after the nerve terminals were exposed to α-DTX (100 nM) for 5 min (Fig. 3-8), suggesting that both K^+ channels are not α-DTX sensitive.

3.3.1.3.4 Sensitivity to K^+ channel blocker β-dendrotoxin

β-DTX, another toxin (same size as α-DTX) from the green mamba venom, has been documented to preferentially block the non-inactivating K^+ channel in brain synaptosomes (Benishin *et al.*, 1988), and smooth muscle cells (Ren *et al.*, 1994). Concentrations of 250 nM of β-DTX did not reduce the outward K^+ current significantly, indicating that in the neurohypophyseal nerve ending K^+ channels are β-DTX resistant (Fig. 3-9).

3.3.1.4 Inactivation of the outward K^+ current

In order to analyze the steady-state inactivation of I_f, the membrane potentials were adjusted to different levels (-100 mV to +10 mV) for 1.5 sec before the application of a test pulse to +10 mV for 1.5 sec. Five cells were used for this experiment. At most depolarized holding potentials the transient outward current

decreased in amplitude (Fig. 3-10). The current at the end of the voltage step reaches a plateau, which is used as an index of the steady-state current. The amplitude of the outward currents (the difference between the peak current and plateau current) were normalized to the value which was activated from the most negative membrane potential (-100 mV). The steady-state plot showed a dependence on the holding potential with 50% inactivation ($V_{1/2}$) occurring at -49.6 mV (k = 10.9).

3.3.2 The Voltage-Dependent Outward K^+ Currents in Neurohypophyseal Nerve Terminals with High Free $[Ca^{2+}]_i$

When the pipettes were filled with low (1.1 mM) EGTA solution, and with 10 mM Ca^{2+} in the bath solution (the concentrations of the other solution components remained unchanged), stepwise depolarization of cells from a holding potential of -80 mV elicited an outward K^+ current. The currents were activated at a membrane potential of around -40 to -30 mV (Fig. 3-11). About 4/5 (35 in 44) of the nerve terminals exhibited non-inactivating components and minimal, or no, fast-inactivating components, while 1/5 (9 in 44) exhibited both. The non-inactivating current amplitude measured at a membrane potential of +20 mV at 5, 10, 15, 20, and 30 minutes after the whole-cell configuration was established were shown in Fig. 3-12. There was basically no noticeable decline in the current amplitude for at least 30 min following penetration of the membrane. By applying the Ca^{2+} channel blockers nifedipine (10 μM) or Cd^{2+} (300 μM) to the bath solution, this outward current was

decreased to 81.19 ± 3.30% (n = 5) and 78.66 ± 5.07% (n = 6), respectively (Fig. 3-13), revealing the Ca^{2+}-activated component that contributed to the development of a small, but significant, portion of the outward current, i.e. the Ca^{2+}-activated K^+ channel current played a role. The activation of this Ca^{2+}-activated K^+ current was apparently secondary to the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channels.

3.4. DISCUSSION

3.4.1 Characteristics of the Outward Current in Rat Neurohypophyseal Nerve Terminals

This group of studies described the characteristics of voltage-dependent outward K^+ currents in the membranes of rat posterior pituitary nerve endings. When cells were dialysed with an intracellular solution containing 11 mM BAPTA and an extracellular solution containing 2 mM Ca^{2+}, the macroscopic K^+ currents were found to be a combination of fast- and non-inactivating components. The occurrence of the two components in different cells is not uniform. Upon depolarization of the cells to 0 to 20 mV from a holding potential of -30 mV, both I_f and I_n can be observed in 1/3 of the cells. Even though both transient and non-inactivating K^+ channels have been documented, there is not any indication of the distribution of these two K^+ channels in rat neurohypophyseal nerve terminals. In the study of Bielefeldt *et al.* (1992), the K^+ channels were investigated in the

membranes of nerve terminals in thin slices prepared from the rat posterior pituitary. This raised the possibility that the recording from an intact or dissociated preparation is different. In a similar study (Thorn *et al.*, 1991), nerve terminals of the posterior pituitary were acutely dissociated from CD rats, which is different from the SD strain used in this study. They observed the transient K^+ current in every cell. Also, the use of 0.25 mM cyclic adenosine 3',5'-monophosphate (cAMP) in their intracellular solutions suggest that cAMP may play a role in the activation of the fast-inactivating K^+ channel in neurohypophyseal nerve terminals (see succeeding chapters).

The Cl^- channel, which also can be activated by membrane depolarization, is classified as an outward rectifier which is kinetically similar to the delayed rectifier K^+ channels (Solc and Wine, 1991; Widdicombe and Wine, 1991). It is worthwhile to consider the contribution of Cl^- conductance to the outward current recorded, because of the Cl^- concentration gradient across the membrane. However, the finding that the amplitude of the outward current generated from the nerve endings of the posterior pituitary were not affected by the Cl^- channel blocker SITS, excluded any possible contribution of the Cl^- channel to the outward currents. Measurement of the reversal potential with a constant Cl^- gradient, the correspondence of the reverse potential with the K^+ equilibrium potential, and the shift of the reversal potential in a positive direction following increases in the external K^+ concentration to reduce the chemical driving force for K^+, further proved that K^+, not Cl^-, was the primary conducting ion that passed through this channel with a characteristic voltage-dependence. The deviation of measured reversal potential at 15 mM $[K^+]_o$, which

was -49.64 mV, from calculated value of -56.25 mV may be due to the osmolarity changes caused by increasing $[K^+]_o$.

The dissociated terminal inactivating current half-inactivation was seen at -49.6 mV. This $V_{1/2}$ was very similar to that reported by others in a similar preparation (Thorn *et al.*, 1991), while more depolarized than that reported in the thin slice preparation (Bielefeldt *et al.*, 1992). One likely explanation is that there are differences between K^+ channels when studied in different preparations, such as isolated nerve terminals or slices.

As mentioned in the introduction, three potassium channels have been classified in rat posterior pituitary nerve terminals (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992): one is the transient, one is the non-inactivating, and the third is the Ca^{2+}-activated K^+ channel. However, the neurohypophyseal terminal Ca^{2+}-activated K^+ channel may be a novel type of K_{Ca} since charybdotoxin and apamin fail to affect it (Bielefeldt *et al.*, 1992; Wang G. *et al*, 1992). Two types ("N_t" and "L") of high-threshold, voltage-activated Ca^{2+} channels have been identified in nerve terminals of the rat neurohypophysis (Wang X. *et al.*, 1992, 1993*a*). The non-responsiveness of the K^+ current to the Ca^{2+} channel antagonists nifedipine, La^{3+} and Cd^{2+}, and the Ca^{2+} chelator EGTA, further supported the finding that there was no contribution to the macroscopic outward K^+ currents by the Ca^{2+}-activated K^+ channels in strongly Ca^{2+} buffered (with 11 mM BAPTA in the pipette and 2 mM Ca^{2+} in the bath) conditions. However, in the present experiments when cells were buffered with a lower pipette concentration of EGTA (1.1 mM),

combined with a higher extracellular Ca^{2+} concentration of 10 mM, in order to achieve a high intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), the Ca^{2+} channel blockers nifedipine and Cd^{2+} reduced the macroscopic outward current amplitude by 20%, indicating a possible contribution of the Ca^{2+}-activated component(s), i.e. the Ca^{2+}-activated K^+ channel(s). The activation of this Ca^{2+}-activated K^+ channel(s), thus, appears to be secondary to the entry of extracellular Ca^{2+}. According to the Ca^{2+} hypothesis of exocytosis, the entry of Ca^{2+} into nerve terminals of the posterior pituitary activates a Ca^{2+}-activated cation channel (this is most likely to be the Ca^{2+}-activated K^+ channel) in the membranes of neurosecretory vesicles which contain the hormone, and this promotes the entry of cations and anions into the vesicle to induce swelling of vesicle, and to cause exocytosis (Lemc *et al.*, 1989; Lee *et al.*, 1992; Nordmann and Artault, 1992). Therefore, the Ca^{2+}-activated component is very important in the membrane of the vesicles, but it may not be as crucial to the establishment of cellular excitation in neurohypophyseal nerve endings.

Interestingly, in low conditions of $[Ca^{2+}]_i$, the K^+ currents rundown at the rate of 1%/min, while in conditions of high $[Ca^{2+}]_i$, there was no obvious rundown. One possible reason is that the cells are more stable with physiological $[Ca^{2+}]_o$. Nevertheless, this must be considered when testing the efficiency of various drugs.

3.4.2 Pharmacology of the Outward K^+ Current in Low $[Ca^{2+}]_i$

TEA was the first, and is still the most frequently used K^+ channel blocker. It can elicit a blockade of most K^+ channels at millimolar concentrations and has been

widely used to discriminate different types of K^+ channels based on their TEA-sensitivity (Rudy, 1988). However, no general rule can be followed concerning the potency and the side of application of TEA (i.e. extracellular versus intracellular) for the block of different K^+ channels in different cells. TEA blocks voltage-gated K^+ channels by acting at two distinct TEA receptors, an external and an internal TEA receptor, located at opposite ends of the aqueous pore. With respect to the external TEA receptor, blockade is relatively independent of membrane potential, gating, or external K^+, and the kinetics of the K^+ channels remain relatively unchanged (Koppenhofer and Vogel, 1969; Armstrong and Hille, 1972). The internal TEA receptor lies in the pore and is accessible to cytoplasmic drugs only when the channel is opened by a depolarizing pulse (Armstrong, 1974; Stanfield, 1983). The presence of a TEA ion at one side reduces the affinity of the other by 4-5 fold (Newland *et al.*, 1992). TEA (5 mM) has been reported to block K^+ channels in thin slice preparations of the posterior pituitary (Bielefeldt *et al.*, 1992), but has no effect on K^+ channels in isolated nerve terminals of the neurohypophysis (Thorn *et al.*, 1991). In the present studies, we found that TEA applied extracellularly blocked the outward K^+ current in a dose-dependent manner, which is consistent with the sensitivity of hormone release to TEA (Bondy *et al.*, 1987; Hobbach *et al.*, 1988).

4-AP constitutes the next most commonly used K^+ blocker. It is more selective and usually more potent in its K^+ channel-blocking activity than TEA. Since it is membrane permeable, 4-AP can be applied to either side of membrane, although its binding site is probably located on the inner side (Yeh *et al.*, 1976).

However, 4-AP is generally a more potent inhibitor of I_A than of the other K^+ currents (Thompson and Aldrich, 1980; Rudy, 1988), and it has consequently become a useful tool in dissecting out the different K^+ currents contributing to the outward K^+ current. 4-AP has been reported to block the transient outward current with an IC_{50} of 3 mM in neurohypophyseal nerve terminals, and the total current including the non-inactivating component, was blocked by 5 mM of 4-AP (Thorn *et al.*, 1991). Therefore, we chose the nerve endings exhibiting both fast- and non-inactivating currents to expose to 4-AP for 5 minutes. Low concentrations (3 mM) of 4-AP inhibited both components by 40-50%, and high concentrations (6 mM) of 4-AP blocked the fast-inactivating component by 95% while blocking the non-inactivating component by 55%. This result is consistent with those reported previously, i.e. the fast-inactivating outward K^+ current was very sensitive, while the non-inactivating K^+ channel was partially sensitive to 4-AP in nerve terminals of rat neurohypophysis. 10 minutes after application of 4-AP (3 mM), the fast-inactivating K^+ current amplitude can be reduced [illegible] (data not shown).

Dendrotoxins from the green mamba snake (*Dendroaspis angusticeps*) venom are a structurally homologous family of 57 to 61 amino acid, single-chained polypeptides, having their N-terminus blocked by pyroglutamic acid (Dolly *et al.*, 1984; Harvey and Anderson, 1985). Some species of dendrotoxins have been found to facilitate transmitter release due to the blocking of certain components of the nerve terminal voltage-dependent K^+ channels (Harvey and Karlsson, 1982; Dolly *et al.*, 1987; Anderson and Harvey, 1988). Fractionation of the venom by size exclusion

chromatography and cation exchange high performance liquid chromatography (HPLC) yielded four 7000-dalton polypeptides (namely α-, β-, γ-, and δ-dendrotoxins), which blocked different types of voltage-dependent K^+ channels in synaptosomes. Sequence analysis revealed a homologous C-terminal segment for all four toxins. Regarding to the N-terminal portion, α- and δ-DTX were similar to each other, while β- and γ-DTX were similar to each other but not to α- and δ-DTX (Benishin *et al.*, 1988). α- and δ-DTX (100 nM) preferentially blocked the fast-inactivating K^+ channels in rat brain synaptosomes (Benishin *et al.*, 1988), and hippocampus neurons (Dolly *et al.*, 1984, Halliwell *et al.*, 1986), while β- and γ-DTX (100 nM) preferentially blocked the non-inactivating K^+ channels in rat brain synaptosomes (Benishin *et al.*, 1988), and smooth muscle cells (Ren *et al.*, 1994), indicating that the N-terminal rather than the C-terminal regions are possibly responsible for affecting the K^+ channel activity. The finding of structurally similar dendrotoxins with preferential activities toward different subtypes of K^+ channels raises the possibility of using these toxins as valuable K^+ channel antagonists, despite a differing structure from that of other known K^+ channel antagonists, to distinguish different populations of voltage-dependent K^+ channels. However, we found that both fast- and non-inactivating K^+ channels were resistant to both α- and β-DTX in isolated nerve endings of the rat posterior pituitary, while Bielefeldt *et al.* (1992) reported that non-inactivating component was dendrotoxin sensitive in the thin slice preparation. Since it is α-DTX which is referred to as merely dendrotoxin in the literature (Benishin *et al.*, 1988), the effect of α-DTX on neurohypophyseal nerve

terminals in the thin slice preparation is conflicting with the α-DTX effect on brain synaptosomes. The discrepancy may due to the difference between the strain of the rats, the cell types, the region studied, and the preparation procedure.

3.4.3 Conclusions

From a holding potential of -80 mV (which is similar to the normal terminal resting potential), depolarization of the membrane elicited a K^+ current. Based on the activation and inactivation kinetics, Ca^{2+} sensitivity and antagonist sensitivity, at least three distinct K^+ channels have been classified in the membrane of rat neurohypophyseal nerve terminals in this investigation. The fast-inactivating K^+ current quickly reached a peak and then decayed within 50 ms. This transient outward current showed steady-state inactivation at more depolarized (than -80 mV) holding potentials with 50% inactivation occurring at -49.6 mV; it is 4-AP sensitive and α-DTX resistant. The non-inactivating current reached its peak more slowly and did not inactivate within 280 ms; it can be blocked by TEA and 4-AP, but not by α- and β-DTX. It worthwhile to point out that this channel exhibited rundown at the rate of 1%/min. The Ca^{2+}-activated K^+ channel was also recorded. The rundown is not significant under conditions of high $[Ca^{2+}]_i$, suggesting that Ca^{2+} increases the stability for K^+ channel gating. These studies provide useful information in determining the role of voltage-dependent K^+ channels in controlling neurotransmitter release from the neurohypophysis in response to various agents.

The macroscopic currents reported here are similar in some respects to those

described by Thorn *et al.*, (1991), Bielefeldt *et al.*, (1992), Wang G. *et al.* (1992). However, the fast-inactivating current recorded was much smaller than we expected. Another difference was evident in the sensitivity of the currents recorded to TEA and the dendrotoxins. Several factors may account for these discrepancies: 1. the strain of animal used -- in the Thorn *et al.* (1991) and Wang G. *et al.* (1992) studies, CD (Charles River) rats were used, while in the present study, SD rats were used. 2. the isolation procedure -- Bielefeldt *et al.* (1992) used the thin slice preparations of the rat posterior pituitary, whereas an acute dissociation procedure was employed in the present study. The contradictory results raise the possibility that various treatments may affect K^+ channels differently, or that there are differences between the vasopressin and oxytocin terminals, which could be sampled from unequally, depending on whether one is recording from an intact, or dissociated preparation. Therefore, caution need to be taken in extrapolating the electrophysiological evidence from single cells to the intact tissue. Further studies employing single-channel recording would help to identify the different subtypes of K^+ channels involved.

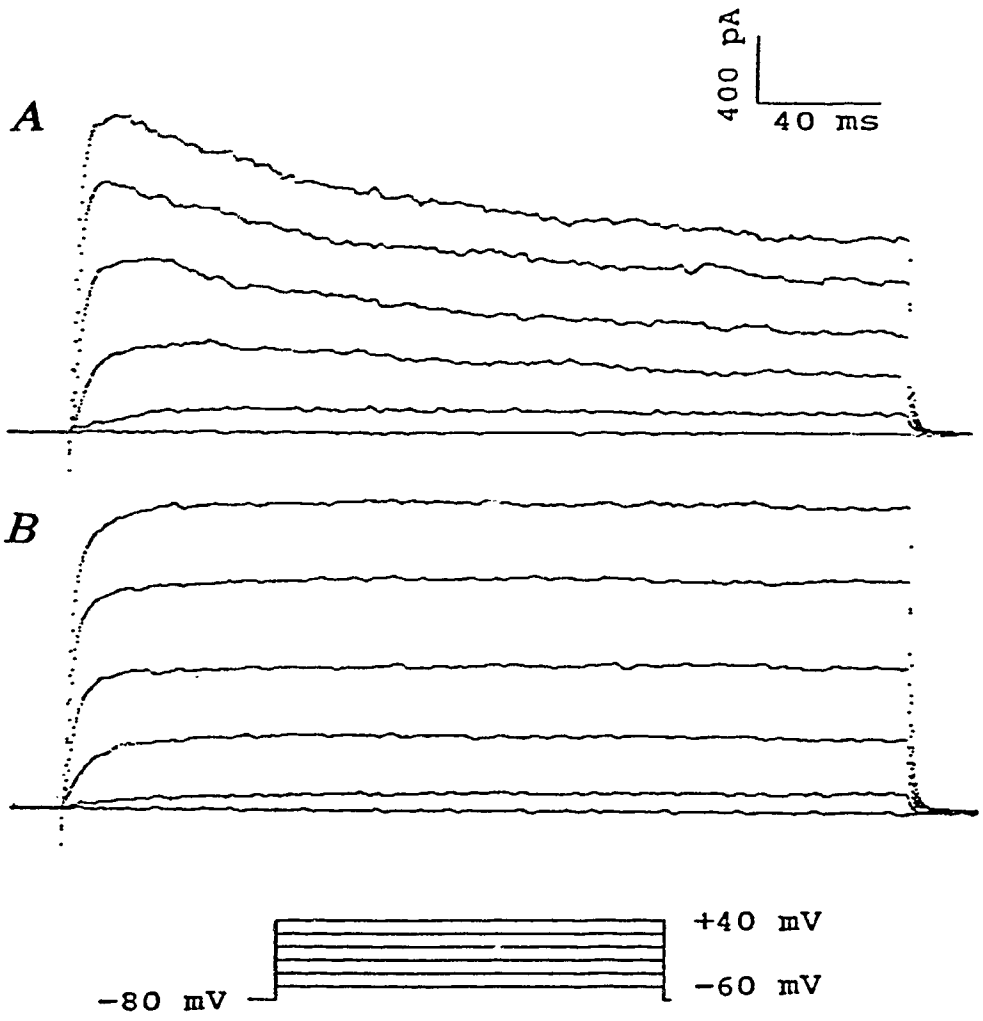

Figure 3-1. Two examples of the outward K^+ currents recorded from neurohypophyseal nerve terminals and the corresponding voltage-clamp pulse protocol. The pipette solution contained 11 mM BAPTA and $[Ca^{2+}]_o$ was 2 mM (to obtain a low $[Ca^{2+}]_i$). The current was activated by depolarizing the cell from a holding potential of -80 mV to various test potentials. *A* shows a typical recording exhibiting the fast-inactivating component and non-inactivating component; *B* shows a typical recording exhibiting a non-inactivating component and minimal or no fast-inactivating component. About 1/3 of the nerve terminals exhibit K^+ currents as shown in *A*, and 2/3 as shown in *B* under control conditions.

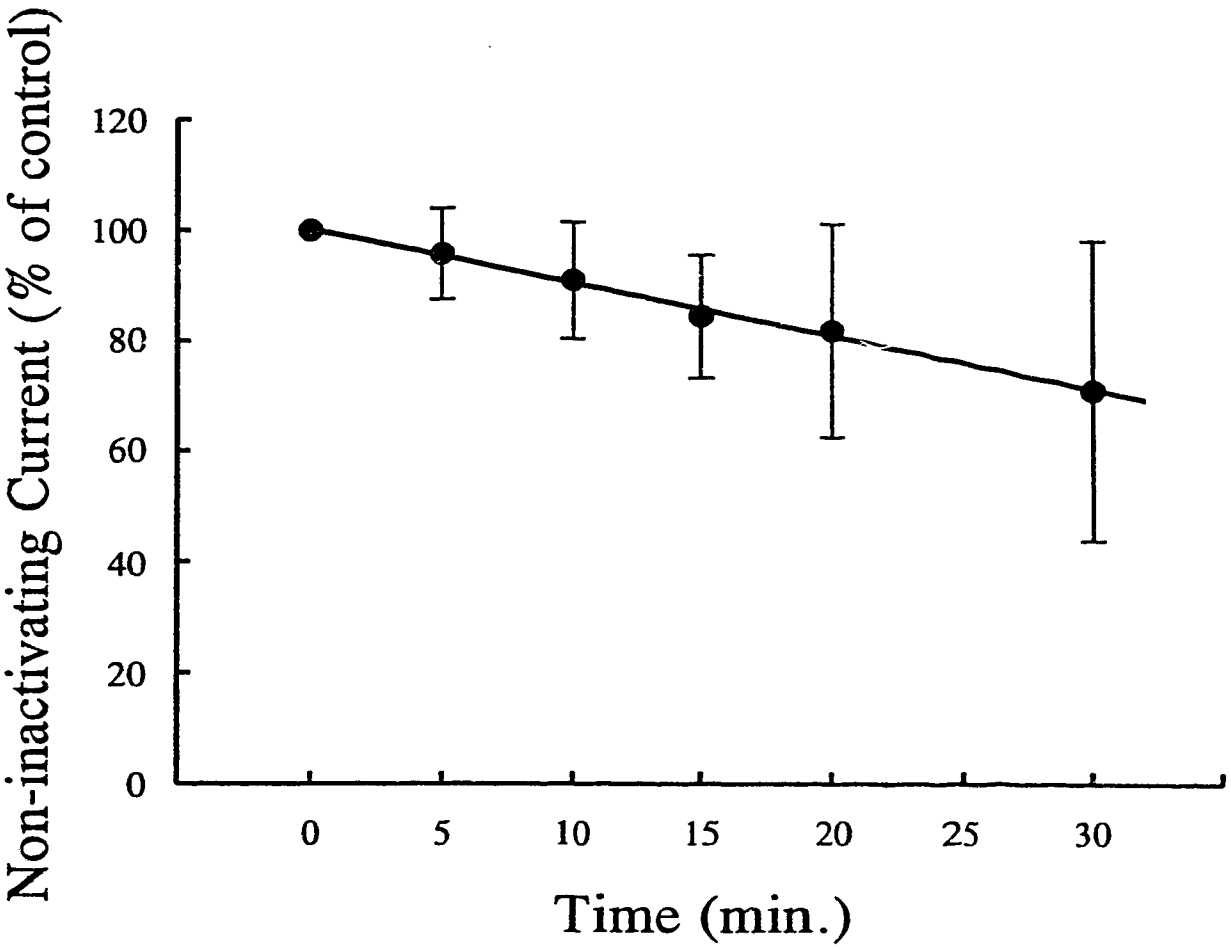

Figure 3-2. The time course of the K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. The non-inactivating current amplitude was measured at a test potential of +20 mV at 5 (n = 13), 10 (n = 12), 15 (n = 9), 20 (n = 5), and 30 (n = 4) min after the whole-cell configuration was established, and was normalized to the corresponding control (current recorded 3-5 min after penetrating the membrane).

