National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

MODULATION OF ION CHANNEL ACTIVITY IN VASCULAR SMOOTH MUSCLE CELLS BY NEUROPEPTIDE Y

Zhi-Gang XIONG

Department of Pharmacology
Faculty of Medicine
University of Ottawa
Ottawa, Ontario, CANADA

A thesis submitted in conformity with the requirements of the
Doctor of Philosophy Degree at the University of Ottawa

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

ISBN 0-612-07869-8

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

TABLE OF CONTENTS

FIGURE LEGENDS

ABSTRACT

Neuropeptide Y (NPY) is a 36 amino acid peptide that is co-stored and co-released with noradrenaline from perivascular sympathetic nerves. It modulates vascular contraction by acting on specific pre- and postsynaptic receptor sites. Presynaptically, it inhibits transmitter release by acting on Y2 receptors on sympathetic nerve endings. Postsynaptically, it has a direct contractile effect and potentiates the response to other vasopressor agents. The vasoconstrictive effect of NPY is dependent on extracellular Ca^{2+} and is sensitive to Ca^{2+}-channel blockers. These observations suggest that voltage-dependent Ca^{2+}-channels may participate in the vasopressor effect of NPY. In the present project, I tested the hypothesis that the vasopressor effect of NPY is related to its modulation of ion channel activities.

The effects of NPY on Ca^{2+}-channel currents in freshly isolated vascular smooth muscle cells (VSMC) from the rat tail artery were studied with the perforated-patch recording technique. Using Ba^{2+} (10 mM) as the charge carrier, inward currents sensitive to Cd^{2+} and nifedipine were potentiated by NPY in a concentration-dependent manner. The threshold concentration for the potentiating effect of NPY was 50 nM and reached a maximum at 150 nM. At a testing potential of +10 mV, the amplitude of inward current was increased by 46% in the presence of NPY 150 nM. NPY also shifted the steady-state activation curve toward less positive membrane potentials by about 6 mV so that the potentiating effect was most prominent near the activation threshold of the current.

The effects of NPY on both Ca^{2+} current amplitude and steady-state activation were much weaker compared to the dihydropyridine agonist Bay K 8644. At a testing potential of

+10 mV, a maximum of 300% increase in current amplitude was observed in the presence of Bay K 8644 1 μM. A 20 mV shift in steady-state activation was recorded in the presence of Bay K 8644 0.5 μM. Unlike Bay K 8644 which shifted steady-state inactivation curve toward less positive membrane potential by 14.3 mV, NPY had no significant effect on steady-state inactivation of the current. A synergistic action between NPY and Bay K 8644 was observed as the combined effect of NPY and Bay K 8644 was larger than the sum of NPY and Bay K 8644 alone.

The effects of NPY on the Ca^{2+}-activated K^+ channel [$K_{(Ca)}$] in VSMCs from the rat tail artery were also studied by whole-cell and single channel recording technique. In the presence of nifedipine (1 μM), whole-cell outward currents through $K_{(Ca)}$ channels were inhibited by NPY in a dose-dependent manner from 20 to 200 nM. A maximum inhibition to about 48% of the control current could be achieved. Recordings form outside-out patches showed that the open probability of $K_{(Ca)}$ channels were similarly inhibited by NPY. At 200 nM NPY, the open probability was reduced to about 36% of control. NPY did not affect the open times or current amplitude, but increased significantly the short (from 0.49 to 0.58 ms) and long (from 441 to 728 ms) close times.

An inhibition of $K_{(Ca)}$ channels was also observed in cell-attached patches when NPY was applied in the bath solution, indicating involvement of diffusible second messenger. The effect of NPY on $K_{(Ca)}$ channels was ATP-dependent. Omission of ATP or replacement by the non-hydrolysable ATP analogue ß,γ-methylenene ATP abolished the inhibitory effect of NPY on $K_{(Ca)}$ currents. Similar inhibitory effect of NPY on $K_{(Ca)}$ channels was observed when ATP was replaced by ATP-γ-S, but the inhibition of the current remained even after NPY was washed

out. The effect of NPY on $K_{(Ca)}$ current was not altered by protein kinase inhibitor (1 μM) in the intracellular solution. However, in the presence of genistein, a tyrosine kinase inhibitor, NPY had no inhibitory effect on $K_{(Ca)}$ channels.

Tyrosine kinase inhibitors (genistein, lavendustin A, and tyrphostin A25) by themselves potentiated $K_{(Ca)}$ channel currents in a dose-dependent manner. At a testing potential of +30 mV and in the presence of genistein 15 μM, whole-cell current amplitude of $K_{(Ca)}$ increased by 143%. Similarly, single channel open probability increased by 120%. The potentiating effect of genistein on $K_{(Ca)}$ channels was ATP-dependent. Daidzein, an inactive analogue of genistein had no effect on $K_{(Ca)}$.

It is concluded that NPY potentiates vasoconstriction by a synergistic effect on Ca^{2+} and $K_{(Ca)}$ channels in VSMCs. NPY can promote Ca^{2+} entry by increasing Ca^{2+} channel activity. Inhibition of $K_{(Ca)}$ channel would further increase the influx of Ca^{2+} by promoting membrane depolarization and/or prolonging the duration of action potentials. It is also concluded that the effect of NPY on $K_{(Ca)}$-channel is mediated by a phosphorylation-dependent pathway, probably involving tyrosine phosphorylation.

INTRODUCTION

I. Structure of NPY

Neuropeptide Y (NPY) was first isolated from porcine brain extracts (Tatemoto, 1982; Tatemoto et al., 1982). It contains 36 amino acids with tyrosine residue on the C-terminal. Since the single letter code for tyrosine is Y, and the fact that it was first isolated from neuronal tissue, this peptide was named neuropeptide Y. NPY has now been isolated from many species including human, rat, rabbit, porcine, bovine, sheep and guinea-pig. Amino acid analysis demonstrated that there are three forms of NPY. Human, rat, rabbit, and guinea-pig forms of NPY are identical and they possess a methionine residue at 17, which is readily oxidised. Porcine and bovine NPY are identical to mammalian forms described above except that the methionine residue in the latter at position 17 is replaced by a leucine residue which is not oxidised. NPY isolated from the sheep differs from all the earlier characterised mammalian forms by having an asparagine residue instead of a glutamine residue at position 10. At position 17 it has a leucine residue as do both porcine and bovine forms of NPY (see MeDermott et al., 1993).

NPY shows structural similarities to both peptide YY (PYY) (70% homology) and pancreatic polypeptide (PP) (50% homology) (Tatemoto, 1982). These three peptides are members of pancreatic polypeptide (PP)-fold family of peptides. This family is different from other similar sized peptides in having a rigid conformation and a high degree of secondary structure in aqueous solution (Schwartz et al., 1990). One member of the

family, avian PP (APP), has been characterized in detail using X-ray crystallography to establish its three-dimensional structure (Glover et al., 1983). NPY and APP share about 50% homology, the residues which are common to both peptides are the C-terminal residues and the core residues which are important for the stabilization of PP-fold (Glover et al., 1984; Schwartz et al., 1990). Based on the known three-dimensional structure of APP, assumption can be made about the three-dimensional structure of NPY. The residues of the N-terminus from a left-handed polyprolin II-like helix which lies in close proximity of an alpha helix formed by residues 14 to 32. A beta turn is responsible for drawing together the two helical configurations. Residues 33 to 36 form the alpha helix at a 90 degree angle.

II. Distribution of NPY

(i). Central Nervous System

Radioimmunoassay studies and immunocytochemical studies have demonstrated a widespread distribution throughout the brain and spinal cord. In the rhinencephalon, NPY-immunoreactivity (NPY-ir) is found in numerous olfactory nuclei (Bonn, 1990) and in all layers in the telencephalon of the cerebral cortex. Especially dense NPY-ir is found in the hippocampus (Martel et al., 1990). Many cell bodies and a few fibers containing NPY-ir are found within the caudate-putamen and the nucleus accumbens (Salin et al., 1990). The amygdala demonstrates dense NPY-ir as does the septal area, the preoptic areas, the hypothalamus and the stria terminals which connects the amygdala to the latter three structures (Reuss et al., 1990).

Within the hypothalamus NPY-ir containing cell bodies are most dense in the arcuate nucleus (Gray & Morley, 1986; Mormede et al., 1990). Within the thalamus, NPY-ir is found in a number of discrete nuclei (Danger et al., 1990). Dense NPY-ir was found also within the locus coeruleus (Pammer et al., 1990), ventral tegmentum area, the substantia nigra, the inferior colliculus and the reticular formation (Bons et al., 1990; Martel et al., 1990). Significant amounts of NPY-ir were found in the nucleus tractus solitarius and area postrema (Martel et al., 1990) implicating NPY as an important regulator of cardiovascular parameters (Vallejo & Lightman, 1986). In the spinal cord the highest density of NPY-ir in terminals is in superficial layers (I-II) of dorsal horn (Gray & Morley, 1986; Marterl., 1990).

(ii). Peripheral Nervous System

Shortly after NPY was isolated from porcine brain, NPY-ir was localized in peripheral noradrenergic neurons (Lundberg et al., 1982) and in other tissues (Furness et al., 1983; Sundler et al., 1983; Stjernquist et al., 1983; Edvinsson et al., 1983). In the peripheral nervous system of guinea pig, cat, pig and man, high levels of NPY-ir were found in sympathetic ganglia, gastrointestinal, respiratory and urogenitary tracts, and in tissues which receive a dense sympathetic innervation, such as vas deferens, heart, spleen, kidney and blood vessels. NPY-ir was also found in the adrenal medulla. The immunohistochemical staining results demonstrating colocalization of NPY with NA in noradrenergic neurons were obtained using antibodies raised against NPY as well as dopamine-beta-hydroxylase (DBH), which is responsible for noradrenaline synthesis in

these neurons (Allen et al., 1986a).

In addition to sympathetic nervous sestem, there is also evidence of the localization of NPY in the parasympathetic nervous system. For example, NPY was found to co-exist with vasoactive intestinal polypeptide (VIP) and acetylcholine (Ach) in parasympathetic cerebrovascular nerves of sympathectomized rats (Suziki et al. 1990). Moreover, Yokoyama et al. (1990) found NPY-ir nerves innervating the prostate gland of monkey which they believed were not sympathetic or parasympathetic due to the lack of Ach and NA.

In addition to NA, NPY can exist with a number of other peptides and neurotransmitters. NPY is co-localized with GABA in the cortex and striatum (Aoki & Pickel, 1990), with somatostatin in the same fibers in various regions of the brain such as the basal ganglia, hypothalamus and striatum (Chronwall et al., 1984; Anderson & Reiner, 1990). In parasympathetic cerebrovascular nerves NPY is co-localized with VIP and Ach (Suziki et al., 1990). NPY is also co-localized with VIP in submucosal plexus neurons of the small intestine (Pataky et al., 1990). In some noradenergic neurons, enkephalines are costored with NPY (Kong et al., 1990). However, the substance most commonly co-localized with NPY in the sympathetic nervous system is NA.

(iii). Cardiovascular System

The heart of several species such as dog, man, cat, guinea-pig , rat, and mouse have been shown to contain high levels of NPY-ir (Wharton & Gulbenkian, 1989; Han et al., 1989; Lundberg et al., 1983; Whartn et al., 1990). The distribution of NPY has

been characterized most extensively in the heart of the guinea pig in which the peptide was identified in all region of the organ (Sternini & Bercha, 1985; Uddman et al., 1985). Highest concentration of NPY-ir was found in nerve fibers around coronary vessels and in the atria, particularly near the sinus and atriventricular node conductive tissues, and in the endocardial layer. In human heart, NPY-ir are distributed throughout the myocardium, being more abundant in the atria than in the ventricles, and are concentrated around the small arteries and arterioles at the adventitial-medial border (Gulbenkian et al., 1990).

Among the blood vessels, particularly dense staining for NPY-ir has been noted in the aorta and coronary vessels (Sternini & Brecha, 1985). Dense plexuses of NPY-ir have been identified around cerebral arteries of cat, guinea pig, rat, and human (Edvinsson et al., 1984a; Uddman et al., 1985). NPY is widely distributed in the gastrointestinal tract, in smooth muscles, in muscularis mucosa, and in surrounding blood vessels (Lee et al., 1985). NPY-ir have been shown to be associated with vascular smooth muscle in the respiratory tract and in arteries and arterioles of the lung (Lundberg et al., 1982). The peptide is widely distributed within the male and female reproductive systems. NPY-ir fibers have been identified in the human fallopian tube, supplying vascular and non-vascular muscle (Heinrich et al., 1987). In the urinary system, NPY-ir has been found throughout the ureter in the vicinity of blood vessels (Allen et al., 1990). The peptide is also associated with blood vessels supplying the pancreas (Sundler et al., 1983). Nerve fibers immunoreactive for NPY surrounds the blood vessels and follicles of thyroid gland of several mammalian species (Grunditz et al., 1984). NPY is

colocalized with NA in some postaganglionic neurons innervating arteries and arterioles, but the immunoreactive NPY nerves which innervate the islets and exocrine parenchyma seem to be non-adrenergic (Ekblad et al., 1984). Non-adrenergic neurons containing NPY are also found in the heart.

NPY-ir has also been identified in platelets, which are by far the largest potential source of circulating NPY in the rat (Myers et al., 1990). Furthermore, it has been reported that NPY-ir is present in endothelial cells of the rabbit ear artery after long term electrical stimulation of the perivascular nerves (Loesch et al., 1992), but the functional significance of this finding has yet to be established.

III. Receptors for NPY

NPY acts on at least two receptor subtypes, tentatively referred to as Y1 and Y2 (Wahlestedt et al, 1990). The Y1-type receptor seems to be located postjunctionally and is able to evoke vasoconstriction or potentiate the effect of other vasoconstrictors (Grundemar & Hankanson, 1994). Two second messenger responses frequently associated with Y1 receptors are influx of Ca^{2+} and inhibition of cAMP accumulation (Wahlestedt et al., 1990; Wahlestedt et al., 1992). Cloning and expression studies demonstrated that human Y1 receptor consists of 384 amino acids and has seven putative transmembrane domains like other membranes of the G-protein-coupled superfamily of receptors (Larhammar et al., 1992). Y2-type receptor seems to be mainly located prejunctionally and is able to suppress the stimulated release of NA. The Y2 receptor recognizes not only the parent molecule but also truncated C-terminal fragments of NPY.

For example, NPY 13-36 is only slightly less potent than NPY 1-36. The Y1 receptor requires the whole NPY molecule in order to become fully activated and the C-terminal fragments are inactive or much less potent. Both Y1 and Y2 receptors recognize peptide YY with minor differences in relative potencies. [Pro^{34}]-NPY is a recently developed analogue that acts as a selective Y1-type receptor agonist (Dumont et al., 1990), while NPY 13-36 has been considered as a selective Y2-type receptor agonist.

In addition to Y1 and Y2 receptors, there seems to exist at least an additional NPY-responsive receptor, which was tentatively designated as a Y3-type NPY-receptor. This receptor can be activated by NPY and C-terminal segments but not by PYY and may mediate cardiovascular responses to microinjection into the neucleus of the solitary tract (Wahlestedt et al., 1992).

IV. Cardiovascular Effects of NPY

NPY exercises its control of cardiovascular system via the central nervous system by acting on the hypothalamus, medulla oblongata and parts of the midbrain including the nucleus tractus solitarii (NTS). Microinjection of NPY into the posterior hypothalamic nucleus resulted in an increase in blood pressure (Westfall et al., 1988) while intramedullary microinjection into the nucleus tractus solitarii and general i.c.v. administration of NPY resulted in a decrease in the heart rate, systolic and diastolic blood pressure due to decrease in the release of NA from catecholamine neurons by NPY.

The main effects of intravenous administration of NPY in vivo in anaesthetized rats are an increase in the mean arterial pressure and total peripheral resistance, a

decrease in cardiac index and stroke volume (Zukowska-Grojec et al., 1987). The basal heart rate was decreased in studies of conscious animals (Minson et al., 1987; Minson et al., 1989; Minson et al., 1990; Zukowska-Grojec et al., 1987; Itoi et al., 1986). In studies of isolated hearts the same observation were made (Balasubramaniam et al., 1988; Allen et al., 1983; Made et al., 1987; Franco-Cereceda et al., 1985). NPY has been shown to decrease the contractility in the atria and ventricles of the heart by exhibiting negative inotropic and chronotropic effects (Balasubramaniam et al.,1988; Piper et al., 1989; Rigel et al., 1989). The mechanism for this is thought to be a coupling of NPY to adenylate cyclase via an inhibitory G-protein and a resultant decrease in cAMP level (Kassis et al., 1987; Piper et al., 1989; Miller et al., 1988). Decrease in the contractility of the heart may also be due to presynaptic inhibition of sympathetic nerves since NPY decrease release of NA from sympathetic nerves innervating the heart (Revington & McCloskey, 1990; Michel et al., 1989). In this way, acting presynaptically, NPY can decrease the cardiac responses evoked by field stimulation without affecting the chronotropic and inotropic responses to exogenous NA (Lundberg et al., 1984).

The main function of NPY on the blood vessels, increasing vascular tone, was observed upon the intravenously administration of a low dose of NPY (Lundberg & Tatemoto, 1982). NPY causes vasoconstriction in many isolated organs including spleen (Saxena et al., 1989), kidney (Minson et al., 1989), Lung (Martling et al., 1990), heart (Komaru et al., 1990). In some isolate blood vessels, NPY has potent direct contractile effects but in most isolated vessels, it has poor direct contractile effects (Lundberg et al., 1989). Strong vasoconstriction in the presence of NPY has been observed in coronary

arteries from man (Tseng et al., 1988), dog (Allen et al., 1986b), pig (Rudehill et al., 1986), rabbit (Allen et al., 1983; Edvinsson et al., 1987a), and rat (Zukowska-Grojec et al., 1987). It is believed that the coronary vasoconstrictor effect of NPY is the result of a direct action on vascular smooth muscle as this action is resistant to blockade of alpha-adrenergic (Aizawa et al., 1987; Rudehill et al., 1986) and serotonergic receptors (Aizawa et al., 1987). Based on the fact that NPY constricts coronary vessels directly, it has been hypothesized that the peptide can provoke coronary vasospasm in vivo (Allen et al., 1986b).

In the cerebral circulation, NPY is also a potent vasoconstrictor, as shown in dog (Suziki et al., 1988), cat (Edvinsson et al., 1984a), rat (Edvinsson et al., 1987a; Tuor et al., 1990; Allen et al., 1984), and rabbit (Nilsson, 1991). The vasoconstrictive response is not affected by blockade of adrenoceptors or antagonists for 5-HT (Edvinsson et al., 1983). In some other vascular beds, a direct constrictor effect of NPY has also been reported, for example in the small arteries of gluteus maximus muscle from man (Pernow et al., 1987; Pernow & Lundberg, 1988), in human forearm resistance vessels (Clarke et al., 1991), in the iliac and femoral vein of the guinea-pig and rat (Edvinsson et al., 1987b), in the uterine artery from guinea-pig (Morris, 1991), and in pig spleen artery (Pernow & Lundberg, 1988).

In most peripheral vessels, NPY alone has no vasoconstrictive effect, however it can potentiate the effect of other vasoconstrictive agents including agonist at alpha-adrenergic receptors, histamine, angiotensin II, 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$, and endothelin (Edvinsson et al., 1984b). This behaviour has been observed in saphenous

vein from dog (Hieble et al., 1989), in ear (Daly & Hieble, 1987), pulmonary (Wahlestedt et al., 1987), and femoral arteries from rabbit (Edvinsson et al., 1984b), and in tail arteries (Neild, 1987; Meclean & McGrath, 1990; Small et al., 1992) and mesenteric arteries from the rat (Westfall et al., 1987). In these vessels the response to other vasoconstrictive agents were enhanced in the presence of NPY. The potentiating effect of NPY is manifested as a leftward shift in the dose-response curve to various vasoconstrictors with no increase in the maximal tension in response to the agonist (Walker et al., 1991; Small et al., 1992). NPY also potentiates the contraction elicited by nerve stimulation (Cheung, 1991; Lundberg et al., 1989).

It was generally believed that both direct and indirect vasoconstrictive effects induced by NPY were through activation of vascular Y1 receptor (Wahlestedt et al., 1990). However, evidence consistent with the existence of postsynaptic vascular Y2 receptors has been obtained. In rat isolated femoral artery ring, NPY (13-36) was shown to be equipotent as NPY and [Leu, Pro]-NPY in eliciting vasoconstriction (Tessel et al., 1993), suggesting that both Y1 and Y2 receptors were involved in the vasoconstrictive effect. NPY and NPY (13-36) have also been shown to increase perfusion pressure in the isolated vascularly perfused pig spleen with potencies that are consistent with selective Y2 receptor activation (Lundberg et al., 1988). Contractions induced by NPY in human skeletal muscle arteries and pig splenic arteries (Pernow & Lundberg, 1988; Pernow, 1989) and rat tail artery (Small et al., 1992) have been shown to be independent of endothelium.

V. Effects of NPY on the Nervous System

(i). Peripheral Nervous System (PNS)

The effects of NPY have been studied extensively on sensory, sympathetic and enteric neurons. In sensory neurons, NPY inhibits the release of both substance P and acetylcholine (Ach) from dorsal root ganglion (DRG) in culture (Ewald et al., 1989). In sympathetic neurons, NPY was shown to inhibit the release of NA from sympathetic nerve terminals (Wahlestedt et al., 1990). However, the detailed transduction pathway is not well understood. In both sensory and sympathetic neurons, NPY has been shown to inhibit N-type Ca^{2+} channel currents (Ewald et al., 1989; Plummer et al., 1991). The inhibition of NPY on Ca^{2+} channels is via Y2 receptor and a pertussis toxin-sensitive G-protein. Actions of NPY have also been studied in myenteric plexus neurons in primary cell culture (Hirning et al., 1990). Similar to sensory and sympathetic neurons, NPY inhibited N-type Ca^{2+} currents in myenteric plexus neurons. It was also demonstrated that NPY did not act via an easily diffusible second messenger, as the activity of single N-type Ca^{2+} channel recorded in the cell-attached mode was unaffected by bath application of NPY (Hirning et al., 1990). Inhibition of neuronal Ca^{2+} channel currents by NPY may possibly explain its presynaptic inhibition of neurotransmitter release. However, since the majority of studies were performed on neuronal cell bodies, it may not satisfactorily explain the presynaptic inhibition of neurotransmitter release which takes place at the nerve terminals. Recent studies by Toth et al. (1993) has provided a direct evidence that presynaptic inhibition produced by NPY at sympathetic nerve terminals is indeed associated with an inhibition of N-type Ca^{2+} channels. In their

studies, superior cervical ganglion (SCG) cells grown in culture with atrial myocytes formed synaptic connections between axon terminals and target cells. The spontaneous activities of the myocytes (Ca^{2+} oscillations and the associated contraction frequency) were inhibited by stimulation of the presynaptic neuron (trains of action potentials were induced by stimulation with a patch pipette on the cell body). Trains of action potentials elevated $[Ca^{2+}]_i$ in the terminal region, but not in the axon. Application of NPY reversibly reduced the intraterminal $[Ca^{2+}]$ elicited by the action potential. The effect of NPY was prevented by ω-conotoxin. These results indicate that NPY causes presynaptic inhibition at sympathetic nerve terminal largely by inhibition of N-type Ca^{2+} channel (Toth et al., 1993).

Other mechanisms, in addition to an inhibition of N-type Ca^{2+} channel, have also been suggested by some investigators. In guinea-pig vas deferens, NPY prevented nerve action potential invasion of the nerve terminals (Cheung & Dukkipati, 1991). The mechanism of presynaptic action by NPY was shown to be different from that of ω-conotoxin and α_2-adrenoceptor activation (Cheung & Dukkipati, 1991). NPY (50-1000 nM) also failed, in cortical and hippocampal synaptosomes, to modify Ca^{2+} influx via N- or L-type Ca^{2+} channels (Lundy & Frew, 1991). In PC-12 cells, Chen & Westfall observed that NPY inhibited K^+-evoked dopamine release by reducing Ca^{2+} influx. The inhibitory effect was via Y2 receptor and was pertussis toxin-sensitive. However, the Ca^{2+} influx inhibited by NPY was not through L- or N-type Ca^{2+} channels since NPY produced further inhibition of dopamine release in the presence of nifedipine and ω-conotoxin (Chen et al., 1994).

(ii). Central Nervous System (CNS)

Effects of NPY on the electrophysiology of neurons of CNS have been examined in several brain regions. To date, most observed effects of NPY on central neurons are inhibitory. In hippocampus, NPY has been shown to selectively inhibit excitatory synaptic transmission in CA1 neurons by suppression of glutamate release (Klapstein & Colmers, 1992; Colmers & Bleakman, 1994). As with the studies on PNS, it seems that Y2 receptor mediates this inhibitory action of NPY, and a decrease of Ca^{2+} influx was suggested for NPY action (Klapstein & Colmers, 1992). NPY also inhibits both excitatory and inhibitory synaptic potentials in 5-HT-containing cells of the dorsal raphe nucleus and reduces spontaneous firing rate and hyperpolarization in the locus coruleus (Grundemar & Hakanson, 1994).

VI. Signal Transduction Pathways Involved in NPY Effects

(i). G-proteins

Possible involvement of G-proteins in NPY action has been demonstrated in studies of the GTP-dependence of ligand binding. In rat cardiac membranes, ^{125}I-NPY bound specifically with high affinity to both atrial and ventricular membranes. The binding of ^{125}I-NPY to ventricular membranes was sensitive to GTP, suggesting G-protein coupling (Sheriff et al., 1990). GTP-γ-S, a non-hydrolysable GTP analogue has also been used to test the involvement of G-protein in NPY action. Toth et al. reported that, inclusion of GTP-γ-S in the patch pipette rendered the effects of NPY on Ca^{2+} influx at sympathetic nerve terminal irreversible, consistent with a G-protein involvement

(Toth et al., 1993). Many studies have also shown that the effects of NPY can be abolished by pretreatment with pertussis toxin. For example, the inhibitory effect of NPY on agonist-induced cAMP accumulation in both heart and vascular preparations have been shown to be PTX-sensitive, indicating an involvement of inhibitory G-protein (G_i) (Kassis et al., 1987; Rocha-Singh et al., 1988). Similarly, pretreatment with PTX have been shown to abolish the vasoconstrictive effect of NPY in different vascular preparations (Morris, 1991; Andriantsitohaina et al., 1990). Involvement of G-protein (G_o) has also been suggested in the effect of NPY on neuronal Ca^{2+} channels (Hirning et al., 1990; Wiley et al., 1990).

(ii). Adenylate Cyclase

Inhibition of agonist-stimulated cyclic AMP accumulation by NPY has been demonstrated in feline cerebral blood vessels (Fredholm et al., 1985), rabbit pulmonary artery vascular smooth muscle (Reynolds & Yokota, 1988), rat ventricular and atrial cells (Rocha-Singh et al., 1988; Kassis et al., 1987), rat vas deferens (Haggblad & Fredholm, 1987), bovine adrenal chromaffin cells (Zhu et al., 1992), pig spleen (Lundberg et al., 1988), rat striatum and brain slices (Westlind-Danielsson et al., 1988 McAuley et al., 1991), human neuroepithelioma cells (Lobaugh & Blackshear, 1990), and a human neuroblastoma cell line SMS-KAN (Shigeri & Fujimoto, 1994). In VSM cells from the rabbit pulmonary artery, NPY dose-dependently inhibited forskolin-stimulated cyclic AMP formation with an EC_{50} of 0.3 nM. A maximal inhibition of forskolin-induced cAMP accumulation by 65% was achieved in the presence of NPY 100 nM. Studies with

IBMX, an inhibitor of mammalian cell phosphodiesterase, demonstrated that it did not attenuate the action of NPY. Thus, it is likely that NPY exerted its effect by inhibiting adenylate cyclase activity (Reynolds & Yokota, 1988). It is not clear how inhibition of adenylate cyclase may mediate the vasoconstrictor effects of NPY. Elevation of cAMP in vascular smooth muscle leads to vasorelaxation, and thus a general mechanism of NPY action in VSM may be to inhibit cyclic nucleotide-mediated relaxation mechanisms (Reynolds & Yokota, 1988). However, since NPY only inhibits agonist-stimulated cAMP accumulation and does not affect the basal level of cAMP, this effect of NPY may not be a prerequisite for the functional vasoconstrictive response to NPY (Lundberg et al., 1988).

(iii). Inositol Phosphates

Increase of intracellular calcium concentration through inositol phosphate signalling pathway has been demonstrated for many vasoconstrictor agents such as noradrenaline, angiotensin II and vasopressin (Cottechia et al., 1985; Alexander et al., 1985). Activation of the inositol phosphate signalling system by NPY in the cardiovascular system has not been demonstrated conclusively. In cultured vascular smooth muscle cells from rabbit pulmonary artery, Reynolds & Yokota (1988) did not observe any change in phosphoinositide (PI) hydrolysis or the elevation of cytosolic calcium in the presence of NPY even though NPY attenuated cAMP accumulation in those cells. In primary culture of vascular smooth muscle cells from azygous veins of neonatal WKY rat, Erne & Hermsmeyer (1988) also failed to observe any changes in

intracellular calcium by NPY. An increase in intracellular calcium in vascular smooth cells from porcine aorta was reported by Mihara et al. in 1989. In their study, NPY induced a dose-dependent and transient rise in intracellular calcium and this effect was not blocked by extracellular EGTA or calcium channel blockers, therefore it seems that the main source of NPY-induced increase of calcium was from the intracellular calcium storage site. However, unlike angiotensin II and bradykinin (BK), NPY induced intracellular calcium increase without any change in inositol 1,4,5-triphosphate generation. Although NPY attenuated forskolin-induced accumulation of cyclic AMP, forskolin and IBMX-induced alteration in intracellular cAMP did not affect the NPY-induced $[Ca^{2+}]_i$ rise. It is suggested that NPY induced increase in $[Ca^{2+}]_i$ is not mediated by intracellular messengers such as IP_3 and cyclic AMP (Mihara et al., 1989).