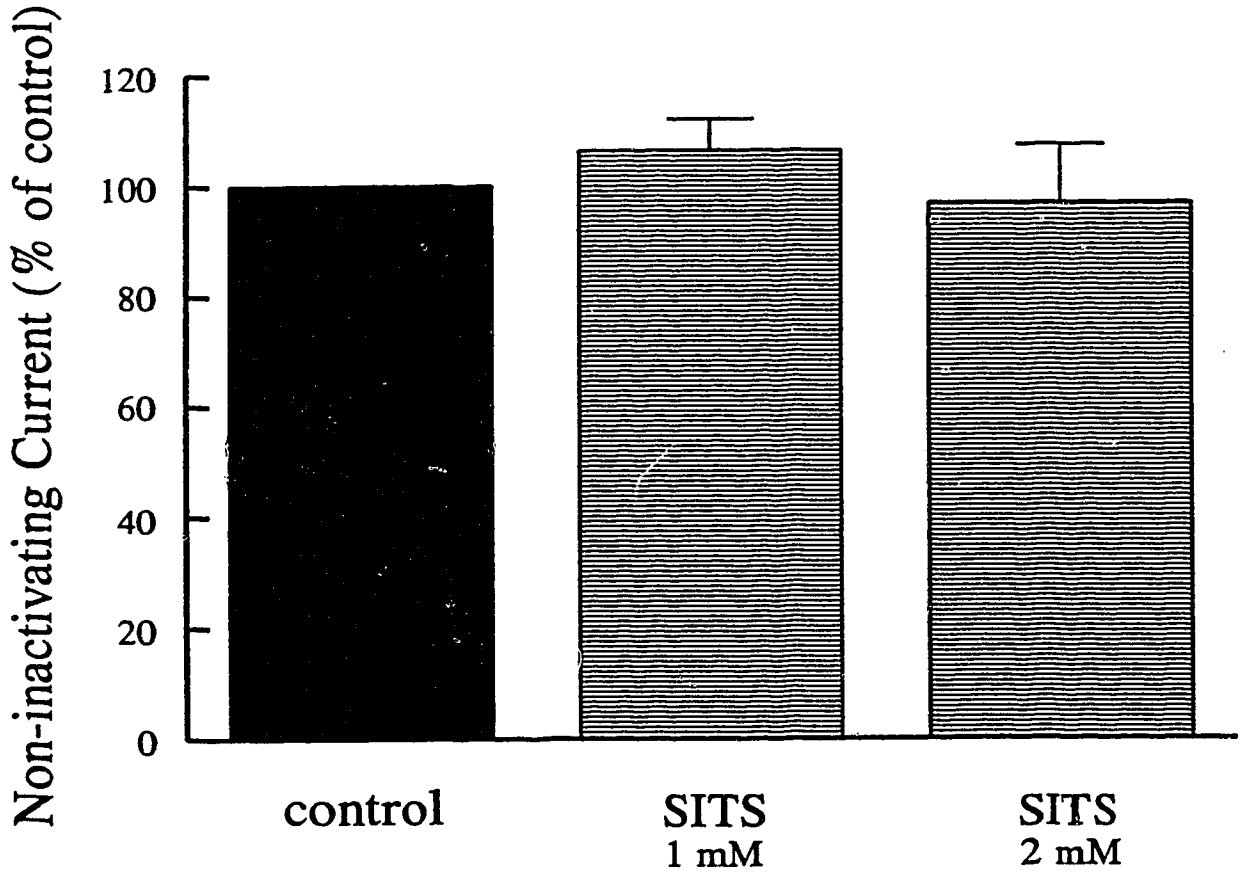

Figure 3-3. Effects of the Cl^- channel blocker SITS on the non-inactivating K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. Cells were treated with 1 mM SITS (n = 3) or 2 mM SITS (n = 3) for 5 min. Current amplitudes were measured at a test potential of +20 mV and were normalized to the corresponding control (current before the addition of the antagonist). $p > 0.05$ compared with the control.

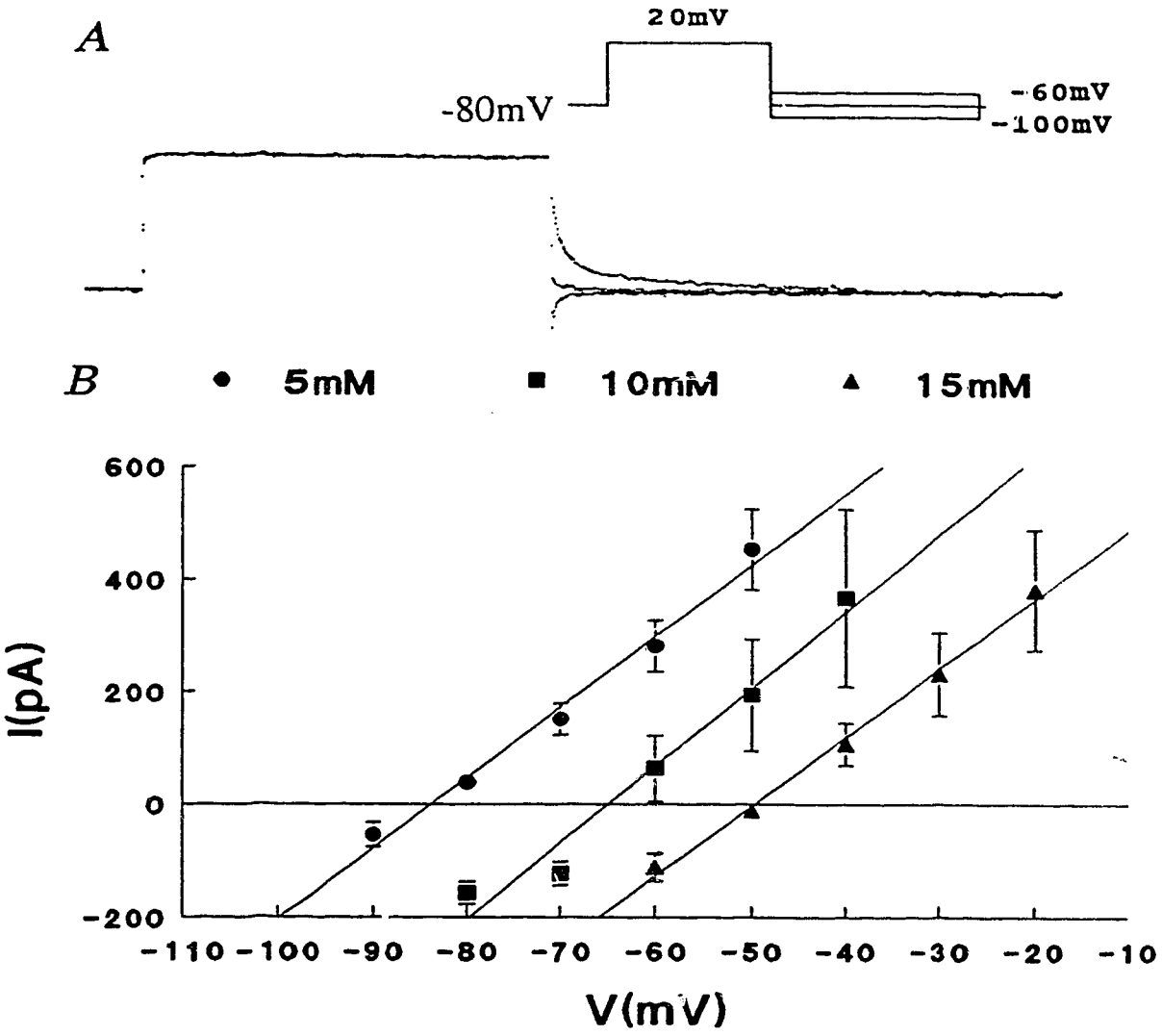

Figure 3-4. Effect of extracellular K^+ on the reversal potential of the K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. *A*, the currents were elicited by voltage steps from a holding potential of -80 mV to a potential of +20 mV and then stepping back to a range of potentials between -100 and -20 mV; *B*, the relationship between tail current amplitude and step membrane potential measured was approximately linear and was fitted using linear regression to determine a reversal potential. The circles represent the results of an experiment using an inside $[K^+]$ of 140 mM, and an outside $[K^+]$ of 5 mM (n = 8). The results of subsequent experiments with varying outside K^+ concentration of 10 mM (n = 5) and 15 mM (n = 4) are shown by the squares and the triangles, respectively.

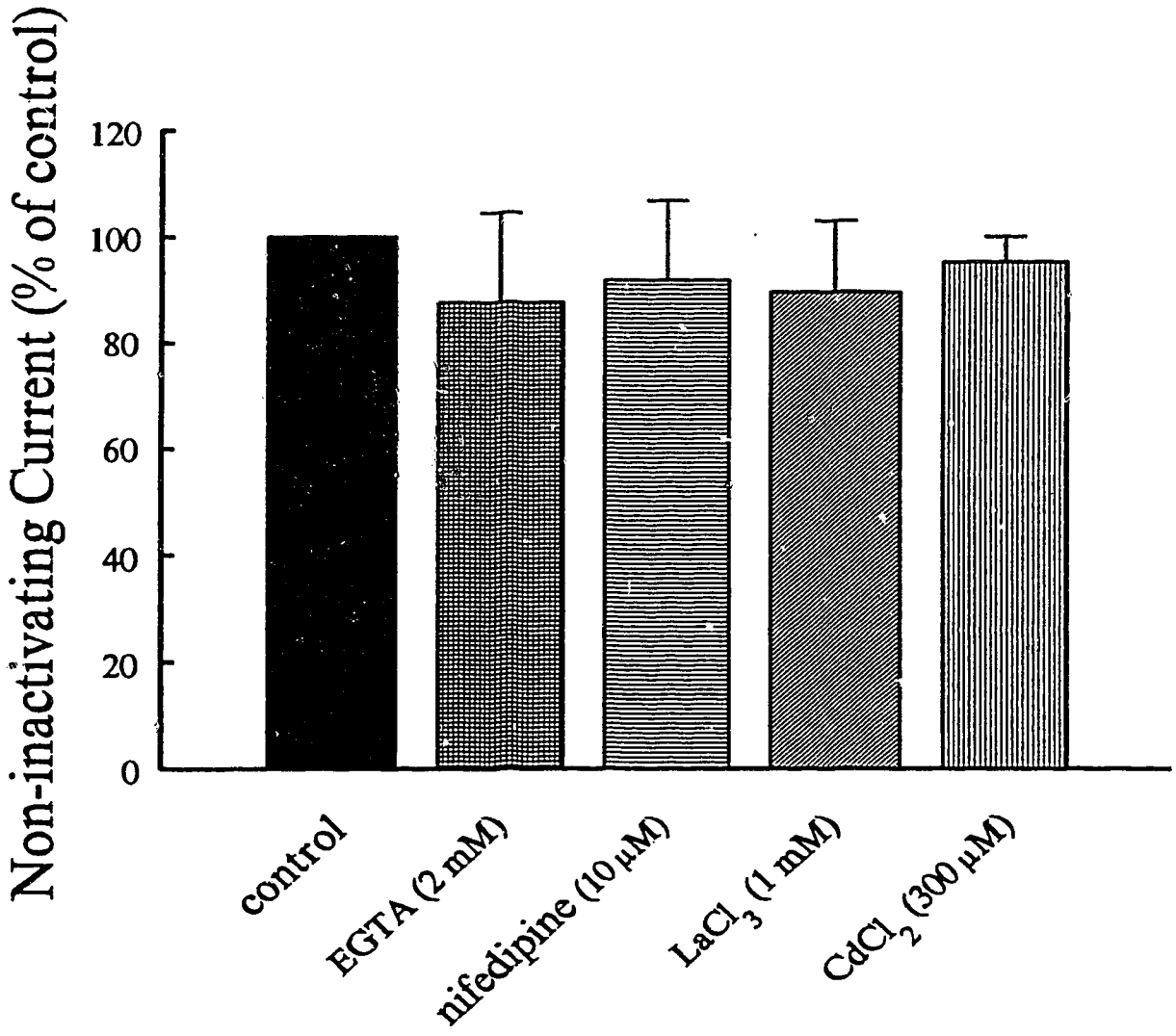

Figure 3-5. Effects of EGTA, nifedipine, $LaCl_3$ and $CdCl_2$ on the non-inactivating K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. 5 min after external application of 2 mM EGTA (n = 3), 10 μM nifedipine (n = 4), 1 mM La^{3+} (n = 4) or 300 μM Cd^{2+} (n =4), current amplitudes were recorded at a test pulse of 0 mV and normalized to the value prior to the addition of the drugs, $p > 0.05$ compared with control.

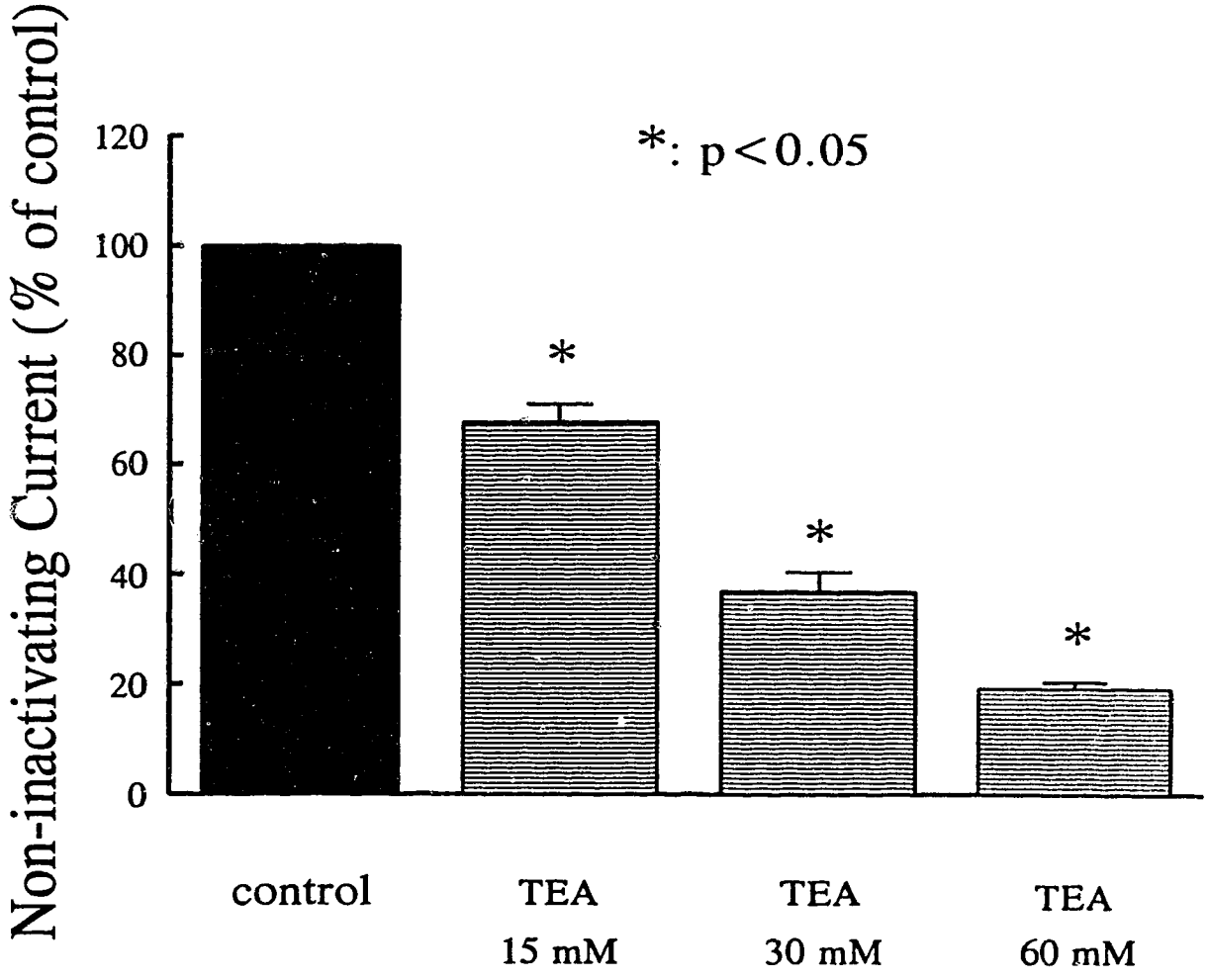

Figure 3-6. Effects of TEA on the non-inactivating K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. The cells were treated with 15 mM (n = 5), 30 mM (n = 4) or 60 mM (n = 4) TEA for 5 minutes. Current amplitudes were measured at test potentials of +20 mV and were normalized to the corresponding control (prior to the addition of the antagonist). *: $p < 0.05$ *vs.* control.

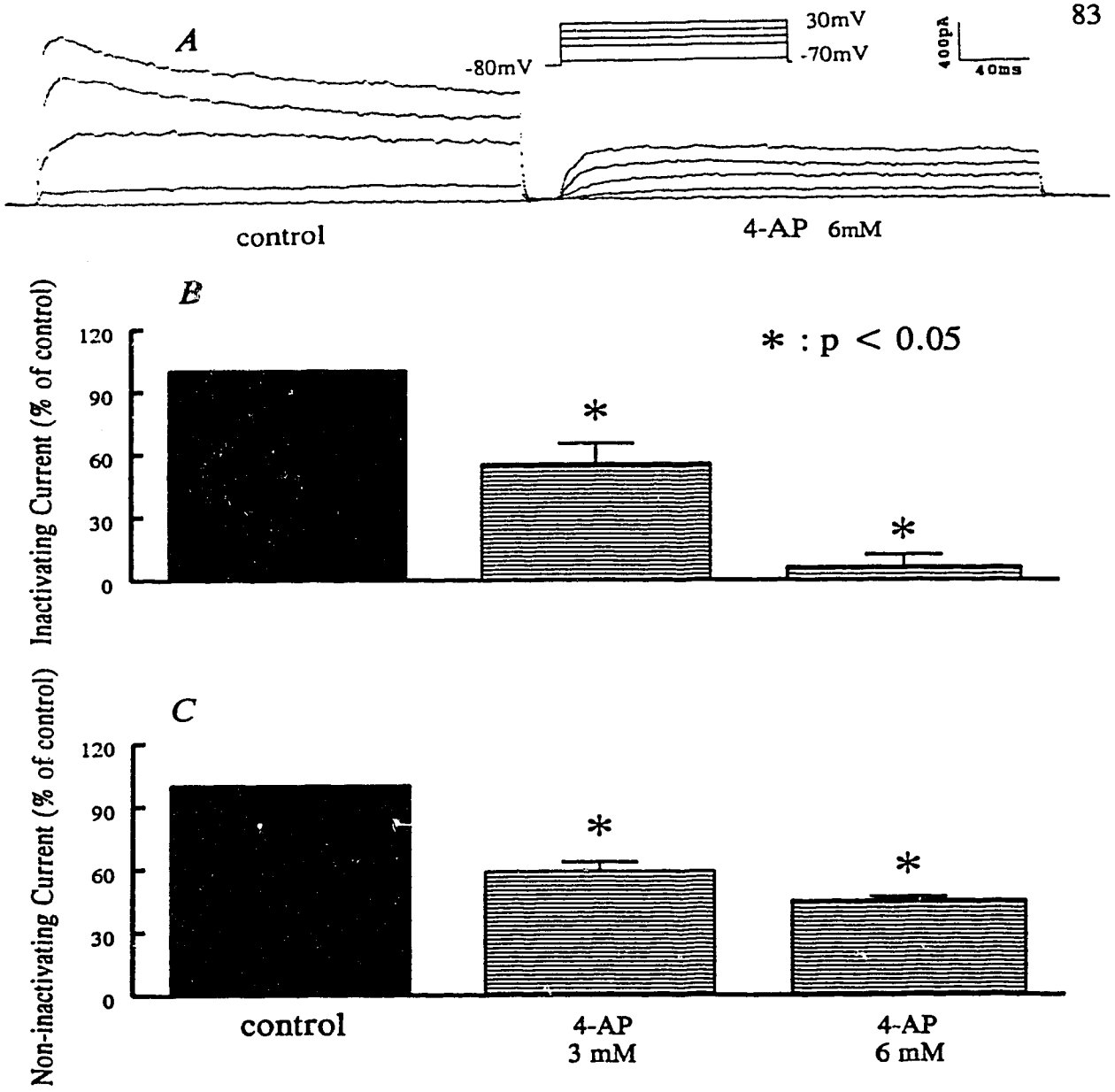

Figure 3-7. Effects of 4-AP on the K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. Cells, exhibiting both fast- and non-inactivating components, were treated with 4-AP (3 mM, n = 4; or 6 mM, n = 6) for 5 minutes. Current amplitudes were measured at a test potential of +10 mV and were normalized to the corresponding control values (current before the treatment). *A*, a typical original outward K^+ current records (leakage corrected) and the voltage-clamp pulse protocol; *B*, effect of 4-AP on the inactivating component; *C*, effect of 4-AP on the non-inactivating component. *: $p < 0.05$ compared with the corresponding control value.

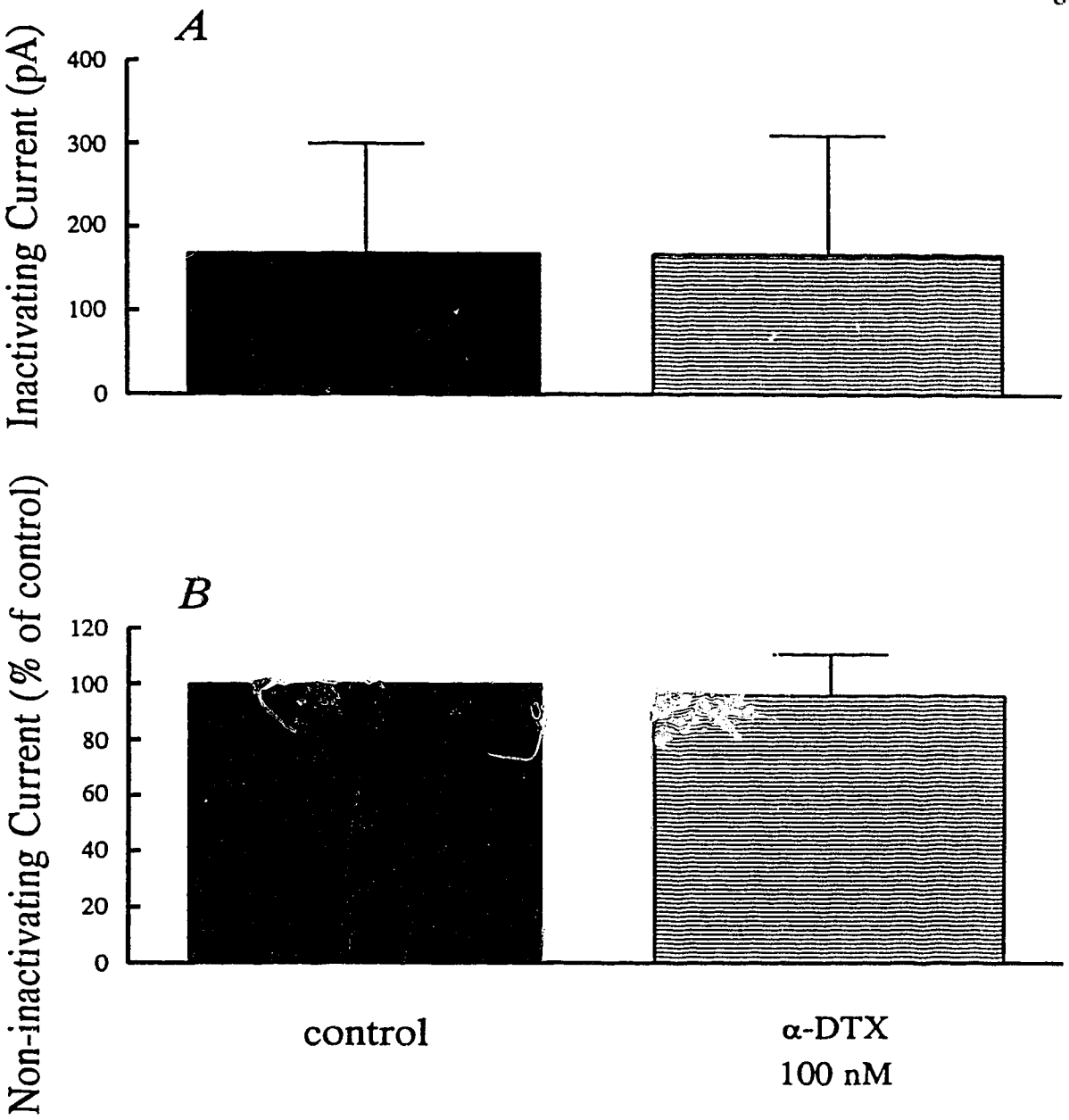

Figure 3-8. Effect of α-DTX on the K^+ currents recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. Cells exhibiting both fast- and non-inactivating components were selected for this group of experiments. α-DTX (100 nM, n = 3) was added to the bath solution and the current was recorded at test potential of +10 mV after 5 minutes, and compared with the corresponding control record. *A*, effect of α-DTX on the inactivating component; *B*, effect of α-DTX on the non-inactivating component. $p > 0.05$ compared with corresponding control value.

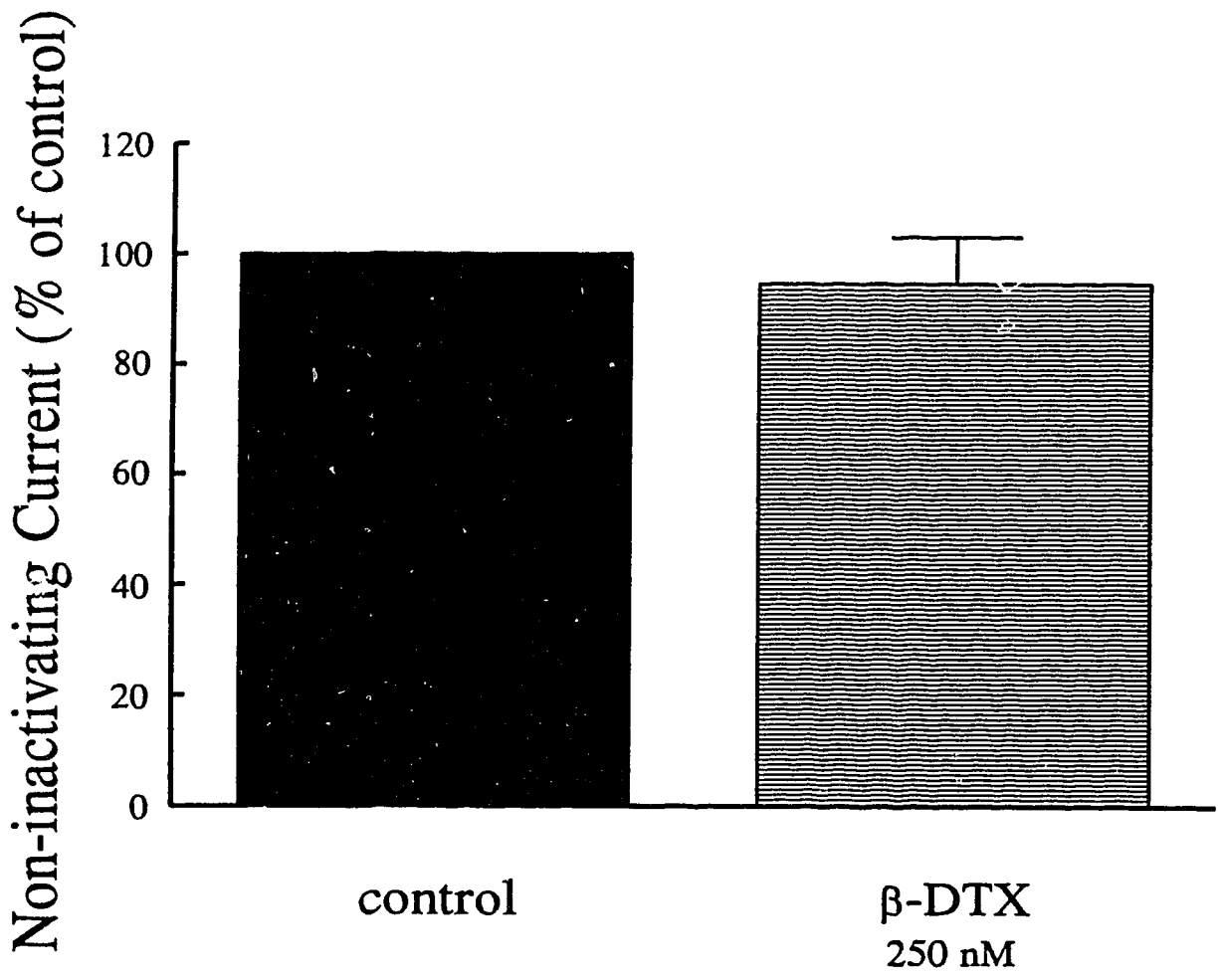

Figure 3-9. Effect of β-DTX on the non-inactivating K^+ current recorded from neurohypophyseal nerve terminals with low $[Ca^{2+}]_i$. The current was measured 5 min after 250 nM β-DTX was added to the bath solution during a test pulse of +10 mV. $p > 0.05$ compared with corresponding control value ($n = 4$).

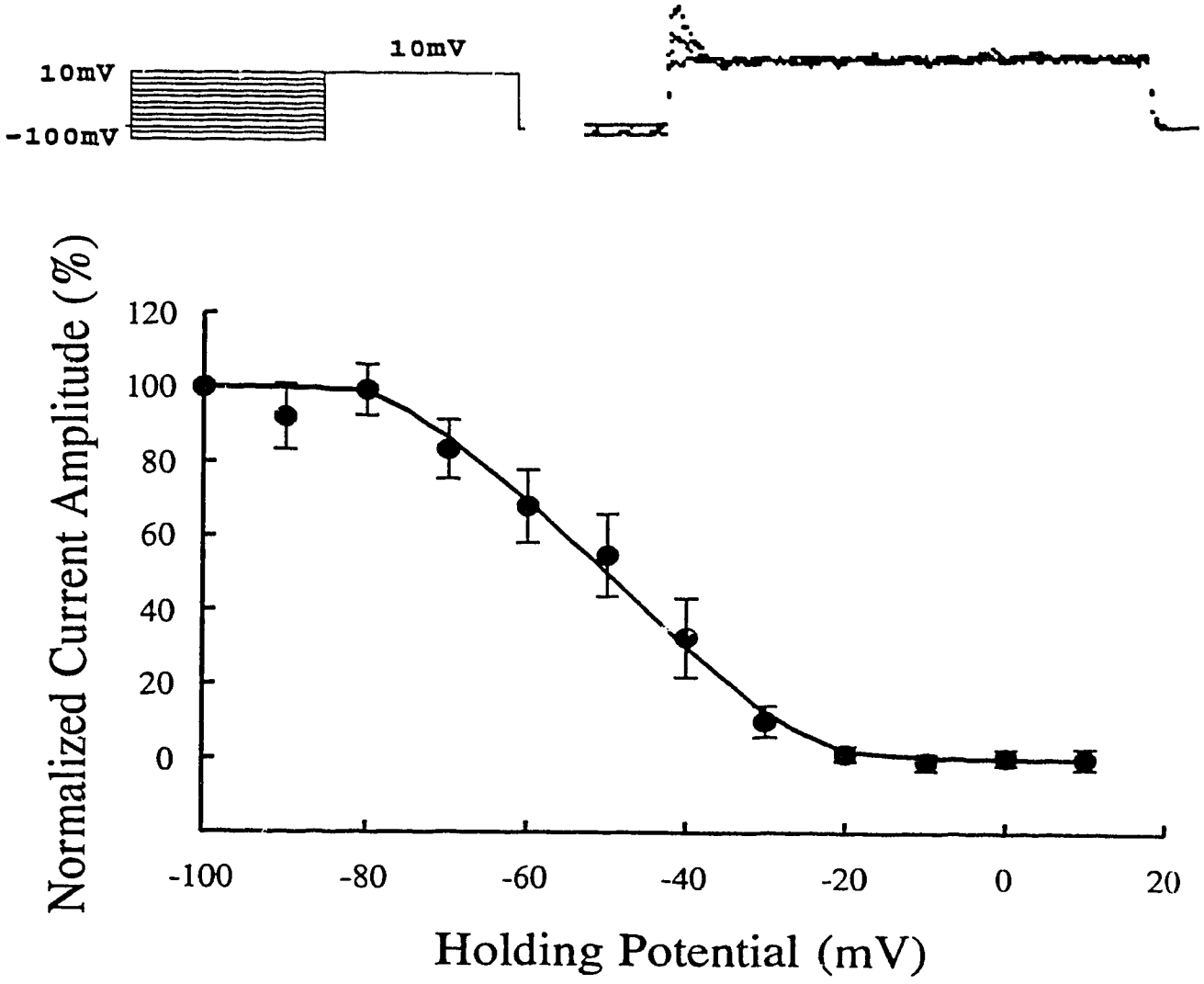

Figure 3-10. Steady-state inactivation of the fast-inactivating current. Following varying the holding potential from -100 mV to +10 mV for 1.5 second, cells were depolarized to test potential of +10 mV (n = 5). The outward currents (the difference between the peak current and plateau current) were normalized to the value which was activated from the most negative holding potential (-100 mV). The line is fitted to the Boltzmann equation $I/I_{max} = [1 + exp(V-V_{1/2}/k)]^{-1}$, where I is the peak current measured, I_{max} is the peak current measured at holding potential -100 mV, V is the holding potential, $V_{1/2}$ is the half-maximal activation voltage, and k is the slope factor.

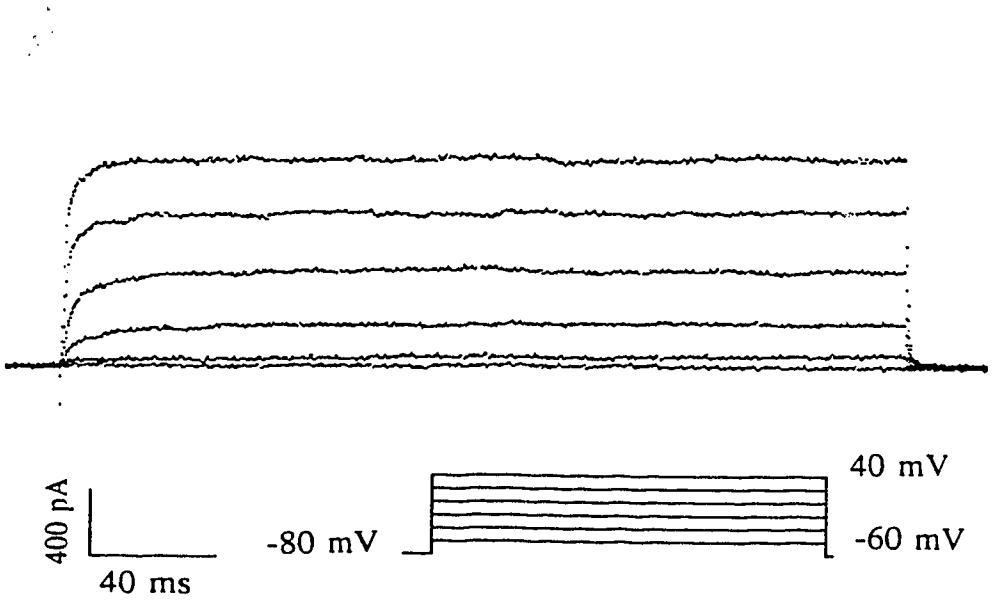

Figure 3-11. The outward K^+ current recorded from neurohypophyseal nerve terminals and the voltage-clamp pulse protocol used. The pipette solution contained 1.1 mM EGTA and the $[Ca^{2+}]_o$ was 10 mM (for high $[Ca^{2+}]_i$). The current was activated by depolarizing the cell from a holding potential of -80 mV to various test potentials. The current was activated at about -40 mV.

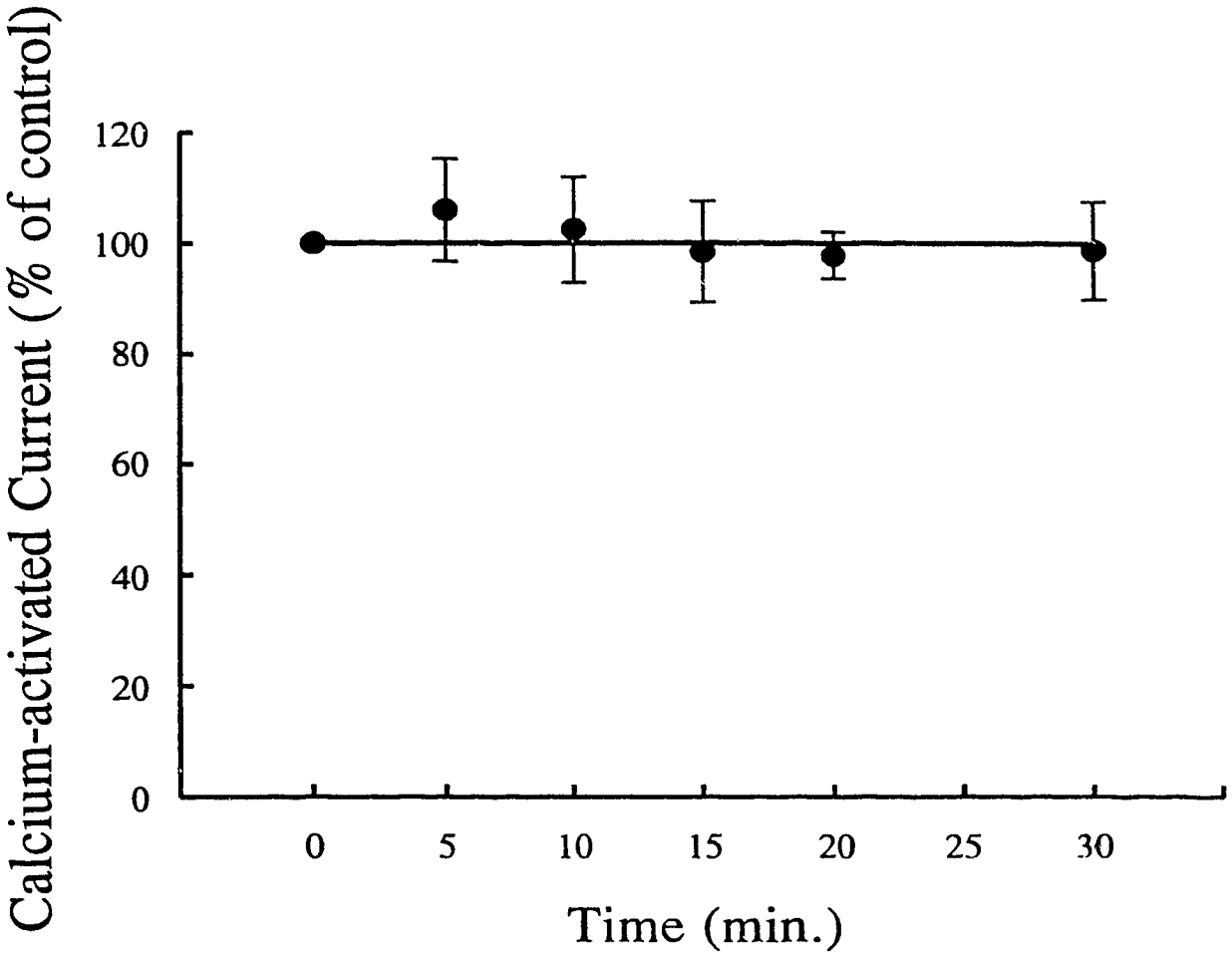

Figure 3-12. The time course of the K^+ currents recorded from neurohypophyseal nerve terminals with high $[Ca^{2+}]_i$. The current amplitude was measured at a test potential of +20 mV at 5 (n = 5), 10 (n = 5), 15 (n = 5), 20 (n = 4) and 30 (n = 3) minutes after the whole-cell configuration was established, and was normalized to the corresponding control (current recorded 3-5 min after penetrating the membrane).

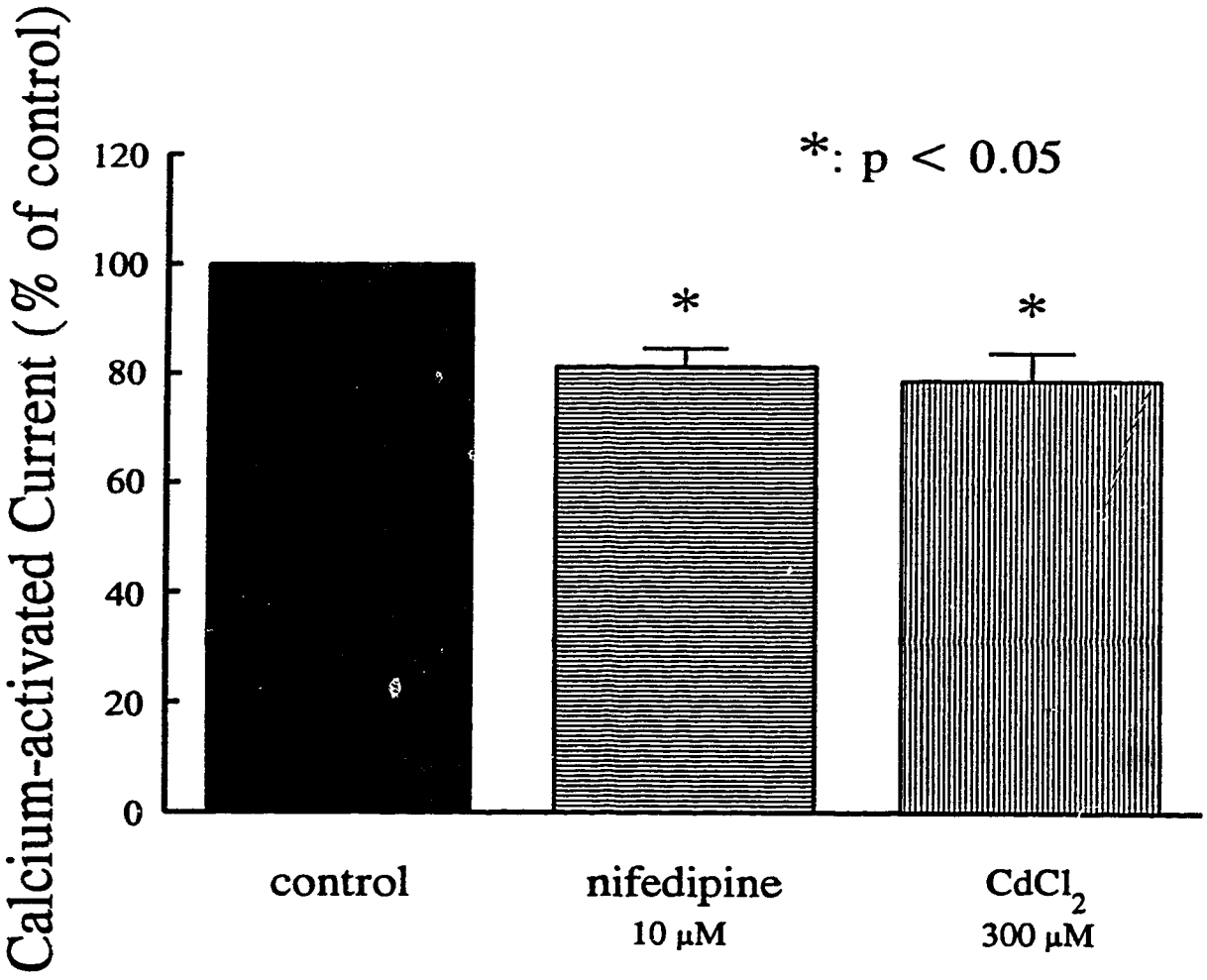

Figure 3-13. Effect of nifedipine and $CdCl_2$ on the K^+ current recorded from neurohypophyseal nerve terminals with high $[Ca^{2+}]_i$. The cells were treated with nifedipine 10 μM (n = 5) or $CdCl_2$ 300 μM (n = 6) for 5 minutes. Current amplitudes were measured at a test pulse of +20 mV and were normalized to the corresponding control. *: $p < 0.05$ compared with control.

CHAPTER IV

THE EFFECTS OF PGE_2 AND THE INVOLVEMENT OF SECOND MESSENGER ON VOLTAGE-DEPENDENT K^+ CHANNELS IN NEUROHYPOPHYSEAL NERVE TERMINALS

4.1. INTRODUCTION

The posterior pituitary contains large amounts of prostaglandin E_2 (PGE_2) (Horton, 1969; Narumiya *et al.*, 1982; Ogorochi *et al.*, 1984; Watanabe *et al.*, 1985). PGE_2 acts through its specific membrane receptors (Shimizu *et al.*, 1979), which may be similar to the peripheral prostaglandin receptors (Gagnon *et al.*, 1973), in an autocrine or paracrine manner to regulate neurohypophyseal function (Haluska *et al.*, 1989; Smith, 1989). However, the effect of PGE_2 on neurohypophyseal hormone release is still controversial. It has been documented that PGE_2 increases vasopressin (ADH) and/or oxytocin (OXT) secretion in humans (Gillespie, 1972), dogs (Yamamoto *et al.*, 1976), guinea pigs (Ishikawa *et al.*, 1981), rats (Hashimoto *et al.*, 1988), and rabbits (Desai and Raghavan, 1982; Hashimoto *et al.*, 1989). The fact that administration of indomethacin (IM), an inhibitor of cyclooxygenase, could decrease ADH and/or OXT excretion in dogs (Yamamoto *et al.*, 1976 and 1978; Hoffman *et al.*, 1982), humans (Glasson *et al.*, 1979; Glasson and Vallotton, 1980), guinea pigs (Ishikawa *et al.*, 1981), goats (Cooke and Homeida, 1984) and rats (Guzek *et al.*, 1986), combined with the discovery that administration of

meclofenamate, an PGE_2 receptor antagonist, results in a consistent fall in plasma ADH levels in dogs (Walker, 1983), supports the hypothesis that endogenous PGE_2 is an important mediator to enhance neurohypophyseal hormone release. Reports that PGE_2 induces a significant increase in transmitter release when incubated with rat neural lobes *in vitro* (Gagnon *et al.*, 1973; Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989), and that IM inhibits release in similar experimental conditions (Bojanowska and Guzek, 1991), may, therefore, suggest that PGE_2 serves as a modulatory factor involved in the mechanism of neurohypophyseal hormone release, and that it acts, at least in part, on the neurohypophysis. In contrast to those reports, there has been a failure to observe any change in ADH release in *in vitro* studies when the rat neurohypophysis is incubated with PGE_2 (Vilhardt and Hedqvist, 1970; Ruoff, 1974; Hashimoto *et al.*, 1988 and 1989), or superfused with IM (Knepel *et al.*, 1985). Moreover, PGE_2 markedly inhibited ADH and/or OXT release by superinfusion of the neurointermediate lobe of the hypophysis (Knepel *et al.*, 1985) and by injection into the lateral ventricle (Fujimoto *et al.*, 1978), also IM enhanced ADH release in rats (Kasting *et al.*, 1985) and dogs (Rutecki *et al.*, 1982). It would, therefore, appear that PGE_2 in some way exerts either a stimulatory or an inhibitory influence on the neurohypophyseal hormone release, although the present data do not allow one to precisely evaluate the participation of PGE_2. The physiological roles and the mechanisms of action of prostaglandins in the posterior pituitary still remain to be elucidated.