Mobilization of intracellular Ca^{2+} by NPY has also been reported in human erythroleukemia cells (HEL) (Motulsky & Michel, 1988; Deniels et al., 1989), rat dorsal root ganglion (DRG) neurons (Perney & Miller, 1989), and a human neuroblastoma cell line (Shigeri & Fujimoto, 1994). In HEL cells and DRG neurons, an increase in the formation of inositol phosphates was observed, suggesting that the mobilization of Ca^{2+} from intracellular stores in these cells was secondary to the generation of inositol phosphates (Deniels et al., 1989; Perney & Miller, 1989). In human neuroblastoma cell line SMS-KAN, NPY attenuated angiotensin II (Ang-II) and bradykinin (BK)-induced inositol 1,4,5-trisphosphate (IP_3) production and Ca^{2+} release from intracellular stores, in addition to inhibit forskolin-induced cAMP accumulation and Ca^{2+} influx through N-type Ca^{2+} channels. The effect of NPY on Ang-II and BK-induced Ca^{2+} mobilization

was not affected by activation or down-regulation of protein kinase C but abolished by herbimycin, a tyrosine kinase inhibitor, suggesting that tyrosine phosphorylation is possibly involved in NPY action (Shigeri & Fujimoto, 1994).

(iv). Ion Channels

In neurons, several studies have demonstrated that NPY can inhibit N-type Ca^{2+} channels (Schofield & Ikeda, 1988; Ewald et al., 1989; Perney & Miller, 1989; Hirning et al., 1990; Wiley et al., 1990; Bleakman et al., 1991). In most cases, the effect of NPY on neuronal Ca^{2+} channels was mediated by Y2 receptor and a pertussis toxin-sensitive G-protein (G_o). In myenteric neurons, NPY has been shown to inhibit Ca^{2+} channels via a direct receptor-G-protein-channel coupling (Hirning et al, 1990). More recently, Foucart et al. (1993) showed that the inhibitory effect of NPY on N-type calcium currents in acutely dissociated neurons from adult rat superior cervical ganglia was mimicked by the fragment NPY (13-36) but not by peptide YY, indicating that a Y3 receptor was involved.

In rat ventricular cardiomyocytes, NPY has been shown to modulate transient outward K^+ current (I_{to}) and slow inward Ca^{2+} current (I_{si}) (Miller et al., 1991). In this preparation, 10^{-7} M NPY reversibly activated the 4-AP sensitive I_{to}. The amplitude of the current was enhanced to 165% of the control value without affecting the time constants of the activation and inactivation. I_{si} was also reversibly increased by NPY, the peak amplitude was increased to 151% of the control value in the presence of NPY 10^{-7} M. The effects of NPY on I_{to} and I_{si} were consistent with both positive and

negative contractile effects of NPY on ventricular cardiomyocytes they have observed. In their studies, NPY attenuated the increase in the contractile response induced by isoprenaline. This effect of NPY could be abolished by the presence of I_{to} inhibitor, 4-aminopyridine (4-AP, 0.5 mM) and pretreatment of the cells with pertussis toxin. In the absence of isoprenaline, but in the presence of 4-AP, NPY exerted stimulatory effect on the cardiomyocytes. This stimulating effect of NPY could be abolished by I_{si} inhibitor verapamil but not by pertussis toxin pretreatment. The results indicate that in the rat the antiadrenergic negative contractile effect of NPY results from its action on the I_{to} which involves a pertussis toxin-sensitive G protein. Blockade of this current by 4-AP unmasks a positive contractile effect of NPY that is related to activation of the I_{si}.

An opposite effect of NPY on calcium channel current was reported by Bryant et al. (1991). In left ventricular myocytes isolated from guinea pig, NPY, with concentration higher than 10^{-6}M, induced a shortening of action potential duration (APD), a reduction in L-type calcium current, and a fall in developed contraction. These effects of NPY were mediated, at least in part, via an inhibitory G-protein G_i.

In canine Purkinje fibres, both NPY and VIP have been shown to modulate pacemaker current (I_f) (Chang et al., 1991). VIP reversibly increased, whereas NPY reversibly decreased I_f at the concentration of 200 nM. This effect of NPY on I_f could also explain the negative chronotropic effect on the heart.

In vascular smooth muscle cells, involvement of voltage-dependent Ca^{2+} channels in the action of NPY has also been implicated. NPY potentiates only the component of the contraction sensitive to Ca^{2+}-channel blockers. Thus nifedipine and other Ca^{2+}-

channel blockers are very effective in abolishing the potentiating effect of NPY on contractions in response to neural stimulation and to applied pressor agents (Andrianstohaina & Stoclet, 1988; Cheung, 1991; Pernow et al., 1986). In the rat tail artery, NPY was found to be more effective in potentiating the contraction in response to potassium depolarization than noradrenaline (Small et al., 1992). In calcium-free solution, NPY's potentiation of NA-induced vasoconstriction in rabbit mesenteric arteries was absent and could only be observed upon the readdition of calcium (Oshita et al., 1989). These studies have provided strong evidence that voltage-dependent Ca^{2+} channels may participate in the potentiating effect of NPY in VSMC.

VII. Ion Channels and Physiological Functions in Vascular Smooth Muscle.

(i). Calcium Channels.

Three major types of voltage-operated Ca^{2+} channels (T, L, and N) have been described in neurons (Nowycky et al., 1985a). Evidence has recently been presented for a possible fourth type of calcium channel in cerebral Purkinje cells, the P channel (Llinas et al., 1989). In cardiac and smooth muscle only T- and L-type channels have been identified (Reuter et al., 1982; Benham et al., 1987). Voltage-operated Ca^{2+} channels in vascular preparations open when the membrane potential is depolarized from resting to a threshold ranging from -50 to -30 mV. This results in a slow maintained contraction (Droogmans et al., 1977; Bolton et al., 1984). The activation threshold and the unitary conductance of Ca^{2+} channels identified from patch-clamp studies of smooth muscles show considerable diversity. This might reflect different distributions of Ca^{2+} channels

in these preparations. T-type channels are distinguished by a faster time course of inactivation and more negative activation and inactivation ranges compared to L-type Ca^{2+} currents. Reports of the unitary conductance of T-type channels range from 6 to 10 pS (Caffrey et al., 1986; Benham et al., 1987). On the other hand, a 15-25 pS channel is thought to underlie the more sustained L-type Ca^{2+} current in smooth muscle (Aaronson et al., 1986; Benham et al., 1987). The L-type channel can also be differentiated from the smaller T-type channel by its higher affinity for the organic Ca^{2+} channel antagonists (e.g. dihydropyridines). L-type Ca^{2+} currents appear to predominate in the vasculature. However, preparations showing contribution from both Ca^{2+} channels include guinea-pig aorta (Caffrey et al., 1986), rabbit mesenteric and ear artery (Aaronson et al., 1986; Benham et al., 1987), dog saphenous vein (Yatani et al., 1987) rat azygous vein (Sturek & Hermsmeyer, 1986), and rat aorta (Toro & Stefani, 1987).

Smooth muscle contraction evoked by elevated KCl or direct electrical stimulation is thought to be due to opening of voltage-operated Ca^{2+}-channels. The threshold level of depolarization required to elicit contraction of VSM correlates reasonably well with that for Ca^{2+} channel opening. In the guinea pig mesenteric artery, contraction occurs with depolarization to about -45 mV from a resting potential of -60 mV (Bolton et al., 1984). Reports from the rat tail artery exhibit contraction at threshold of -49 mV (Cheung, 1984). The magnitude of cell contraction is also modulated by activation of voltage and Ca^{2+}-dependent K^+ channels which repolarize the membrane potential and hence causes closing of Ca^{2+} channels. Thus, any factors which shift the balance between these opposing conductance (g_{Ca} versus g_K) will ultimately determine the

contractile response of vasculature to membrane depolarizing stimuli.

The role of dihydropyridine insensitive T-type Ca^{2+} channels in VSM is still uncertain. Sturek & Hermsmeyer (1986) suggested a possible role for the T-type channel in mediating pacemaker activity in certain vessels. An inhibitory effect of Ni^{2+} on the latter phase of diastolic depolarization has been observed in pacemaker cells of the rabbit sino-atrial node (Hagiwara et al., 1988). In muscular arteries, which are electrically quiescent at rest, it has been proposed that T-type channels contribute to the excitatory junction potential (Benham et al., 1987). However, direct pharmacological evidence of their contribution to electrical activities in the blood vessels is lacking. This is largely attributed to the apparent lack of a highly selective and potent pharmacological probe. Speculation of a development role of the T-type channel has recently been proposed (Bean, 1989). This is based largely on the prominence of T-type currents in embryonic cells of skeletal and smooth muscles (Beam & Knudson, 1988; Sturek & Hermsmeyer, 1986).

Because of its sensitivity to DHPs, the L-type Ca^{2+} channel is still the only type of Ca^{2+} channel for which structure information is available. Recent molecular biology studies have shown that the L-type Ca^{2+} channel in muscle cell contains five subunits, known as α_1, α_2, ß, γ and δ. α_1 subunit (about 170 kD in molecular weight) contains substantial hydrophobic domains which can span the membrane to form the ion conducting pore. Similar to sodium channels, α_1 subunit for Ca^{2+} channel consists of four homologous domains, each containing six putative membrane-spanning sequences. This subunit contains the dihydropyridine binding sites and important phosphorylation

sites for cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin kinase (Nastainczyk et al., 1987; Rohrkasten et al., 1988; Hosey & Lazdunski, 1988; Tuana & Murphy, 1990). α_2 subunit (143 kD) is a large glycoprotein, which does not contain any known drug-binding sites. ß subunit (54 kD) does not have a hydrophobic domain but contains important phosphorylation sites for PKA, PKG and PKC (Catteral, 1988; Ruth et al., 1989). γ subunit (30 kD) has a carbohydrate content of about 30% and extensive hydrophobic domain(s). δ subunit is a 24-27 kD glycopeptide which is linked by sulfhydryl groups to the α_2 subunit (Spedding & Paoletti, 1992; Catterall, 1988).

Functional Ca^{2+} channels have been successfully expressed in Xenopus oocytes (Biel et al., 1991) or in Chinese hamster ovary (CHO) cells (Bosse et al., 1992) by injecting either cDNA or mRNA encoding the Ca^{2+}-channels. Although α_1 cDNA alone is sufficient for stable expression of functional calcium channels, coexpression of α_1 subunit with other subunits can dramatically affect the channel gating kinetics and the dihydropyridine binding activities (Singer et al., 1991; Varadi et al., 1991). The experiment by Singer et al. showed that coexpression of α_1 with α_2 and ß subunits increased the current density. While coexpression with γ subunit shifted the steady state inactivation curve toward negative membrane potentials. Wei et al. (1991) reported that coexpression of ß subunit with α_1 subunit not only caused an increase in peak currents but also a leftward shift in the current-voltage curve and an increase in the rate of activation compared with the expression of α_1 subunit alone. The experiments of Varadi et al. even indicated that the available number of dihydropyridine receptor sites increased

more than 10-fold when coexpressed α_1 subunit with ß subunit, whereas the affinity of the drug binding remained unchanged. The increase in the number of functionally active binding sites may be due to an enhancement of membrane insertion and/or to stabilization of a specific conformation of the α_1 subunit in the membrane (Varadi et al., 1991).

L-type Ca^{2+} channels can be modulated either by a direct ligand binding on α_1 subunit or by phosphorylation on α_1 and/or ß subunits. The extent of phsophorylation of the α_1 and ß subunits showed good correlation of the rate of ^{45}Ca flux and the number of functional channels in reconstituted phsopholipid, suggesting that appropriate phosphorylation is important for functional activity of Ca^{2+}-channels. The phosphorylation process has been shown to be involved in the modulation of Ca^{2+} channels by a variety of neurotransmitters which operate through multiple second messengers and G-protein-linked pathways (Miller & Fox, 1990).

(ii). Potassium Channels.

Potassium channels form the broadest and most diverse class of membrane channels found in mammalian cells. Identification of the properties of different types of K^+ channels in smooth muscle has provided a better understanding of their roles in regulating various cellular processes. In the following discussion the K^+ channels distributed in VSM are classified into five major families. These include: (1) delayed rectifier K^+ channels, (2) transient outward K^+ channels, (3) Ca^{2+}-activated K^+ channels, (4) inward rectifier K^+ channels, and (5) ATP-dependent K^+ channels.

Although a *delayed rectifier* was recognized in giant squid axon by Hodgkin and

Huxley (1952) some 40 years ago, only recently has a similar conductance been identified in blood vessels (Beech & Bolton, 1989a; Volk et al., 1991). The delayed rectifier K^+ channel displays a relatively low unitary conductance (5-20 pS) yet accounts for the majority of outward current for depolarizations negative to 0 mV. Therefore, opening of delayed rectifier channels is likely to play an essential role over the range of membrane potentials encountered during cell excitation. As shown in nerve and skeletal muscle, the current through delayed rectifier channels turns on with a brief delay following the onset of a membrane depolarization (Hille, 1984). The time course of current development, modelled by a fourth order exponential (Hodgkin and Huxley, 1952), occurs with faster kinetics at more depolarized potentials. This is thought to reflect transitions through four closed channel states which are governed by voltage-dependent rate constants. Much of the rectification of the macroscopic current can therefore be attributed to this time- and voltage-dependent gating mechanism. During sustained membrane depolarization, decay of the outward current occurs over a period of several seconds reflecting slow inactivation of this type of K^+ channel in smooth muscles. However, the time course and the number of components which constitute the inactivation process varies amongst different tissues. In this respect, it is unlikely that delayed rectifier currents can be interpreted as due to a single uniform population of channels (Dubois, 1981). Furthermore, it is uncertain whether inactivation of this type of K^+ channel operates by a mechanism different from that of transient channels (i.e. those which carry the A current). A highly potent and selective pharmacological probe for delayed rectifier currents has not been identified for vascular tissues. The K^+-channel blocker 4-AP is considerably more

effective than TEA on the classical delayed rectifier K^+ currents when applied to the external bathing solutions. However, TEA and related quaternary ammonium derivatives show higher blocking affinities when applied internally. These compounds are thought to stabilize an open blocked state of the channel by accessing a site near to the inner pore (see Armstrong, 1975). The presence of a 4-AP and TEA insensitive component of delayed rectifier current has been reported in rabbit coronary artery (Volk et al., 1991). This might reflect the existence of more than one population of delayed rectifier channels in this tissue. A blocking action of phencyclidine on this type of current has been reported in rabbit portal vein (Beech & Bolton, 1989a). Unfortunately, other voltage-dependent K^+ channels (i.e. A currents) show variable affinities for these compounds (Okabe et al., 1987; Beech & Bolton, 1989b). Resolution of the single channel activities which mediate the voltage-dependent K^+ currents in VSM should provide a more simple means of distinguishing these different channel subtypes.

The voltage dependence of the delayed rectifier channel makes it capable of exerting a repolarizing effect on the action potential. The magnitude and kinetics of channel activation are probably major determinants of action potential duration in VSMCs. Therefore, delayed rectifier channels could govern the extent to which Ca^{2+} channels open in the presence of a supra-threshold stimulus. In smooth muscles which show a prolonged plateau, a significant delayed rectifier current does not develop (Lang, 1989). Therefore, a much larger inward current is observed in this preparation. Since the inward current underlying the upstroke of the action potential in VSM is carried by Ca^{2+}, opening of Ca^{2+}-sensitive K^+ channels could also function to initiate the

repolarization. In ventricular cells, elevated cytosolic Ca^{2+} potentiates the delayed rectifier currents (Tohse, 1990). However, the effects of Ca^{2+} on the function of delayed rectifier K^+ channels has not been demonstrated in VSM.

A *transient outward current* (the A current) was first described in molluscan neurons (Connor & Stevens, 1971). Currents through A type channels are activated by membrane depolarization but rapidly decay while the depolarization is maintained. The classical A type K^+ channel displays a steeper voltage-dependence and more negative inactivation range compared to other voltage-gated channels (i.e. delayed rectifiers). Accordingly, steady-state inactivation of the A type channel would be pronounced at the normal resting membrane potential of these preparations. However, A currents can be elicited after short hyperpolarizing pre-pulses. Recovery of the channels from an inactivated state occurs more rapidly with application of very negative membrane potentials. Therefore, recovery of the A type channels is thought to occur only during the after-hyperpolarization of the action potential. The outward A current would in turn, slow the depolarization to threshold of each subsequent spike (Connor & Stevens, 1971). In tissues which display regenerative electrical activity, A currents are thought to regulate the rate of action potential discharge and thereby control pacemaker activity. In smooth muscle, a transient outward current has been reported in guinea pig ureter, rabbit pulmonary artery and rabbit portal vein (Lang, 1989; Ohya et al., 1986; Hume & Leblanc, 1989; Beech & Bolton, 1989b). However, the steady-state inactivation of these currents generally occur at more depolarized membrane potentials than neuronal A currents. Therefore, A type channels in smooth muscle could be partially available at

physiological membrane potentials. Accordingly, the A current may contribute to repolarization as it does in cardiac cells (Giles & van Ginneken, 1985). It has also been suggested that A type channels function to oppose the inward Ca^{2+} current around the threshold of the spike and delay firing (Ohya et al., 1986; Imaizuma et al., 1990). This might account for the difficulty in generating action potentials from most vascular preparations. A transient outward current which displays characteristics of the classical neuronal A current has been reported from rabbit portal vein (Beech & Bolton, 1989b). However, its role remains somewhat obscure since significant after-hyperpolarizations necessary for channel recovery rarely occur in these tissues.

Like the delayed rectifiers in smooth muscle, A currents show a greater affinity for 4-AP than TEA (Okabe et al., 1987; Beech & Bolton, 1989b). In addition to variations in their distribution and inactivation properties, this has made it particularly difficult to separate these two types of voltage activated K^+ currents in whole cell recordings. Dendrotoxin (α-DTX) isolated from snake venom, has been shown to produce a selective block of A currents in hippocampal neurons (Halliwell et al., 1986). However, in other preparations the blocking actions of DTX appear as diverse as the aminopyridines (Penner et al., 1986; Corrette et al., 1991). Therefore, DTX may not be useful in the separation of transient and sustained components of outward K^+ currents in smooth muscles. Single channel activities underlying the transient component of macroscopic currents have yet to be identified in VSMCs. However, a unitary conductance of about 14 pS has been reported in ureteral smooth muscle (Imaizuma et al., 1990). Further studies of this nature will be necessary to explain the diverse composition

of voltage-dependent K^+ currents in VSM.

The defining feature of *inward (or anomalous) rectifiers* is their ability to pass larger inward than outward current. The presence of inward rectifier channels has been reported in mammalian skeletal and cardiac muscle (Adrian, 1969; Noble, 1984). Rectification of macroscopic currents is thought to be due to a voltage-dependent gating process and the asymmetry of the open channel currents. An analogous conductance in smooth muscle was first recognized in cells of the rabbit jejunum (Benham et al., 1987). Upon hyperpolarization of the membrane, inward current develops with both instantaneous and time-dependent components. It is thought that inward rectifier channels remain closed at membrane potentials positive to the resting value. With a hyperpolarizing step, the channels reopen thereby generating the additional time-dependent inward current. Instantaneous rectification of the inward currents in cardiac cells is thought to be due to a voltage-dependent block of the open channel by intracellular Mg^{2+} (Horie et al., 1987; Ishihara et al., 1989). With a physiological $[Mg^{2+}]_i$ little outward current is passed, whereas, the current-voltage relationship becomes nearly linear in the absence of cytoplasmic Mg^{2+}.

The ionic selectivity of the inward rectifier currents varies considerably among different tissues. This could dictate the particular role that each type of inward rectifier channel plays in modulating membrane excitability. In smooth muscle, both highly K^+-selective (Edwards et al., 1988; Edwards & Hirst, 1988) and non-selective currents (Benham et al., 1987) have been recorded. The inward rectifier K^+ channel in arterioles may underlie the resting membrane potential (Edwards et al., 1988; Edwards & Hirst,

1988). Furthermore, a contribution to the latter phase of action potential repolarization has been proposed in these tissues. The relatively non-selective inwardly rectifying current recorded from rabbit jejunum has been shown to have a depolarizing effect on the membrane (Benham et al., 1987). It is thought to function during the slow waves of potential changes in longitudinal smooth muscle and promote action potential discharge. This current may be due to activation of channels which pass K^+, Na^+ and Cl^- ions.

Changes in the external $[K^+]$ have profound effects on the current amplitude and activation parameters of classical inward rectifiers. Thus, opening of the inward rectifier channels is governed not only by membrane potential but also by the K^+ driving force. A shift in the activation range of the inward rectifier in the arterioles is believed to be at least partially responsible for membrane depolarization with low external K^+ (Edwards et al., 1988). However, changes in external $[K^+]$ may have less effect on the activation parameters of non-selective inward rectifier currents (Benham et al., 1987). While displaying a lack of sensitivity to external TEA, inward rectifier K^+ channels in smooth muscle are blocked by Ba^{2+} and Cs^+. This could explain the potent depolarizing effects of external Ba^{2+} on VSM membranes (Harder & Sperelakis, 1979).

Ca^{2+}-activated K^+ channels $[K_{(Ca)}]$ are the most commonly observed class of K^+ channels in VSM, due to both their wide distribution in different tissues and the technical ease with which the large single channel current can be measured. $K_{(Ca)}$ channels in VSM can be subdivided into two main groups: large conductance "BK" channels and low conductance "SK" channels (Blatz & Magleby, 1987). The former have been identified in several vascular preparations including rabbit portal vein (Beech &

Bolton, 1989a), porcine carotid artery (Desilets et al., 1989), rabbit mesenteric artery (Benham et al., 1986), and rat tail artery (Bolzon et al., 1993). In addition, BK channels are present in smooth muscle of the intestine (Benham et al., 1984), trachea (McCann & Welsh, 1986; Kume et al., 1989) and ureter (Lang, 1989). BK channels, often denoted as "Maxi-K^+ channels" have unitary conductances of 150-200 pS in symmetrical K^+ solutions. Pharmacologically, they are blocked by millimolar concentration of TEA and nanomolar concentration of the scorpion toxin, charybdotoxin (CTX; Barrett et al., 1982; Miller et al., 1985). Evidence of a SK channel analogous to that described in skeletal muscle (Blatz & Magleby, 1987) has been reported in smooth muscle of urinary bladder (Isenberg & Klockner, 1986). In addition to a lower unitary conductance (2-6 pS), these channels can be distinguished by their weak voltage dependence and high sensitivity to intracellular Ca^{2+} compared to BK channels. Pharmacologically, SK channels are blocked by apamin but are much less sensitive to CTX than the large conductance K^+ channels (Lang & Ritchie, 1990). An apamin sensitive conductance, however, has not been reported in VSM. The lack of evidence for SK channel activities in these tissues may be partly related to technical challenge of resolving such small unitary currents.

$K_{(Ca)}$ channels in VSM contribute to membrane repolarization following Ca^{2+} influx during the upstroke of action potentials. Since the normal resting [Ca^{2+}] in VSMCs is in the order of 10^{-7} M, the BK channel will likely remain closed over physiological potentials negative to 0 mV. However, with concurrent elevation of cytosolic Ca^{2+} and membrane depolarization, opening of these channels would exert a strong repolarizing tendency on the membrane potential. Large outward currents through

BK channels might partially account for the shorter duration of action potentials in skeletal and smooth muscle compared to cardiac preparations. Due to their large unitary conductance, activation of only a few channels would be necessary to substantially increase the K^+ conductance of the membrane and initiate the downstroke. Opening of large $K_{(Ca)}$ channels would have significant effects in smooth muscle cells which generally display a high membrane resistance. Although SK channels are thought to mediate the after-hyperpolarization in skeletal muscle (Barret et al., 1981; Romey & Lazdunski, 1984), their contribution to spike activity in smooth muscle is still uncertain.

ATP-dependent K^+ channel (K_{ATP}) was first demonstrated by Noma (1983) in cardiac muscle. The main feature of this type of K^+ channel is that it can be inhibited by cytoplasmic ATP. K_{ATP} channels are sensitive to the blockade by hypoglycemic drugs such as glybenclamide and show various unitary conductance (Ashcroft, 1988). The contribution of this type of channel to membrane conductance depends on the normal cytosolic ATP to ADP ratio and the concentration of ATP required to maintain channel closure in each tissue. K_{ATP} channels have been shown to be present in VSM of rat mesenteric artery (Standen et al., 1990), rabbit portal vein (Beech & Bolton, 1989c) and pulmonary arteries (Clapp & Gurney, 1992). It is generally believed that, similar to skeletal and cardiac muscles, K_{ATP} channels in VSM remain closed with normal physiological level of cytosolic ATP. However, recent studies in rat mesenteric and pulmonary arteries have suggested the opening of K_{ATP} channels in the resting condition, suggesting that this type of K^+ channel may contribute to the resting membrane potential (Quayle & Standen, 1994). It is also suggested that opening of K_{ATP} channel is largely

responsible for hypoxia-induced coronary vasodilation (Daut et al., 1990).

(iii). Chloride Channels

The existence of Cl^- channels in VSM has been demonstrated in rabbit and rat portal vein (Byrne & Large, 1988; Pacaud et al., 1989), rabbit ear artery (Amedee et al., 1990); Wang & Large, 1991) and rat aorta (Kokubun et al., 1991). Since the Cl^- equilibrium potential lies positive to the normal resting potential of most VSMCs (Kuriyama et al., 1971), opening of Cl^- channels would mediate an inward depolarizing current. In rat portal vein, inward Cl^- currents are sensitive to cytosolic Ca^{2+} concentration (Pacaud et al., 1989). It is thought, therefore, that this conductance might be an important determination of the duration of Ca^{2+}-based action potentials in this preparation. Addition of NA to rabbit portal vein and guinea pig mesenteric vein also activates a Cl^- conductance (Byrne & Large, 1988; van Helden, 1988). Compounds such as papaverine which affect caffeine-sensitive intracellular Ca^{2+} stores are able to modulate NA-induced inward current (Wang & Large, 1991). In rat aorta, patch clamp studies demonstrate large conductance Cl^- channels which are blocked by application of disulphonic stilbene derivatives such as 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) and 4-acetamido-4-isothiocyanostilbene-2,2'-disulphonic acid (SITS) (Kokubun et al., 1991). These channels display a similar voltage dependence and unitary conductance (300 pS) to those distributed in skeletal and cardiac muscle membrane (Blatz & Magleby, 1983; Coulombe et al., 1987).

VIII. Modulation of Ion Channels in VSM by Protein Phosphorylation.

There is now abundant evidence that ion channels are the substrates for protein kinases and phosphoprotein phosphatases. Ion channel phosphorylation can influence profoundly the electrical properties of excitable cells including neurons, cardiac cells, and smooth muscle cells. Phosphorylation of proteins on serine, threonine, and tyrosine residues is an ubiquitous mechanism of regulating the activity of proteins, including ion channels.

Recent studies have demonstrated that, in some ion channels, protein kinases are tightly associated with the channel protein to form stable regulatory complexes. The close association of protein kinases with ion channels has been demonstrated for L-type Ca^{2+} channels and $K_{(Ca)}$ channels. In skeletal muscle, an endogenous protein kinase activity was found to be copurified with the L-type Ca^{2+}-channels in partially purified preparation, indicating a close association between kinase and Ca^{2+} channel proteins (Tuana et al., 1988). Studies by Johnson et al. (1994) have also demonstrated a physical association between L-type Ca^{2+} channels and protein kinase A in skeletal muscle. It was suggested that protein kinase A is anchored within approximately 150 nm of the Ca^{2+} channel and can give rapid phosphorylation in response to brief depolarizing stimuli. Similarly, close association between protein kinase and $K_{(Ca)}$ channels has been reported by Chung et al. (1991). In their study, the $K_{(Ca)}$ channel from rat brain was incorporated into planar lipid bilayers. The activity of the incorporated $K_{(Ca)}$ channel was increased by the addition of Mg-ATP to the cytoplasmic side of the channel. This effect took place without the addition of protein kinase, and was not mimicked by non-

hydrolysable ATP analog. Furthermore, addition of phosphatase 1 reversed the modulation by Mg-ATP. These results indicate that an endogenous protein kinase activity is firmly associated with $K_{(Ca)}$ channels (Chung et al., 1994).

(i) Modulation of Ca^{2+}-channels by Protein Phosphorylation.

Similar to neurons and cardiac cells, Ca^{2+} channels in VSMCs have been shown to be regulated by different protein kinases including cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase C (PKC) and protein tyrosine kinase (PTK). Phosphorylation by PKA is known to enhance the Ca^{2+} channel currents in cardiac cells (Kameyama et al., 1986; Trautwein et al., 1987). This mechanism can explain a positive inotropic and chronotropic effects by ß-adrenoceptor agonist such as isoproterenol (ISO). The role of PKA in the regulation of Ca^{2+} currents in VSM, however, is still controversial. In smooth muscle cells from porcine coronary artery, Fukumitsu et al. (1990) reported that ISO, which increases intracellular cAMP level and stimulates PKA, increased L type Ca^{2+} currents. Similar enhancement of Ca^{2+} current by ISO was observed in cultured A7r5 VSM cells (Marks et al., 1990). In contrast to these observations, Droogmans et al. (1987) reported that in rabbit ear artery smooth muscle cells, ISO inhibited Ca^{2+} currents. Similarly, Xiong et al. (1994a) reported that in rabbit portal vein VSM cells, cAMP (1-3 mM) and forskolin (10-100 µM) inhibited Ca^{2+} current. This inhibitory effect was mimicked by the catalytic subunit of PKA (Xiong et al., 1994a). Recent studies by Xiong et al. (1994b) suggested that ISO can modulate the Ca^{2+} channels through two different mechanisms. They observed that,

in rabbit portal vein smooth muscle cells, low concentration of ISO (< 100 nM) stimulated the Ca^{2+} current, whereas higher concentration of ISO (1-100 μM) inhibited the current. The stimulatory effect of ISO on Ca^{2+} channels was mimicked by activated G-protein α subunit and not blocked by non-specific protein kinase inhibitor H-7, suggesting a direct G-protein coupling. The inhibitory effect of ISO, however, was completely abolished by H-7, suggesting an involvement of protein phosphorylation (Xiong et al., 1994b).