Since K^+ channels play a fundamental role in cellular excitability, it can be

speculated that PGE_2 may exert its neurohypophyseal actions through the modulation of K^+ channels. It has been reported that PGE_2 increases K^+ conductance in kidney cells (Steidl *et al.*, 1991), promotes the opening of the ligand-gated K^+ channels in cortical synaptosomes (Zoltay and Cooper, 1994), and activates cyclic adenosine 3',5'-monophosphate (cAMP)-mediated K^+ channels (Mori *et al.*, 1989) as well as adenosine 5'-triphosphate (ATP)-gated K^+ channels (Guillemare *et al.*, 1994) in *Xenopus* oocytes; conversely, PGE_2 can block the Ca^{2+}-activated K^+ channel in neurons (Fowler *et al.*, 1985) and in brain synaptosomes (Ren and Benishin, 1994), inhibit the delayed rectifier K^+ channel in smooth muscle cells (Ren, 1994), and decrease K^+ conductance in lymphocytes (Bastin *et al.*, 1990). As previously described, K^+ channels are subject to modulation by second messengers. The interaction of PGE_2 with prostaglandin E receptors (EPs) activates the regulatory guanine nucleotide binding proteins (G proteins), which are either directly coupled to an ion channel or coupled to a second messenger system(s). Uncoupled G protein α-subunit couples to the adenylate cyclase, and, in turn, catalyses the conversion of adenosine 5'-triphosphate (ATP) to cAMP. cAMP has been implicated in the control of ion channel activity either directly or indirectly via the cAMP-dependent protein phosphorylation. It has been demonstrated that EPs are coupled to adenylate cyclase activity in the posterior pituitary (Malet *et al.*, 1982), and it is commonly believed that PGE_2 enhances cAMP generation in various types of cells (Samuelsson *et al.*, 1978; Coleman *et al.*, 1994*b*). Although PGE_2 can also activate the phospholipase C (PLC)-protein kinase C (PKC) cascade (Haluska *et al.*, 1989; Goureau *et al.*,

1992) and phospholipase D (PLD) (Wu *et al.*, 1992), it has been suggested that the modulation of K^+ channel conductance by PGE_2 can be attributed to the accumulation of cytosolic cAMP and a subsequent phosphorylation of K^+ channels by cAMP-dependent protein kinase (PKA) (Mori *et al.*, 1989; Bastin *et al.*, 1990; Dixon *et al.*, 1990; Ren *et al.*, 1995*a*). However, there is an absence, to date, of any direct evidence investigating the action of PGE_2 on K^+ channels in the nerve endings of neurohypophysis. In an effort to gain a better understanding and an explanation of the effect of PGE_2 on the regulation of hormone release from posterior pituitary, the present study has investigated the effects of PGE_2 on K^+ channel currents in rat neurohypophyseal nerve terminals.

4.2. EXPERIMENTAL DESIGN

The purpose of this group of experiments was to investigate the effects of PGE_2 on the voltage-dependent K^+ channels in neurohypophyseal nerve terminals, -- the characteristics of which were presented in the previous chapter. Meclofenamate, a PGE_2 receptor antagonist, was used to verify the involvement of PGE_2 receptors, because PGE_2 has been considered to exert its action through a specific membrane receptor. Guanosine-5'-O-(2-thiodiphosphate) (GDPβS), a nonhydrolysable guanosine 5'-diphosphate (GDP) analogue, was applied to the pipette to inhibit G protein activity, in order to examine whether the PGE_2 receptor is coupled to a G protein(s). Regarding the second messenger systems which may be involved in the action of

PGE_2 on K^+ channels, we concentrated on the cAMP-PKA pathway, since PGE_2 has been commonly believed to increase cAMP generation. Forskolin, an adenylate cyclase activator, and the cAMP agonists dibutyl adenosine 3',5'-cyclic monophosphate (Db-CAMP), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-CAMP) and adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPs), as well as the cAMP antagonists adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPs), were employed to determine the role of cAMP in PGE_2 action in the nerve endings of the posterior pituitary.

4.3. RESULTS

4.3.1 Effects of PGE_2 on K^+ Currents in Neurohypophyseal Nerve Terminals under Low Free $[Ca^{2+}]_i$

By extracellular application, PGE_2 could initially increase the amplitude of the non-inactivating component (I_n) from a holding potential of -80 mV (Fig. 4-1). 15 to 20 minutes after PGE_2 administration, the amplitude of the fast-inactivating component (I_f) was increased, and the non-inactivating current (I_n) was decreased. The data presented in Fig. 4-2 shows that I_n, measured at test potential of +10 mV, normalized to its respective control (before treatment) and plotted as a function of the concentration of PGE_2, was increased 5 minutes after the administration of PGE_2. The results indicated that the enhancement of I_n was concentration dependent, within PGE_2 concentration ranges of 50 μM to 500 μM. For the limitation of the drug

supply, no further experiments were conducted by applying PGE_2 at concentration higher than 500 μM. The time course of PGE_2-induced effects on the K^+ currents in nerve endings of the posterior pituitary are shown in Fig. 4-3. At a concentration of 500 μM, PGE_2 increased I_n, and this increase peaked 5 min after application (Fig. 4-3*B*). However, this effect was transient. There was a return to control levels after 15 to 20 minutes. In the meantime, PGE_2 gradually facilitated I_f (Fig. 4-3*A*); 20 min after PGE_2 treatment, I_f was significantly enlarged, compared to the corresponding values both before and 5 min after treatment, while I_n was significantly attenuated, compared to the corresponding value 5 min after treatment.

4.3.2 Signal Transduction Involved in the Effects of PGE_2 on K^+ Currents in Neurohypophyseal Nerve Terminals under Low Free $[Ca^{2+}]_i$

4.3.2.1 Involvement of PGE_2 receptors in PGE_2 effects on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

To determine how PGE_2 might exert its action on K^+ currents in nerve endings of the posterior pituitary, meclofenamate, a PGE_2 receptor antagonist, was added to the cell bath 10 min prior to the addition of 500 μM PGE_2. Fig. 4-4 shows the effect of sodium meclofenamate on PGE_2-induced changes to both I_f and I_n. Meclofenamate (1 mM) itself did not have any effect on either I_f or I_n. However, the expected effects of PGE_2 on either I_f or I_n, 5 and 20 minutes after PGE_2 administration, were abolished by pretreatment with meclofenamate. Although there was a noticeable decrease in I_n after exposure to meclofinamate and PGE_2, it was not

significant and was within the rate of normal rundown. These results clearly indicate that the action of PGE_2 is through the PGE_2 membrane surface receptor(s).

4.3.2.2 Involvement of G proteins in PGE_2 effects on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

In order to decide whether any G proteins are coupled to the PGE_2 receptor, GDPβS, employed as a G protein inhibitor, was applied to the pipette solution. Cells were pretreated intracellularly with 1 mM GDPβS, a nonhydrolysable GDP analogue, for 5 min before being exposed to 500 μM PGE_2 (Fig. 4-5). From a holding potential of -80 mV, current amplitudes were recorded 5 and 20 min after exposure to PGE_2 at test pulses of +20 mV. Following GDPβS pretreatment, no increases in I_n were observed 5 min after PGE_2 administration, also PGE_2 was not found to induce an increase in I_f after 20 min. Even though an attenuation of I_n was observed 20 minutes after PGE_2 addition, it was not significant, compared to the corresponding value prior to PGE_2 application, and was within the rate of normal rundown. The finding that GDPβS prevented the expected effects of PGE_2 on both I_f and I_n suggests that a G protein is very likely to be involved in the action of PGE_2.

4.3.2.3 Involvement of cAMP cascade on PGE_2 effects on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

This group of experiments was designed to determine whether the cAMP pathway was involved in PGE_2-induced signal transduction modulating K^+ channels

in the nerve endings of the posterior pituitary.

4.3.2.3.1 The effect of the adenylate cyclase activator forskolin on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

To examine the idea that the activation of adenylate cyclase was involved in the mechanism of PGE_2-induced long-term modulation of K^+ channels in the isolated nerve terminals of rat posterior pituitary, forskolin, an adenylate cyclase activator, was applied into the extracellular solution 5 min before recording (Fig. 4-6). Forskolin increased the fast-inactivating component and simultaneously decreased the non-inactivating component. The effects induced by forskolin were very similar to the long-term action of PGE_2 on the K^+ currents, i.e. the increase of I_f and the decrease of I_n 20 min after PGE_2 administration. Fig. 4-7 shows the current-voltage (I-V) relationship of the effect of forskolin on both I_f and I_n. It is assumed from these experiments that PGE_2 elicits its effects on K^+ channel modulation via the activation of adenylate cyclase, since forskolin can mimic part of the PGE_2-induced effects.

In order to decide whether the effects of forskolin on K^+ currents in neurohypophyseal terminals were 4-aminopyridine (4-AP) sensitive, 4-AP was employed to the external solution 10 min prior the administration of forskolin. As described in chapter 3, 4-AP can primarily block the fast-inactivating component, and may block some of the non-inactivating component, as well. In the presence of 4-AP, forskolin can still decrease I_n (Fig. 4-8). However, the increase of I_f elicited by

forskolin in the absence of 4-AP did not appear. This data suggests that the enhanced I_f is 4-AP sensitive.

In order to determine whether the effect of forskolin on K^+ channels in the nerve endings of the posterior pituitary is Ca^{2+} dependent, cadmium (300 μM) was added to the bath, 5 min prior to the employment of forskolin, to block Ca^{2+} channel activity. Fig. 4-9 demonstrates that cadmium itself did not affect either I_f or I_n, as described in chapter 3. With the treatment of cadmium, forskolin also enlarged I_f and attenuated I_n, simultaneously. Therefore, the effects of forskolin on both I_f and I_n were not Ca^{2+}-sensitive. These results were supported by similar results obtained using lanthanum (1 mM) to block Ca^{2+} channels or using strontium (2 mM) to replace Ca^{2+} (2 mM) in the extracellular solution (data not shown).

4.3.2.3.2 The effect of the cAMP agonist 8-Br-cAMP, Db-cAMP and Sp-cAMPs on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

The support for the concept that the cAMP cascade was involved in the mechanism of PGE_2-induced long-term regulation of K^+ channels came from studies on the effects of cAMP agonists. In these experiments, the current amplitudes were measured 5 min after application of 8-Br-cAMP, Db-cAMP or Sp-cAMPs. 8-Br-cAMP (5 and 10 μM), the most potent cAMP agonist among these three, was able to mimic the long-term modulation of K^+ channel activity induced by PGE_2, i.e. increased I_f and decreased I_n, in a dose dependent manner (Fig. 4-10). Db-cAMP (300 μM, Fig.4-11) and Sp-cAMPs (1 mM, Fig. 4-12) had similar effects to 8-Br-

cAMP. These data suggest that cAMP may play an important role in modulating K^+ channels, especially fast-inactivating K^+ channel activity in nerve terminals, which may be one of the most important media regarding PGE_2-induced long-term modulatory effects on K^+ channels.

4.3.2.3.3 The effect of the cAMP antagonist Rp-cAMPs on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$

In order to confirm the involvement of the cAMP pathway in part of the regulating effects of PGE_2 on K^+ channels in the nerve terminals of the posterior pituitary, Rp-cAMPs, an antagonist of cAMP, was added to the internal solution to block the cAMP cascade. PGE_2 (500 μM) was applied to the external solution 5 minutes after penetrating the membrane. Fig. 4-13 demonstrates that Rp-cAMPs (100 μM), was not able to eliminate the enhancement of I_n induced by PGE_2 5 min after administration. However, 20 min after application of PGE_2, the expect enlargement of I_f did not appear. Also, the decrease in I_n was not significant, when compared to the value 5 min after PGE_2 treatment, thus, Rp-cAMPs had no effect on I_n. To check that the blocking effect of Rp-cAMPs on PGE_2-induced long-term modulation was through the inhibition of cAMP, 8-Br-cAMP, an agonist of cAMP, was employed to the bath (Fig. 4-14). There were no notable changes to either I_f or I_n 5 min after 8-Br-cAMP addition. These results suggested that the long-term modulation of K^+ channel activity (long-term increase in I_f and decrease in I_n) induced by PGE_2 was conferred by the activation of adenylate cyclase and, thus,

increases in cAMP production, whereas the mechanism underlying the short-term modulation, i.e. the facilitation of I_n, remained unknown.

4.3.3 Effects of PGE_2 on K^+ Currents in Neurohypophyseal Nerve Terminals under High Free $[Ca^{2+}]_i$

Under conditions of high $[Ca^{2+}]_i$, the Ca^{2+}-dependent K^+ channels were activated by depolarizing the membrane to +10 mV, as described in chapter 3. 5 min after external application of PGE_2, there were not any notable changes in neither the fast-inactivating component, nor the Ca^{2+}-activated component (Fig. 4-15). However, 20 min after administration, PGE_2 (250 μM) was able to increase the fast-inactivating component, while the Ca^{2+}-activated component remained unaffected. The data confirmed that the enhancement effect of PGE_2 on I_f was not Ca^{2+} dependent, and indicated that PGE_2 had no effects on Ca^{2+}-activated K^+ channels.

4.4. DISCUSSION

4.4.1 Characterization of the Effects of PGE_2 on K^+ Channels in Neurohypophyseal Nerve Terminals

PGE_2 has been demonstrated to block certain kinds of K^+ channels in lymphocytes (Bastin *et al.*, 1990), neurons (Fowler *et al.*, 1985), brain synaptosomes (Ren and Benishin, 1994), and smooth muscle cells (Ren, 1994), and, conversely, to promote the activity of some K^+ channels in cortical synaptosomes (Zoltay and

Cooper, 1994) and *Xenopus* oocytes (Mori *et al.*, 1989; Guillemare *et al.*, 1994). The apparent discrepancies with regard to the effects of PGE_2 on K^+ channels may depend on the tissues and species studied. Up-to-date, there have not been any reports with respect to the effect of PGE_2 on K^+ channels in neurohypophyseal nerve terminals. The present study showed that under conditions of low $[Ca^{2+}]_i$, PGE_2, a putative local hormone, initially facilitated the non-inactivating component in the nerve endings of the posterior pituitary, in a concentration-dependent fashion. After administration of PGE_2 for 15 to 20 min, there was an increase in the fast-inactivating component, and a decrease in the non-inactivating component of the K^+ current. The decline of the non-inactivating component was greater than the rate of rundown (1 %/min), therefore, the attenuation of non-inactivating component could not account for normal rundown. It is reported that ethanol at concentrations of 200 mM and above could affect K^+ channel conductance and inactivation (Treistman *et al.*, 1991). However, the final concentration of ethanol used as a solvent in this experiment was about 30 mM, and ethanol did not exert an obvious effect at this concentration (data not shown). Therefore, it is not possible that the observed regulating action elicited by PGE_2 was caused by ethanol.

Under conditions of high $[Ca^{2+}]_i$, no significant enhancement of the non-inactivating component was observed 5 min after PGE_2 application. The discrepancy between PGE_2 effects on the non-inactivating component under high or low $[Ca^{2+}]_i$ conditions may due to the activation of Ca^{2+}-activated K^+ channels. Ca^{2+}-activated K^+ channels may not be sensitive to PGE_2, since they are activated by high $[Ca^{2+}]_i$

when depolarizing the membrane. The other possibility is that the facilitation of the non-inactivating component induced by PGE_2 is varied by different $[Ca^{2+}]_i$. 20 min after PGE_2 administration, the fast-inactivating component was increased dramatically, which is similar to the PGE_2 effect on the fast-inactivating component under low $[Ca^{2+}]_i$, while non-inactivating component was decreased (but not significantly). Because there is no rundown under high $[Ca^{2+}]_i$, as described in chapter 3, the attenuation of non-inactivating component may not be accounted for by rundown. There was a 20% contribution of the Ca^{2+}-activated K^+ current to the overall non-inactivating currents, as analyzed in chapter 3. Ca^{2+}-activated K^+ channels may not respond to PGE_2. Therefore, PGE_2 has the tendency to inhibit non-inactivating components, even if it is not statistically significant.

According to present data, PGE_2 may not affect Ca^{2+}-activated K^+ channels. After a short period of time, which is necessary to allow PGE_2 to diffuse evenly in the bath, PGE_2 can enlarge the non-inactivating component, and this effect may be Ca^{2+}-sensitive. After a long period of time, the enlarged non-inactivating component declined, while the fast-inactivating component increased. These effects are not Ca^{2+}-dependent. This is consistent with the results observed when using a cAMP analogue to mimic the long-term action of PGE_2 (described below). The long-term effects of PGE_2 are mimicked by cAMP analogue, but not the short-term enhancement of I_n.

The activation of fast-inactivating K^+ channels, which comprise the major current responsible for terminal repolarization after a spike, opposes any

depolarization tendencies (Thorn *et al.*, 1991). By retarding the production of successive spikes in a train, the transient K^+ current tends to act as a brake on spike discharge as well as limiting spike frequency (Connor and Stevens, 1971; Rogawski, 1985; Jan and Jan, 1989), and, thus decreases transmitter release. Opening the non-inactivating K^+ channel could raise the threshold for action potential generation in neurohypophyseal nerve endings (Bielefeldt *et al.*, 1992), and, then, limit secretion toward the end of a long burst (Bicknell *et al.*, 1984). The activation of this non-inactivating K^+ channel could play a role in the fatigue of stimulus-secretion coupling in the posterior pituitary. These two distinct K^+ channels, therefore, independently control transmitter release.

K^+ channels are distributed on neurohypophyseal nerve terminal plasma membranes, where they play a key role in controlling many cellular functions, and most importantly, the secretion of hormones (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). The modulatory actions of PGE_2 on K^+ channels in the nerve endings may give a helpful clue to explain the controversial effects of PGE_2 in the neurohypophysis. PGE_2 has been demonstrated to induce either an increase in transmitter release (Gagnon *et al.*, 1973; Ishikawa *et al.*, 1981; Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989; Bojanowska and Guzek, 1991) or a decrease (Fujimoto *et al.*, 1978; Rutecki *et al.*, 1982; Kasting *et al.*, 1985; Knepel *et al.*, 1985). As presented in this chapter, PGE_2 regulates the various components of K^+ channel activity differently in a time-course dependent manner, and, thus, it is assumed that PGE_2 differently modulates transmitter release. For example, Knepel

et al. (1985) reported that ADH secretion was not altered when the neurointermediate lobe was incubated with PGE_2 for a short period of time, but was inhibited by longer incubations. This differential activity, which is dependent on the time course, coincides with our findings. Under physiological conditions, which are more like the present recordings under high $[Ca^{2+}]_i$, PGE_2 does not affect any K^+ channels 5 min after application; the fast-inactivating component, however, is increased significantly after longer incubations. The enhanced fast-inactivating K^+ channel is capable of slowing down the action potential generation, thus diminishing transmitter release. The facilitated neuropeptide release induced by PGE_2 (Negro-Vilar, *et al.*, 1985) could be accounted for by the decreased non-inactivating component in present studies, which is able to lower the threshold for action potential generation, after same duration of incubation. It is hypothesized that the overall role of PGE_2 on the posterior pituitary is dependent upon the modulation of K^+ channels and which effect is predominant.

It is noteworthy that caution must be used when applying the results of single cell recordings to the intact tissue. Due to the various experimental procedures and conditions used in the references cited, it is very difficult to compare and evaluate the controversial results from all the references. In some experiments, PGE_2 was introduced *in vivo* (Fujimoto, *et al.*, 1978; Rutecki *et al.*, 1982), whereas some experiments used *in vitro* application (Gagnon *et al.*, 1973; Ishikawa *et al.*, 1981; Knepel *et al.*, 1985; Negro-Vilar *et al.*, 1985). Therefore, it is impossible to estimate accurately the duration of the tissue exposure to PGE_2. In the references

cited, transmitter release was measured either under resting conditions (Gagnon *et al.*, 1973; Fujimoto *et al.*, 1978; Ishikawa *et al.*, 1981; Bojanowska *et al.*, 1985; Negro-Vilar *et al.*, 1985), or the secretion of transmitter was elicited by electrical stimulation (Knepel *et al.*, 1985) or elevated concentrations of K^+ (Bojanowska and Guzek, 1991). With repeated electrical stimulation, the inactivation of transient K^+ channel would build up, leading to a reduction in the available channels and a subsequent broadening of the spike (Bourque, 1990). Thus, the effectiveness of such bursts of incoming activity in eliciting maximal release of transmitters from the neurohypophyseal terminals must be put into consideration. Alternately, depolarization caused by excessive K^+ may end up by changing the resting membrane potentials. It has been indicated that the activity of K^+ channels in the neurohypophysis can be enhanced by hyperpolarization, and virtually eliminated by depolarization (Bielefeldt *et al.*, 1992). Even relatively small changes of membrane potential may alter action potentials and the efficiency of secretion. Nevertheless, present data would not allow one to elucidate the physiological roles and the mechanisms of PGE_2 action.

4.4.2 Signal Transduction Involved in the Modulation of K^+ Channels by PGE_2 in Neurohypophyseal Nerve Terminals

PGE_2 has been consistently considered to be the most potent prostaglandin receptor agonist (Melien *et al.*, 1988; Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990). It is common believed that the actions of PGE_2 are mediated by interaction with its

specific surface receptors, such as EP_1, EP_2, EP_3 and EP_4 (Coleman *et al.*, 1987, 1994*a*). Meclofenamate, a potent PGE_2 membrane receptor antagonist (Zelinski-Wooten *et al.*, 1990), completely diminished the regulatory effects of PGE_2 on K^+ channels. It has been proven that the modulatory effects of PGE_2 on K^+ channels in nerve endings are through binding to its receptors, and this finding is consistent with the already well-established receptor-mediated model of PGE_2 action.

It is speculated that following the interaction of PGE_2 with its receptor, signal transduction system(s) are triggered via the activation of G proteins. GDPβS, an analogue of GDP, is able to compete with endogenous guanosine 5'-triphosphate (GTP), and, thus, antagonizes G protein-mediated effects of neurotransmitters or hormones on K^+ channel currents (Vaca *et al.*, 1992; Fan *et al.*, 1993; Hamada *et al.*, 1993; Wu and Assmann, 1994). The abolishment of PGE_2 effects induced by GDPβS strongly implied that the regulatory action of PGE_2, exerted on the K^+ channels, was very likely to be mediated by G protein(s). However, the question of whether G proteins exert a local, direct action on the K^+ channels, or whether any soluble second messenger cascades are involved, leads to further investigation.

It is believed that PGE_2, like most neurotransmitters and hormones, induces its effects via cytosolic second messengers, such as cAMP, Ca^{2+}, inositol triphosphate (IP_3) and diacylglycerol (DG), which are essential for the signal transduction of various physiological processes (Ho *et al.*, 1988; Di Marzo *et al.*, 1991), including modulating the activity of membrane ion channels. A micromolar concentration range of PGE_2 was found to be high enough to affect cellular processes

(Yamaguchi *et al.*, 1988; Mori *et al.*, 1989; Goureau *et al.*, 1992; Ren, 1994). It was hypothesized that the modulatory effects of PGE_2 on K^+ channels in neurohypophyseal nerve endings are mediated by cAMP-cascade, due to the fact that PGE_2 increases cAMP generation in various types of cells (Samuelsson *et al.*, 1978; Coleman *et al.*, 1994*b*).

Modulation of K^+ channel activity by cAMP has been widely documented. In *Xenopus* oocytes and the node of Ranvier, addition of cAMP amplifies the K^+ current (Seelig and Kendig, 1982; Van Renterghem *et al.*, 1985). In smooth muscle cells, cAMP partially prevents the attenuation of the transient outward K^+ current induced by repetitive stimulation (Ohya *et al.*, 1987). Forskolin or cAMP suppresses a voltage-dependent K^+ current in neurones (Dunlap, 1985; Grega and Macdonald, 1987), lymphocytes (Choquet *et al.*, 1987; Choquet and Korn, 1988) and oligodendrocytes (Soliven *et al.*, 1988). It has been hypothesized that cAMP exerts its action by a subsequent phosphorylation of K^+ channels via PKA (Levitan, 1985), while a possible direct effect of cAMP on K^+ channels should not be excluded.

It has been proven previously that cAMP activity and subsequent PKA phosphorylation of the channel protein mediated modulation of K^+ channels by PGE_2 in human lymphocytes (Bastin *et al.*, 1990), *Xenopus* oocytes (Miledi and Woodward, 1989; Mori *et al.*, 1989) and rat smooth muscle cells (Ren, 1994; Ren *et al.*, 1995*a*). Intracellular cAMP concentrations are dependent upon both the activity of adenylate cyclase, which converts ATP to cAMP, and phosphodiesterase, which degrades cAMP to 5'-AMP. In the present study, the adenylate cyclase activator, forskolin

(de Souza *et al.*, 1983; Henquin *et al.*, 1983; Laurenza *et al.*, 1989), was found to mimic the long-term effects of PGE_2 on K^+ channels, i.e. the enlargement of the fast-inactivating component and the simultaneous inhibition of the non-inactivating component, and the latter is much greater than the rate of rundown, suggesting that these actions of PGE_2 are mediated by the activation of cAMP. Enhancement of the fast-inactivating component induced by forskolin is 4-AP sensitive. Also, both the increase of the fast-inactivating component and the decrease of the non-inactivating component are not Ca^{2+}-dependent. A lack of dependence on Ca^{2+} is consistent with the finding that PGE_2 facilitates the fast-inactivating component, and had the tendency to attenuate the non-inactivating component under high $[Ca^{2+}]_i$ conditions. However, the report that forskolin-induced block of delayed rectifying K^+ channels in pancreatic β-cells is not mediated by cAMP (Zunkler, *et al.*, 1988) raises the question as to whether the effects of forskolin on K^+ channels in the nerve endings of the posterior pituitary are through cAMP accumulation or a direct interaction with channel proteins.

To answer this question, subsequent investigations were performed. 8-Br-cAMP, a membrane-permeable analogue of cAMP (Brautigan *et al.*, 1991; Chneiweiss *et al.*, 1991), was also able to increase the fast-inactivating component and simultaneously to decrease the non-inactivating component in a dose dependent manner. Similar results were obtained by application of Db-cAMP, a cell permeable cAMP analogue which preferentially activates PKA (Posternak and Weimann, 1974; Hei *et al.*, 1991), to the extracellular solution. Sp-cAMPs, an activator of PKA

(Dostmann *et al.*, 1990; Sammak *et al.*, 1992), induced similar effects to forskolin on K^+ channels activity, too. The fact that cAMP analogues 8-Br-cAMP, Db-cAMP and Sp-cAMPs had similar effects to forskolin on K^+ channels supports that cAMP was indeed the second messenger which mediates long-term modulation induced by PGE_2. This was consistent with the finding that cAMP, when employed intracellularly, increased the rate of activation of the transient K^+ channel in rat neurohypophyseal nerve endings (Thorn *et al.*, 1991; also see chapter 3). Rp-cAMPs is a competitive inhibitor of PKA, which is not hydrolysed by mammalian phosphodiesterase (Butt *et al.*, 1990; Dostmann *et al.*, 1990; Wang L-Y. *et al.*, 1991). The long-term enlargement of the fast-inactivating component did not appear, when cells were pretreated intracellularly by Rp-cAMPs to block the stimulated phosphorylation by PKA. Although there was a fall in the non-inactivating component 20 min after PGE_2 addition, it was not significant, and was within the range of normal rundown. The finding that Rp-cAMPs abolished the long-term regulatory effects of PGE_2 and totally diminished the effect of 8-Br-cAMP on K^+ channels, further established that the cAMP cascade was involved in the modulation of K^+ channels, and that the long-term regulatory actions induced by PGE_2 were mediated by the activation of cAMP and the subsequent activation of PKA.

The results that intracellular application of Rp-cAMPs did not affect the short-term enhancement of the non-inactivating component induced by PGE_2 were consistent with the finding that the enhancement of the non-inactivating component was not seen with the treatment of forskolin. These observations suggested that this

short-term action of PGE_2 may be mediated by another second messenger system(s).

4.4.3 Conclusions

There is accumulating experimental evidence indicating that PGE_2 plays a critical role in controlling transmitter secretion from the neurohypophysis. This effect could be largely attributed to the modulatory action of PGE_2 on posterior pituitary K^+ channel activity. The results presented in this chapter have revealed that PGE_2 modulates the voltage-dependent K^+ channels in neurohypophyseal nerve terminals, through a receptor-mediated process involving the activation of G protein(s). A short time after application, PGE_2 stimulated the non-inactivating component under conditions of low $[Ca^{2+}]_i$. This action seems to be Ca^{2+}-sensitive, since it disappeared under high $[Ca^{2+}]_i$ conditions. The mechanism(s) underlying this effect remain, as yet, unknown. A longer period of time after PGE_2 addition, the fast-inactivating component increased, while the non-inactivating component declined to control levels. These processes were not Ca^{2+}-dependent. The present data suggests that these long-term regulatory effects induced by PGE_2 involve the cAMP-PKA cascade. It is hypothesized that PGE_2 binds to its specific membrane receptor(s) to activate G protein(s). The uncoupling of a G protein from the PGE_2 receptor(s) could lead to the subsequent activation of adenylate cyclase, and, hence, catalyses the conversion of ATP to cAMP, which, in turn, activates PKA. K^+ channel activities are subsequently subject to modulation by protein phosphorylation via PKA. PGE_2 may not, however, alter the function of Ca^{2+}-activated K^+ channels. Nevertheless,

these observations have provide strong evidence that PGE_2 might contribute to the regulation of hormone(s) release from the posterior pituitary by modulating K^+ channel activities. The possibility that PGE_2 might act through more than one signal pathway must not be excluded. It is hypothesized that stimulatory or inhibitory actions of PGE_2 on hormone release demonstrated by the literature cited, may be dependent on the second messenger status of the cell.

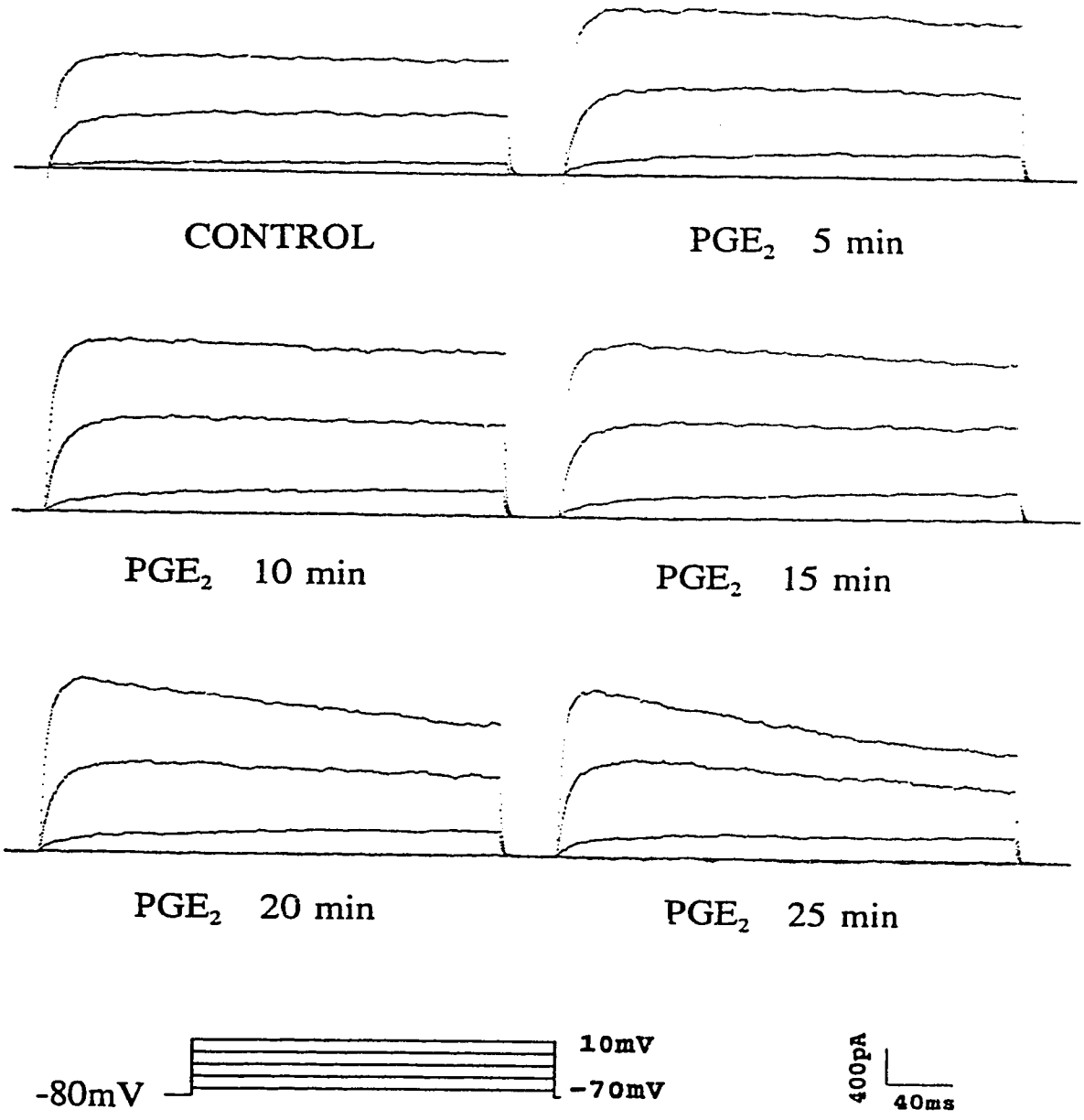

Figure 4-1. The original traces (leakage corrected) of the effects of PGE_2 on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. PGE_2 (500μM) was added to the extracellular solution. I_n initially increased, then gradually declined after 15 to 20 min. I_f increased 15 to 20 min after PGE_2 administration.

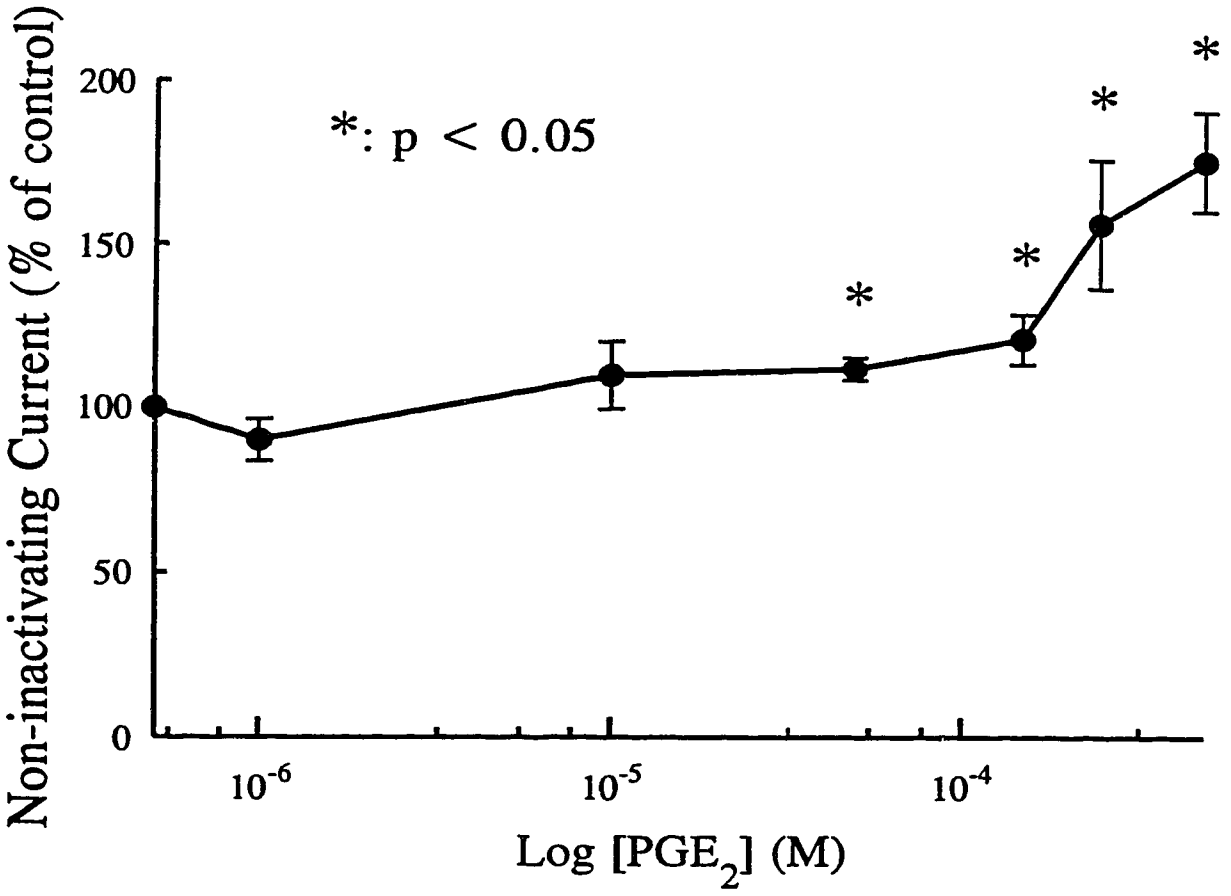

Figure 4-2. The excitatory effect of PGE_2 on I_n in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. PGE_2 at concentrations of 1 μM (n = 5), 10 μM (n = 5), 50 μM (n = 5), 150 μM (n = 5), 250 μM (n = 4) or 500 μM (n = 5) was applied to the bath. The currents were measured 5 minutes after PGE_2 administration at a test pulse of +10 mV, from a holding potential of -80 mV. *: $p < 0.05$ compared to the corresponding value prior to the PGE_2 treatment.

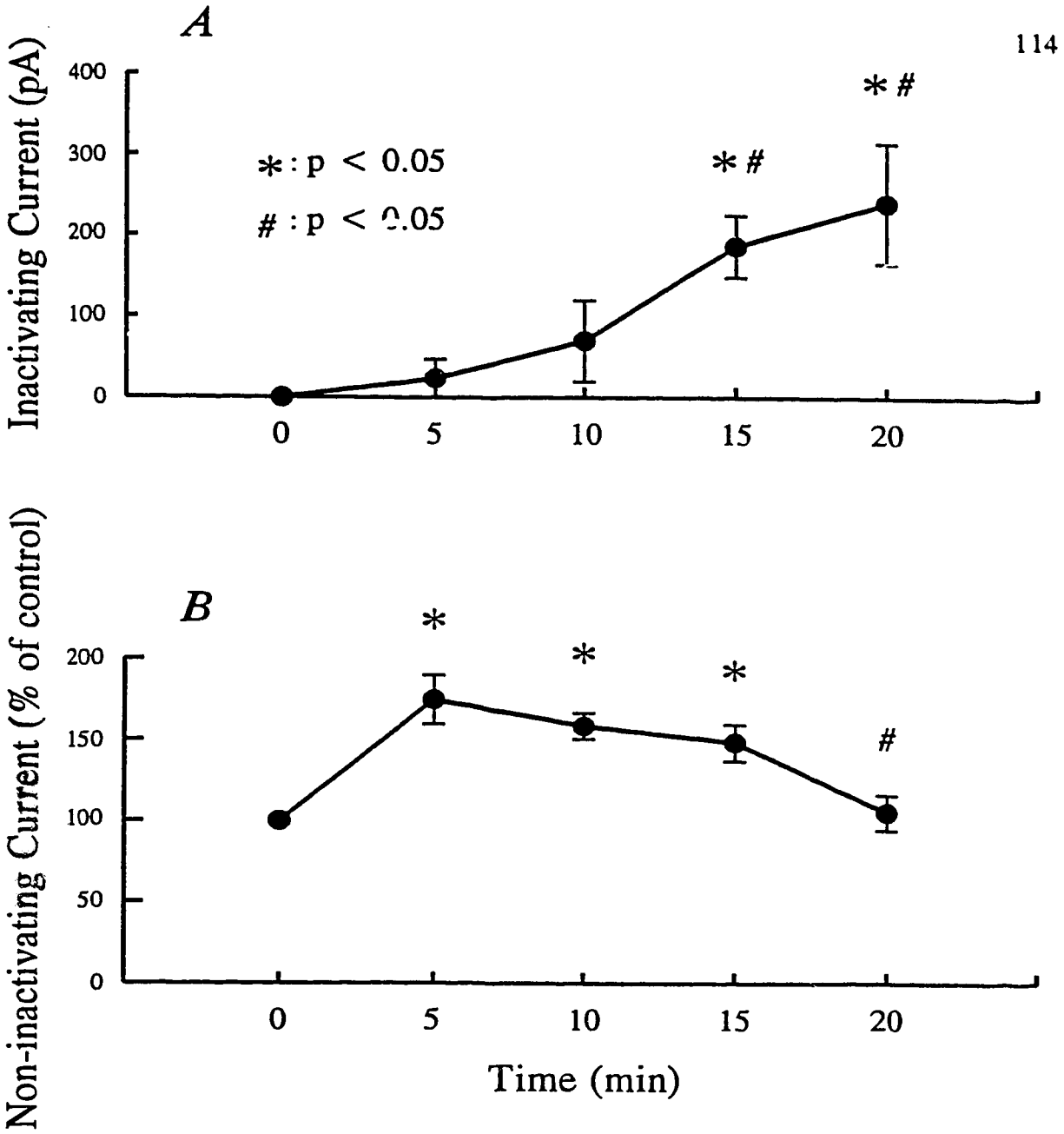

Figure 4-3. Time course of the effects of PGE_2 on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. The currents were recorded before (n = 5), and 5 min (n = 5), 10 min (n = 5), 15 min (n = 4) and 20 min (n = 4) after injection of PGE_2 (500 μM) into the external solution. The current amplitudes were measured after depolarizing the membrane to +10 mV, from a holding potential of -80 mV. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ *vs.* control (prior to PGE_2); #: $p < 0.05$ *vs.* PGE_2 at 5 min.

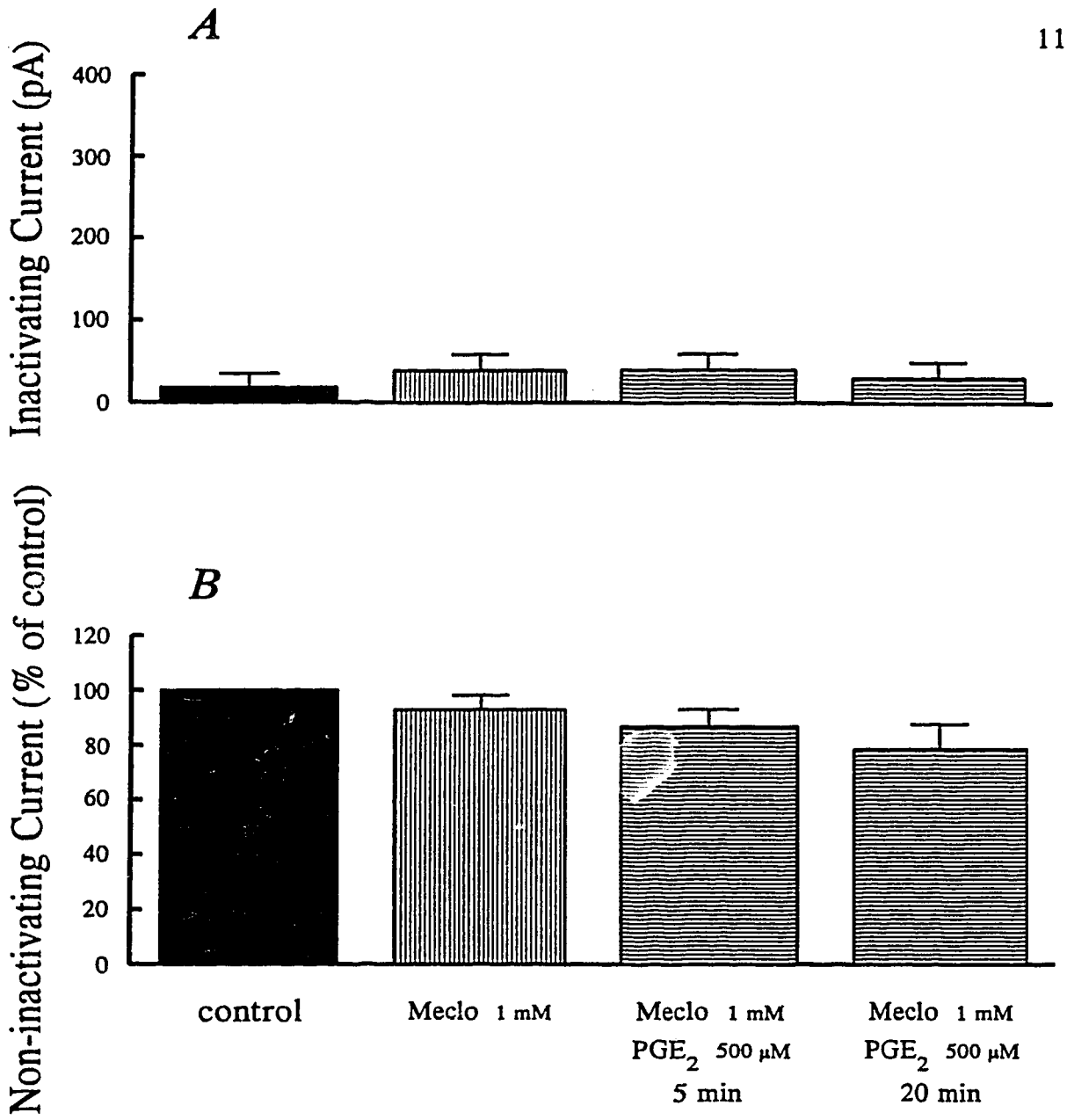

Figure 4-4. Effect of meclofenamate (Meclo) on PGE_2-induced modulation of K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. The cells were pretreated with 1 mM meclofenamate, added to the bath solution, for 10 minutes, then exposed to PGE_2 (500 μM). Currents were recorded prior to the addition of meclofenamate (control, n = 6) and prior to PGE_2 application (Meclo, n = 6), and 5 min (n = 6) or 20 min (n = 5) after PGE_2 administration. The current amplitudes were measured following a test pulse of +20 mV, from a holding potential of -80 mV. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. $p > 0.05$ *vs.* control; $p > 0.05$ *vs.* Meclo.