Elevation of cellular cGMP concentration and activation of PKG was shown to inhibit Ca^{2+} currents in cardiac cells (Levi et al., 1989; Mery et al., 1991). Similarly, Ca^{2+} currents in VSM have been shown to be inhibited by cGMP-dependent mechanism (Clapp & Gurney, 1991; Sperelakis & Ohya, 1990; Blatter & Wier, 1994). Clapp & Gurney observed that in rabbit pulmonary arterial smooth muscle cells, nitroprusside (NP) inhibited a nifedipine-sensitive whole-cell Ca^{2+} channel currents. Since NP was known to activate soluble guanylate cyclase and increase cellular cGMP level, and the inhibitory effect of NP on Ca^{2+} currents required the presence of ATP (in their experiment, ATP was not required for the basal activity of Ca^{2+} channel, but it was required for NP effect), it was suggested that cGMP-dependent protein phosphorylation was possibly involved in NP-induced inhibition of Ca^{2+} current (Clapp & Gurney, 1991). Similarly, in cultured smooth muscle cells from rabbit aorta, dibutyryl analogs of cGMP was shown to suppress the slow Ca^{2+} channel currents (Bkaily et al., 1988). Inhibition of Ca^{2+} channel currents by cGMP-dependent mechanism may partially explain vasorelaxation effect by atrial natriuretic peptide and nitro-compounds which can increase intracellular

cGMP concentration.

Phorbol esters, the activators of PKC, have been shown to induce contraction of vascular muscle that is dependent at least partially on $[Ca^{2+}]_o$ (Itoh & Lederis, 1987). This effect implies that activation of PKC may increase Ca^{2+} entry through Ca^{2+} channel. Direct evidence in support of PKC involvement in the modulation of Ca^{2+} channels came from patch-clamp experiment which showed an enhancement of Ca^{2+} currents in A7r5 VSMCs by phorbol esters (Fish et al., 1988). Stimulation of PKC has been suggested to be involved in NA and Ang-II induced increase of Ca^{2+} channel currents in VSMCs (Mironneau, 1991; Sperelakis & Ohya, 1991).

(ii). Modulation of K^+-channels by Protein Phosphorylation.

Among the potassium channels, delayed rectifier, $K_{(Ca)}$ and K_{ATP} all have been shown to be modulated by protein phosphorylation. In cardiac cells, delayed rectifier K^+ currents were enhanced by activation of both PKA and PKC (Walsh & Kass, 1988, Duchatelle-Gourdon et al., 1991). In VSM, delayed rectifier has also been suggested to be regulated by protein phosphorylation process. In rabbit pulmonary artery smooth muscle cells, elevation of intracellular ATP levels by flash photolysis of caged ATP augmented the delayed rectifier K^+ current (Evans et al., 1994). On the other hand, removal of substrates for ATP synthesis, and the presumed reduction in intracellular ATP concentration inhibited delayed rectifier K^+ current in rat portal vein (Noack et al., 1992). Furthermore, Edwards et al. (1993) demonstrated that the delayed rectifier K^+ current was substantially reduced when the specific protein kinase A inhibitor (PKI) was

included in the intracellular solution. This result provided a strong evidence of PKA involvement in the regulation of delayed rectifier K^+ channels in VSM. Studies by Edwards et al. (1993) also demonstrated that calphostin, a potent and highly selective inhibitor of PKC had no effect on the current, suggesting that delayed rectifier K^+ current in VSM may not by regulated by PKC (Edwards et al., 1993). Bkaily (1990) reported that in aorta smooth muscle cells, atrial natriuretic factor (ANF) activated delayed rectifier K^+ currents. Since ANF was known to stimulate particulate guanylate cyclase and increase the level of cGMP, it was suggested that delayed rectifier K^+ channles may be regulated by PKG. Studies by Huang et al. (1993) demonstrated that PTK is involved in the modulation (inhibition) of a delayed rectifier K^+ channels co-expressed with m1 receptor in Xenopus oocytes and mammalian cells. Stimulation of m1 receptor by carbachol inhibited the delayed rectifier current. This effect of carbachol was largely blocked by PTK inhibitor genistein, suggesting that PTK was involved in the modulation of delayed rectifier K^+ channels. A direct tyrosine phosphorylation of channel protein was also observed following the application of carbachol (Huang et al., 1993).

Regulation of $K_{(Ca)}$ channels in smooth muscle cells by protein phosphorylation have been well studied and it seems that this channel can be regulated by all known serine (threonine) kinases. Modulation of $K_{(Ca)}$ channel activity by PKA have been demonstrated in canine colon smooth muscle (Carl et al., 1991), tracheal myocytes (Kume et al., 1989), and VSMCs from rat aorta (Sadoshima et al., 1988). An upregulation of $K_{(Ca)}$ channel activity was usually observed following the activation of PKA.

Furthmore, okadaic acid, a phosphatase inhibitor has been shown to further increase the $K_{(Ca)}$ channel activity in the presence of PKA, indicating that the channel activities are controlled by both protein phosphorylation and dephosphorylation processes (Carl et al., 1991). Activation of PKA and increase $K_{(Ca)}$ channel activity has been suggested to be involved in the vasorelaxation effect induced by ß-adrenoceptor agonists such as isoproterenol (Sadoshima et al., 1988).

A group of vasodilators such as the nitro-compounds and atrial natriuretic peptide (ANP), which elevate the intracellular cGMP level, are known to cause hyperpolarization and relaxation of vascular smooth muscle (Itoh et al., 1987). This effect may be partially explained by an upregulation of $K_{(Ca)}$ activity by cGMP-dependent protein phosphorylation (Kubo et al., 1994; Taniguchi et al., 1993). Studies by Taniguchi et al. (1993) demonstrated that in canine coronary artery smooth muscle cells, application of G kinase to the cytoplasmic face of the inside-out membrane patchs substantially enhanced $K_{(Ca)}$ channel activities in the presence of cGMP and Mg-ATP. The voltage-dependence of the channel was shifted in the negative direction, and the mean number of open channels was increased by G kinase.

In contrast to PKA and PKG, activation of PKC has been shown to inhibit the $K_{(Ca)}$ channel activity in VSMCs. Minami et al. (1993) reported that, in cultured smooth muscle cells from porcine coronary artery, bath application of PKC activator phorbol 12-myristate 13-acetate (PMA) or 1-oleoyl-2-acetylglycerol (OAG) significantly decreased the open probability of the $K_{(Ca)}$ channel in cell-attached recording. Similar decrease of $K_{(Ca)}$ channel activity was observed when PKC was directly applied to the

cytoplasmic face of inside-out membrane patchs. Moreover, the inhibitory effect on $K_{(Ca)}$ channels by PMA, OAG and PKC can be blocked by staurosporine, a relatively specific PKC inhibitor (Minami et al., 1993). These results clearly demonstrated that protein phosphorylation by PKC down-regulates $K_{(Ca)}$ channel activity. This mechanism may partially explain porbol esters induced vascular smooth muscle contraction.

Modulation of K_{ATP} channels by PKA was first reported in pancreatic ß-cells (Ribalet et al., 1989). Activation of PKA was suggested to increase the K_{ATP} channel activity in this preparation. Similar result was observed in VSMCs. Miyoshi & Nakaya (1993) reported that, in cultured smooth muscle cells from the porcine coronary artery, bath application of isoproterenol or forskolin activated a 30 pS, ATP and glybenclamide-sensitive K^+ channel in cell-attached recording. In inside-out recording, application of ATP (1 mM) to the cytoplasmic face of the membrane inhibited the channel activity. Addition of cAMP to the bath had no effect on the channel. However, when PKA was applied in the presence of ATP and cAMP, channel activity was substantially increased. Application of the catalytic subunit of PKA without cAMP also activated the channel. These result indicates that protein phosphorylation by PKA upregulates K_{ATP} channel activity. This mechanism may also contribute to the hyperpolarization and vasorelaxation effect of ß-adrenoceptor agonist. Calcitonin gene related peptide (CGRP) is one of the most potent known vasodilators which can cause hyperpolarization and relaxation of VSM. In rabbit mesenteric artery, CGRP was shown to activate K_{ATP} channels (Quayle et al., 1994). Activation of K_{ATP} currents by CGRP was mimicked by the catalytic subunit of PKA. In addition, CGRP activation of K_{ATP} was abolished by inhibitors of

PKA. These results indicate that cAMP-dependent protein phosphorylation is involved in the CGRP induced K_{ATP} activation (Quayle et al., 1994).

In contrast to an upregulation of K_{ATP} channel by PKA, activation of PKC has been shown to inhibit the K_{ATP} channel activity in smooth muscle cells. Bonev & Nelson (1993) reported that in urinary bladder smooth muscle cells, stimulation of muscarinic receptor carbachol inhibited whole-cell K_{ATP} channel activity. The effect of carbachol was mimicked by PKC activators phorbol 12-myristate 13-actate and diacyglycerol analogue. On the other hand, blockers of PKC (Bisindolylmaleimide GF-109203X and calphostin C) greatly reduced carbachol induced inhibition of K_{ATP} currents. This result suggests that protein phosphorylation by PKC is involved in the inhibition of K_{ATP} channels by muscarinic receptor agonist. Several vasoconstrictor agents such as endothelin, vasopressin and angiotensin II have been reported to inhibit K_{ATP} channel currents in coronary artery smooth muscle cells. Since these agents can activate PKC, phosphorylation through PKC may be the mechanism underlying their inhibitory effect on K_{ATP} channels (see Quayle & Standen, 1994). Recent study by Kubo et al. (1994) demonstrated that K_{ATP} channel in cultured smooth muscle cells from rat thoracic aorta was activated by ANF and isosorbide dinitrate, the activators of particulate and soluble guanylate cyclase, suggesting a possible role of PKG in the modulation of K_{ATP} channel activity.

IX. Protein-tyrosine Phosphorylation and Smooth Muscle Function.

In recent years, a group of protein kinases that selectively phosphorylates protein

tyrosine residues has been identified. These phosphotyrosine residues are much less abundant than phosphoserine or phosphothreonine residues, accounting for < 1% of phosphoamino acid residues in most cell types. Despite the low level of phosphotyrosine-containing proteins, tyrosine-specific protein kinases (PTKs) have been shown to play an important role in regulating many cellular processes including cell growth and proliferation, secretion, T-cell activation, focal adhesion, and platelet aggregation (Hunter & Cooper, 1985).

High levels of tyrosine kinase activities have been detected in smooth muscle (Di Salvo et al., 1988), suggesting possible role of tyrosine phosphorylation in regulating smooth muscle function. Recent studies have indeed demonstrated that tyrosine phosphorylation are involved in regulating smooth muscle functions including cell growth, smooth muscle contraction and ion channel modulation.

(i). Cell Growth.

Similar to other cell types, growth factors such as EGF, and PDGF have been shown to stimulate the growth of smooth muscle cells (Bilder et al., 1991; Erlinge et al., 1993). In cultured smooth muscle cells from rabbit aortic and carotid arteries, EGF and PDGF stimulated the growth of VSMC as well as DNA synthesis in a dose-dependent manner. These effects can be abolished by tyrosine kinase inhibitor tyrphostins, indicating that tyrosine phosphorylation plays important role in cell growth (Bilder et al., 1991). Moreover, recent studies have suggested that enhanced PTK activities are associated with hyperproliferative diseases such as cancer, atherosclerosis and psoriasis

(Levitzki & Gilon, 1991).

(ii). Smooth Muscle Contraction.

In addition to an important role in cell growth, tyrosine phosphorylation has been shown to be involved in smooth muscle contraction (Merkel et al., 1993; Yang et al., 1992). In isolated rabbit aortic rings, EGF induced a dose-dependent contraction with a half maximal concentration of 7.4 $\pm$ 1.4 nM. This contraction by EGF was completely blocked by the tyrosine kinase inhibitor RG50864 (10 μM) (Merkel et al., 1993). Transforming growth factor-a (TGF-a) has been shown to induce contraction of gastric smooth muscle which was blocked by tyrosine kinase inhibitors tyrphostin and genistein (Yang et al., 1992). Similarly, PDGF induced contraction of rat aortic smooth muscle can be attenuated by tyrphostin (Sauro & Thomas, 1993).

In addition to growth factors-induced contraction, tyrosine phosphorylation has been reported to be involved in smooth muscle contraction induced by various vasoconstrictive agents including norepinephrine, angiotensin II and prostaglandin $F_{2\alpha}$ (Di Salvo et al., 1993; Yang et al., 1992). Studies by Di Salvo et al. (1993) demonstrated that contraction of canine carotid arterial smooth muscle induced by norepinephrine, phenylephrine or carbachol were markedly and reversibly inhibited by tyrosine-kinase inhibitors genistein and geldanomycin. In guinea-pig gastric smooth muscle, angiotensin induced contraction of longitudinal muscle strips was completely blocked by genistein and tyrphostin (Yang et al., 1993). These results suggest that tyrosine phosphorylation plays very important role in regulating smooth muscle

contraction by activation of receptors that do not contain intrinsic tyrosine kinase activities.

Similar to growth factors such as EGF, PDGF and insulin (Nishibe et al., 1990; Downing et al., 1989), direct biochemical studies have demonstrated that vasoconstrictive agents including Ang-II, norepinephrine and vasopressin stimulate protein-tyrosine phosphorylation of several cellular proteins in vascular smooth muscle cells. Studies by Tsuda et al. (1991) showed that, in cultured rat aortic smooth muscle cells, angiotensin II induced tyrosine phosphorylation of at least 9 proteins with molecular masses of 190, 117, 105, 82, 77, 73, 45, and 40 kDa in time- and dose-dependent manners. Other vasoconstrictors such as [Arg]-vasopressin, 5-hydroxytryptamine and norepinephrine induced the tyrosine phosphorylation of the same set of proteins as angiotensin II (Tsuda et al., 1991).

(iii). Modulation of Ion Channels.

Modulation of ion channels in vascular smooth muscle cells by tyrosine kinase has also been demonstrated by Wijetunge et al. (1992). Using whole-cell voltage-clamp, calcium channel currents in single smooth muscle cells from rabbit ear artery were recorded. Tyrosine kinase inhibitors tyrphostin 23 and genistein produced dose-dependent inhibition of Ca^{2+} channel current amplitude without obvious changes in activation and inactivation. Daidzein, an inactive analogue of genistein, had little effect on this current, consistent with an action of these agents at a tyrosine kinase(s).

(iv). Possible Signal Transduction Pathway.

Two types of tyrosine kinases have been identified in smooth muscle cells: receptor tyrosine kinases and non-receptor tyrosine kinases. Receptor-PTKs are transmembrane proteins, capable of receiving signals from the extracellular environment and directly initiating stimulus-response coupling within cell. For example, membrane receptors for EGF, PDGF, and insulin contain an extracellular ligand-binding domain, a membrane spanning domain and a cytoplasmic tyrosine kinase catalytic domain (Ullrich & Schlessinger, 1990). In contrast to these receptor PTKs, so-called non-receptor PTKs can function at various location within the cell. For example, members of the src family of PTKs can be associated with the inner face of the plasma membrane by a process dependent on specific amino-terminal amino acids as well as a myristic acid residue (Resh, 1994). In this location, they are strategically placed to relay signals from transmembrane receptors that have no intrinsic kinase activity to cytoplasmic or cytoskeletal proteins that participate in downstream signalling events. The detailed cellular events leading to cell growth or contraction following stimulation of tyrosine kinase is not fully understood. Phosphorylation of phospholipase C (PLC) has been shown to be an early event following EGF, and PDGF stimulation of receptor tyrosine kinase. Tyrosine phosphorylation of PLC increases its kinase activity and stimulates phosphatidylinositol 4,5-diphosphate (PIP_2) hydrolysis to form inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol. The latter compounds represent second messengers important in activating protein kinase C and in mobilizing calcium from intracellular stores (Margolis et al., 1989). Elevation of $[Ca^{2+}]_i$ and activation of PKC will lead to

cell contraction or proliferation. However, another growth factor, colony stimulating factor-1 (CSF-1) has been shown to stimulate mitogenesis without stimulating PLC and phosphatidylinositide breakdown (Downing et al., 1989).

Unlike EGF, PDGF or insulin receptors, Receptors for Ang-II, noradrenaline and vasopressin have seven transmembrane segments which belong to G-protein coupled superfamily and contain no tyrosine kinase domain. Enhanced tyrosine phosphorylation of cellular proteins induced by these vasoconstrictive agents would be likely through activation of non-receptor tyrosine kinases. Recent studies by Polte et al. (1994) has suggested a possible role of focal adhesion kinase (FAK), a non-receptor PTKs, in the regulation of VSMC growth and function. Using immunohisochemiclal techniques, it was observed that in the developing mouse embryo, FAK was particularly abundant in the walls of developing blood vessels shortly after or concomitant with their formation. The expression of FAK was most prominent in the medial layer of arteries populated by smooth muscle cells. In vitro studies using cultured rat aortic VSMCs also demonstrated that FAK phosphotyrosine content was dramatically elevated in response to plating cells onto the adhesive glycoprotein, fibronectin, and treatment of quiescent VSMC with the vasoconstrictor angiotensin II. Thus, in vascular smooth muscle cells, like fibroblasts, FAK appears to play a role in signalling mechanisms induced by extracellular matrix components as well as G-protein coupled receptor agonists. The combined results of this study suggest that signalling through FAK may play an important role in blood vessel morphogenesis and function (Polte et al., 1994).

HYPOTHESIS

The vasopressor effect of NPY is related to its modulation of ion channel activities.

OBJECTIVES

1. To study the effect of NPY on Ca^{2+}-channel currents in VSMCs.

2. To study if NPY has effect on other major ion channels in VSMCs.

3. To determine possible mechanism of NPY's effect on the ion channels.

METHODS

I. Cell Isolation.

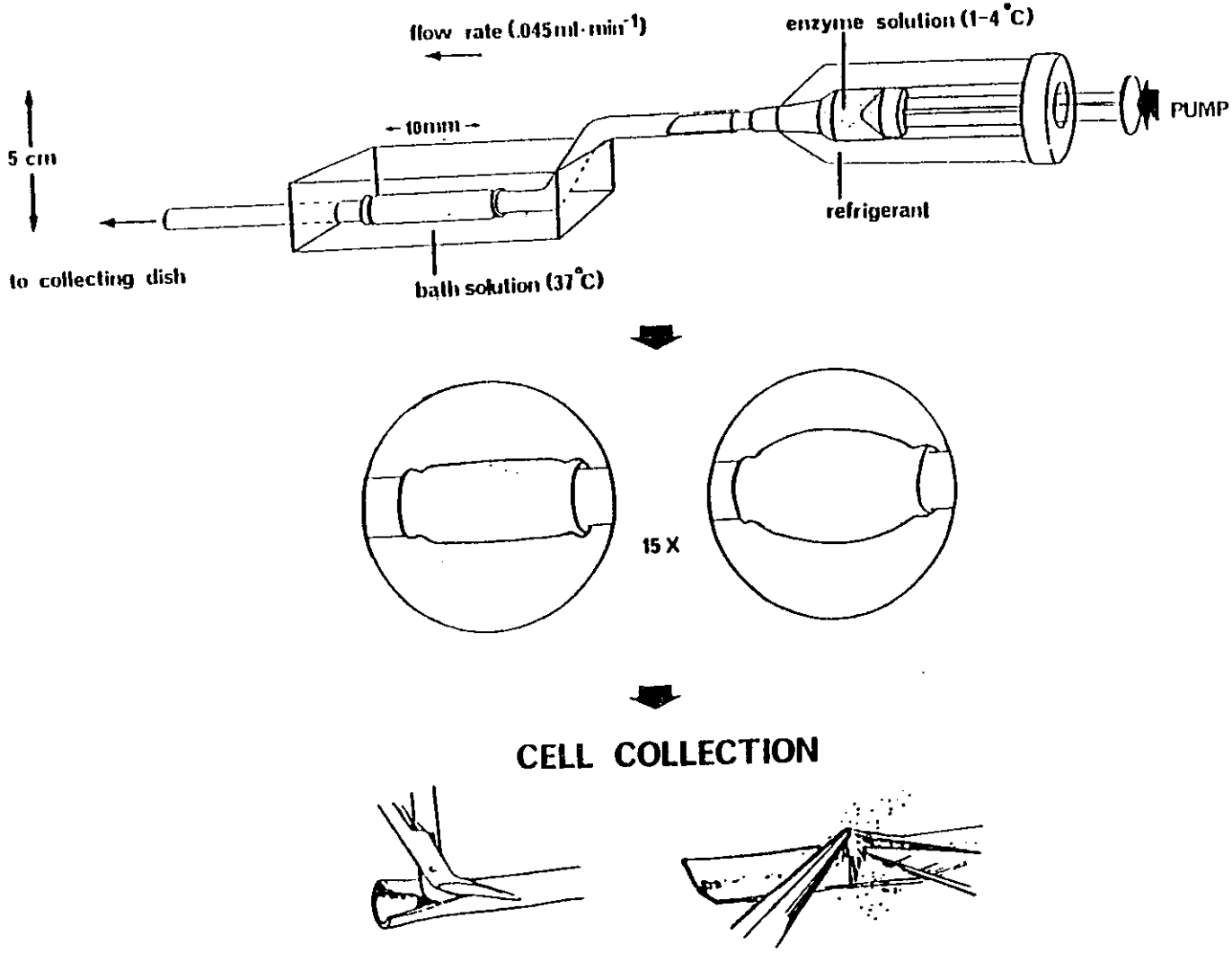
All studies were performed with freshly isolated single cells from the rat tail artery, a preparation which contains only Y1 receptor (Tschopl et al., 1993). Using a technique developed in our laboratory (Bolzon & Cheung, 1989), tail arteries from male Wistar rats (12-14 weeks, Charles River Labs. Inc., Wilmington, Mass.) were internally perfused with a Ca^{2+}- and Mg^{2+}-free HEPES buffer solution at 37°C for 90 mins followed by perfusion with the same solution containing 0.02% collagenase (type II, Sigma, 150 U/ml), 0.1% papain (BDH, 20 U/ml) and 4 mM dithiothreitol (Sigma) for 60-70 mins. Single cells were then harvested on 35x35 mm culture dishes by gentle teasing of the vessels with a pair of fine forceps (Fig. 1). These cells, averaging 110 μM in length, were Ca^{2+}-tolerant and contracted in response to potassium depolarization, noradrenaline, and purinergic stimulation (Small et al., 1992). Potentiation of contractions to these agents by NPY was also observed (Small et al., 1992).

II. Recording Technique.

All experiments were performed within the first 3-4 hours following cell isolation. Culture dishes containing single cells were mounted on the stage of an inverted phase-contrast microscope and constantly superfused with bathing solutions at a rate of 2 ml/min. All electrophysiological studies were performed at room temperature using the tight-seal patch-clamp technique (Hamill et al., 1981). Patch electrodes were pulled from borosilicate glass tubings (o.d. 1.5 mm, i.d. 0.8 mm, A-M Systems, Everett, Wash.) with

Figure 1. Experimental Apparatus for Single Cell Isolation.

Segment of rat tail artery (about 1 cm in length) was tied using nylon line onto polyethylene tubing. The vessel was immersed in a bath constantly perfused with Ca^{2+} and Mg^{2+}-free Hank's solution at $37^{o}C$. Using an infusion pump, the vessel was internally perfused with enzyme solution containing collagenase, papain and dithiothretol at a rate of 0.05 - 0.1 ml/min. To maintain the enzyme activity for the duration of the isolation, the syringe that contains the enzyme solution was encased in a 50 c.c. centrifuge tube filled with frozen gel. During enzyme digestion, the distal tubing was kept 5 cm higher than the bath in order to pressurize the vessel. Following 60 mins digestion, the vessel was cut open longitudinally and the cells were harvested in 35 mm culture dish by gentle teasing of the artery with a pair of fine forceps.


flow rate (.045ml·min⁻¹)
enzyme solution (1-4 °C)
PUMP
10mm
5 cm
refrigerant
to collecting dish
bath solution (37°C)
15 X
CELL COLLECTION

a Flaming Brown P-80 pipette puller (Sutter Instruments, Novato, Calif.). Tips of electrodes were polished prior to the experiment by heating with a platinum wire coated with glass. The resistance of the pipettes ranged between 2 and 5 MΩ when filled with pipette solutions. The liquid junction potential was zeroed electronically prior to seal formation. A hydraulic micromanipulator (MO-102, Narishige Scientific Instruments) was used to position recording pipettes near the mid-region of each cell. The pipette-membrane seal was in the range of 5 - 15 GΩ.

For conventional whole-cell recording, the patch membrane was disrupted by application of a brief pulse of suction after formation of gigaohm seal with the membrane (Hamill et al., 1981). Recordings from outside-out membrane patches were obtained by gradual withdrawal of the pipette from the cell until the large capacitive current associated with the whole cell disappeared. For cell-attached recording, cells were initially perfused with normal Hank's solution for 10-20 min and subsequently perfused with high K^+ solution. The cells were depolarized by high K^+ solution to zero the resting membrane potential so that the real membrane potential during voltage clamp can be determined. Inside-out patches were obtained by withdrawal of the pipette after establishing the cell-attached configuration. To minimize the area of excised membrane patches and thereby reduce the number of active channels, pipettes with smaller tips (resistance of 4-5 MΩ) were used for single channel recordings.

For perforated-patch recording (Horn & Marty, 1988), patch membrane was not disruptured by suction but gradually perforated by the incorporation of nystatin into the patch membrane to form conducting pores. Nystatin (Sigma) freshly dissolved in

dimethylsulphoxide (DMSO; Sigma) was included in the pipette solution at a final concentration of 100-150 μg/ml. After formation of a gigaohm seal on the cell membrane, capacitive transients were monitored by applying 10 mV hyperpolarizing voltage steps from a holding potential of -70 mV. Cell capacitance was obtained by integrating the area under the capacitive transient and divided by the voltage step. The decay of the capacitive transient was fitted with a single exponential to obtain the time constant. Series resistance was calculated by dividing the time constant by the cell capacitance (Hamill et al., 1981) (Fig. 2). The formation of low-resistance pathway with nystatin was indicated by a gradual decrease in the series resistance accompanied by an increase in inward current amplitude. The series resistance stabilized to a value of 9.4 $\pm$ 1.1 MΩ (n=8) in about 15 mins and remained unchanged for the duration of the experiment (Fig. 3). This was higher than the 4.2 $\pm$ 0.6 MΩ (n=5) obtained with conventional whole-cell recording. In general, about 80% series resistance compensation was achieved. The leak current was negligible and was not subtracted from the records.

III. Solutions and Chemicals.

(i). Solution for cell isolation. The composition of the Ca^{2+}- and Mg^{2+}-free solution used for cell isolation was (in mM): NaCl 137; KCl 5.5; KH_2PO_4 0.4; $NaHCO_3$ 4.2; NaH_2PO_4 0.4; glucose 5.6; HEPES 10 and pH was adjusted to 7.4 with NaOH.

(ii). Solutions for recording Ca^{2+}-channel current. The ionic composition of the extracellular bathing solution used in recording of Ca^{2+}-channel current was (in mM):

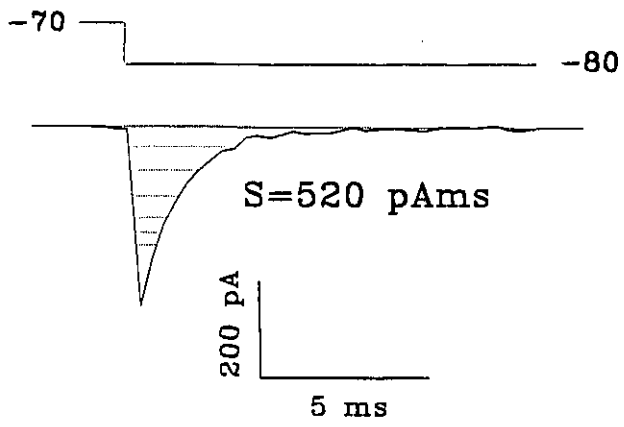
Figure 2. Method for Cell Capacitance and Series Resistance Measurement.

Panel A. Integrating. A capacitive transient was induced by 10 mV hyperpolarizing pulse from a holding potential of -70 mV. The area (S) under the transient was obtained by integration using pClamp 5.5.1. program.

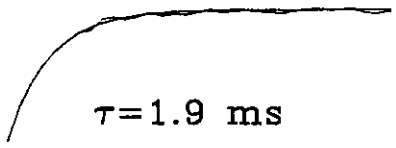
Panel B. Curve fitting. The decay of capacitive transient was well fitted by a single exponential and a time constant (τ) was obtained.

Panel C. Cell capacitance (C_m) was calculated by dividing the area (S) by voltage step (v). Series resistance (R_s) was calculated by dividing the time constant of decay (τ) by cell capacitance.