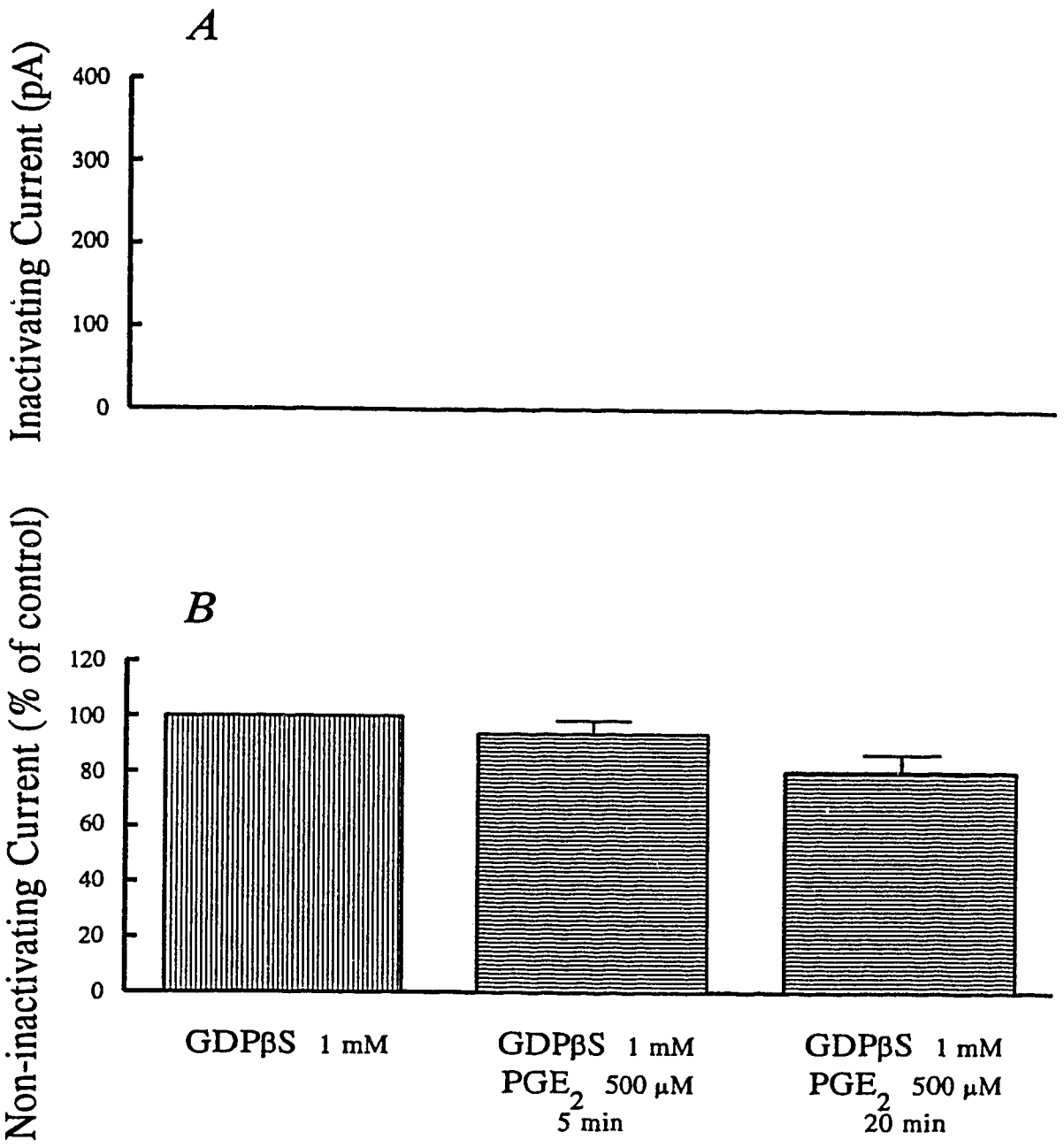

Figure 4-5. Effect of GDPβS on the modulation of K^+ currents by PGE_2 in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. The cells were pretreated for 5 minutes with intracellular application of GDPβS (1 mM), then exposed to PGE_2 (500 μM). Currents were recorded at a test potential of +20 mV, 5 min (n = 5) and 20 min (n = 5) after PGE_2 treatment. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. $p > 0.05$ *vs.* GDPβS.

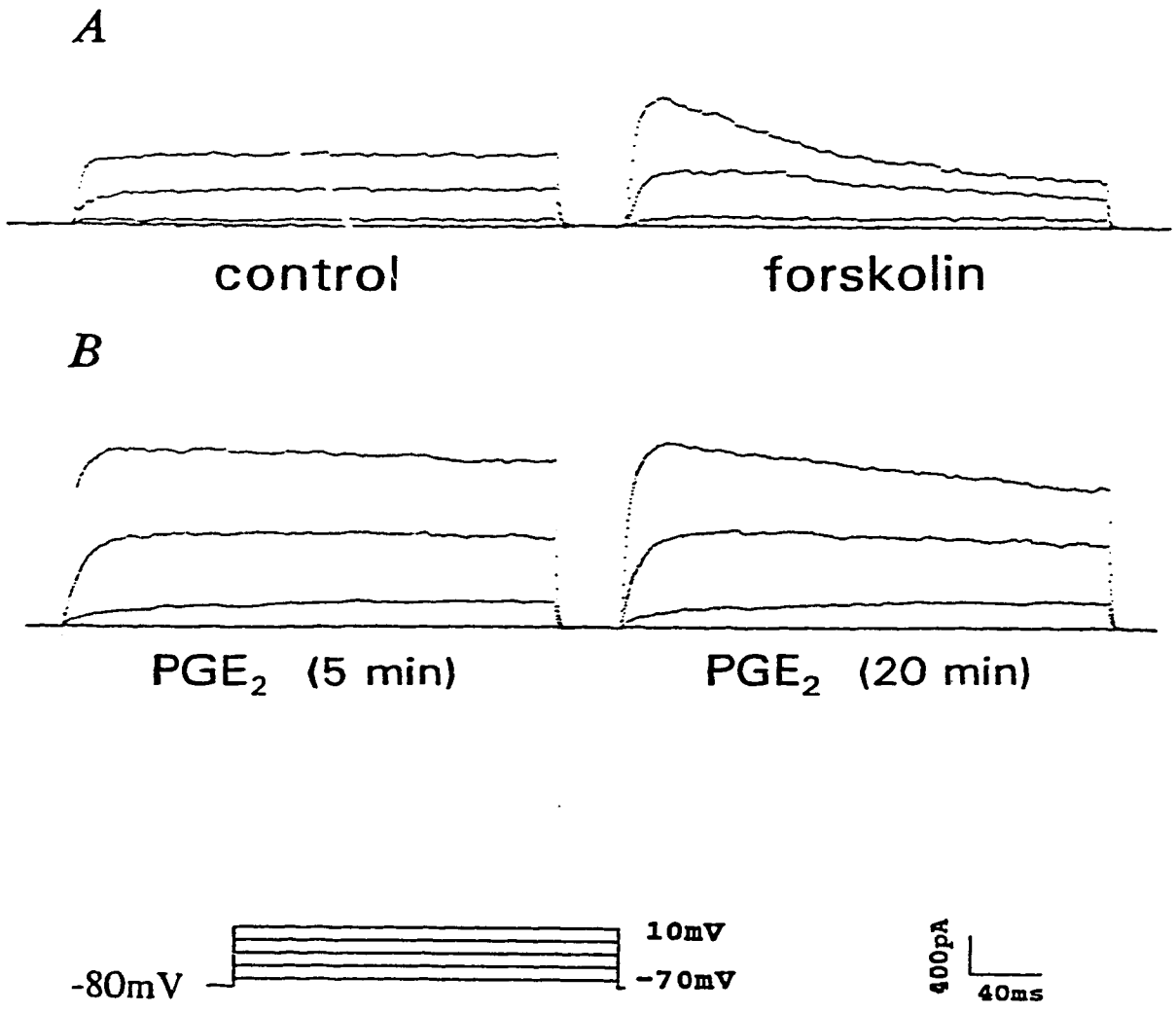

Figure 4-6. Effect of forskolin on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. *A*, The original currents were recorded (leakage corrected) 5 min after forskolin (1.65 μM) administration to the extracellular solution; *B*, The original currents were recorded (leakage corrected) 5 and 20 min after PGE_2 (500 μM) treatment.

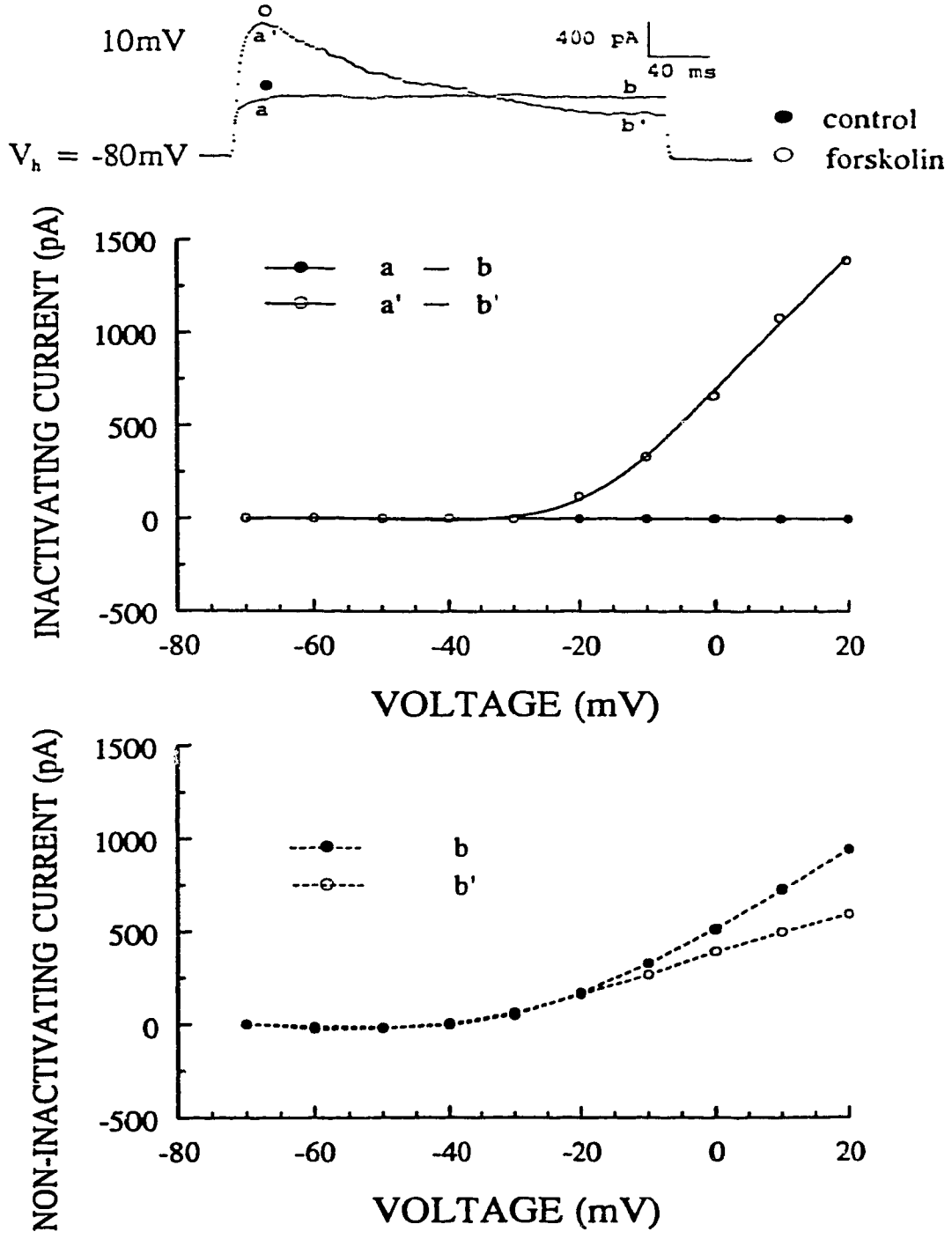

Figure 4-7. I-V relationship of the effect of forskolin on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. Forskolin (1.65 μM) was administered to the external solution. Solid circles represent the current before treatment with forskolin, and open circles represent the current after administration of forskolin. *A*, fast-inactivating current; *B*, non-inactivating current.

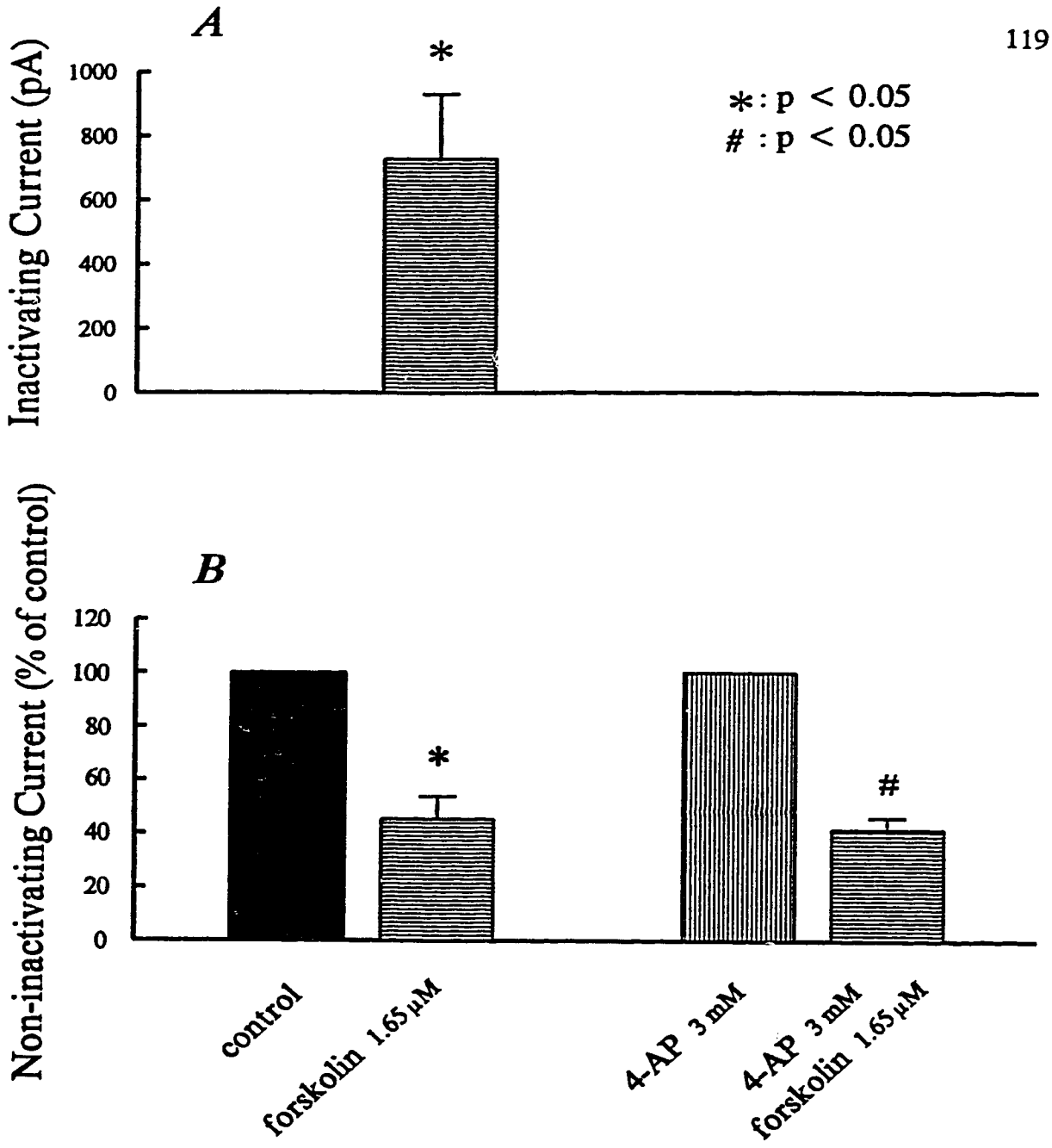

Figure 4-8. Summary of the effects of forskolin and 4-AP on K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. 4-AP (3 mM) was added to the bath for at least 10 min to pretreat the cells. Forskolin (1.65 μM) was added to the bath either with untreated cells (control, n = 5) or 4-AP pretreated cells (4-AP, n = 3). The current amplitudes were measured 5 min after forskolin administration when depolarizing the membrane to +10 mV. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ *vs.* control; #: $p < 0.05$ *vs.* 4-AP.

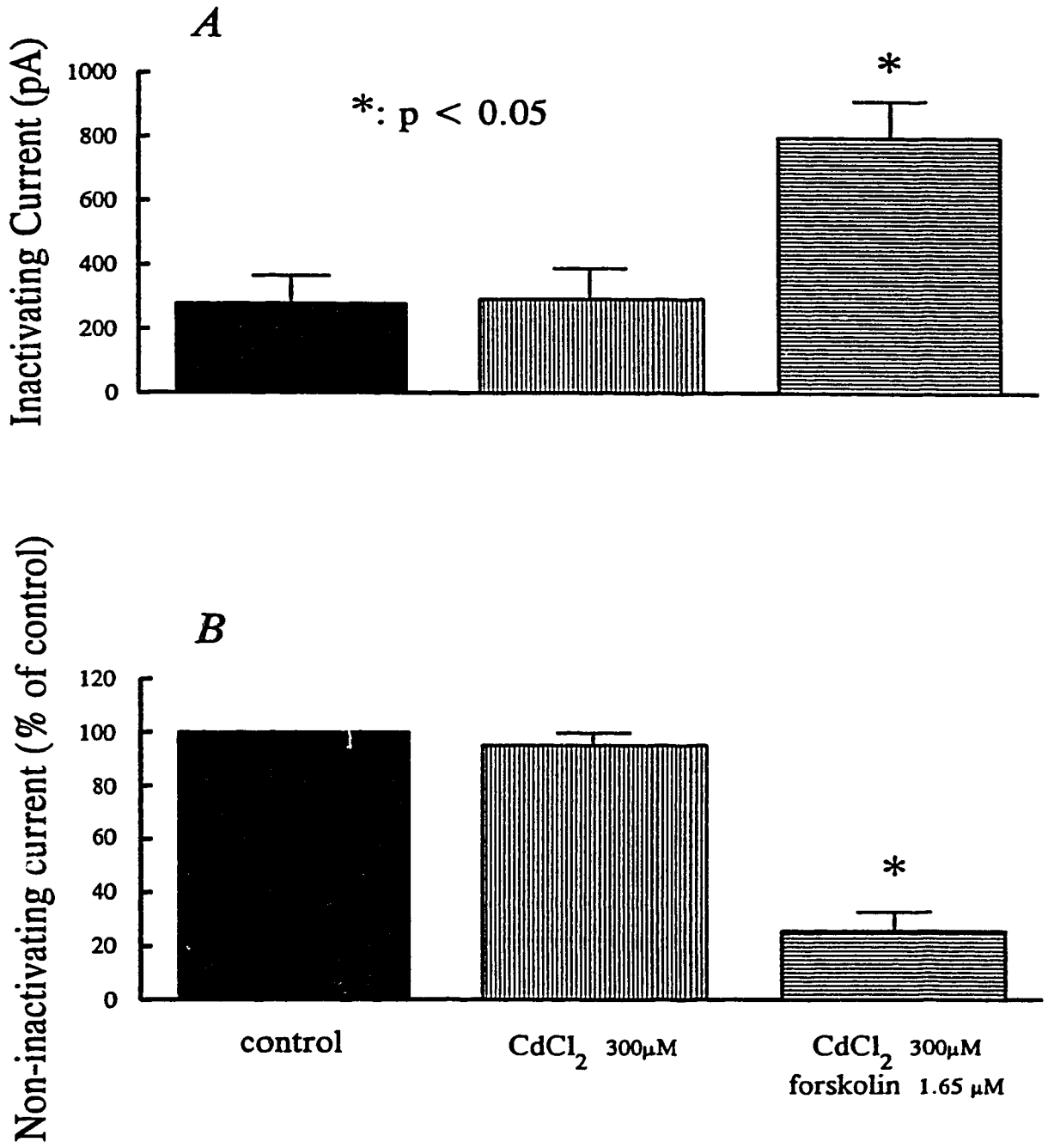

Figure 4-9. Effect of $CdCl_2$ on forskolin-induced changes of K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. The cells were treated with $CdCl_2$ (300 μM, n = 3) for 5 minutes before being exposed to forskolin (1.65 μM, n = 3). The amplitudes of the currents were recorded at a test pulse of 0 mV, from a holding potential of -80 mV. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ *vs.* control or $CdCl_2$.

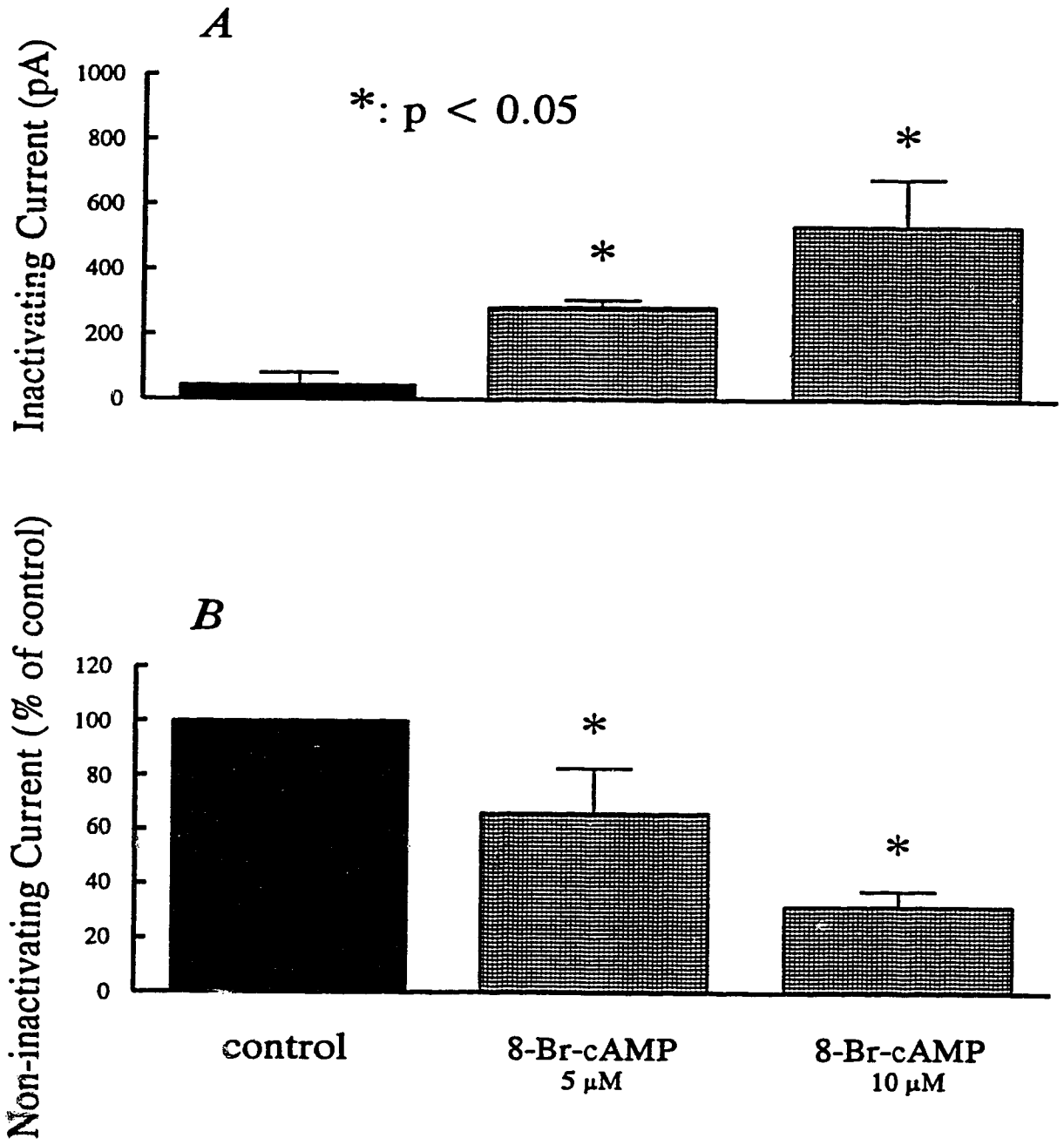

Figure 4-10. Effect of 8-Br-cAMP on K^+ currents recorded from neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. 8-Br-cAMP (5 μM or 10 μM) was added to the bath and the amplitudes of the currents were recorded at a test potential of +20 mV after 5 minutes. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ compared with corresponding control. ($n = 5$)