A Integrating



B Curve fitting



C Calculation

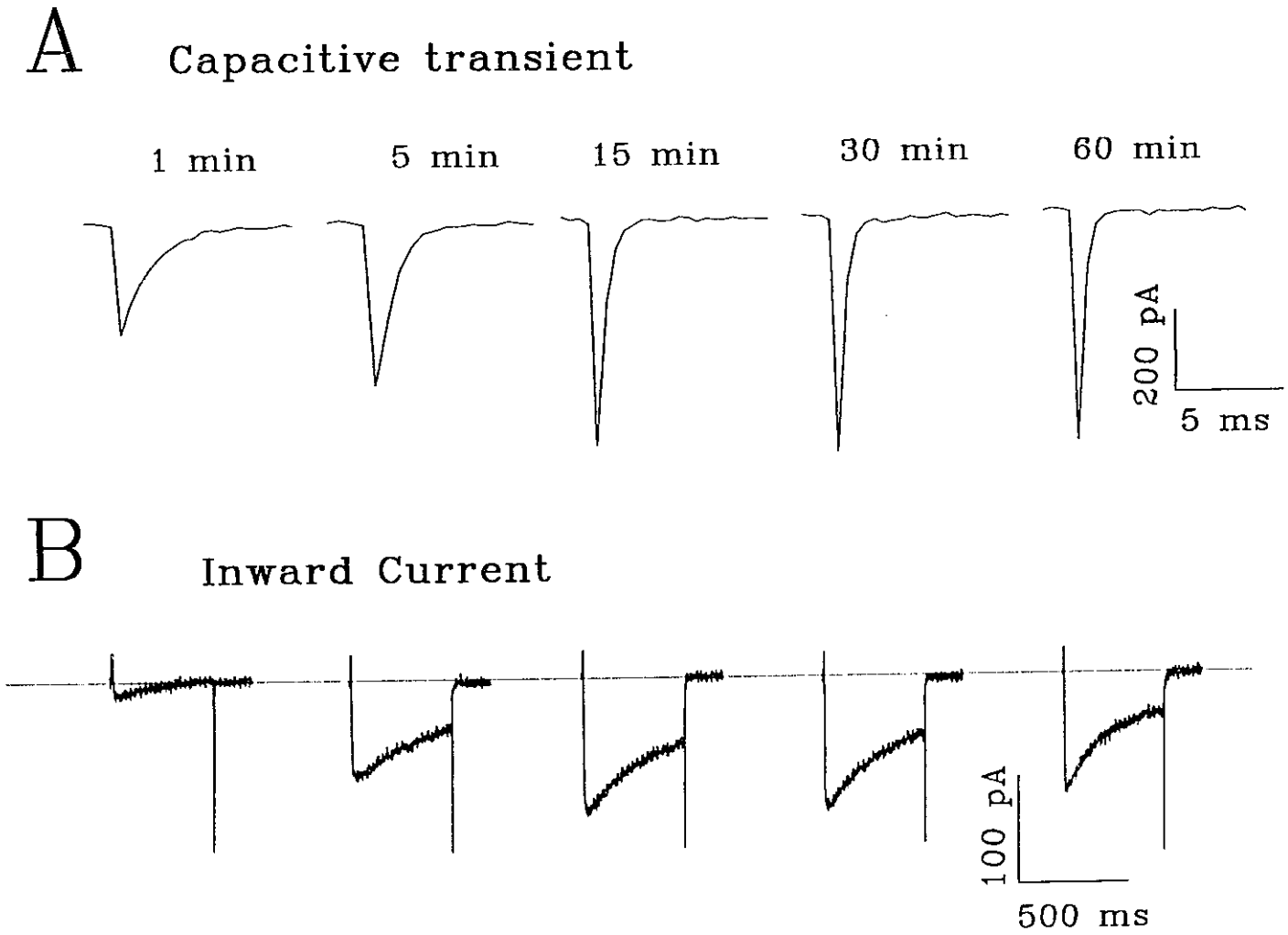
C_m= S/V=520 pAms/10 mV=52 pF

R_s= τ/C_m=1.9 ms/52 pF=36 MΩ

Figure 3. Time Course of Capacitive Transient and Inward Current in Perforated-patch Recording.

Panel A. Representative traces showing an example of time-dependent change of capacitive transient with perforated patch recording technique. Capacitive transient was induced by 10 mV hyperpolarizing pulse from a holding potential of -70 mV. The decay of capacitive transient gradually accelerated within 1 to 15 min after establishment of gegaseal, indicating a gradual decrease in series resistance. Nystatin 150 μg/ml was used in pipette solution.

Panel B. Concomitant recording of inward current with capacitive transient shows that the amplitude of Ba^{2+} inward current gradually increased within 15 min and remained stable after 15 min. Currents were recorded at a test potential of +10 mV from a holding of -70 mV.


A
Capacitive transient
1 min
5 min
15 min
30 min
60 min
200 pA
5 ms
B
Inward Current
100 pA
500 ms

$BaCl_2$ 10; tetraethylammonium chloride (TEA-Cl) 135; glucose 10; HEPES 10; pH was buffered to 7.3 with Tris. The composition of the pipette filling solution for Ca^{2+}-channel current was (in mM): CsCl 120; $MgCl_2$ 4; TEA 20; EGTA 4; ATP 2; HEPES 10; pH was buffered to 7.2 with Tris.

(iii). Solutions for recording K^+-channel current. The ionic composition of the extracellular bathing solution for the recording of Ca^{2+}-activated K^+ channel currents with whole-cell and outside-out configuration was: NaCl 137; KCl 5.5; $CaCl_2$ 1.8; $MgCl_2$ 1.0; KH_2PO_4 0.4; $NaHCO_3$ 4.2; NaH_2PO_4 0.4; glucose 5.6; HEPES 10; pH was titrated to 7.4 with 1N NaOH. To block Ca^{2+}-channel activities, nifedipine 1 μM was added to the extracellular solution. The pipette solution contained (in mM): KCl 150; $MgCl_2$ 1.0; Na_2-ATP 1.0; $CaCl_2$ 3.0; EGTA 5.0; HEPES 10; pH was buffered to 7.3 with Tris. Free Ca^{2+} in the pipette solution was calculated to be 240 nM according to Fabiato programme (Fabiato, 1988).

The ionic composition of the pipette solutions for both inside-out and cell-attached recordings was the same as the extracellular bathing solution used for whole-cell and outside-out recordings. The ionic composition of the extracellular bathing solution for inside-out recording was the same as the pipette solutions for whole-cell and outside-out recordings. The extracellular bathing solution for cell-attached recording contained (in mM): KCl 150; $CaCl_2$ 1.8; $MgCl_2$ 1.0; glucose 5.6; HEPES 10; pH was buffered to 7.4 with Tris. Nifedipine 1 μM was also added to this solution.

To study ATP-dependent effect of NPY on $K_{(Ca)}$ channels, the "Death brew"

solutions were used to eliminate ATP production. The bath solution in this case was similar to that for whole-cell and outside-out recordings except that glucose was removed and the osmolarity was supplemented with sodium ions. For the pipette solution, ATP was replaced with equimolar adenylimidodiphosphate (AMP-PNP), a non-hydrolysable ATP analogue that binds to the active site of protein kinase and produces dead-end inhibition by formation of an unproductive enzyme-AMP-PNP complex (Pelzer et al., 1990)). 2-deoxyglucose (1 mM) and carbonyl cyanide-M-chlorophenyl hydrozone (CCCP) (10 μM) were also added to pipette solution.

NPY (Peninsula Lab, Belmont, Calif.) and charybdotoxin (Alomone Lab., Jerusalem, Israel) were prepared as 20 μM stock solution in water. Tetraethylammonium (TEA) (Sigma Chemical Co., St. Louis, USA) was prepared as 2 M stock solution in water. Nifedipine and Bay K 8644 (Miles Lab. Inc., Elkhart, Ind.) were prepared as 2 mM stock solution in DMSO. Genistein (Sigma), tyrphostin A25 (Calbiochem, La Jolla, CA) and Lavendustin A (Biomol, Plymouth Meeting, PA) were prepared as 50 mM stock solution in DMSO. The final concentration of DMSO in the bath solution was less than 0.1%. This concentration was found to have no direct effect on membrane currents. All other chemicals were purchased from Sigma Chemical Co. and were dissolved directly in the perfusion solutions.

IV. Data Acquisition.

Currents were recorded with an Axopatch-1D amplifier (Axon Instruments, Foster City, Calif.). Data acquisition and analysis were performed using the pClamp 5.5.1

programme (Axon Instruments). In some case, pClamp 6 was also used for analysis of single channel current. For whole-cell recording, the current traces were electronically compensated for series resistance and cell capacitance and filtered at 2.0 kHz with a sampling rate of 3.0 kHz. For cell-attached, outside-out and inside-out recordings, single channel activity was continuously recorded by a SE3000 FM tape recorder (SE Labs, Middlesex) with a filtering rate of 2.0 KHz.

V. Data Analysis.

All values are presented as means ± standard error of mean (SEM). Student's t-test was used for statistical comparison and $P < 0.05$ was considered to be significant. Figures and curve-fitting were made using SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA.). The amplitude of whole-cell inward Ca^{2+} current was measured as the difference between the peak and the zero current level. For whole-cell outward K^+ current, the amplitude was determined as the difference between steady state current level and the zero current level. In cases where large oscillatory currents were recorded, the trace was imported to SigmaPlot and an average value was obtained.

Activation of Ca^{2+}-channel currents was determined from the relative amplitude of currents elicited at the different test potentials. The activation curves were constructed according to the peak currents (Isenberg & Klockner, 1982):

$$G_N = I_{Ca}[G_{Ca}(Vm-V_{Rev})]^{-1}$$

where G_N is normalized conductance at the test potential V_m, I_{Ca} is the peak of the inward current at V_m, G_{Ca} is the maximal conductance, and V_{Rev} is the reversal

potential. The normalized curves were fitted with a Boltzmann distribution (Hodgkin & Huxley, 1952)):

$$I = I_{Ca}\{1+\exp[(V_m-V_h)/k]\}^{-1}$$

where I is the relative current, I_{Ca} is the peak current, V_h is the potential at which the current is half maximal and k is a measure of the slope of the curve.

Steady-state inactivation of the Ca^{2+} currents were studied by holding the cells at a various potentials for 5 seconds before stepping to a test potential of +10 mV. The curves were similarly fitted with Bolzmann equation.

For the analysis of single channel currents. Records were played back from tape recorder with a sampling rate of 10 kHz and collected with a personal computer for further analysis with the pClamp program. Single channel amplitude was determined by the mean of the difference between the baseline level and the open level. These determinations were made from Gaussian fits to amplitude histograms. Channel open probability (P_o) was calculated as the fraction between the open time and the total time and the threshold for event detection was set at 50% of the average channel amplitude. In patches with multiple channels, open probability was calculated by the following relation:

$$P_o = (T_1 + T_2 \ldots + T_n)/NT_{tot}$$

where T is the amount of time that n channels are open and N is the maximum number of levels observed in the patch.

RESULTS

PART I. Characterization of Inward Current

I. Whole-cell Recording of Inward Current

Figure 4A shows the inward currents recorded with physiological external solution containing 1.8 mM Ca^{2+}. Currents were elicited by step depolarization to different test potentials from a holding potential of -70 mV. Following a capacitive transient, inward currents were activated with a test potential positive to -40 mV. The amplitude of the inward current increased gradually with test potentials from -40 to +10 mV and declined at more positive potentials. At test potentials negative to -20 mV, the inward current developed slowly to the peak without obvious decay with time. At more positive test potentials, the current developed much faster and showed a time-dependent decay (Fig. 4A). Plotting of peak inward current against the test potential showed a " V " shape of current-voltage relationship (Fig. 4C). The inward current was activated at potentials positive to -40 mV and reversed at the membrane potential around +60 mV. The maximum inward current was recorded at a test potential of about +10 mV (Fig. 4C).

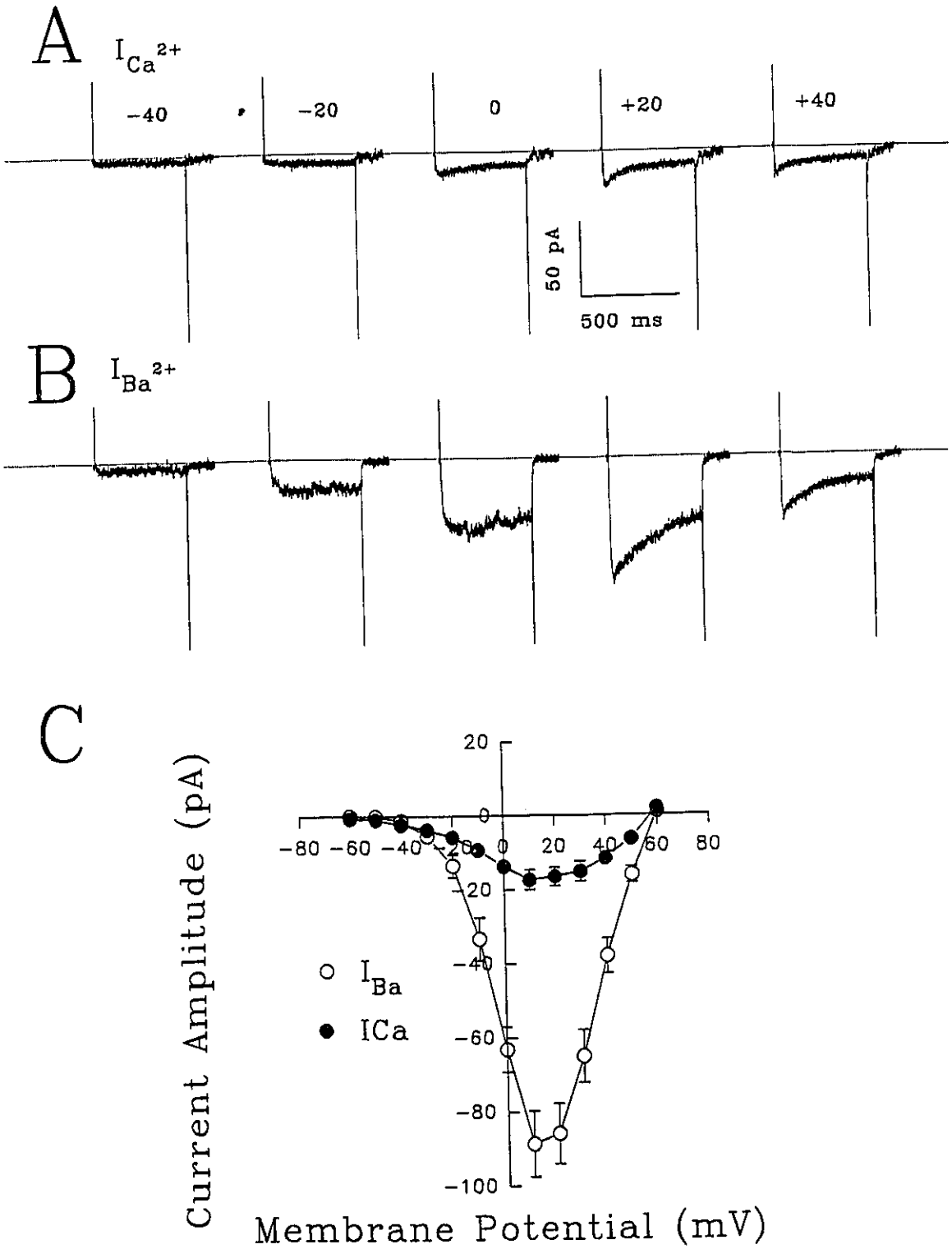
Because of the small current carried by Ca^{2+}, experiments were then carried out using Ba^{2+} (10 mM) as the charge carrier. The amplitude of inward currents increased substantially at all membrane potentials when Ba^{2+} solution was used (Fig. 4B). No apparent shift in the I-V relationship was observed with the 10 mM Ba^{2+} solution. However, the peak inward current was significantly increased compared to the current amplitude in the physiological bath solution (17.3 $\pm$ 2.7 pA in 1.8 mM Ca^{2+} solution,

Figure 4. Inward Currents Carried by Ca^{2+} and Ba^{2+}.

Panel A. Whole-cell inward currents elicited from a holding potential of -70 mV to different test potentials ranging from -40 to +40 mV with 1.8 mM Ca^{2+} in extracellular solution.

Panel B. Inward currents elicited from a holding potential of -70 mV to different test potentials ranging from -40 to +40 mV with 10 mM Ba^{2+} in extracellular solution.

Panel C. Current-voltage (I-V) relationships of the peak inward currents elicited from holding potential of -70 mV. Symbols represent the mean amplitude of the inward current for 10 cells with 1.8 mM Ca^{2+} as charge carrier (●) or 12 cells with 10 mM Ba^{2+} as charge carrier (○).


A
I_{Ca}^{2+}
-40
-20
0
+20
+40
50 pA
500 ms
B
I_{Ba}^{2+}
C
Current Amplitude (pA)
Membrane Potential (mV)
I_{Ba}
ICa
20
0
-20
-40
-60
-80
-100
-80 -60 -40 -20 0 20 40 60 80

n=10; 88.7 ± 8.9 pA in 10 mM Ba^{2+} solution, n=12).

The amplitude of the inward current recorded with conventional whole-cell configuration was not stable and changed with time. In the first 5-10 min after the establishment of whole-cell recording, the currents increased significantly. Thereafter, the currents gradually declined with time (Fig. 5A, C). At 30 and 40 min of whole-cell recording, current amplitude decreased to 77.5% and 44.6% (n=6) of that obtained at 10 min of the recording. This "run up" followed by "run-down" of the inward current was typical of whole-cell recordings in vascular smooth muscle cells (Clapp & Gurney, 1991).

II. Perforated-Patch Recording

(i). Time Course of Inward Current

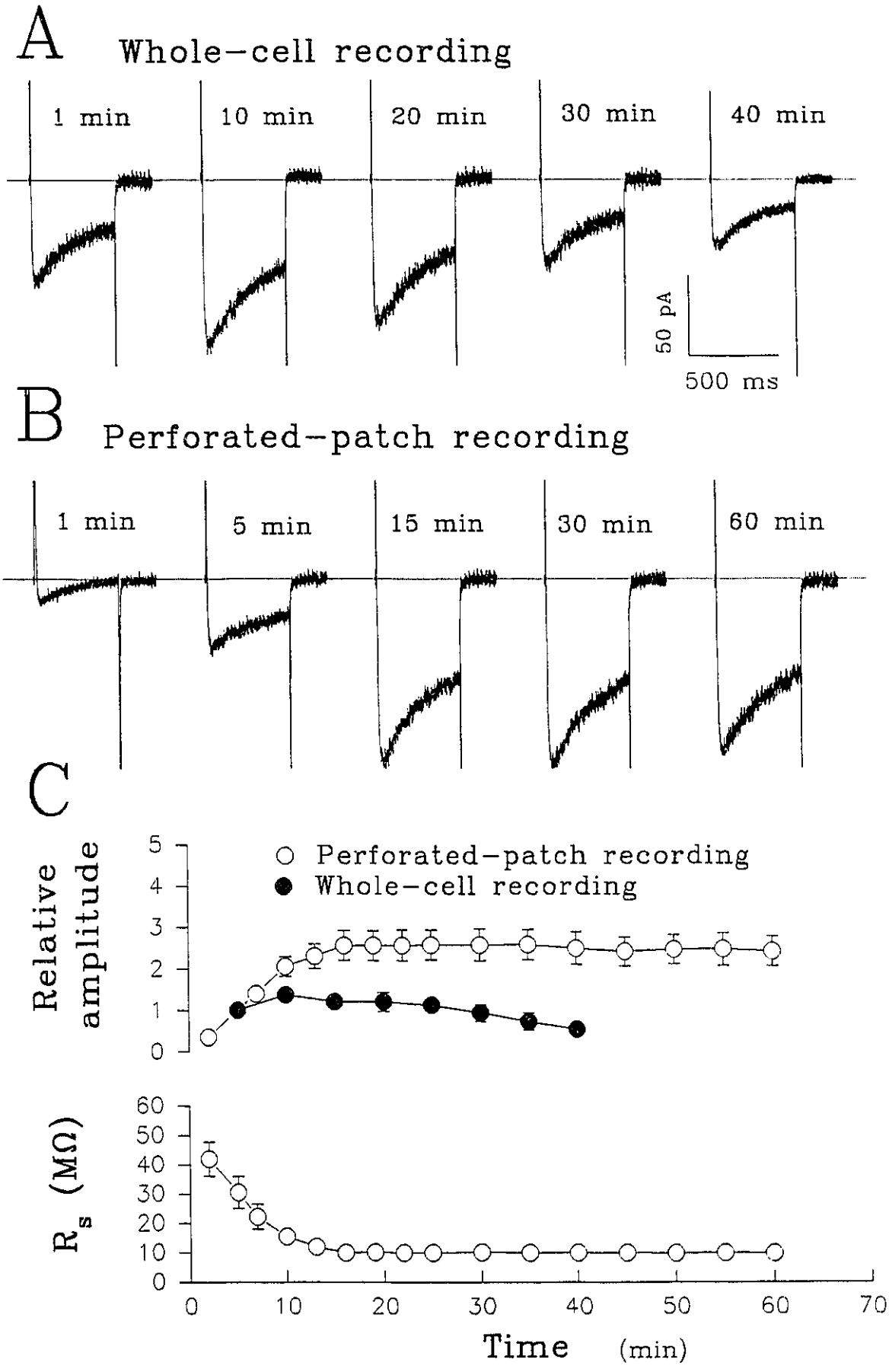
Because of the variation of the current with time using conventional whole-cell recording technique, it was difficult to assess and quantify the effect of NPY. A satisfactory method to record the inward Ca^{2+}-channel current was realized with the perforated-patch recording technique (Horn & Marty, 1988). Figure 5B shows the time-dependent change in current amplitude recorded with nystatin perforated-patch recording technique. With this technique, patch membrane was not disruptured by suction but gradually perforated by the incorporation of nystatin into the patch membrane to form cation-selective ion channels (Horn & Marty, 1988). After forming a gigaohm seal in the cell-attached configuration, the series resistance gradually decreased as the membrane became perforated by nystatin (100-150 μg/ml) in the pipette solution (Fig. 5C). This

Figure 5. Whole-cell vs. Perforated-patch Recording.

Panel A. Changes in inward current with time using conventional whole-cell recording technique with 10 mM Ba^{2+} as charge carrier. Holding potential = -70 mV; test potential = +10 mV.

Panel B. Changes in inward current with time using the perforated-patch recording technique. Note the slow capacitive transient in the first minute. Holding potential = -70 mV, test potential = +10 mV.

Panel C. Graph showing the change in current amplitude with time in whole-cell (●) and perforated-patch (○) recording configurations. The change in current amplitude with time was compared to that at 5 min of recording. The time-dependent increase in current amplitude with perforated-patch recording was associated with a decrease in series resistance. Currents were recorded from a holding potential of -70 mV to a test potential of +10 mV. n=6 for whole-cell recording; n=5 for perforated-patch recording.


A
Whole-cell recording
1 min
10 min
20 min
30 min
40 min
50 pA
500 ms
B
Perforated-patch recording
1 min
5 min
15 min
30 min
60 min
C
Relative amplitude
Perforated-patch recording
Whole-cell recording
R_s (MΩ)
Time (min)

was accompanied by an increase in current amplitude. Both the series resistance and the inward current stabilized after 15 min and remained at the same level for up to at least 60 min. At 70 min of recording, current was 97% of that obtained at 15 min of the recording (n=5). The series resistance gradually decreased from 42 ± 5.8 to 10 ± 1.1 MΩ (n=5) within first 15 mins of the recording and stabilized thereafter for more than an hour. At 60 min, series resistance was 9.8 ± 1.3 (n=5) MΩ. In all subsequent experiments, data were obtained only from perforated-patch recordings within the 15 to 60 min period when there were no significant changes in the current amplitude.

(ii). Two Components of Inward Current

In the majority of cells, recordings demonstrated only one component of inward current. This current had the characteristics of L-type calcium current as indicated by: (a) Slow inactivating process. At a test potential of +10 mV, the time constant of inactivation was about 500 ms (Fig. 6B). (b) High sensitivity to nifedipine blockade. In most cells the inward currents were completely abolished by 1 μM nifedipine (Fig. 7). (c) Resistant to inactivation by changing the holding potential from -70 to -40 mV (Fig. 6A, B). However, a transient component was also observed in about 10% of cells. This transient component had the characteristics of T-type inward current as it has a fast inactivation process (time constant about 50 ms at +10 mV) and it could be inactivated by a holding potential positive to -40 mV (Fig. 6). It was also resistant to the blockade by nifedipine (Fig. 7). Figure 6 shows an example of two components of the inward current. At a holding potential of -70 mV, the inward current at +10 mV was composed

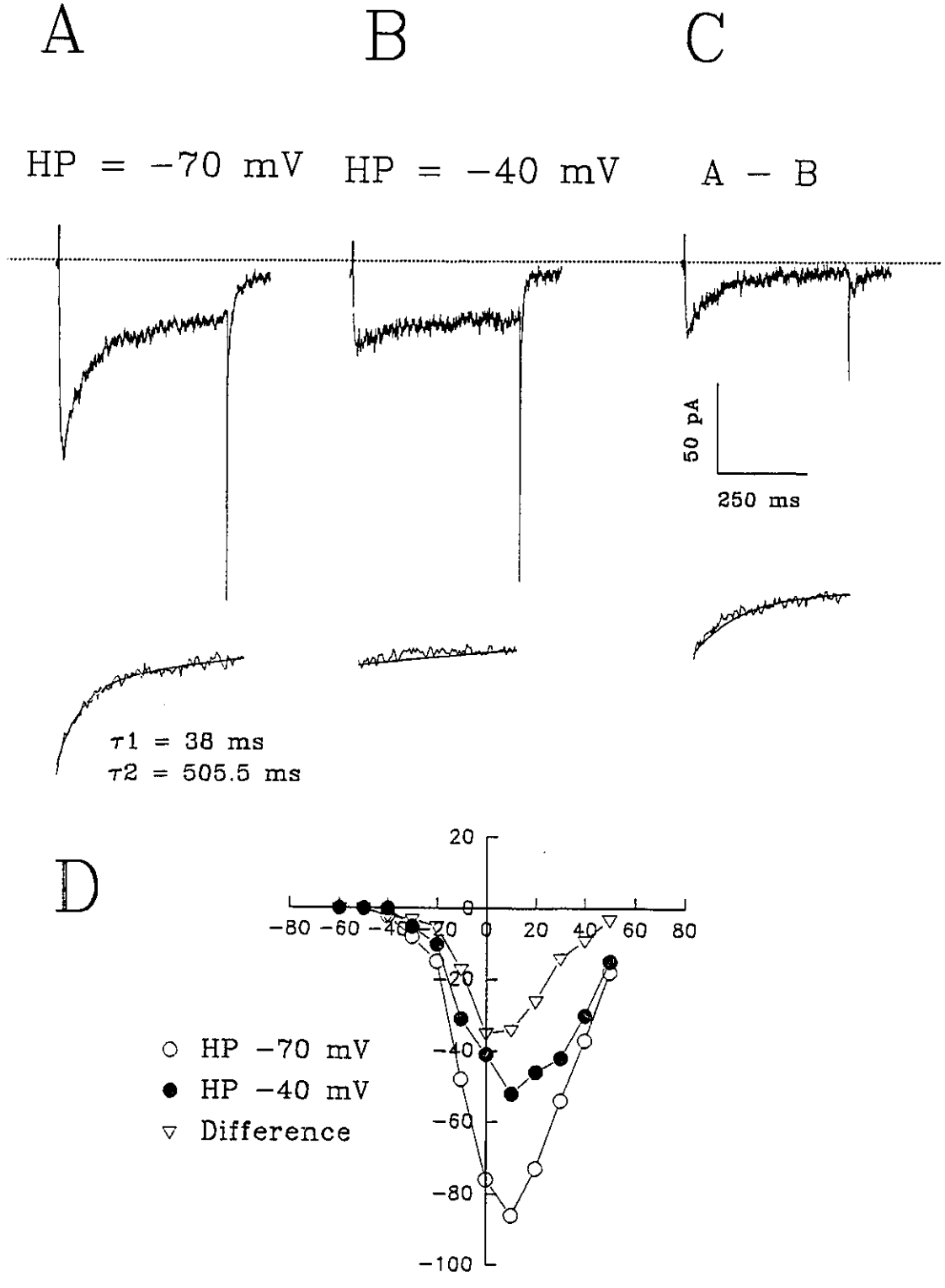
Figure 6. Two Components of Inward Current.

Panel A. Upper panel: inward currents were recorded from a holding potential of -70 mV to a test potential of +10 mV. The decay of inward current was composed of fast and slow components. Lower panel: the decay of inward current was well fitted by two exponentials with a time constant of 38 ms and 505.5 ms respectively.

Panel B. Upper panel: inward currents were recorded from a holding potential of -40 mV to a test potential of +10 mV. Only the slow inactivating component of inward current was observed. Lower panel: the decay of the inward current was fitted by a single exponential.

Panel C. Upper panel: subtracting the currents recorded from a holding potential of -70 mV by that with a holding potential of -40 mV revealed a transient and fast inactivating component corresponding the T-type inward current. Lower panel: the decay of the inward current was fitted with a single exponential.

Panel D. The I-V curves for the inward currents recorded from holding potential of -70 mV (○); from holding potential of -40 mV (●); and the currents obtained by subtracting the inward current from holding potential of -70 mV by that from holding potential of -40 mV (▽).