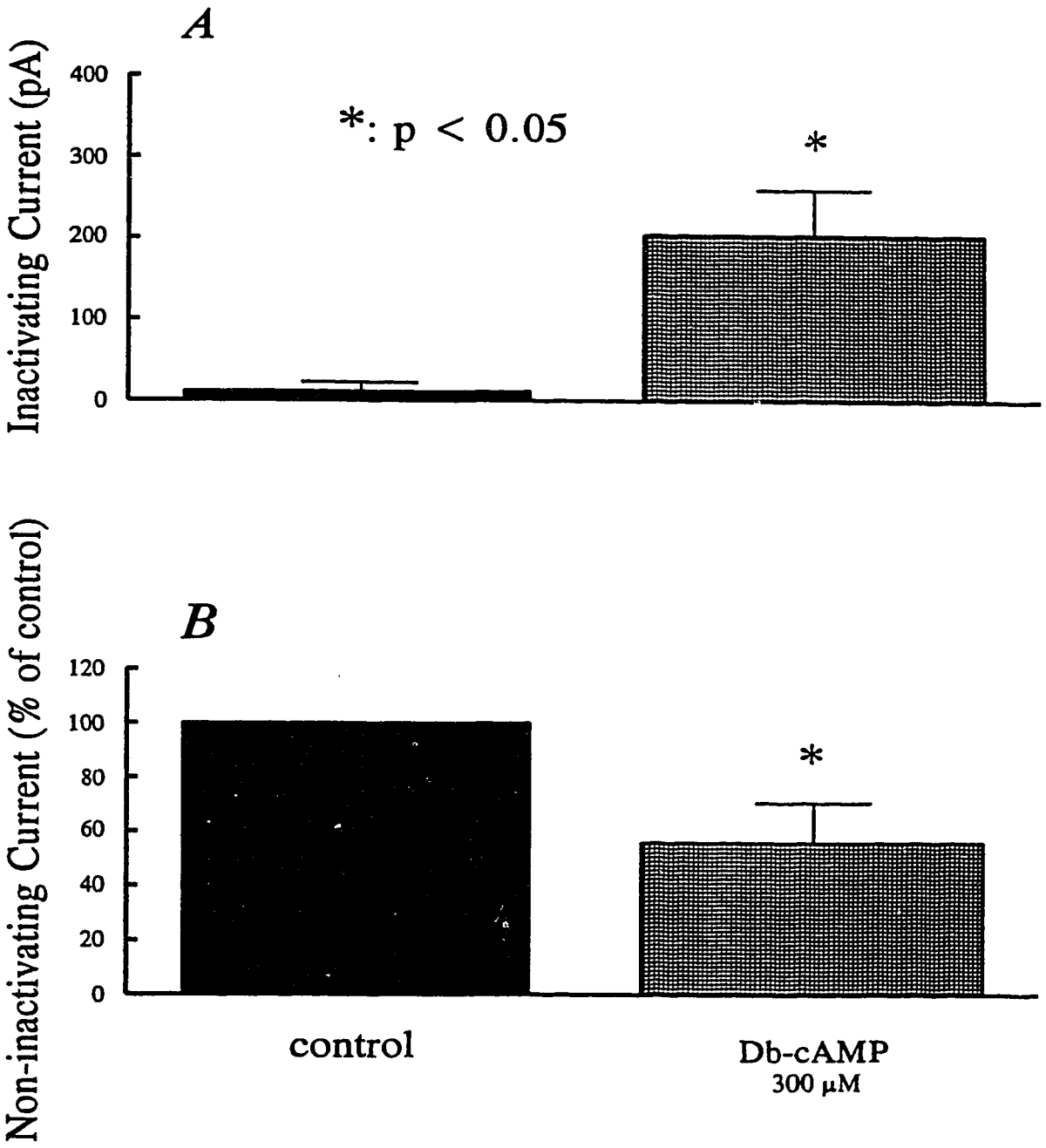

Figure 4-11. Effect of Db-cAMP on K^+ currents recorded from neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. Db-cAMP 300 μM was added to the bath and the current amplitudes were measured at a test pulse of +20 mV 5 min later. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ *vs.* control. ($n = 5$)

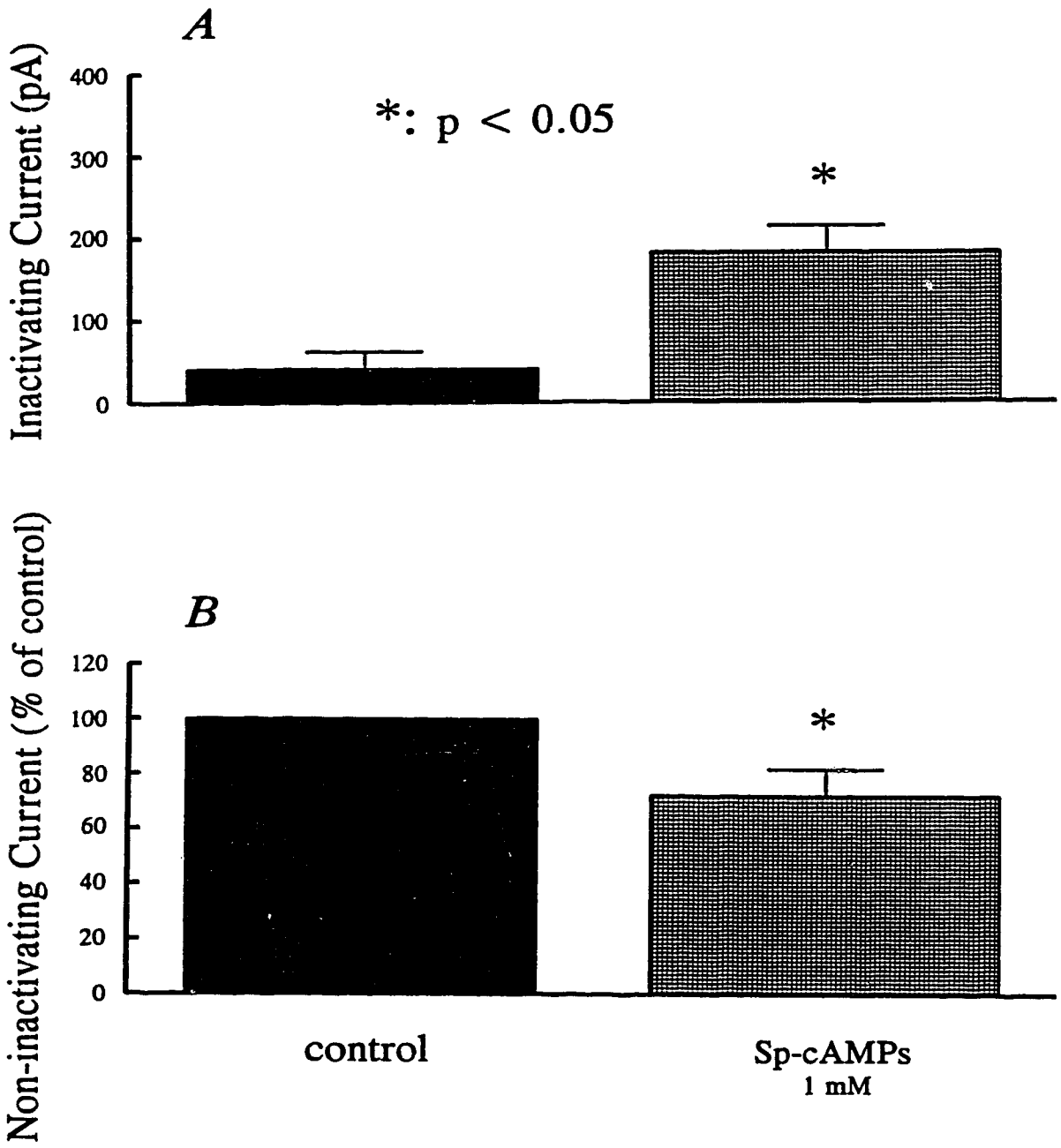

Figure 4-12. Effect of Sp-cAMPs on K^+ currents recorded from neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. Sp-cAMPs (1 mM) was added to the bath and the current amplitudes were measured at a test potential of +20 mV after 5 min. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ compared with corresponding control. ($n = 8$)

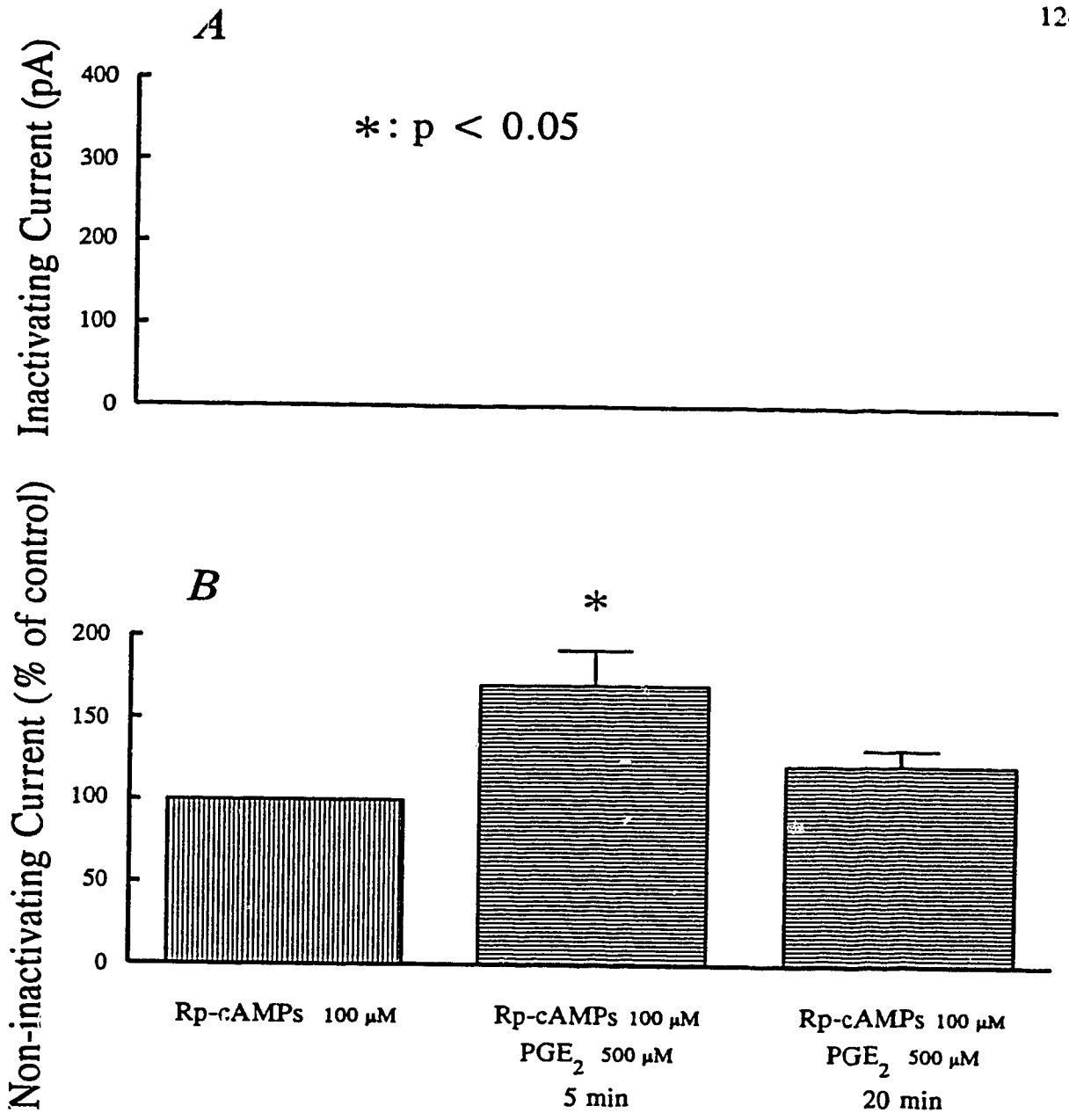

Figure 4-13. Effect of Rp-cAMPs on PGE_2-induced modulation of K^+ currents in neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. Rp-cAMPs (100 μM) was applied via the pipette solution. 5 minutes after penetrating the membrane, PGE_2 (500 μM) was added to the bath. The amplitudes of the currents were measured at a test potential of +20 mV prior to PGE_2 application (Rp-cAMPs, n = 5) and 5 min (n = 5) or 20 min (n = 5) after PGE_2 administration (PGE_2). *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *:$p < 0.05$ *vs.* Rp-cAMPs.

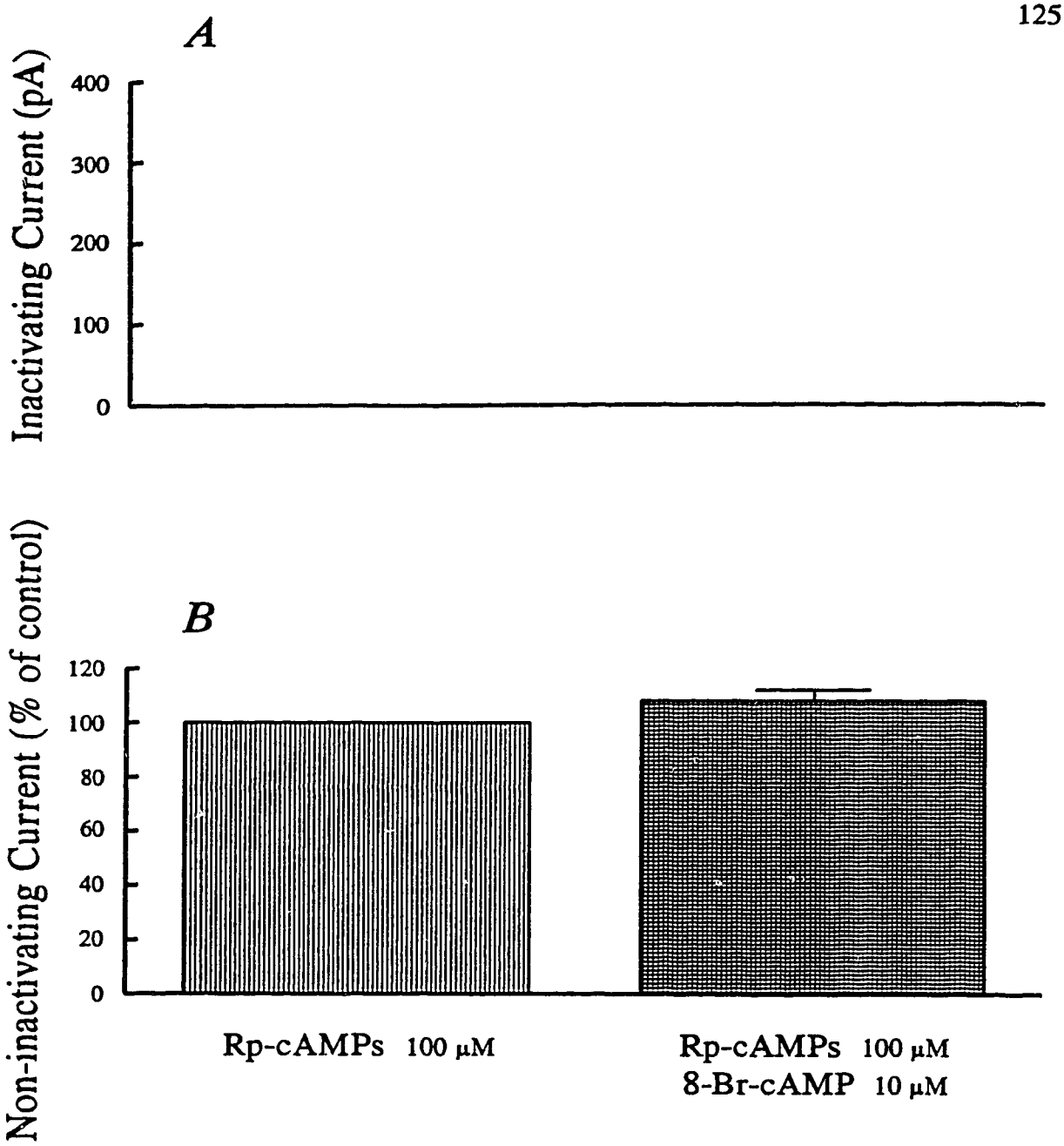

Figure 4-14. Effect of 8-Br-cAMP on K^+ currents in Rp-cAMPs pretreated neurohypophyseal nerve terminals under low $[Ca^{2+}]_i$. Rp-cAMPs (100 μM) was applied via the pipette solution. 8-Br-cAMP (10 μM) was added to the bath 5 min after penetrating the cell membrane. The amplitudes of the currents were recorded at a test pulse of +20 mV before (Rp-cAMPs, n = 6) and 5 min (n = 6) after 8-Br-cAMP administration. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. $p > 0.05$ *vs.* Rp-cAMPs.

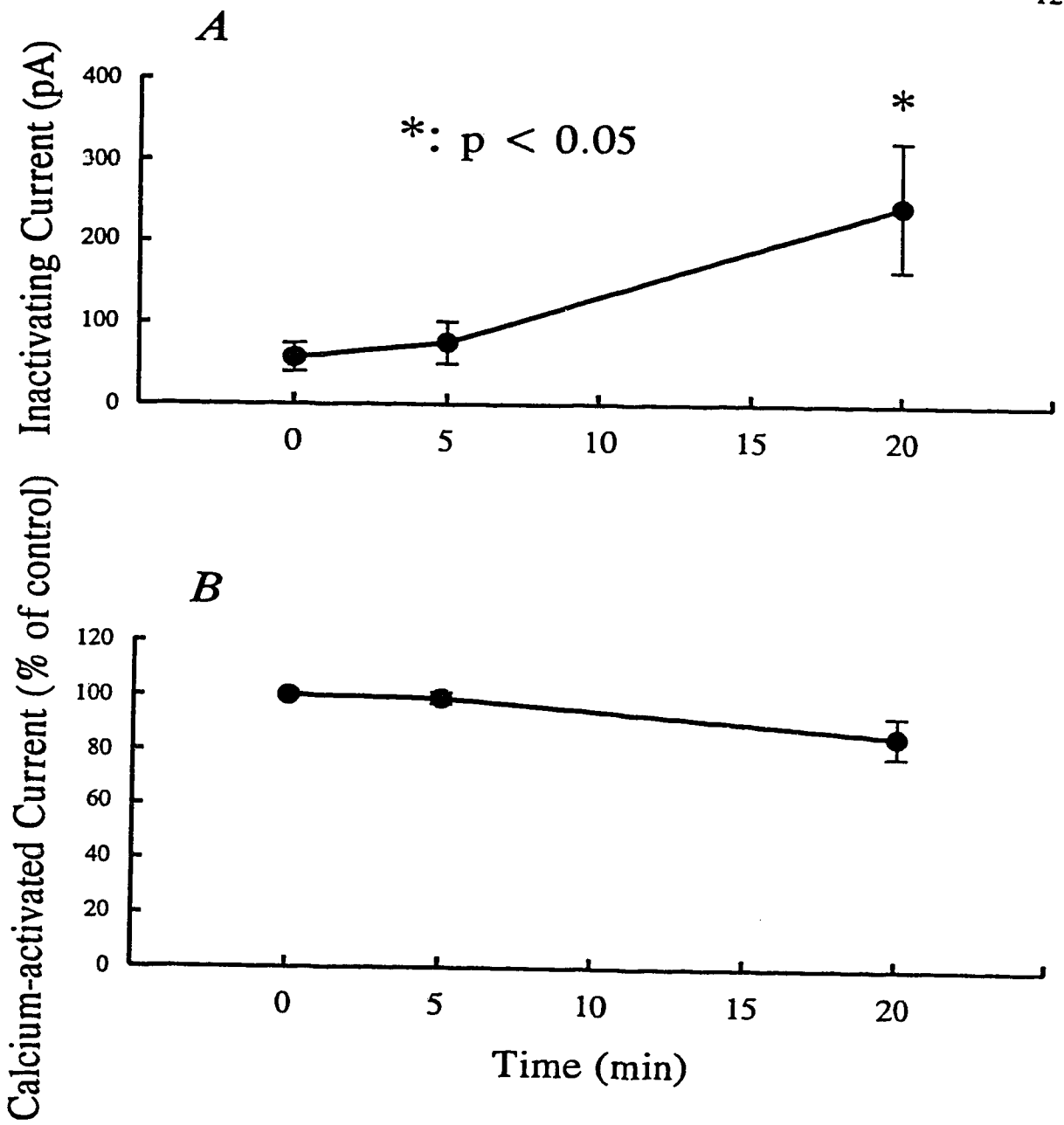

Figure 4-15. Effect of PGE_2 on K^+ currents in neurohypophyseal nerve terminals under high $[Ca^{2+}]_i$. The external solution contains a $[Ca^{2+}]_o$ of 10 mM, and the pipette solution contains 1.1 mM EGTA. PGE_2 (50 μM, n = 5, or 250 μM, n = 5) was added to bath. Currents were recorded at test potential of +10 mV 5 min and 20 min after PGE_2 application. *A*, fast-inactivating current; *B*, non-inactivating current normalized to the corresponding control. *: $p < 0.05$ *vs.* control.

CHAPTER V

GENERAL DISCUSSION AND SUMMARY

5.1. GENERAL DISCUSSION

The nerve terminals of the posterior pituitary are the axon terminals of hypothalamic neurosecretory cells. They store and release the neuropeptides vasopressin (ADH) and oxytocin (OXT), which are synthesized within the cell bodies of the neurons in the supraoptic and paraventricular nuclei of the hypothalamus. The arrival of presynaptic action potentials induces the depolarization of these nerve endings (Salzberg *et al.*, 1983), and hence, promotes the entry of calcium into the nerve terminals to trigger the release of neurosecretory vesicular contents via exocytosis (Nordmann, 1983; Brethes *et al.*, 1987; Nordmann *et al.*, 1987; Dayanithi *et al.*, 1988; Stuenkel and Nordmann, 1993*a*). However, the details of how ionic currents control the release of neuropeptides remain largely unknown.

It has been well demonstrated that this Ca^{2+}-dependent secretion from nerve endings is subject to alteration by previous electrical activity (Bondy *et al.*, 1987; Cazalis *et al.*, 1985; Nordmann and Stuenkel, 1986) and by chemical signals (Bondy *et al.*, 1989). Many substances have been suggested to exert their actions on the posterior pituitary via modulation of K^+ channel activities, which leads to either physiological or pharmacological effects on hormone release. For example, prostaglandin E_2 (PGE_2) has been reported to either facilitate (Gagnon *et al.*, 1973;

Ishikawa *et al.*, 1981; Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989; Bojanowska and Guzek, 1991) or inhibit (Fujimoto *et al.*, 1978; Rutecki *et al.*, 1982; Kasting *et al.*, 1985; Knepel *et al.*, 1985) ADH and/or OXT secretion. Conductances of ion channels such as Ca^{2+} and K^+ channels, are the major determining factor in the establishment of cellular excitability, and thus, synaptic efficiency and hormone release. In this thesis, K^+ channels in the membrane of neurohypophyseal nerve terminals have been characterized, and the effects induced by PGE_2 on K^+ channels have been investigated.

5.1.1 Characterization of the Voltage-Dependent Outward K^+ Channels in Neurohypophyseal Nerve Terminals

K^+ channels have the capacity to serve as transducers, both for signals that are chemical in nature and for signals that are encoded in the frequency or pattern of electrical activity. Blockade of K^+ channels or inactivation of K^+ currents broadens action potentials (Bondy *et al.*, 1987; Salzberg and Obaid, 1988; Jackson *et al.*, 1991), thus facilitating neuropeptide release from the posterior pituitary (Bondy *et al.*, 1987; Hobbach *et al.*, 1988). In order to understand the regulation of secretion from the posterior pituitary, it is of particular importance to investigate how the K^+ channels cooperate with each other in nerve endings, and what the mechanisms are for regulating different types of K^+ channels.

One major focus of this thesis was to characterize the voltage-dependent outward K^+ channels in neurohypophyseal nerve terminals. The small size and

inaccessibility of most nerve endings have precluded direct analysis of membrane ionic currents and their influence on secretion until the method of isolating neurosecretory nerve terminals from the crab sinus gland (Lemos *et al.*, 1986*b*; Nordmann *et al.*, 1986; Stuenkel *et al.*, 1990) and the rat posterior pituitary (Lemos and Nordmann, 1986; Nordmann *et al.*, 1987) had been established. The high density and large size (mean diameter of 2 μm) (Nordmann, 1977) of the rat neurohypophyseal nerve terminals (the release site), which can be easily separated from the axon (the transport site) and from the neuronal cell bodies (the site of synthesis), make this an excellent preparation for studies using the patch-clamp technique. By immunohistochemistry, this preparation has been judged to be nearly exclusively nerve endings (Nordmann *et al.*, 1987; Wang X. *et al.*, 1991*a*, 1993*b*). Two types of Ca^{2+} channels (Lemos and Nowycky, 1989; Wang X. *et al.*, 1992, 1993*a*), three groups of K^+ channels (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992) and one Na^+ channel (Nordmann and Dyball, 1978) have been characterized in rat neurohypophyseal terminals, and various work has been carried out to study the action of different substances, such as ω-conotoxin, funnel web spider toxin, ethanol and tetrandrine on K^+ channels (Treistman *et al.*, 1991; Wang G. and Lemos, 1992; Wang G. *et al.*, 1993), Ca^{2+}-channels (Treistman *et al.*, 1991; Wang X. *et al.*, 1991*b,c*, 1992, 1994; Wang G. and Lemos, 1992, 1994; Wang G. *et al.*, 1993; Lemos *et al.*, 1994) and the Na^+ channel (Wang G. *et al.*, 1993). This work has extended our knowledge of electrical activity in the posterior pituitary.