A
B
C
HP = −70 mV
HP = −40 mV
A − B
50 pA
250 ms
τ1 = 38 ms
τ2 = 505.5 ms
D
HP −70 mV
HP −40 mV
Difference
20
0
−20
−40
−60
−80
−100
−80 −60 −40 −20 0 20 40 60 80

of fast and slow inactivating processes indicating a mixture of T and L currents (Fig. 6A). The decay of the inward current was well fitted by two exponentials with a time constant of 38 and 505.5 ms respectively (Fig. 6A, bottom). When the holding potential was set to -40 mV, only the slow inactivating component persisted (Fig. 6B). Subtracting the currents recorded with holding potential of -70 mV by that recorded with holding potential of -40 mV revealed a transient and fast inactivating component of inward current corresponding to the T-type of inward current (Fig. 6C). The current-voltage relationships for both slow and fast component of inward current were shown in figure 6D. The slow component had a threshold at about -30 mV, while the fast component had a threshold around -40 mV. The maximal inward current was recorded at +10 mV for the slow component while it was recorded at 0 mV for the fast component. Both the slow and fast component had a reversal potential around +60 mV (Fig. 6D). Existence of two components of inward currents was also demonstrated by using L-type calcium channel blocker nifedipine (Fig. 7). After blocking of slow inactivating L-type inward current by nifedipine, a transient inward current was observed in some cells (Fig. 7B). A combination of nifedipine and low holding potential (positive to -40 mV) completely abolished the inward current (not shown).

(iii). Activation and Inactivation

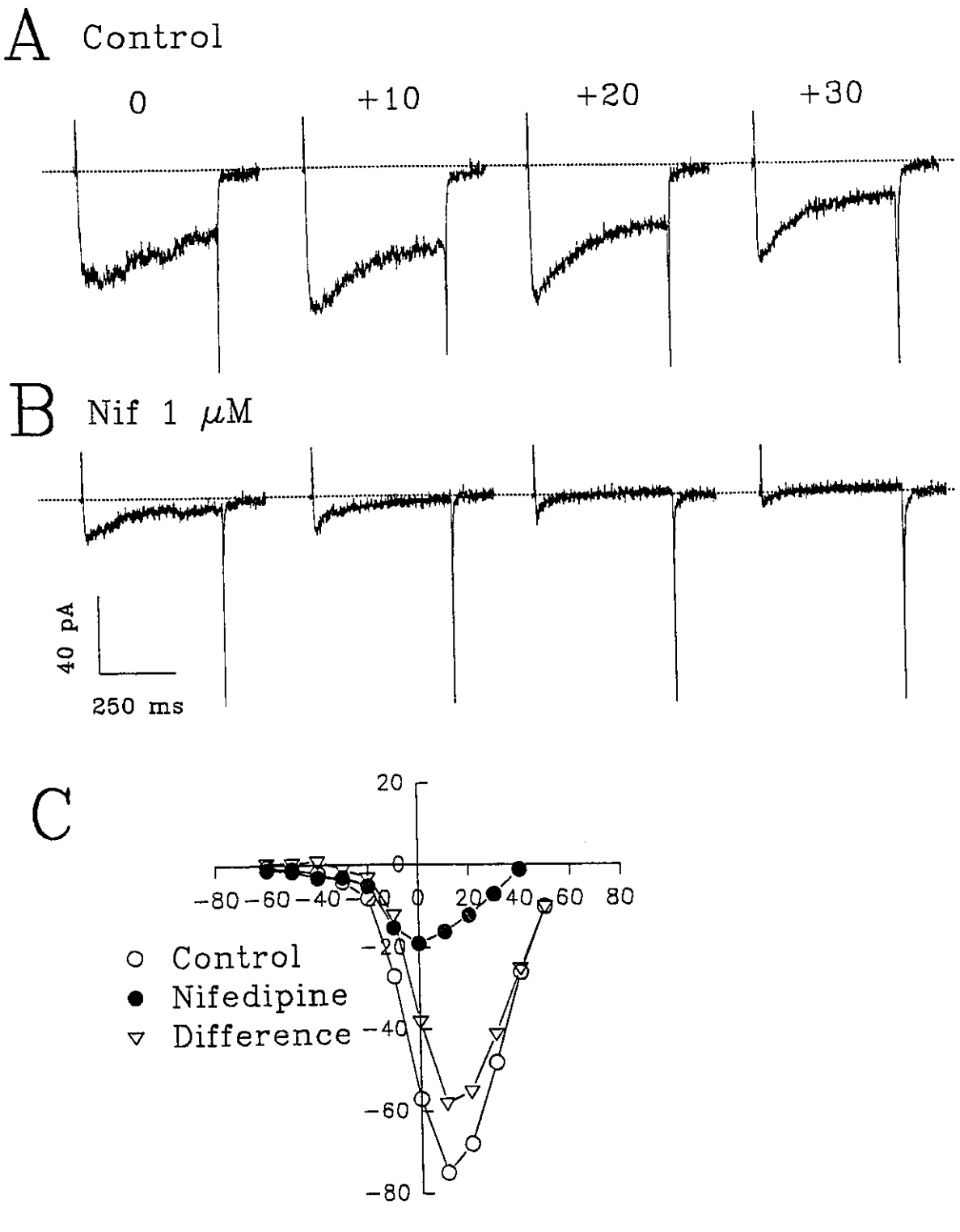
Voltage-dependent activation of inward current was examined by plotting the normalized conductance of inward current as a function of test potential. The peak inward current was used to calculate the conductance at each test potential by dividing the

Figure 7. Effect of Nifedipine on Inward Current.

Panel A. Inward current elicited from a holding potential of -70 mV to the test potentials ranging from 0 to +30 mV. The decay of inward current was composed of fast and slow component.

Panel B. In the presence of nifedipine (Nif) 1 μM, the amplitude of inward current was substantially decreased and only the transient and fast inactivating component was observed.

Panel C. I-V curves for the inward current in control (○); nifedipine 1 μM (●); and the difference between control and nifedipine (▽).


A
Control
0
+10
+20
+30
B
Nif 1 μM
40 pA
250 ms
C
20
0
-80 -60 -40 -20 0 20 40 60 80
-20
-40
-60
-80
Control
Nifedipine
Difference

peak amplitude by the driving force ($V-V_{rev}$). An average of +58 mV was obtained as the reversal potential for the inward current carried by 10 mM Ba^{2+} (n=8). Figure 8A shows an example of voltage-dependent activation of inward current. Currents were elicited by 500 ms depolarizing test pulse to different potentials (V_t) from holding potential of -70 mV. Inward current was observed at the test potential of -30 mV and the amplitude of the current increased at more depolarized potentials (Fig. 8A). Plotting of normalized conductance versus test potential displayed a sigmoid activation curve (Fig. 8C). The threshold of activation was around -40 mV and full activation was reached with a test potential positive to +10 mV (Fig. 8C). The half maximum of activation (V_h, 5.1 mV) and the slope of the activation curve (k, -7.7) were obtained by the fitting with Boltzmann's equation.

The time dependence of the inactivation was studied by using a pre-pulse of variable duration, t_v, followed by a test pulse that maximally activated the inward current. Figure 9 shows an example of the experiment: from a holding potential of -80 mV, a pre-pulse to -20 mV was given, and subsequently a 300 ms test pulse to +10 mV was applied to the cell. The duration of the pre-pulse was varied between 0 and 10 s. The amplitude of inward current activated at the test potential was decreased when pre-pulse duration was increased. This decrease was faster with the pre-pulse duration between 0 and 5 s and almost reached steady-state with the pre-pulse duration longer than 5 s (Fig. 9A). The time dependent inactivation curve was shown in figure 9B (n=11). The curve was well fitted by two exponentials with a time constant of 1.76 and 38.35 sec respectively.

Steady-state inactivation was determined by holding the membrane potential for

Figure 8. Voltage-dependent Activation and Inactivation of Inward Current.

Panel A. Representative traces showing an example of voltage-dependent activation of the inward current. Currents were recorded from a holding potential of -70 mV to different test potentials indicated above each trace. Inward current was observed with test potentials positive to -30 mV, and the amplitude of inward current increased at more depolarized test potential.

Panel B. Voltage-dependent inactivation of inward current. Currents were elicited by a depolarizing step to the test potential of +10 mV from different holding potentials (5 s duration). With holding potentials negative to -40 mV, no obvious inactivation of inward current was observed. However, with holding potentials positive to -30 mV, inactivation of inward current was observed as the amplitude of the current decreased with more positive holding potentials.

Panel C. Activation (○) and inactivation (●) curves of inward current. For activation curve, the peak inward current was used to calculate the conductance at each test potential by dividing the peak amplitude by the driving force ($V-V_{rev}$). An average of +58 mV was used for V_{rev}. The activation curve was constructed by plotting the normalized conductance against the test potential. For inactivation curve, the peak inward current induced from different holding potential was normalized to the inward current recorded with a holding potential of -70 mV and plotted against the holding potential. The smooth curves were obtained by fitting with Boltzmann's equation. n = 5 for both curves.

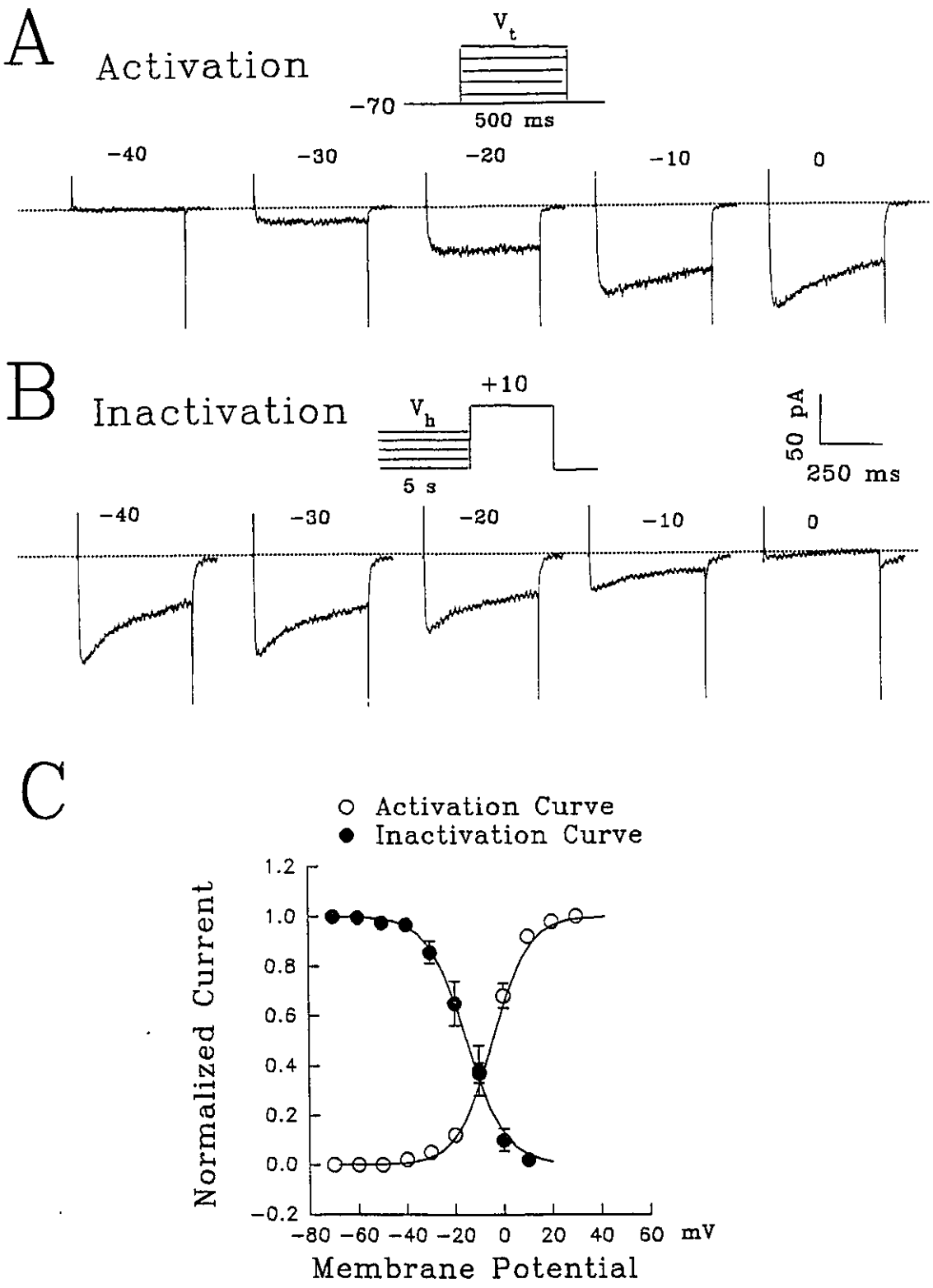
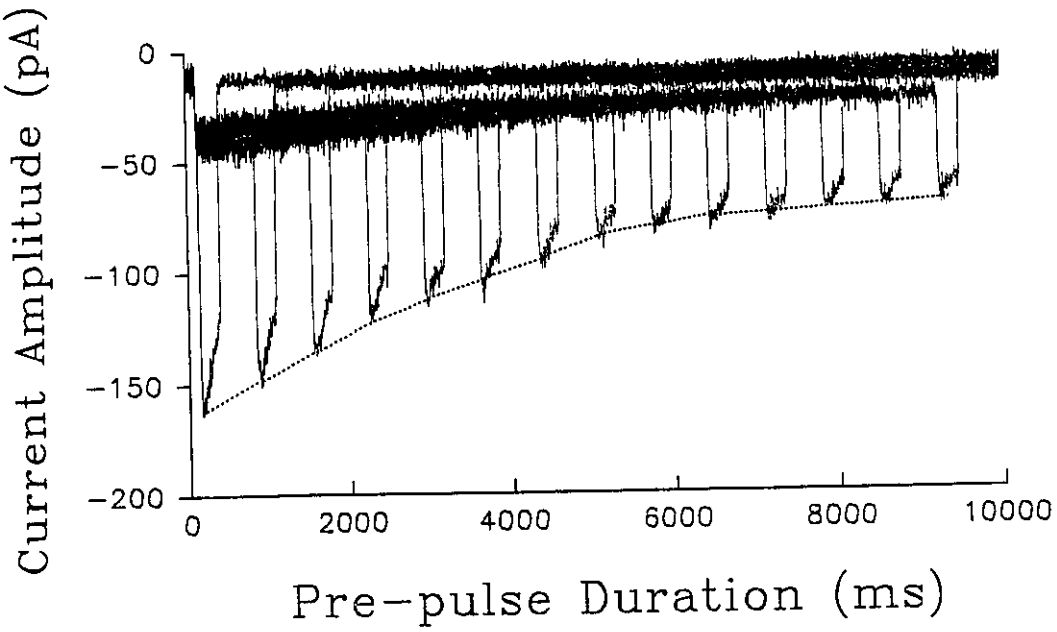

A
Activation
V_t
−70
500 ms
−40
−30
−20
−10
0
B
Inactivation
+10
V_h
5 s
50 pA
250 ms
−40
−30
−20
−10
0
C
Activation Curve
Inactivation Curve
Normalized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−80 −60 −40 −20 0 20 40 60 mV
Membrane Potential

Figure 9. Time-dependent Inactivation of Inward Current.

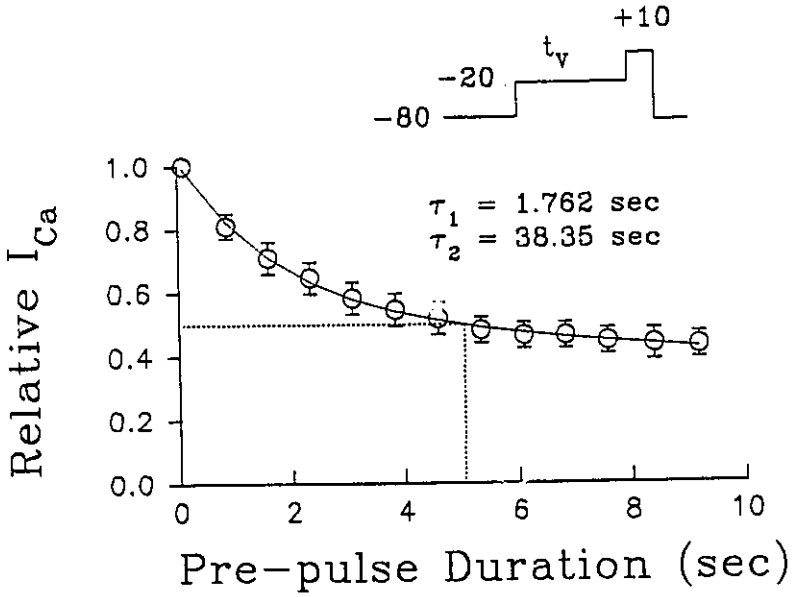
Panel A. Superimposed traces showing the time-dependent inactivation of inward current. From a holding potential of -80 mV, a pre-pulse to -20 mV with durations between 0 to 10 s was given, and subsequently a 300 ms test pulse to +10 mV was applied to the cell. The amplitude of inward current gradually decreased with the increase in the pre-pulse duration. The decrease in current amplitude was fast with the pre-pulse duration between 0 to 5 s, and almost reached steady-state with the pre-pulse duration longer than 5 s.

Panel B. Time-dependent inactivation curve of the inward current. From holding potential of -80 mV, currents were elicited by a 300 ms test pulse to +10 mV following a pre-pulse to -20 mV with various durations from 0 to 10 s. The amplitude of inward currents with different durations of pre-pulse were normalized to the current recorded with no pre-pulse and plotted against the pre-pulse duration. n = 11. The curve was fitted by two exponential with a time constant of 1.762 and 38.350 sec respectively.

A


Current Amplitude (pA)
0
-50
-100
-150
-200
0
2000
4000
6000
8000
10000
Pre-pulse Duration (ms)

B


+10
t_V
-20
-80
Relative I_{Ca}
1.0
0.8
0.6
0.4
0.2
0.0
τ_1 = 1.762 sec
τ_2 = 38.35 sec
0
2
4
6
8
10
Pre-pulse Duration (sec)

a prolonged period (5 s) at various levels, followed by a depolarizing step to +10 mV. The peak inward current induced by this procedure is an estimate of the fraction of channels that have not inactivated. Figure 8B shows an example of inactivation. Inward currents were elicited with 500 ms step pulses to +10 mV from holding potential ranging from -40 to 0 mV. Inactivation of inward current was observed with a holding potential positive to -30 mV as demonstrated by a decrease in current amplitude. As the holding potential decreased, more current was inactivated. With a holding potential of 0 mV, only very small inward current was recorded (Fig. 8B). The inactivation curve was constructed by normalizing the current amplitude recorded with different holding potentials to that recorded with holding potential of -70 mV and plotted against the holding potential (Fig. 8C). Inward current started to be inactivated with the holding potential positive to -30 mV, and almost completely inactivated with the holding potential positive to +10 mV. The half maximum inactivation was -15 mV and the slope factor was 6.9 for the inward current (n=6).

(iv). Effect of nifedipine

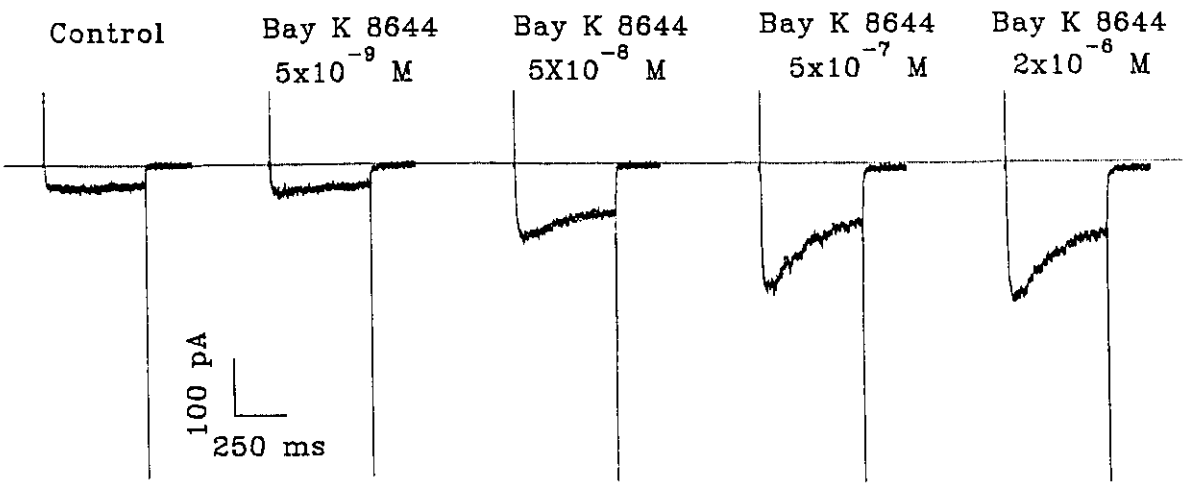
Nifedipine reduced the amplitude of the inward currents at all test potentials (Fig. 7), and complete blockade was achieved with 1 μM nifedipine in majority of the cells. A small transient inward current remained in some cells after nifedipine, corresponding to the T-type Ca^{2+} channel current (Fig. 7B). However, in the most cells, this transient component contributed only a small fraction of the total current (<15%).

Figure 10. Dose-dependent Effect of Bay K 8644 on Inward Current.

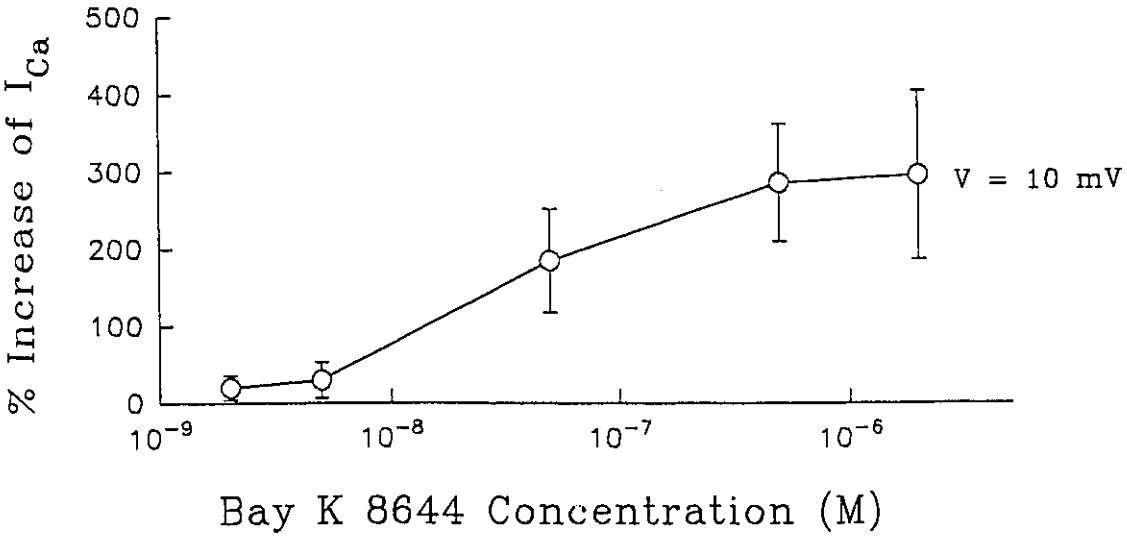
Panel A. Representative recording showing the dose-dependent effect of Bay K 8644 on inward current recorded at a test potential of +10 mV from holding potential of -70 mV. Capacitive currents have been truncated.

Panel B. Graph showing the dose-response curve for the effect of Bay K 8644 on the inward current amplitude. The percentage change was measured by comparing the peak current in control with that after application of Bay K 8644 for 10 mins. n = 4 or 5. Holding potential = -70 mV; test potential = +10 mV.

A


Control
Bay K 8644
5x10^{-9} M
Bay K 8644
5X10^{-8} M
Bay K 8644
5x10^{-7} M
Bay K 8644
2x10^{-6} M
100 pA
250 ms

B


% Increase of I_{Ca}
500
400
300
200
100
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
V = 10 mV
Bay K 8644 Concentration (M)

(v). Effect of Bay K 8644

Bay K 8644 dose-dependently increased inward current amplitude with a threshold concentration of about 2 nM and reached maximum effect at a concentration of 1 μM (Fig. 10A, B). At a testing potential of +10 mV, a threefold (300 ± 110%, n=5) increase of current amplitude was recorded with maximal concentration of Bay K 8644 (Fig. 10B). The percentage increase in current amplitude induced by Bay K 8644 was larger at lower test potentials and declined with more positive test potentials (Fig. 11A). At a testing potential of 0 mV, more than fivefold (560 ± 230%, n=5) increase in current amplitude was recorded. In addition to an increase in current amplitude, Bay K 8644 caused a leftward shift of I-V curve (Fig. 11A). Both the threshold and the maximal inward current were shifted toward more negative membrane potential. The activation and inactivation curves were also shifted toward more negative membrane potential by Bay K 8644 without significant change in the slop factor of the curves (Fig. 11B, C). A 20 mV leftward shift of activation curve was recorded with Bay K 8644 0.5 μM ($n=6$, $p<0.01$) (Fig. 11B). Similarly, a 14.3 mV leftward shift in inactivation curve was recorded with the same concentration of Bay K 8644 (n=6) (Fig. 11C). Bay K 8644 also affected the time course of inactivation of the inward current as demonstrated by an increase in the rate of inactivation (Fig. 12).

Figure 11. Effects of Bay K 8644 on Activation and Inactivation.

Panel A. Current-voltage relationship of the inward current before and after exposure to Bay K 8644 (0.5 μM). The amplitudes of inward current have been normalized to cell capacitance. Holding potential = -70 mV. n = 6.

Panel B. Activation curves of the inward current in control and in the presence of Bay K 8644. The curves were fitted with Boltzmann's equation. n = 6.

Panel C. Inactivation curves of the inward current in control and after treatment with Bay K 8644. Currents were elicited by stepping to a test potential of +10 mV from different holding potentials with a duration of 5 s. The curves were fitted with Boltzmann's equation. n = 6.

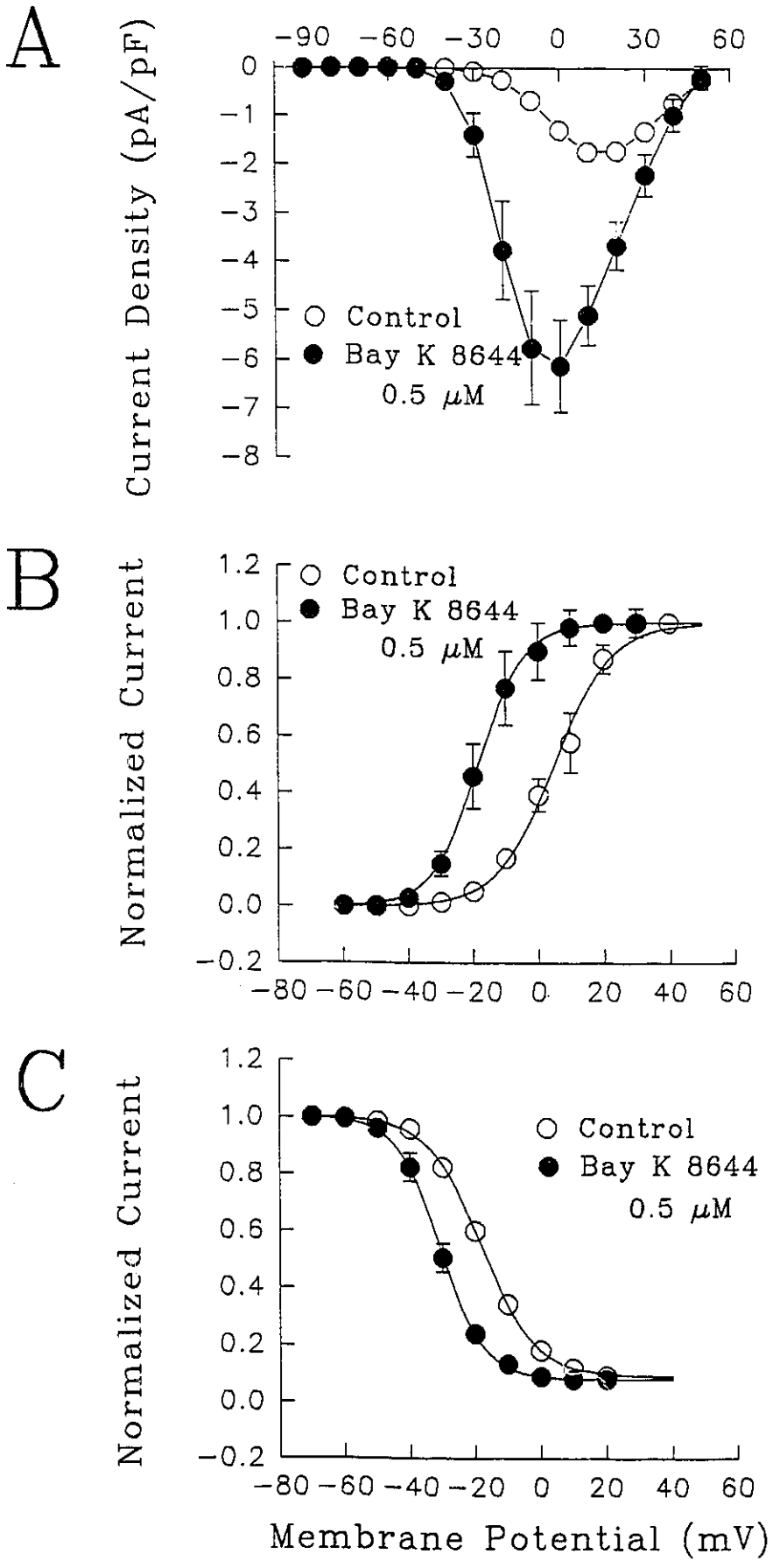
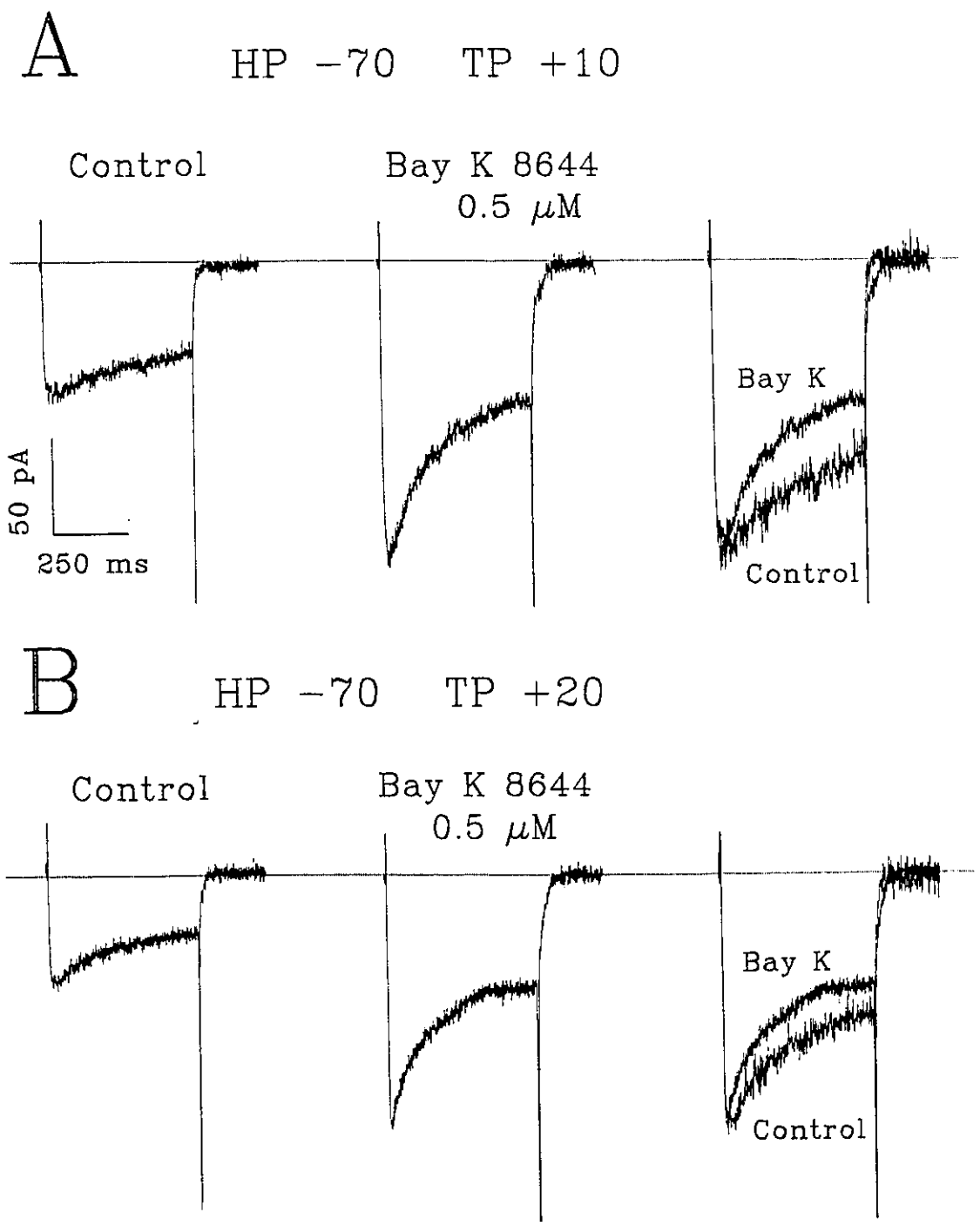

A
Current Density (pA/pF)
-90 -60 -30 0 30 60
0
-1
-2
-3
-4
-5
-6
-7
-8
Control
Bay K 8644
0.5 μM
B
Normalized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Control
Bay K 8644
0.5 μM
-80 -60 -40 -20 0 20 40 60
C
Normalized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Control
Bay K 8644
0.5 μM
-80 -60 -40 -20 0 20 40 60
Membrane Potential (mV)

Figure 12. Effect of Bay K 8644 on the Time Course of Inactivation.

Panel A. Currents were elicited from a holding potential of -70 mV to a test potential of +10 mV. Left panel: control; Middle panel: with Bay K 8644 0.5 μM; Right panel: superimposed traces of the control current rescaled to match that in the presence of Bay K 8644.

Panel B. Currents were elicited from holding potential of -70 mV to test potential of +20 mV. Left panel: control; Middle panel: with Bay K 8644 0.5 μM; Right panel: superimposed traces of the control current rescaled to match that in the presence of Bay K 8644.


A
HP −70 TP +10
Control
Bay K 8644
0.5 μM
Bay K
Control
50 pA
250 ms
B
HP −70 TP +20
Control
Bay K 8644
0.5 μM
Bay K
Control

PART II. Effect of NPY on Inward Current

I. NPY Potentiates Inward Current

Figure 13 shows the effect of NPY on inward current with perforated-patch recording. Currents were elicited by 500 ms depolarizing step to test potentials from -40 to +40 mV with a holding potential of -70 mV. Inward current was observed at the test potential of -20 mV and the amplitude of inward current increased with test potentials from -20 to +10 mV. The maximal current was recorded at a test potential of +10 mV and the current declined at more positive test potentials (Fig. 13, left panel). The inward currents were enhanced with the application of NPY (150 nM) at all test potentials. The relative potentiation effect of NPY on current amplitude was larger at lower test potentials and declined with more positive test potential. At a test potential of -20 mV, about 100% increase in current amplitude was recorded. While at a test potential of +20 mV, only 30% increase in current amplitude was observed. NPY also shifted the threshold of inward current toward more negative test potential. In the absence of NPY the inward current was not detected at a test potential of -30 mV but it was readily observed after NPY application (Fig. 13, middle panel). The maximal inward current was recorded at the test potential of +10 mV both before and after NPY application (Fig. 13, left & middle panel). Figure 14 shows the time course of NPY effect. Maximum potentiation of the inward current by NPY was achieved in about 5 min and the effect of NPY was reversible. The peak of the inward currents was increased by 34% at a test potential of +10 mV ($n=6$, $p<0.05$). Concurrent recording of the series resistance indicates that the effect of NPY was not associated with changes in the series resistance (Fig. 14B, C).

Figure 13. Effect of NPY on Inward Current.

Panel A. Control. Currents were elicited with 500 ms depolarizing pulses from a holding potential of -70 mV to test potentials ranging from -40 to +40 mV. Capacitive currents have been truncated.

Panel B. Currents recorded after the cell had been exposed to NPY (150 nM) for 10 min.

Panel C. Current recorded after the cell had been treated with nifedipine (2 μM) for 5 min.

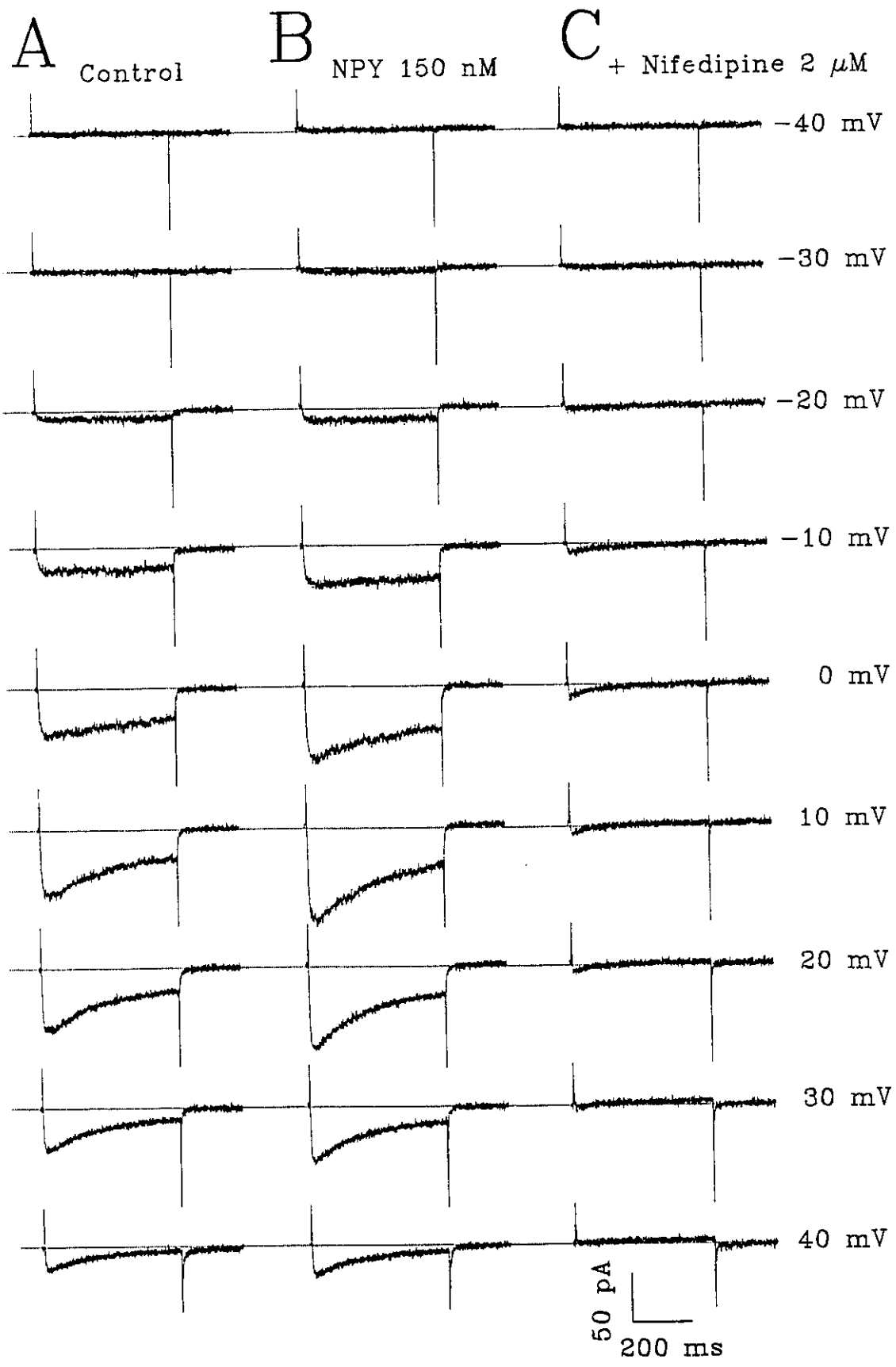
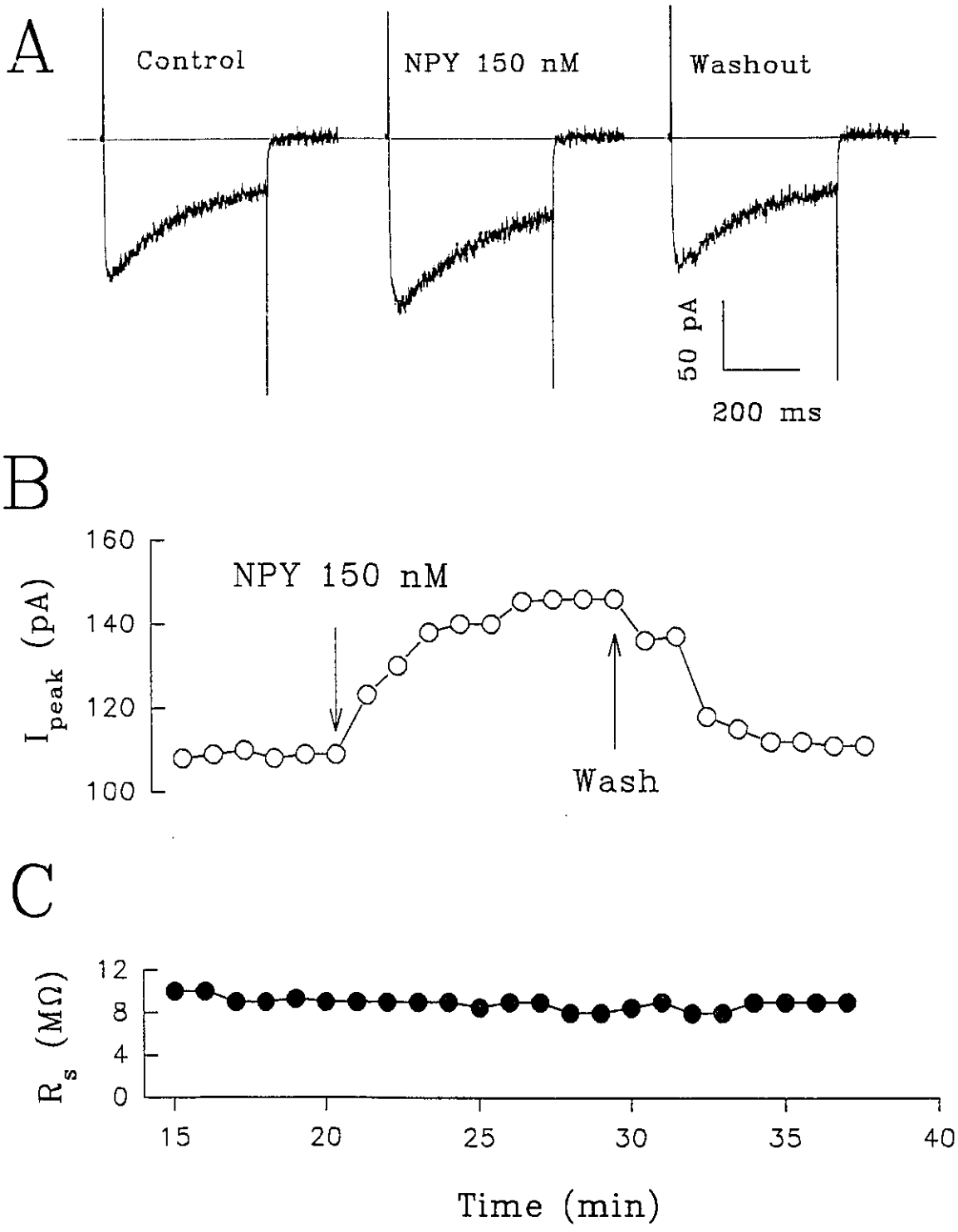

A
Control
B
NPY 150 nM
C
+ Nifedipine 2 μM
−40 mV
−30 mV
−20 mV
−10 mV
0 mV
10 mV
20 mV
30 mV
40 mV
50 pA
200 ms

Figure 14. Time Course of the Effect of NPY on Inward Current.

Panel A. Representative recordings showing the effect of NPY (150 nM) on the inward current and reversal to the control level after washout. Holding potential = -70 mV; test potential = +10 mV. Capacitive currents have been truncated.

Panel B. Graph showing the time-course of the effect of NPY on peak inward current.

Panel C. Changes in the series resistance (R_S) with time. The changes in current amplitude were not associated with changes in R_S.


A
Control
NPY 150 nM
Washout
50 pA
200 ms
B
160
140
120
100
I_{peak} (pA)
NPY 150 nM
Wash
C
12
8
4
0
R_s (MΩ)
15
20
25
30
35
40
Time (min)

II. Dose and Voltage Dependence of the Effect oi NPY

The effect of NPY was dependent on concentration and the test potential applied. At a test potential of +10 mV, a small detectable increase (13 ± 6%, n=5) of the current amplitude could be observed at about 50 nM and the increase reached a plateau at 150 nM (46 ± 10%, n=6, $p<0.01$) (Fig. 15). The percentage increase was maximal at lower test potentials and declined with more positive test potentials. With 150 nM NPY, inward current was increased by 78 ± 14%, 46 ± 10% and 27 ± 6% at test potential of 0, 10 and 20 mV respectively (Fig. 15).

III. Effects of Nifedipine and Cd^{2+}

The inward currents were sensitive to blockade by the divalent cation Cd^{2+}. The peak amplitude of inward currents at the testing potential of +10 mV was reduced to 6.4 ± 0.7% of the control value by Cd^{2+} (5 μM, 5 mins) (n=3). Subsequent addition of NPY 150 nM failed to enhance the inward current (6.6 ± 0.5%; Fig. 16). Similar effects were obtained with nifedipine. Application of nifedipine (1 μM) resulted in a significant reduction in the peak inward current to 20.3 ± 4.6% (n=4). The inward current were not potentiated by the subsequent addition of NPY 150 nM (21.0 ± 4.7%) (Fig. 17). The potentiation effect of NPY on inward current was also abolished by subsequent addition of nifedipine at all test potentials (Fig. 13, right panel). These results suggested that NPY was acting on currents mediated by Ca^{2+}-channels.

Figure 15. Dose and Voltage-dependent Effect of NPY on Inward Current.

Graph showing the dependence of current amplitude on NPY concentration and test potential. Currents were elicited from a holding potential of -70 mV. The percentage change was measured by comparing the peak current in control with that after application of NPY for 10 min. n = 5 or 6.

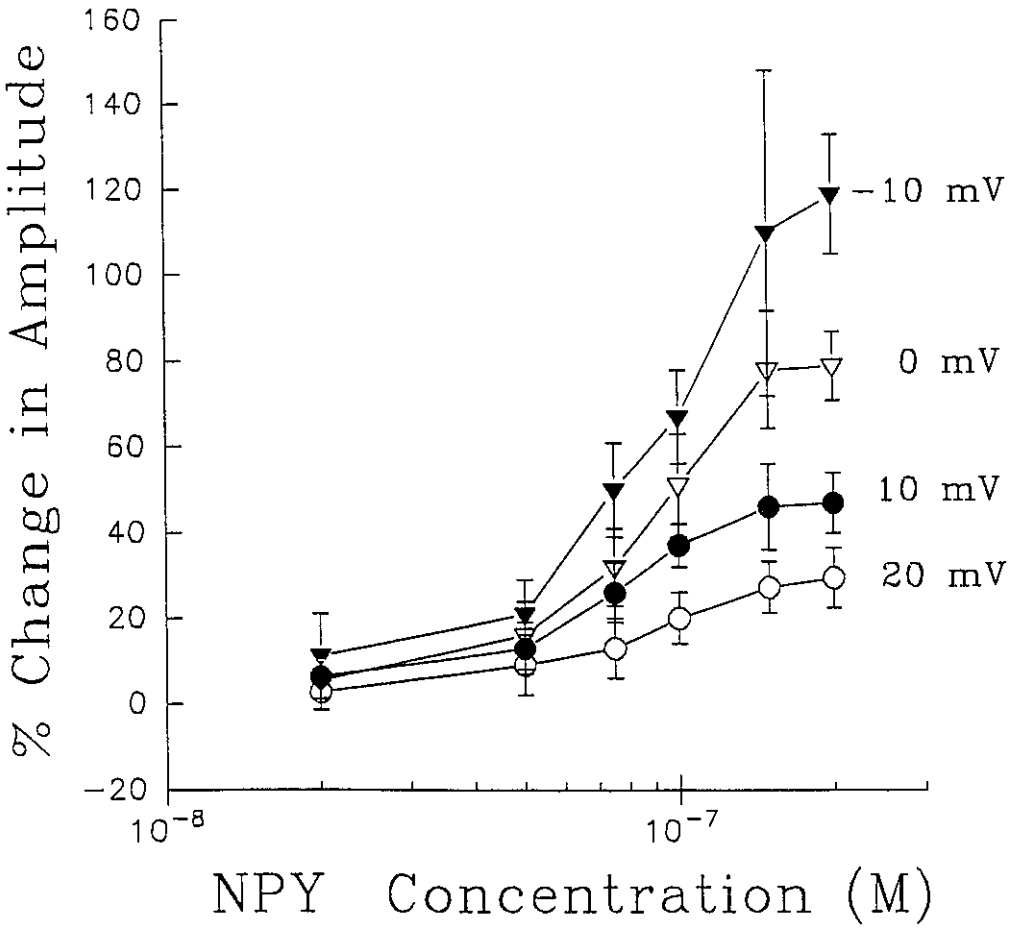
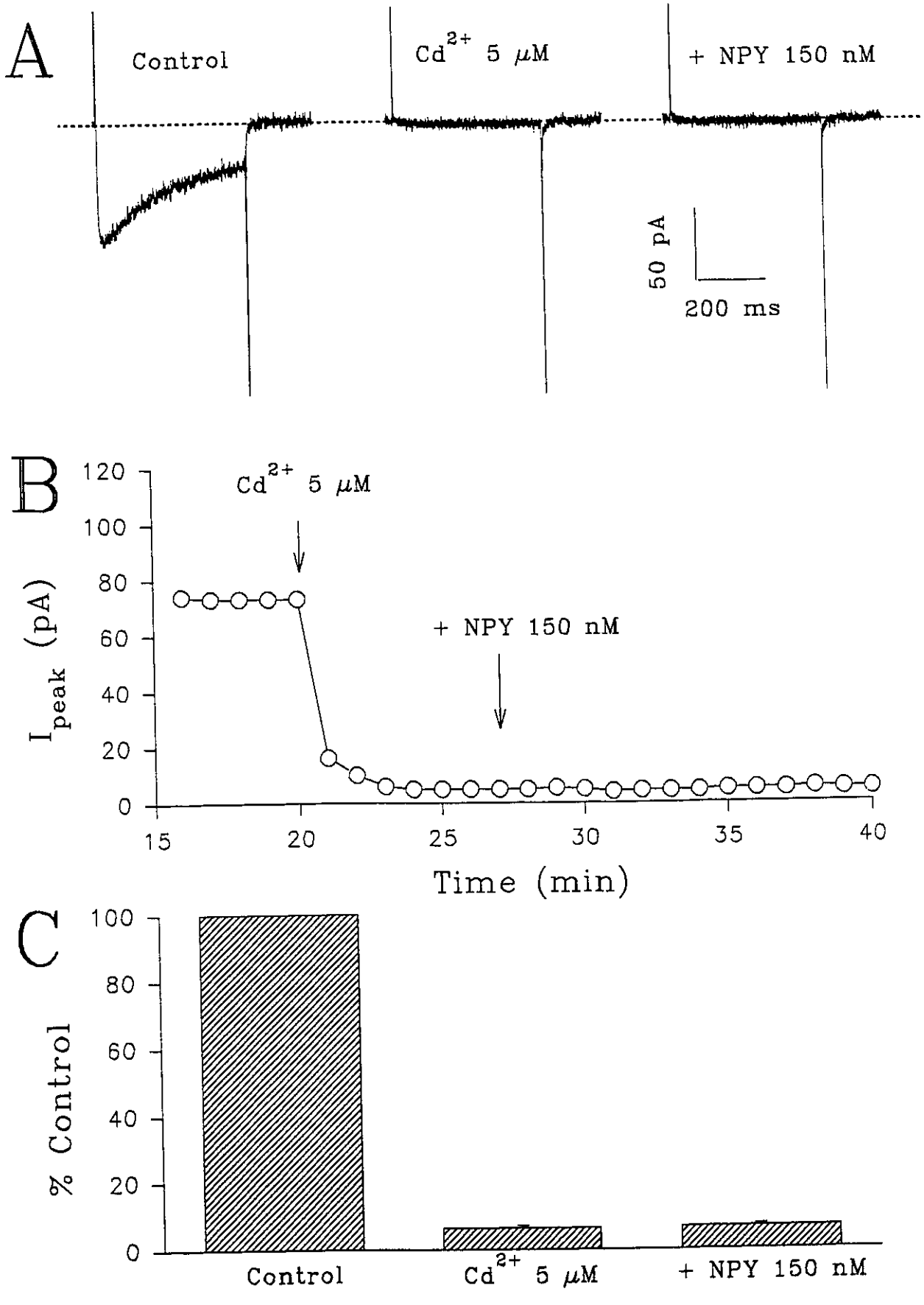

160
140
120
100
80
60
40
20
0
−20
% Change in Amplitude
10^−8
10^−7
NPY Concentration (M)
−10 mV
0 mV
10 mV
20 mV

Figure 16. Effect of Cd^{2+} on Inward Current.

Panel A. Representative recordings showing the effect of Cd^{2+} (5 μM) on the inward current. Subsequent addition of NPY (150 nM) failed to generate additional current. Holding potential = -70 mV; test potential = +10 mV. Capacitive currents have been truncated.

Panel B. Graph showing the time course of the effect of Cd^{2+} on the inward current.

Panel C. Bar graph showing the effect of Cd^{2+} (5 μM) alone and with NPY (150 nM) on the inward current. n = 3.


A
Control
Cd2+ 5 μM
+ NPY 150 nM
50 pA
200 ms
B
Cd2+ 5 μM
+ NPY 150 nM
Ipeak (pA)
120
100
80
60
40
20
0
15
20
25
30
35
40
Time (min)
C
% Control
100
80
60
40
20
0
Control
Cd2+ 5 μM
+ NPY 150 nM

IV. Effect of Changing Holding Potential on NPY Effect

A small transient inward current persisted in the presence of nifedipine (Fig. 17A). This may represent the T-type of Ca^{2+}-channel current that is known to be resistant to nifedipine. The transient current was not affected by 150 nM NPY (Fig. 17). This observation would suggest that NPY acts mainly on the L-type current. To confirm that NPY acts on L-type Ca^{2+}-channels, the potentiating effect of NPY (150 nM) on inward current at +10 mV from different holding potentials was compared. It is well documented that low-threshold T-type Ca^{2+}-channels inactivate below -30 mV while high-threshold L-type channels remained mostly active at these potentials (Wang et al., 1989; Wiley et al., 1990). Figure 18 demonstrates that the effect of NPY (150 nM) was little affected by changing the holding potential from -70 mV to -40 mV (Fig. 18A, B). In fact, the percentage increase of the inward current with NPY remained the same for holding potentials between -70 mV and -30 mV. From a holding potential of -70 mV, inward current at test potential of +10 mV was increased by 33.0 $\pm$ 7.5% (n=4) with NPY (150 nM). A 33.4 $\pm$ 7.8% increase was obtained with a holding potential of -40 mV (n=4) (Fig. 18C). These findings are consistent with an action of NPY on L-type Ca^{2+}-channels. NPY also did not change the time course of the inward currents, as revealed by the superimposed traces of the rescaled control response on that with NPY (Fig. 18A, B, right-hand panels).

V. Effect of NPY on Activation and Inactivation

NPY increased the inward currents most effectively at lower voltage ranges, as

Figure 17. Effect of Nifedipine on Inward Current.

Panel A. Representative recordings showing the effect of nifedipine (Nif) (1 μM) on the inward current. Subsequent addition of NPY (150 nM) failed to potentiate the remaining current. Holding potential = -70 mV; test potential = +10 mV. Capacitive currents have been truncated.

Panel B. Graph showing the time course of the effect of nifedipine on the inward current.

Panel C. Bar graph showing the effects of nifedipine (1 μM) alone and with NPY (150 nM) on the inward current. n = 4.