From a holding potential of -80 mV, which is similar to the normal terminal

resting potential, stepwise depolarization generated a voltage-dependent outward K^+ current. The close agreement between the reversal potential of the outward currents and the K^+ equilibrium potentials under different experimental conditions, and the resistance of this outward currents to the Cl^- channel blocker 4-acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) provided good evidence that K^+ was the primary conducting ion responsible for this outward current. At least three distinct voltage-dependent K^+ channels can be distinguished, based on the activation and inactivation kinetics, Ca^{2+} sensitivity and antagonist sensitivity, in the membrane of rat neurohypophyseal nerve terminals in this investigation. These findings are consistent with those reported elsewhere (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992).

The fast-inactivating K^+ current quickly reached a peak and then decayed within 50 ms. This transient outward current showed steady-state inactivation at more depolarized (i.e. more than -80 mV) holding potentials with 50% inactivation occurring at -49.6 mV, which was very similar to that reported in a similar preparation (Thorn *et al.*, 1991), while more depolarized than that reported in the thin slice preparation (Bielefeldt *et al.*, 1992). 4-aminopyridine (4-AP) could block this fast-inactivating K^+ current in a dose-dependent manner, and the concentrations of 4-AP used were in the same range as reported in a similar preparation (Thorn *et al.*, 1991). However, this fast-inactivating component was resistant to α-dendrotoxin (α-DTX), a venom toxin which preferably inhibits the transient outward K^+ channels (Benishin *et al.*, 1988).

The non-inactivating current reached its peak more slowly and did not inactivate within 280 ms. The non-responsiveness of this K^+ current to the Ca^{2+} channel antagonists nifedipine, La^{3+} and Cd^{2+}, and the Ca^{2+} chelator ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) under the conditions of low $[Ca^{2+}]_i$ used, indicated a lack of Ca^{2+} dependency. This non-inactivating component could be blocked by tetraethyl ammonium chloride (TEA), in a concentration-dependent manner, which was consistent with the results reported from thin slice preparations of the posterior pituitary (Bielefeldt *et al.*, 1992), and with the sensitivity of hormone release to TEA (Bondy *et al.*, 1987; Hobbach *et al.*, 1988), but not with studies using a similar preparation (Thorn *et al.*, 1991). Besides its primary effect on fast-inactivating current, 4-AP could partially inhibit this non-inactivating current. However, neither α- nor β-DTX, a venom toxin which preferably blocks delayed rectifier K^+ channels (Benishin *et al.*, 1988), had an effect on this non-inactivating current. This finding contradicted the reported discovery that α-DTX could block the non-inactivating component in thin slices of the neurohypophysis (Bielefeldt *et al.*, 1992).

The Ca^{2+}-activated K^+ channel was also recorded, when cells were buffered with a lower pipette concentration of EGTA (1.1 mM), combined with a higher extracellular Ca^{2+} concentration of 10 mM, in order to achieve a high intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). This Ca^{2+}-activated K^+ component was sensitive to application of the Ca^{2+} channel blockers nifedipine and Cd^{2+}, suggesting its activation was secondary to the entry of extracellular Ca^{2+}. This neurohypophyseal

terminal Ca^{2+}-activated K^+ channel may be a novel type of K_{Ca}, since apamin, a small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) blocker (Blatz and Magleby, 1987; Cook and Quast, 1990), and charybdotoxin, a big-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) blocker (Valdivia *et al.*, 1988), failed to affect it (Bielefeldt *et al.*, 1992; Wang G. *et al*, 1992).

The macroscopic K^+ currents reported here are similar in some respects to those described by Thorn *et al.*, (1991), Bielefeldt *et al.*, (1992), and Wang G. *et al*, (1992). However, there are some pharmacological discrepancies, such as the relative sensitivity to both TEA and the dendrotoxins. Several factors may account for these discrepancies: 1. the strain of animal used -- in the Thorn *et al.* (1991) and Wang G. *et al.* (1992) studies, CD (Charles River) rats were used, while in the present study, SD rats were used. 2. the isolation procedure -- Bielefeldt *et al.* (1992) used the thin slice preparations of the rat posterior pituitary, whereas an acute dissociation procedure was employed in the present study. The contradictory results recorded from an intact or dissociated preparation raise the possibility that various treatments may affect K^+ channels differently, or that there are differences between the vasopressin and oxytocin terminals, which could be sampled from unequally, depending on whether one is recording from an intact, or dissociated preparation. Therefore, caution needs to be taken in extrapolating the electrophysiological evidence from single cells to the intact tissue.

It is noteworthy that the non-inactivating current exhibited rundown at a rate of 1%/min, when cells were dialysed with an intracellular solution containing 11 mM

BAPTA and an extracellular solution containing 2 mM Ca^{2+} to obtain a low $[Ca^{2+}]_i$. The rundown is not significant under conditions of high $[Ca^{2+}]_i$, suggesting that the cells are more stable with higher level of $[Ca^{2+}]_o$ and that Ca^{2+} increases the stability for K^+ channel gating. Nevertheless, this must be considered when testing the efficiency of various drugs.

The characterization of voltage-dependent K^+ channels in nerve endings provides useful information on the role of voltage-dependent K^+ channels played in controlling neurotransmitter release from the neurohypophysis in response to various agents. Further studies employing single-channel recordings would help to identify the different subtypes of K^+ channels involved.

5.1.2 The Effects of PGE_2 on Voltage-Dependent Outward K^+ Current in Neurohypophyseal Nerve Terminals

The posterior pituitary contains large amounts of PGE_2 (Horton, 1969; Narumiya *et al.*, 1982; Ogorochi *et al.*, 1984; Watanabe *et al.*, 1985). However, the fact that PGE_2, a putative local hormone, elicited either stimulatary (Gagnon *et al.*, 1973; Ishikawa *et al.*, 1981; Negro-Vilar *et al.*, 1985; Bojanowska and Guzek, 1989, 1991) or inhibitory (Fujimoto *et al.*, 1978; Rutecki *et al.*, 1982; Kasting *et al.*, 1985; Knepel *et al.*, 1985) influences on the release of neuropeptide from the neurohypophysis raises questions regarding the physiological roles and the mechanisms of action of PGE_2 in the posterior pituitary.

K^+ channels are distributed on the neurohypophyseal nerve terminal plasma

membranes, where they play a key role in controlling many cellular functions, and most importantly, the secretion of hormones (Thorn *et al.*, 1991; Bielefeldt *et al.*, 1992; Wang G. *et al.*, 1992). PGE_2 has been demonstrated to block certain kinds of K^+ channels in lymphocytes (Bastin *et al.*, 1990), neurons (Fowler *et al.*, 1985), brain synaptosomes (Ren and Benishin, 1994), and smooth muscle cells (Ren, 1994), while enhancing the activity of some K^+ channels in cortical synaptosomes (Zoltay and Cooper, 1994) and *Xenopus* oocytes (Mori *et al.*, 1989; Guillemare *et al.*, 1994). It has been suggested that each of the three types of K^+ channels identified in neurohypophyseal nerve terminals plays a slightly different role in controlling the membrane potential. Therefore, it is speculated that PGE_2 may exert its neurohypophyseal actions through the modulation of K^+ channels. Up-to-date, there are not any reports on the effect of PGE_2 on K^+ channels in neurohypophyseal nerve terminals. In this thesis, the effects of PGE_2 on the voltage-dependent outward K^+ channel currents from the nerve endings of the posterior pituitary have been thoroughly investigated, in order to gain more information on the ionic basis of its neurohypophyseal actions.

The activation of fast-inactivating K^+ channels, which are the major current carrier during neurohypophyseal repolarization after a spike, opposes the tendency toward depolarization (Thorn *et al.*, 1991). By retarding the production of successive spikes in a train, the transient K^+ current tends to act as a brake on spike discharge as well as limits spike frequency (Connor and Stevens, 1971; Rogawski, 1985; Jan and Jan, 1989), and, thus decreases the transmitter release. PGE_2 increased the fast-

inactivating component gradually. These effects were not Ca^{2+}-dependent, which was consistent with the discovery when using cyclic adenosine 3',5'-monophosphate (cAMP) analogues to mimic the long term PGE_2 action (described below). The fact that the time course for the significant enhancement of fast-inactivating K^+ channels, which occurred 20 min after administration of PGE_2 in this investigation, coincides with the time course of inhibited ADH secretion induced by PGE_2 (Knepel *et al.*, 1985), suggests that PGE_2 is capable of slowing down action potential generation, thus diminishing transmitter release from the posterior pituitary.

The activation of the non-inactivating K^+ channel is involved in the fatigue of stimulus-secretion coupling in the posterior pituitary (Bielefeldt *et al.*, 1992). Opening the non-inactivating K^+ channel could raise the threshold for action potential generation in neurohypophyseal nerve endings (Bielefeldt *et al.*, 1992), and, then, limit secretion toward the end of a long burst (Bicknell *et al.*, 1984). PGE_2 initially facilitated the non-inactivating component, and this process is Ca^{2+}-sensitive. Then, the enlarged non-inactivating component declined at a rate which was greater than that of normal rundown (1 %/min). These effects were not Ca^{2+}-dependent, which was consistent with the fact when using cAMP analogue to mimic the long term PGE_2 action (described below). The facilitatory effect of PGE_2 on neurohypophyseal hormone release (Negro-Vilar, *et al.*, 1985) could be accounted for by the decreased non-inactivating component found in present studies. A decrease of this current, which is able to lower the threshold for action potential generation, occurred following a similar duration of PGE_2 incubation as that used in Negro-Vilar *et al.*

(1985) study.

The activation of Ca^{2+}-activated K^+ channels (K_{Ca}) produces a long-lasting hyperpolarization (Jackson *et al.*, 1991), which contributes to action potential repolarization. Ca^{2+}-activated K^+ currents were found to comprise 20% of the overall non-inactivating currents under conditions of high $[Ca^{2+}]_i$, as analyzed in chapter 3. According to the Ca^{2+} hypothesis of exocytosis, the entry of Ca^{2+} into nerve terminals of the posterior pituitary activates a Ca^{2+}-activated cation channel (this is most likely to be the Ca^{2+}-activated K^+ channel) in the membranes of neurosecretory vesicles which contain the hormone, and this promotes the entry of cations and anions into the vesicle to induce vesicle swelling, and to cause exocytosis (Lemon *et al.*, 1989; Lee *et al.*, 1992; Nordmann and Artault, 1992). Therefore, the Ca^{2+}-activated component is very important in the membrane of the vesicles, but it may not be as crucial to the establishment of cellular excitation in neurohypophyseal nerve endings. Also, PGE_2 failed to affect Ca^{2+}-activated K^+ channels in this study, described in chapter 4, indicating that the regulation of hormone release induced by PGE_2 may not through Ca^{2+}-activated K^+ channels.

The modulating actions of PGE_2 on K^+ channels in these nerve endings indicates the feasibility of extrapolating the present findings to the biological effects, *in vivo* or *in vitro*, elicited by PGE_2, on the neurohypophysis. Following different time courses of incubation, PGE_2 regulated different components of the K^+ current differently, which independently controlled the transmitter release, and, thus, it is assumed that PGE_2 differently modulates transmitter release. It is hypothesized that

the overall role of PGE_2 on the posterior pituitary is dependent upon the modulation of K^+ channels and which effect is predominant. However, caution must be used when applying the results of single cell recording to the intact tissue. Because of the various experimental procedures and conditions, it is very difficult to account for these divergent results from all the references cited. The discrepancies may come from the species or sex of the experimental animals and the method of PGE_2 introduction. It is also possible that PGE_2 affects K^+ channels in separate populations of vasopressin and oxytocin terminals differently. The duration of the tissue exposure to PGE_2 and the conditions under which transmitter release was measured may also contribute to the diverse and sometimes opposite effects of PGE_2. Various stimuli used in different experiments may vary K^+ channel activity prior to PGE_2 application, and alter the effects of PGE_2 on K^+ channels as well as hormone release. Although present studies provide a clue to elucidate the physiological roles and the mechanisms of the action of PGE_2 on the posterior pituitary, it does not exclude the possibility that the conflicting effects induced by PGE_2 in different experiments may also be through PGE_2 regulation of other ion channels, such as Ca^{2+} channels and Na^+ channels.

5.1.3 The Mechanism(s) Underlying the Modulation of K^+ Channels by PGE_2 in Neurohypophyseal Nerve Terminals

Further studies on the cellular mechanism of the modulation of K^+ channels induced by PGE_2 demonstrated that the PGE_2 receptor antagonist, meclofenamate

(Zelinski-Wooten *et al.*, 1990), and the regulatory G protein inhibitor, guanosine-5'-O-(2-thiodiphosphate) (GDPβS) (Vaca *et al.*, 1992; Fan *et al.*, 1993; Hamada *et al.*, 1993; Wu and Assmann, 1994), completely blocked the PGE_2-induced modulation of K^+ channels. Taken together, these findings were in agreement with the well-described classical model of action for PGE_2: i.e. as a local hormone, PGE_2 exerts its biological action through binding of the specific surface membrane receptors (EPs) (Shimizu *et al.*, 1979), which may be similar with the peripheral prostaglandin receptors (Gagnon *et al.*, 1973), such as EP_1, EP_2, EP_3 and EP_4 (Coleman *et al.*, 1987, 1994*a*), and activation of certain membrane G proteins (Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990), to regulate neurohypophyseal function (Haluska *et al.*, 1989; Smith, 1989), although which G protein subtype involved in the modulation of K^+ channel activity was not investigated. However, the question whether the G protein(s) are directly coupled to an ion channel or coupled to a second messenger system(s) still remains elusive, although some evidences provided below support the latter.

PGE_2 activity has been well documented to result in the accumulation of cAMP via stimulation of adenylate cyclase in diverse tissues (Samuelsson *et al.*, 1978; Saito *et al.*, 1985; Almawi *et al.*, 1987; Yu *et al.*, 1988; Bernal *et al.*, 1990; Dixon *et al.*, 1990; Dore-Duffy and Donovan, 1991; Hagel-Bradway *et al.*, 1991; Ploeg *et al.*, 1991; Coleman *et al.*, 1994*b*; Kunapuli *et al.*, 1994), although, in rare cases, PGE_2 could inhibit cAMP accumulation (Melien *et al.*, 1988; Nakao *et al.*, 1989; Negishi *et al.*, 1989). cAMP and cAMP dependent protein kinase (PKA)

activation have been reported to regulate certain voltage-dependent K^+ channels in various types of cells (Seelig and Kendig, 1982; Dunlap, 1985; Van Renterghem *et al.*, 1985; Choquet *et al.*, 1987; Grega and MacDonald, 1987; Ohya *et al.*, 1987; Choquet and Korn, 1988; Soliven *et al.*, 1988). It has been shown that cAMP and PKA phosphorylation of the channel protein mediated the modulation of K^+ channels by PGE_2 in human lymphocytes (Bastin *et al.*, 1990), *Xenopus* oocytes (Miledi and Woodward, 1989; Mori *et al.*, 1989) and rat smooth muscle cells (Ren, 1994), and that EPs were coupled to adenylate cyclase activation in the posterior pituitary (Malet *et al.*, 1982). The discovery that application of 0.25 mM cAMP to intracellular solutions (Thorn *et al.*, 1991) increases the incidence of transient K^+ channel activation, while, as shown in this study normally only 1/3 of the cells recorded from exhibit this current, gives a hint that cAMP may play a role as a putative second messenger in the activation of the fast-inactivating K^+ channel in neurohypophyseal nerve terminals. In this thesis, intensive pharmacological studies have been performed to elucidate the involvement of cAMP accumulation and the subsequent activation of PKA in the modulation of K^+ currents induced by PGE_2. Studies using the cAMP agonists, forskolin (de Souza *et al.*, 1983; Henquin *et al.*, 1983; Laurenza *et al.*, 1989), 8-Br-cAMP (Brautigan *et al.*, 1991; Chneiweiss *et al.*, 1991), Db-cAMP (Posternak and Weiman, 1974; Hei *et al.*, 1991) and Sp-cAMPs (Dostmann *et al.*, 1990; Tsien *et al.*, 1992) to mimic, and the PKA inhibitor, Rp-cAMPs (Butt *et al.*, 1990; Dostmann *et al.*, 1990; Wang L-Y. *et al.*, 1991) to abolish the long-term effects of PGE_2 on K^+ channels (i.e. the increase of the 4-AP sensitive fast-

inactivating component), and the simultaneous inhibition of the non-inactivating component, provided direct evidence that the cAMP cascade is involved in the modulation of K^+ channels, and that the long-term PGE_2-induced regulatory actions are mediated by the activation of cAMP and the subsequent activation of PKA, although a possible direct effect of cAMP on K^+ channels should not be excluded. The finding that neither the increase of the fast-inactivating component, nor the decrease of the non-inactivating component induced by forskolin were Ca^{2+}-dependent was consistent with the lack of Ca^{2+}-dependency of the long-term effects of PGE_2 on K^+ channels. This was in line with the finding that G protein(s) are involved in the modulation of K^+ channels by PGE_2, as uncoupling of G protein subunits directly leads to the activation of adenylate cyclase.

The fact that intracellular application of Rp-cAMPs, to block PKA stimulated phosphorylation, did not affect the PGE_2-induced short-term enhancement of the non-inactivating component ruled out the possibility that the cAMP-PKA cascade mediated this effect. The mechanism(s) underlying this short-term effect remain unknown.

It is commonly believed that the diverse and sometimes opposite effects of PGE_2 on cell function could be attributed to its interaction, via G proteins, with not only the cAMP cascade but also the protein kinase C (PKC) cascade (Haluska *et al.*, 1989; Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990; Goureau *et al.*, 1992). Similar to the cAMP-PKA system, PKC is another signalling system in which that G protein(s) may be involved to couple the PGE_2 receptor to phospholipase C (PLC) (Gilman, 1987). Studies have demonstrated that PGE_2 can stimulate PLC activation

and thus induce phosphatidyl inositol (PI) hydrolysis, resulting in the formation of diacylglycerol (DG) and inositol trisphosphate (IP_3) which leads to the activation of PKC and elevation of $[Ca^{2+}]_i$ (Sonnenburg and Smith, 1988; Yamaguchi *et al.*, 1988; Yokohama *et al.*, 1988; Aboolian *et al.*, 1989). There has also been some evidence suggesting that PGE_2 could modulate K^+ channel activity through the activation of PKC in rat brain synaptosomes (Ren and Benishin, 1994) and in rat tail artery vascular smooth muscle cells (Ren *et al.*, 1995*a*). In addition to interaction with these two signal transduction pathways, PGE_2 has also been described to activate phospholipase D (PLD) (Wu *et al.*, 1992). Activation of PLD, however, is able to generate DG, and thus, activate PKC (Nishizuka, 1994). Although coupling of the PGE_2 receptors to the adenylate cyclase complex, through either G_s or G_i, is one possible explanation for modulation of K^+ channels and neuropeptide release induced by PGE_2, other signal transduction pathway(s) and "cross-talk" cannot be excluded. It is possible that the activation of other second messenger system(s) other than the cAMP-PKA cascade, eg. PKC pathway, can either alter various K^+ channel activities differently, or affect the activation of the cAMP-PKA pathway, and, thus contributes to the contradictory results of neurohypophyseal hormone secretion induced by PGE_2.

PGE_2 has previously been reported to induce membrane depolarization in NG-108-15 hybrid cells (Higashida *et al.*, 1984), neurones (Matsumura *et al.*, 1993) colon cells (Siemer and Gogelein, 1993) and smooth muscle cells (Ren *et al.*, 1995*b*), or hyperpolarization in *Xenopus* oocytes (Mori *et al.*, 1989) and epitheloid cells (Steidl *et al.*, 1991). The mechanism of PGE_2-induced depolarization is a result of

activation of inward cation channels (Matsumura *et al.*, 1993), while PGE_2-induced hyperpolarization is credited to the activation of Ca^{2+}-activated K^+ channels (Steidl *et al.*, 1991). PGE_2-induced depolarization or hyperpolarization, can in part explain its biological actions. Whether the effects of PGE_2 on hormone release from neurohypophysis are due to membrane depolarization or hyperpolarization remain to be elucidated.

The possible mechanism(s) of action of the PGE_2-induced modulation of K^+ channels may be as follows: firstly, PGE_2 binds to its specific surface membrane receptor; secondly, the binding of PGE_2 to its membrane receptor activates a G protein; thirdly, the uncoupling of G protein subunits may either act directly on K^+ channel proteins or activate second messenger pathway(s). One of the pathways which have been shown to be active in this process is the cAMP-PKA pathway. Briefly, this pathway involves the activation of the adenylate cyclase which converts ATP to cAMP, and, in turn, cAMP activates PKA-induced phosphorylation of K^+ channel proteins. The possibility of the activation of other second messenger pathways cannot be excluded. The precise mechanism of the postreceptor events induced by PGE_2, has not yet been completely elucidated in the neurohypophyseal nerve terminals.

5.2. SUMMARY

The results of the present work can be summarized as follows:

1. This study characterized voltage-dependent outward K^+ channel currents in neurohypophyseal nerve terminals. The pharmacological and kinetic studies identified three distinct K^+ channels. One is the transient K^+ current, which is 4-AP sensitive and α-DTX resistant. Another is the non-inactivating current, which can be blocked by TEA and 4-AP, but not by α- and β-DTX. The third is the Ca^{2+}-activated K^+ channel. These results suggested that the preparation of the isolated nerve endings provided a suitable model for the study of the ionic basis of neuropeptide release from the posterior pituitary.

2. Under whole-cell voltage-clamp conditions, PGE_2 modulated the voltage-dependent K^+ channels in neurohypophyseal nerve terminals, through a receptor-mediated process involving the activation of G protein(s).

3. After short periods of incubation, PGE_2 facilitated the non-inactivating component, and this process seems to be Ca^{2+}-sensitive. The postreceptor activation of subcellular signal transduction machinery underlying this effect remain unknown as yet.

4. Following longer incubation periods with PGE_2, the fast-inactivating component increased, while the non-inactivating component declined. These processes are not Ca^{2+}-dependent, and the cAMP-PKA cascade was involved in the mediation of these long-term regulating effects induced by PGE_2. It is hypothesized that PGE_2 binds to its specific membrane receptor(s) to activate G protein(s). The uncoupling of a G protein from the PGE_2 receptor(s) could lead to the subsequent activation of adenylate cyclase, and, hence, catalyses the conversion of ATP to cAMP, which, in turn, activates PKA. The K^+ channel activities are subject to modulation by protein phosphorylation via PKA.

5. PGE_2 may not alter the function of Ca^{2+}-activated K^+ channels.

6. These observations have provided strong evidence that PGE_2 might contribute to the regulation of hormone release from the posterior pituitary by modulating K^+ channel activities. The possibility that PGE_2 might act through more than one signal pathway cannot be excluded.

5.3. SIGNIFICANCE

1. This is the first report to characterize the modulatory effects of K^+ channels induced by PGE_2 in neurohypophyseal nerve terminals.

2. The present work explores, for the first time, one of the possible mechanism(s) of action of PGE_2 in the regulation of K^+ channels in the posterior pituitary.

3. This thesis attempted to elucidate the ionic basis of the physiological role of PGE_2 on neuropeptide secretion from the neurohypophysis.

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