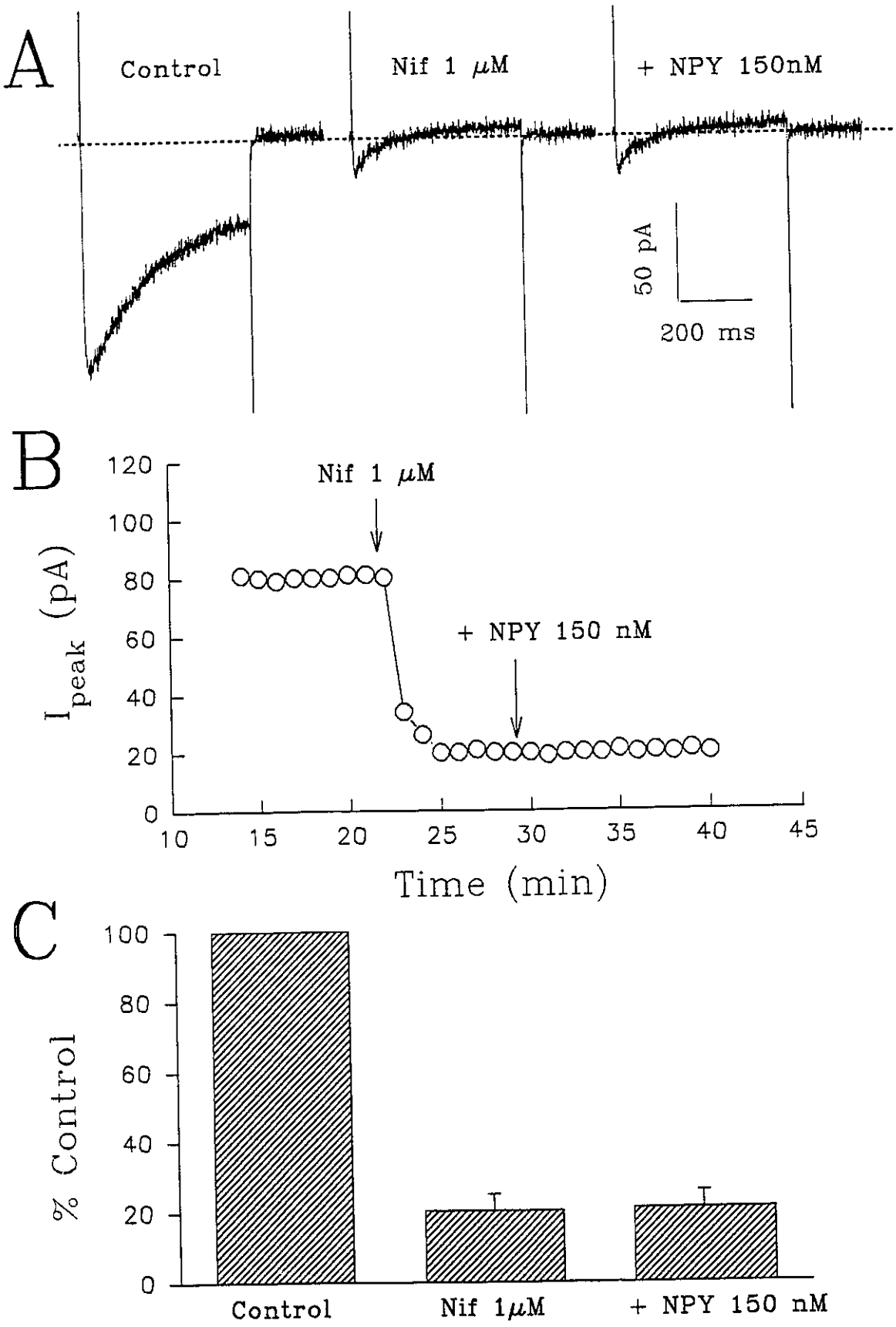
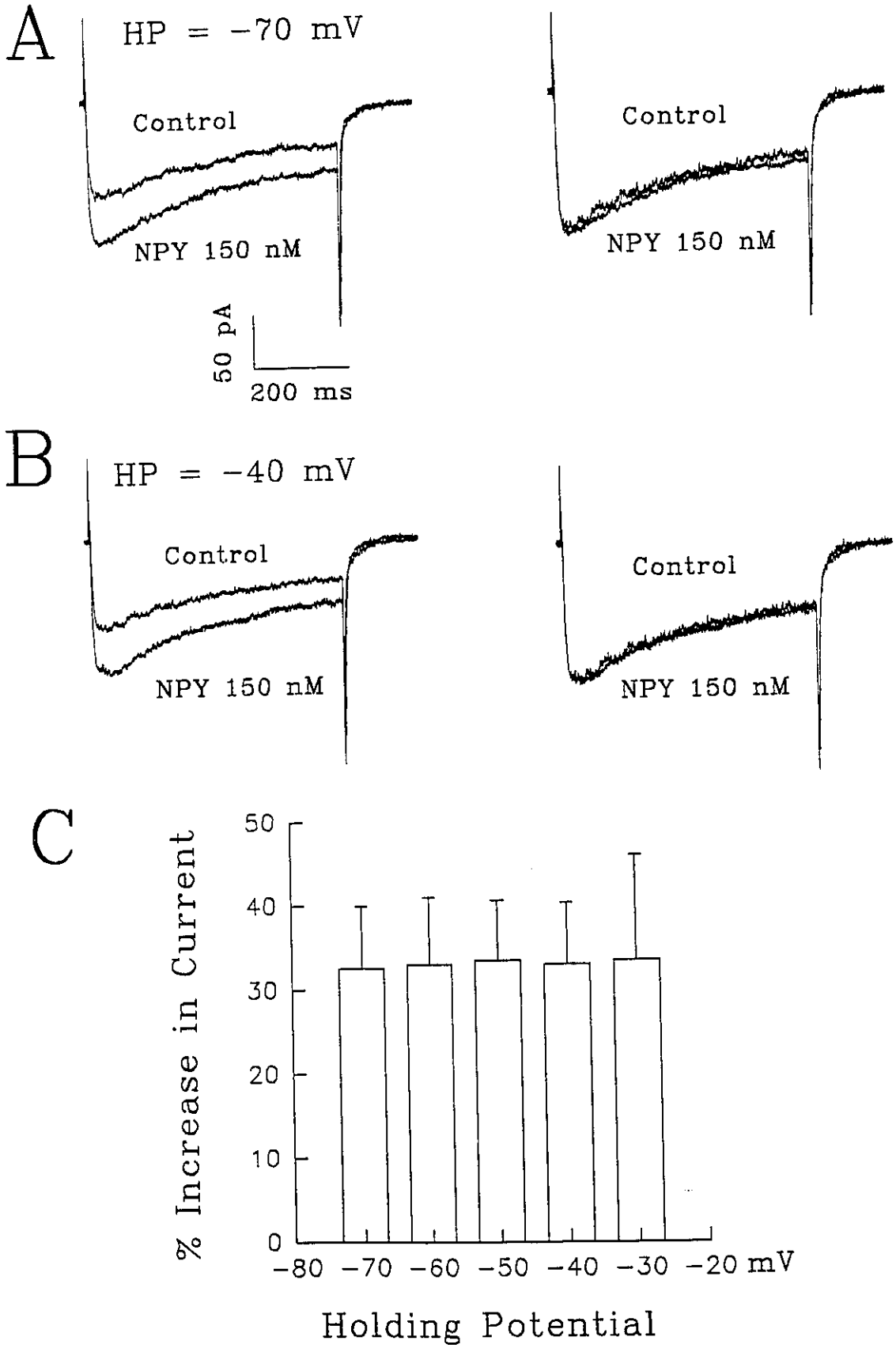

A
Control
Nif 1 μM
+ NPY 150nM
50 pA
200 ms
B
Nif 1 μM
+ NPY 150 nM
I_{peak} (pA)
120
100
80
60
40
20
0
10
15
20
25
30
35
40
45
Time (min)
C
% Control
100
80
60
40
20
0
Control
Nif 1μM
+ NPY 150 nM

Figure 18. Effect of Different Holding Potentials on NPY Effect.

Panel A. Superimposed traces showing the increase in inward current with NPY (150 nM) from a holding potential of -70 mV to a test potential of +10 mV. The right panel shows superimposed traces of the control current rescaled to match that recorded in the presence of NPY.

Panel B. Superimposed traces showing the increase in current amplitude induced by NPY (150 nM) from a holding potential of -40 mV to a test potential of +10 mV. The right panel shows superimposed traces of the control current rescaled to match that recorded in the presence of NPY.

Panel C. Bar graph showing the percentage increase in peak current amplitude induced by NPY (150 nM) at holding potentials from -70 mV to -30 mV to a test potential of +10 mV. n = 4.


A
HP = −70 mV
Control
NPY 150 nM
50 pA
200 ms
Control
NPY 150 nM
B
HP = −40 mV
Control
NPY 150 nM
Control
NPY 150 nM
C
% Increase in Current
50
40
30
20
10
0
−80 −70 −60 −50 −40 −30 −20 mV
Holding Potential

shown in the current-voltage relationship (Fig. 19A). The currents reached a maximum at +10 mV in both the control and in the presence of NPY except that the peak was broader for NPY. NPY did not change the reversal potential of the inward currents (control = +58.3 ± 3.0 mV; NPY = +58.1 ± 3.2 mV; n=5). The effect of NPY on steady-state activation of the Ca^{2+}-channel currents was calculated according to the peak currents (Isenberg & Klockner, 1982) and the activation curves were fitted with a Boltzmann distribution (Hodgkin & Huxley, 1952) (see Method). NPY caused a parallel shift of the steady-state activation curves to less positive potentials, as indicated by a 6 mV shift in V_h (control = 5.1 ± 1.2 mV; NPY 150 nM = -1.0 ± 1.2 mV; n=6; $p<0.01$) (Fig. 19B). There was no significant change in the slope factor (control = 7.7 ± 0.6; NPY = 7.4 ± 0.5).

Steady-state inactivation of the inward currents was studied by holding the cells at various potentials for 5 second before stepping to a test potential of +10 mV. The curves similarly fitted to Bolzmann's equation showed no significant changes in either V_h (control = -15.0±3.0 mV; NPY 150 nM = -16.2 ± 2.1 mV; n=5) or the slope factor (control = -6.9 ± 0.6 mV; NPY = -7.1 ± 0.2) (Fig. 19C).

VI. Interaction Between NPY and Bay K 8644

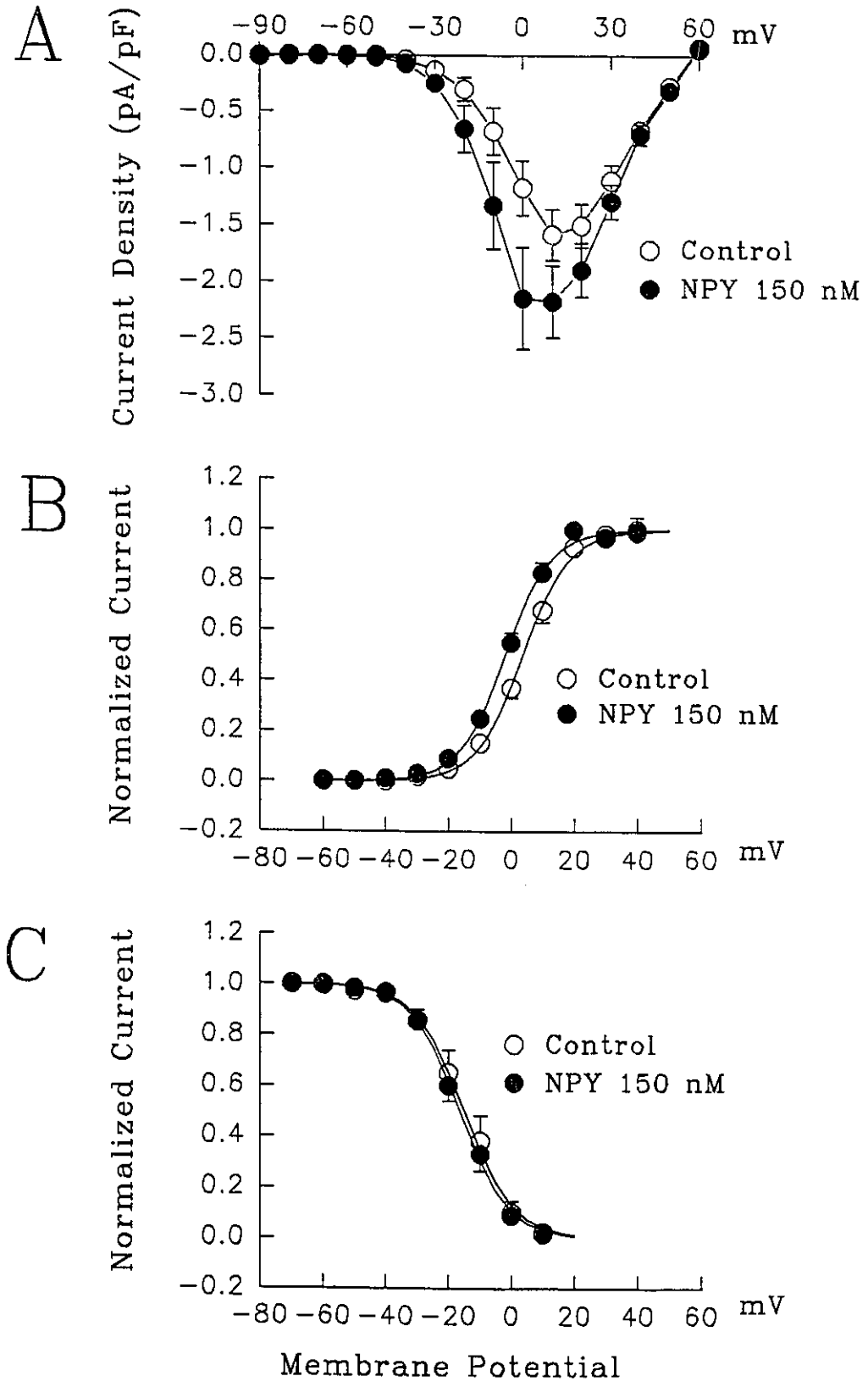
To investigate whether NPY and Bay K 8644 exert their effects on Ca^{2+} channels through a similar mechanism, the possible interaction between NPY and Bay K 8644 on inward currents was studied in three different conditions: 1) Bay K 8644 was applied on top of maximal concentration of NPY (200 nM); 2) NPY was applied on top of maximal

Figure 19. Effects of NPY on Activation and Inactivation of Inward Current.

Panel A. I-V curve of inward current before (○) and after (●) exposure to NPY (150 nM). The amplitude of inward current has been normalized to cell capacitance. To measure the capacitance of each cell, a 10 mV hyperpolarizing step was applied from the holding potential of -70 mV. Cell capacitance was obtained by integrating the capacitive transient and dividing by the voltage step. n = 6.

Panel B. Activation curves of the inward current in control (○) and in the presence of NPY (150 nM) (●). The curves were fitted with Boltzmann's equation (See text). n = 6.

Panel C. Inactivation curves of the inward current in control (○) and after treatment with NPY (●). Currents were elicited by stepping to a test potential of +10 mV from different holding potentials with a duration of 5 s. The curves were fitted with Boltzmann's equation. n = 6.


A
Current Density (pA/pF)
-90 -60 -30 0 30 60 mV
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Control
NPY 150 nM
B
Normalized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-80 -60 -40 -20 0 20 40 60 mV
Control
NPY 150 nM
C
Normalized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-80 -60 -40 -20 0 20 40 60 mV
Control
NPY 150 nM
Membrane Potential

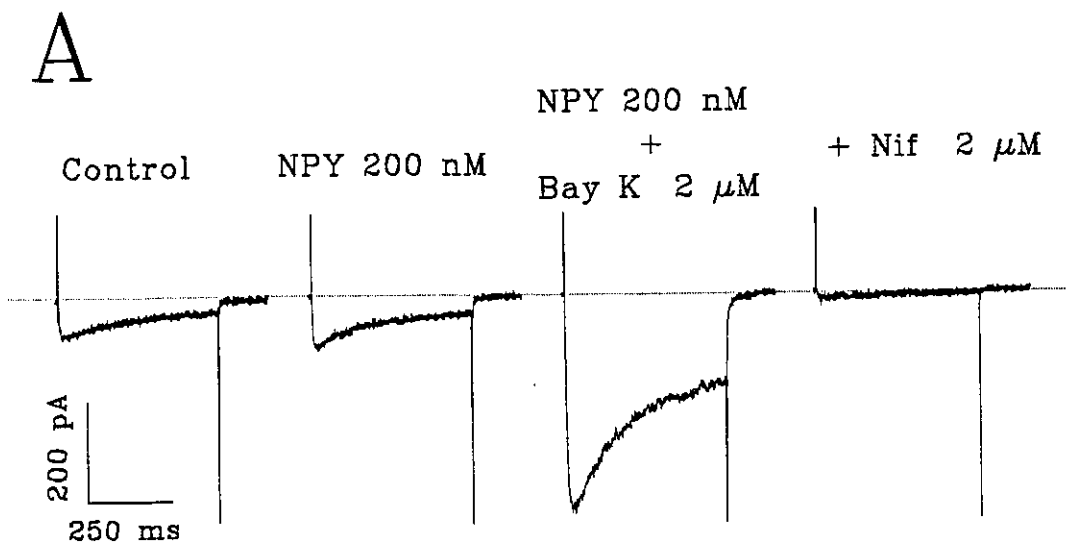
concentration of Bay K 8644 (2 nM). Figure 20 shows the effects of Bay K 8644 (2 μM) on top of maximal concentration of NPY (200 nM). At a test potential of +10 mV, a 42 ± 4% (n=5) increase in current amplitude was produced by NPY 200 nM. Addition of Bay K 8644 2 μM on top of NPY induced a total increase of current amplitude by 409 ± 87% (n=5) (Fig. 20A, B). NPY caused a slight leftward shift of I-V curve. Addition of Bay K 8644 induced a further leftward shift of the I-V curve. Figure 21 shows the effects of NPY on top of maximal concentration of Bay K 8644. Bay K 8644 2 μM induced increase in current amplitude with a leftward shift of I-V curve. Addition of NPY 200 nM on top of Bay K 8644 induced a further increase in current amplitude without further shift in I-V curve (Fig. 21A, B). At a test potential of +10 mV, a 302 ± 74% (n=4) increase in current amplitude was induced by Bay K 8644 (2 μM). Addition of NPY 200 nM produced a significant increase in current amplitude to 383 ± 85% (n=4, $p<0.05$). Figure 22 shows the effects of NPY on inward current in the presence of threshold concentration of Bay K 8644 (2 nM). At a test potential of +10 mV, a 32 ± 18% (n=4) increase in current amplitude was induced by Bay K 8644 2 nM. Subsequent addition of NPY 20, 75, and 200 nM induced a total increase in current amplitude by 43 ± 15%, 56 ± 22% and 79 ± 21% (n=4) respectively (Fig. 22A, B).

Figure 23 shows the additional currents generated by NPY 200 nM in the absence and presence of 2 nM and 2 μM of Bay K 8644. The values were obtained by subtracting the current after NPY by that before NPY with or without Bay K 8644. Preapplication of low concentration of Bay K 8644 (2 nM) did not affect the effect of NPY on the current as indicated by an overlap of I-V curves in the absence and presence

Figure 20. Effect of Bay K 8644 in the Presence of Maximal Concentration of NPY.

Panel A. Representative traces showing the effects of NPY (200 nM) alone and with Bay K 8644 (2 μM) on inward current. Currents were recorded from a holding potential of -70 mV to a test potential of +10 mV. After the cell had been exposed to NPY for 10 mins, Bay K 8644 was applied on top of NPY.

Panel B. I-V curves in control (○); NPY 200 nM alone (●); and NPY 200 nM plus Bay K 8644 2 μM (▽). NPY 200 nM induced increase in current amplitude with a slight leftward shift of I-V curve. Subsequent addition of Bay K 8644 2 μM induced much larger increase in current amplitude and further leftward shift of I-V curve. n=5.


A
Control
NPY 200 nM
NPY 200 nM
+
Bay K 2 μM
+ Nif 2 μM
200 pA
250 ms

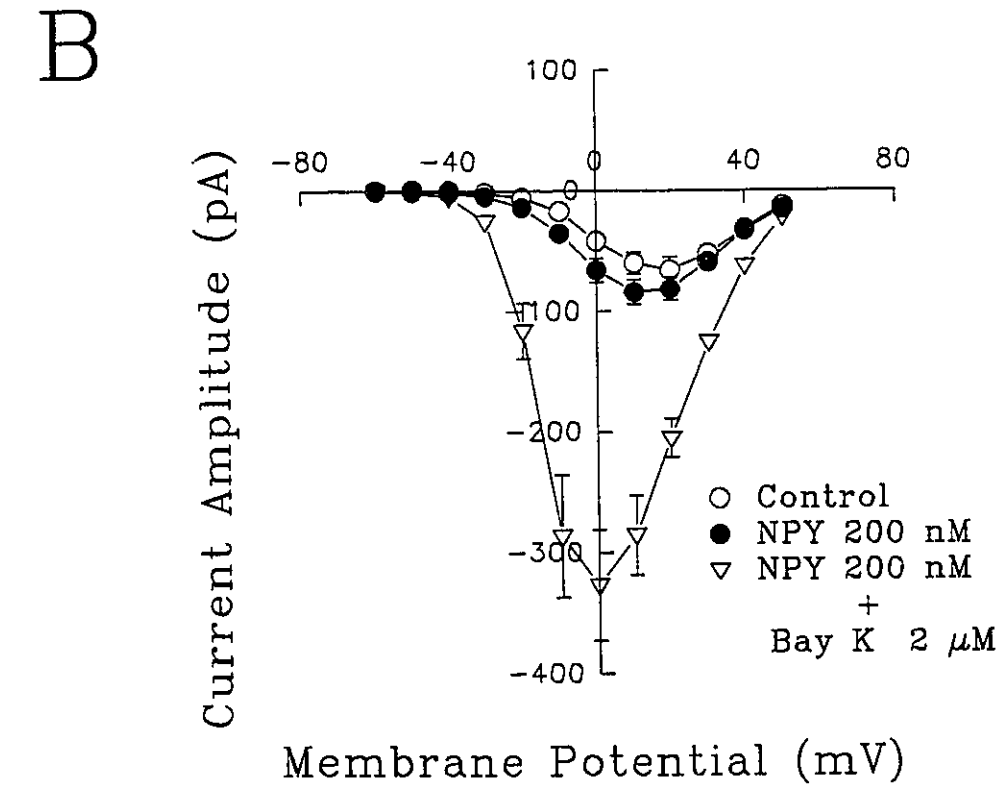
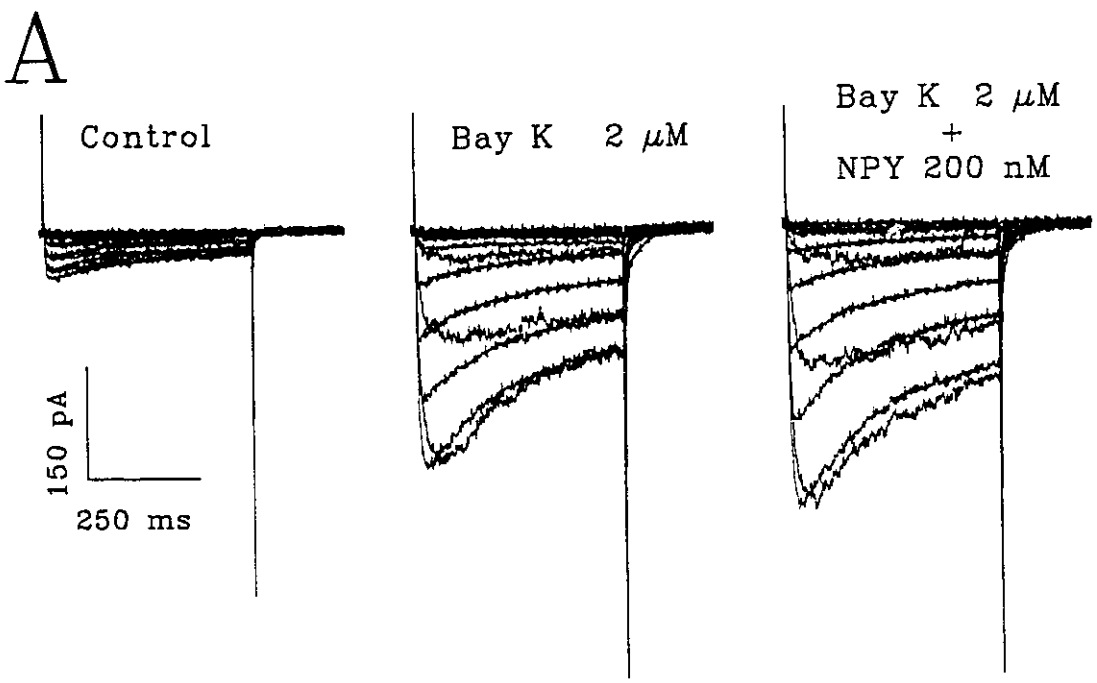

B
100
-80
-40
0
40
80
0
-100
-200
-300
-400
Current Amplitude (pA)
Membrane Potential (mV)
Control
NPY 200 nM
NPY 200 nM
+
Bay K 2 μM

Figure 21. Effect of NPY in the Presence of Maximal Concentration of Bay K 8644.

Panel A. Traces showing the effects of Bay K 8644 (2 μM) alone and with NPY (200 nM) on inward currents. Holding potential = -70 mV. Test potentials = -60 to +50 mV.

Panel B. I-V curves in control (○); Bay K 8644 2 μM alone (●); and Bay K 8644 2 μM plus NPY 200 nM (▽). Bay K 8644 (2 μM) induced large increase in current amplitude with a leftward shift of I-V curve. Subsequent addition of NPY (200 nM) induced further increase in current amplitude without further shift of I-V curve. n=4.


A
Control
Bay K 2 μM
Bay K 2 μM
+
NPY 200 nM
150 pA
250 ms

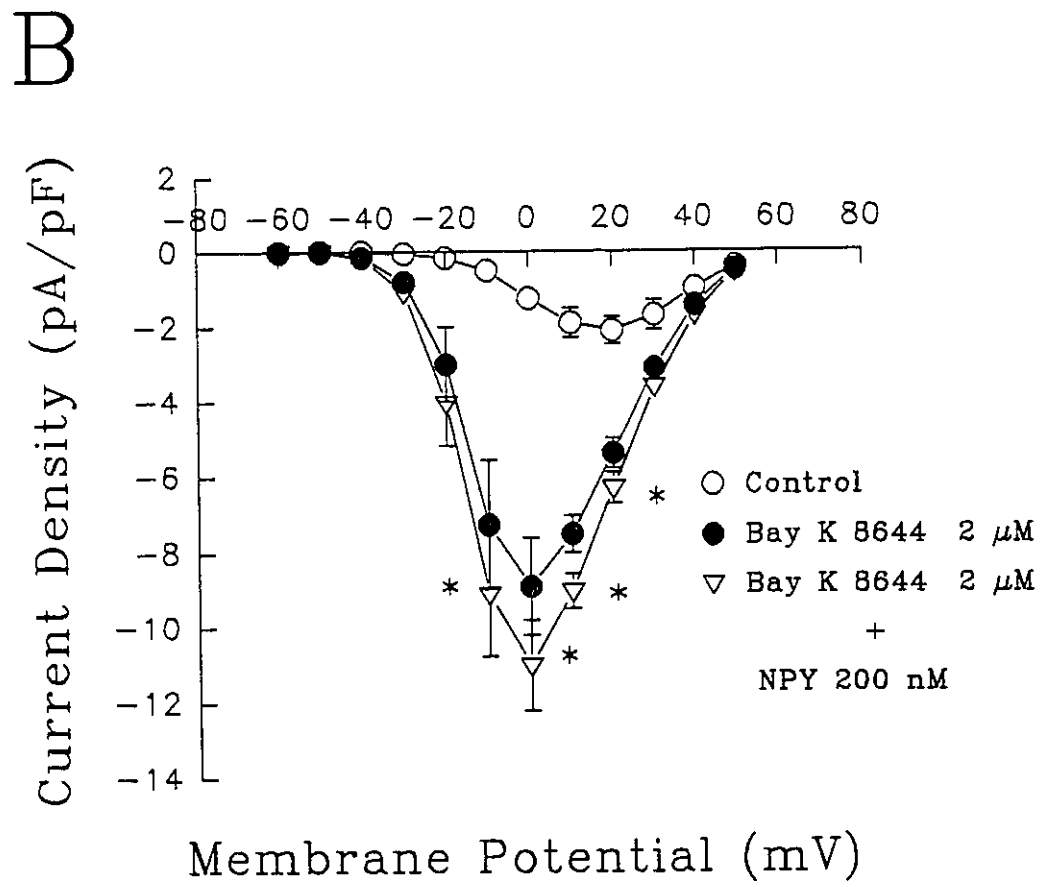
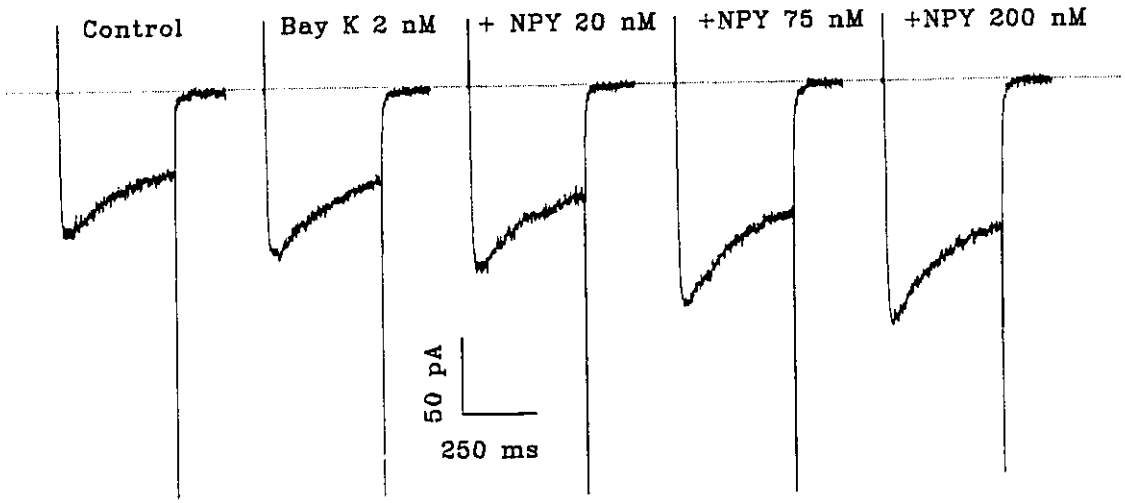

B
Current Density (pA/pF)
2
0
−2
−4
−6
−8
−10
−12
−14
−80 −60 −40 −20 0 20 40 60 80
Membrane Potential (mV)
Control
Bay K 8644 2 μM
Bay K 8644 2 μM
+
NPY 200 nM
*

Figure 22. Effect of NPY in the Presence of Low Concentration of Bay K 8644.

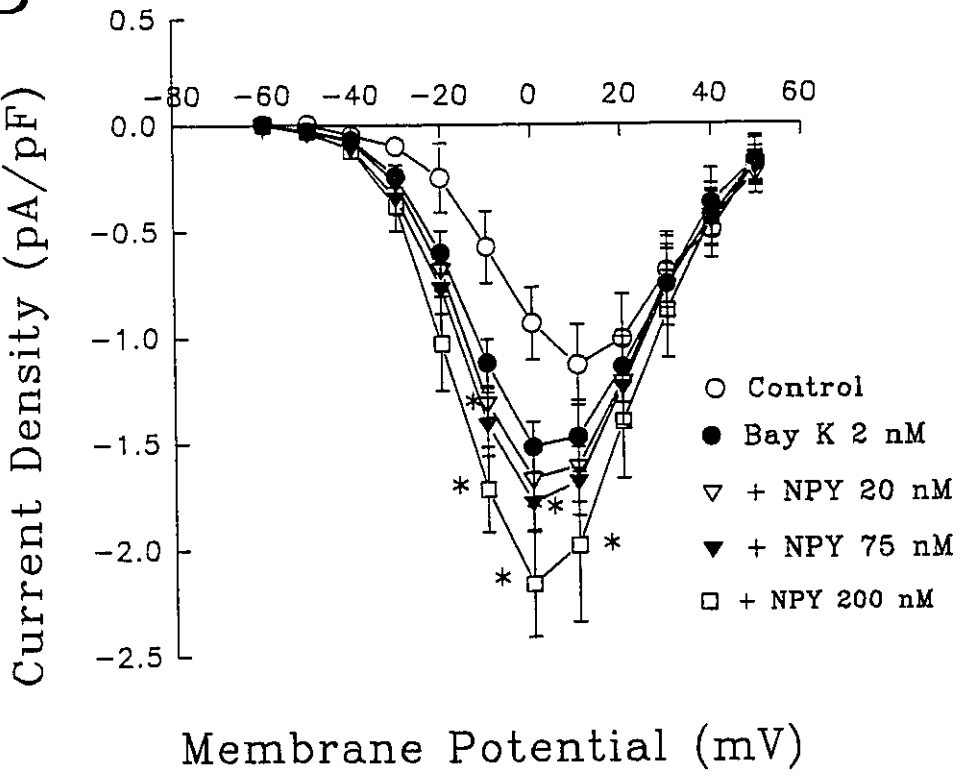
Panel A. Representative traces showing the effect of NPY 20, 75 and 200 nM on inward current in the presence of Bay K 8644 (2 nM). Bay K 8644 (2 nM) induced a small increase in current amplitude. After the effect of Bay K 8644 stabilized for 10 mins, different concentration of NPY was applied to the cell on top of Bay K 8644. Holding potential = -70 mV; test potential = +10 mV.

Panel B. I-V curves in control (○); Bay K 8644 2 nM alone (●); Bay K 8644 2 nM plus NPY 20 nM (▽); Bay 8644 2 nM plus NPY 75 nM (▼); and Bay K 8644 2 nM plus NPY 200 nM (□). n=4.

A


Control
Bay K 2 nM
+ NPY 20 nM
+NPY 75 nM
+NPY 200 nM
50 pA
250 ms

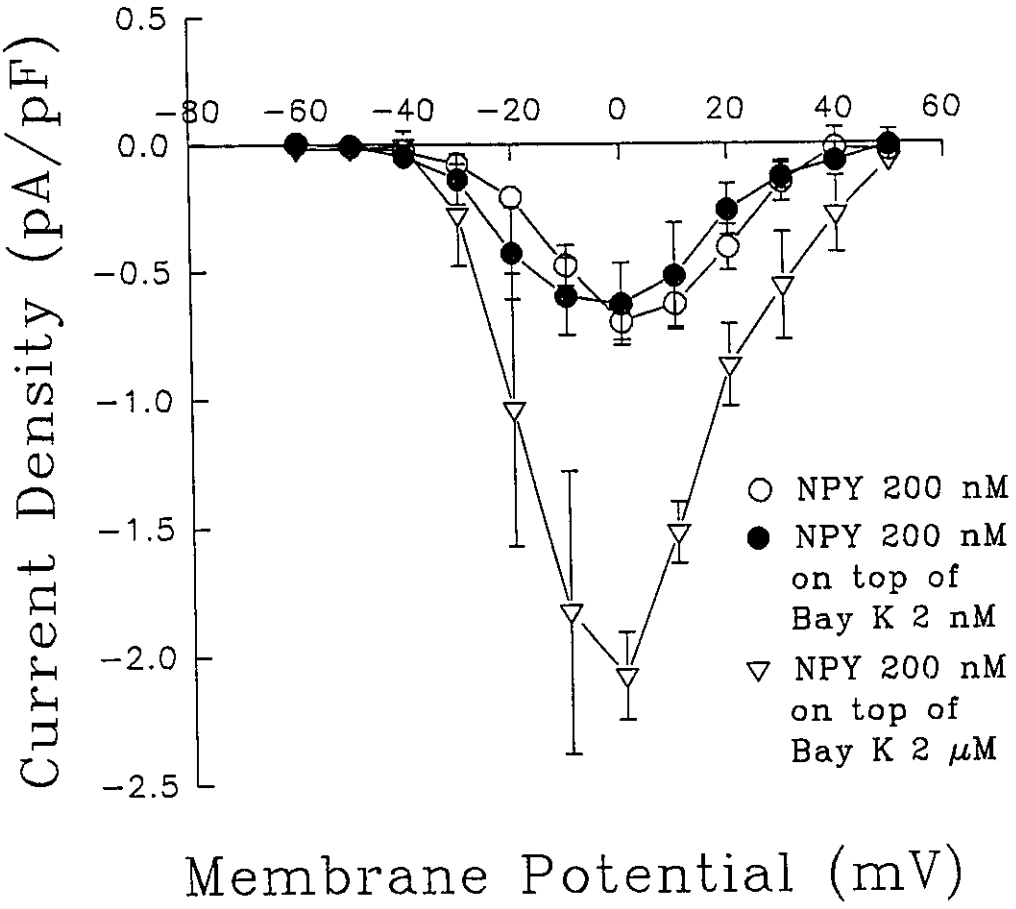
B


0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
−80
−60
−40
−20
0
20
40
60
Current Density (pA/pF)
Membrane Potential (mV)
Control
Bay K 2 nM
+ NPY 20 nM
+ NPY 75 nM
+ NPY 200 nM

of 2 nM Bay K 8644. However, the effect of NPY was significantly potentiated by higher concentration of Bay K 8644 (2 μM) (Fig. 23). At a test potential of 0 mV, NPY 200 nM alone induced a current of only 0.67 pA/pF (n=6). In the presence of 2 μM Bay K 8644, the current density attributable to NPY increased to 2.1 pA/pF (n=4).

Figure 23. Additional current Induced by NPY.

Graph showing the additional current induced by NPY (200 nM) in the absence (○) and in the presence of 2 nM (●) and 2 μM (▽) of Bay K 8644. The values were obtained by subtracting the current after NPY by that before NPY with or without Bay K 8644. Holding potential = -70 mV. n=4 or 6.


0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
−80
−60
−40
−20
0
20
40
60
Current Density (pA/pF)
Membrane Potential (mV)
NPY 200 nM
NPY 200 nM on top of Bay K 2 nM
NPY 200 nM on top of Bay K 2 μM

PART III. Characterization of Outward Currents

I. Whole-cell Recording.

(i). Voltage-dependence of the Outward Currents.

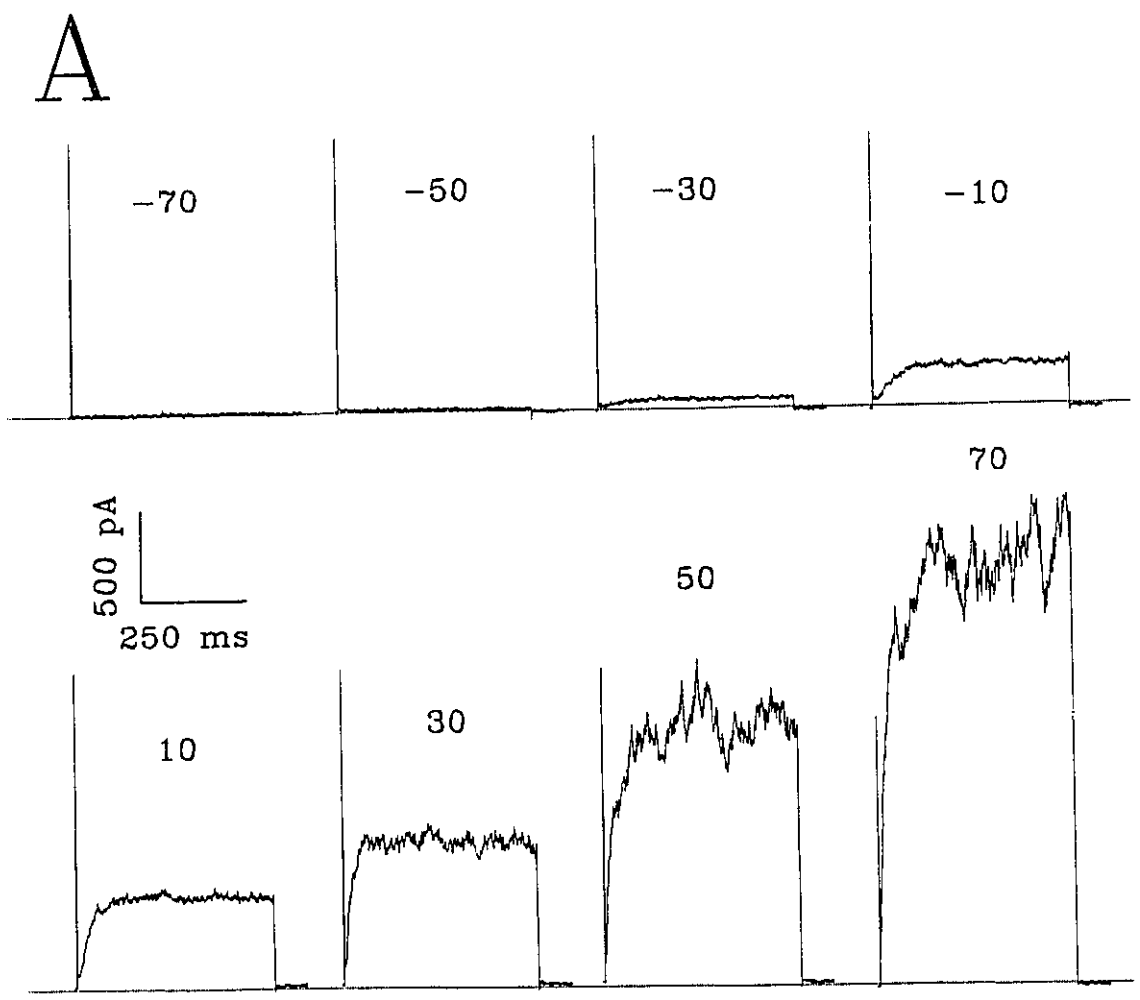
With physiological external bathing solution and high K^+ (150 mM) in the pipette solution, outward currents were induced with step depolarizations in single vascular smooth muscle cells from the rat tail artery, as previously reported (Bolzon et al., 1992; Bolzon et al., 1993). Figure 24 shows an example of voltage-dependent change in outward currents recorded with whole-cell configuration. Currents were elicited by 500 ms depolarizing steps from a holding potential of -80 mV to test potentials ranging from -70 to +70 mV. When a threshold of about -40 mV was reached, an outward current was observed following the capacitive transient. The amplitude of the outward current was dependent on the test potential and increased with more depolarized test potentials (Fig. 24A). Plotting of current amplitude measured at each step pulse against the test potential showed outward rectification in the current-voltage relationship (I-V curve) for the outward current (Fig. 24B).

Unlike the inward calcium current, outward currents recorded with whole-cell configuration were rather stable. Figure 25 shows the change of outward current with time. Current was elicited by 500 ms depolarizing pulse from a holding potential -70 mV to a test potential of +30 mV. The outward current was observed right after the establishment of the whole-cell configuration. The amplitude of the current gradually increased within first 15 min and stabilized thereafter for up to 70 min (Fig. 25A). The time-dependent change in current amplitude from 7 cells was shown in Figure 25B.

Figure 24. Whole-cell Recording of Outward Current.

Panel A. Whole-cell outward currents elicited with 500 ms depolarizing pulses from a holding potential of -80 mV to different test potentials ranging from -70 to +70 mV. Pipette solution contained free Ca^{2+} concentration of 240 nM. Capacitive currents have been truncated.

Panel B. Current-voltage (I-V) relationship of outward current elicited from a holding potential of -80 mV. The outward current was activated with test potential positive to -40 mV and the amplitude of the current increased with more depolarized membrane potentials.


A
−70
−50
−30
−10
500 pA
250 ms
10
30
50
70

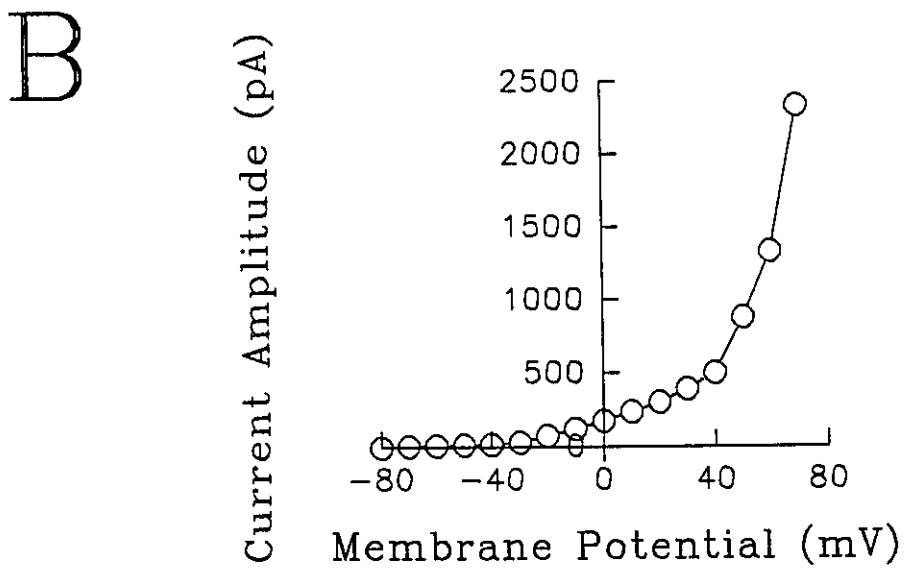
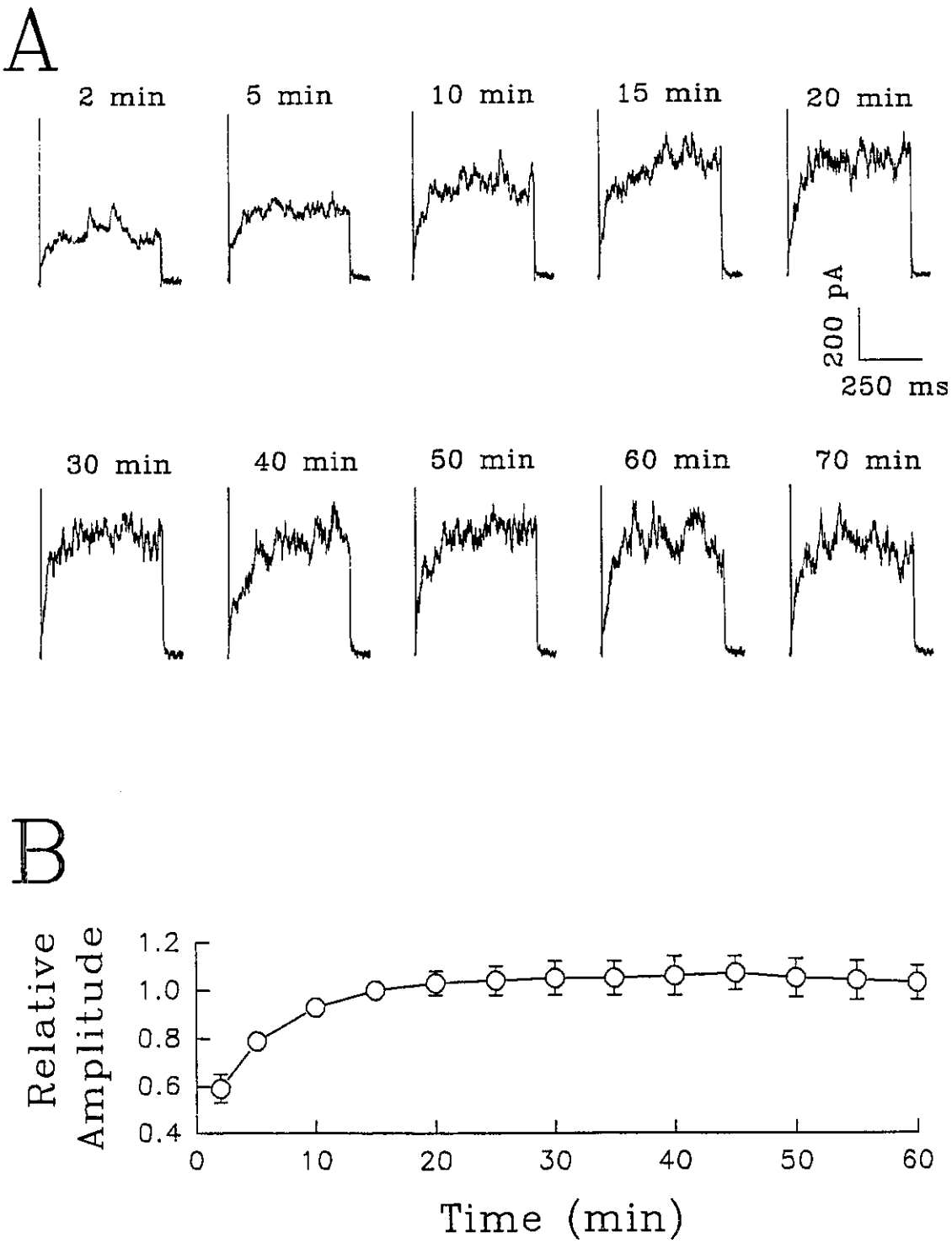

B
Current Amplitude (pA)
2500
2000
1500
1000
500
−80
−40
0
40
80
Membrane Potential (mV)

Figure 25. Stability of the Outward Current.

Panel A. Representative traces showing the change in outward current with time. Current was recorded at different time following the establishment of whole-cell configuration. Holding potential = -70 mV; test potential = +30 mV.

Panel B. Graph showing the change in current amplitude with time. The amplitude of the outward current at different time has been normalized to that recorded at 15 min. Holding potential = -70 mV; test potential = +30 mV. n = 7.


A
2 min
5 min
10 min
15 min
20 min
200 pA
250 ms
30 min
40 min
50 min
60 min
70 min
B
Relative Amplitude
1.2
1.0
0.8
0.6
0.4
0
10
20
30
40
50
60
Time (min)

Current amplitude at different time has been normalized to that recorded at 15 min (in most cells current started to be stable at this time). The relative current amplitude increased from 0.59 ± 0.04 at 2 min to 1.0 at 15 min (n=7) and remained rather stable after 15 min. At 60 min of the recording, it was 1.03 ± 0.07 (n=7) (Fig. 25B). In all subsequent experiments, data were obtained only from the recordings made within the 15 to 60 min period when there were no significant changes in the current amplitude.

(ii). Different Components of the Outward Currents.

Two major components of outward currents were recorded (Fig. 26). These two components had the characteristics of delayed rectifier K^+ channel current (K_{dr}) and Ca^{2+}-activated K^+ channel current (K_{Ca}) according to their sensitivity to inactivation, intracellular Ca^{2+} and specific K^+ channel blockers (Bolzon et al, 1993). From a holding potential of -70 mV, a smooth component was activated at -40 mV. A second noisy component was activated at a threshold of -10 mV (Fig. 24A; 26A, left panel). When holding potential was set at -40 mV, only the noisy component persisted (Fig. 26A, middle panel). On the other hand, when charybdotoxin (CTX, 150 nM) was applied to the bath solution, the noisy component was suppressed, leaving only the smooth component (Fig. 26B). Similar to the noisy component, the I-V curve for the smooth component showed an outward rectification (Fig. 26B, right panel). However, this rectification is much weaker compared to the noisy component.

The noisy component of outward current was sensitive to K^+ channel blocker tetraethylammonium (TEA). Application of low concentration of TEA (2 mM) for 5 min

Figure 26. Two Major Components of Outward Current.

Panel A. Left panel: with a holding potential of -70 mV, both smooth and noisy components of outward current were recorded. Middle panel: when holding potential was set to -40 mV, only the noisy component was observed. Right panel: I-V curves for outward currents recorded with holding potential of -70 mV (○) and -40 mV (●).

Panel B. Left panel: with a holding potential of -70 mV, both smooth and noisy components of outward current were recorded. Middle panel: In the presence of charybdotoxin (CTX) 150 nM, the noisy component was suppressed and only the smooth component remained. Right panel: I-V curves for control (○) and in the presence of CTX 150 nM (●).

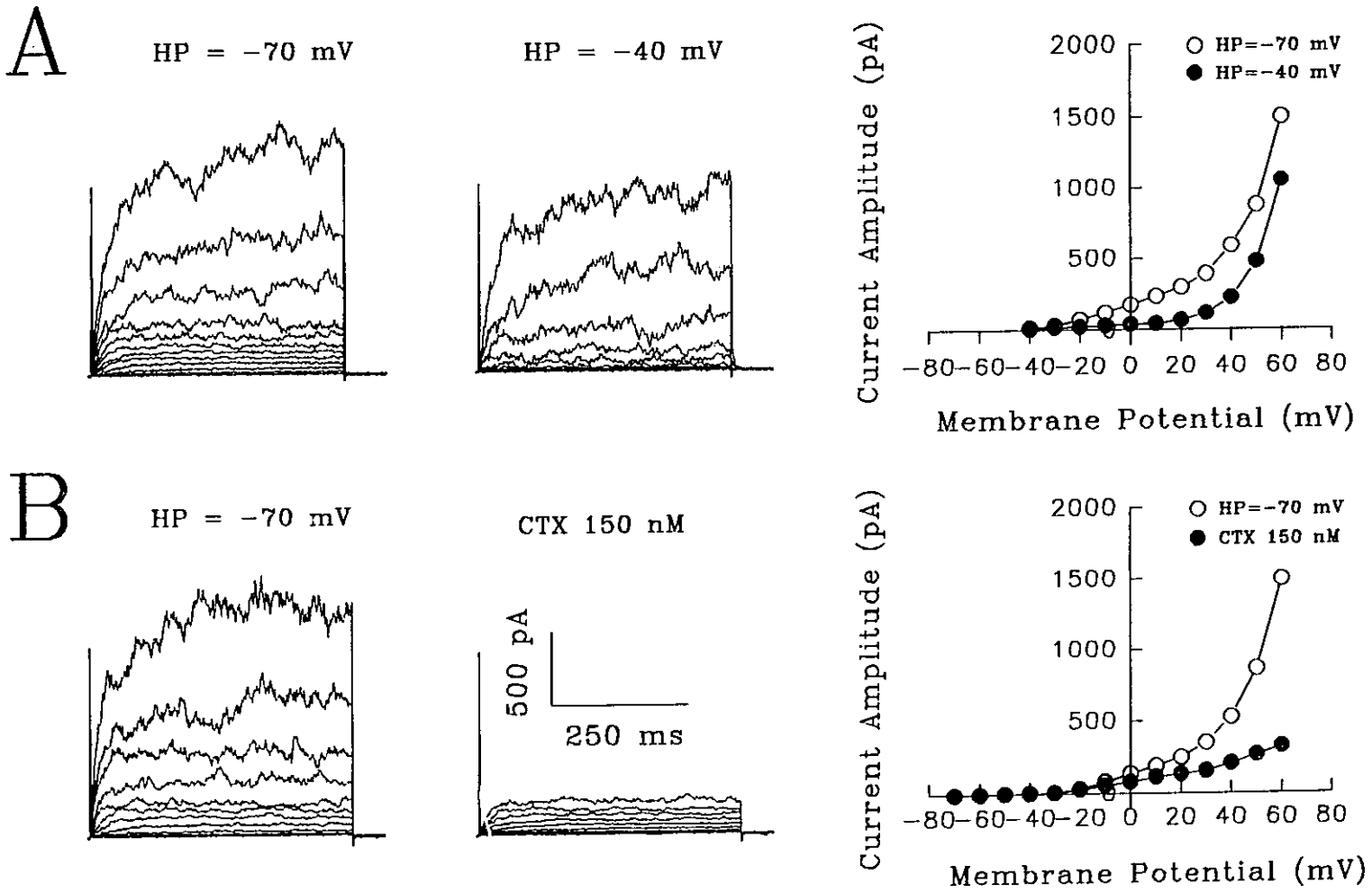
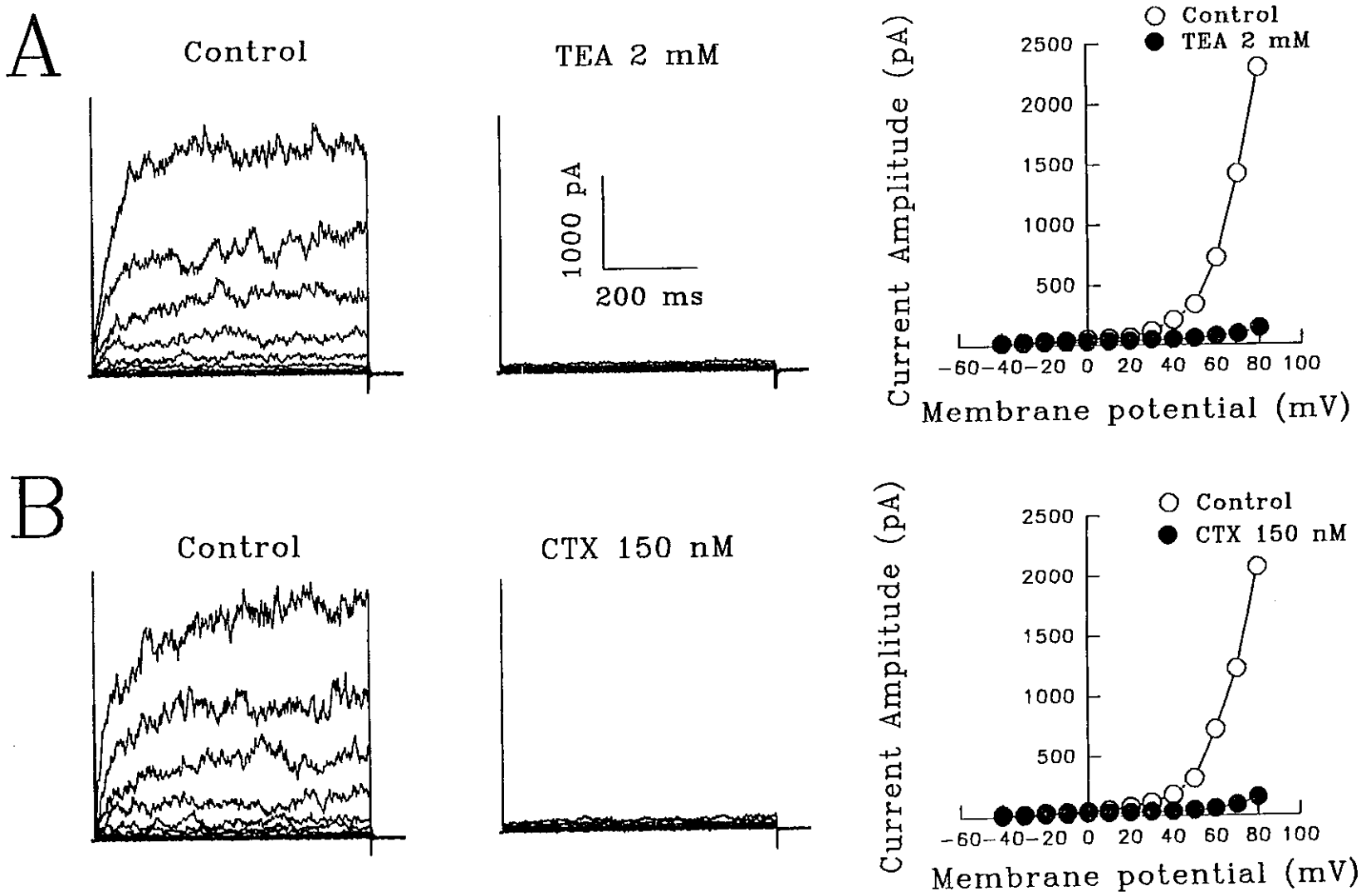

A
HP = −70 mV
HP = −40 mV
Current Amplitude (pA)
2000
1500
1000
500
HP=−70 mV
HP=−40 mV
−80 −60 −40 −20 0 20 40 60 80
Membrane Potential (mV)
B
HP = −70 mV
CTX 150 nM
500 pA
250 ms
Current Amplitude (pA)
2000
1500
1000
500
HP=−70 mV
CTX 150 nM
−80 −60 −40 −20 0 20 40 60 80
Membrane Potential (mV)

Figure 27. Effects of TEA and CTX on Ca^{2+}-activated K^+ Current.

Panel A. Representative traces showing the effect of tetraethylammonium (TEA) 2 mM on the outward current recorded from a holding potential of -40 mV. Left panel: control. Middle panel: after TEA (2 mM) for 5 min. Right panel: I-V curves for control (○) and after TEA (●).

Panel B. Representative traces showing the effect of charybdotoxin (CTX) 150 nM on the outward current recorded from a holding potential of -40 mV. Left panel: control. Middle panel: after CTX (2 mM) for 5 min. Right panel: I-V curves for control (○) and after CTX (●).


A
Control
TEA 2 mM
1000 pA
200 ms
Control
TEA 2 mM
Current Amplitude (pA)
2500
2000
1500
1000
500
-60 -40 -20 0 20 40 60 80 100
Membrane potential (mV)
B
Control
CTX 150 nM
Control
CTX 150 nM
Current Amplitude (pA)
2500
2000
1500
1000
500
-60 -40 -20 0 20 40 60 80 100
Membrane potential (mV)

almost completely abolished the outward current (Fig. 27A). It was also sensitive to blockade by the specific Ca^{2+}-activated K^+ channel blocker CTX. Application of CTX (150 mM) for 5 min inhibited more than 90% of the current. Furthermore, the activity of noisy component depended on intracellular Ca^{2+} concentration (Fig. 28). When lower Ca^{2+} concentration was used in the pipette solution, the amplitude of the current was substantially decreased and the threshold of activation was shifted toward more depolarized membrane potential. With 240 nM Ca^{2+} in the pipette solution, the outward current recorded from a holding potential of -40 mV had a threshold around -10 mV. However, it was about +10 mV with 16 nM Ca^{2+}. At a test potential of +30 mV, the current density was 4.2 pA/pF for 240 nM Ca^{2+}, while it was only 2.0 pA/pF for 16 nM Ca^{2+} (Fig. 28). These results indicated that the noisy component was the current through $K_{(Ca)}$.

The smooth component could be inactivated by changing the holding potential from -70 to -40 mV (Fig. 29A). It was also sensitive to blockade by 4-aminopyridine (4-AP). Application of 4-AP (1 mM) for 5 min inhibited almost 85% of the current (Fig. 29B). These results indicated that the smooth component of the outward current was the current through K_{dr}.

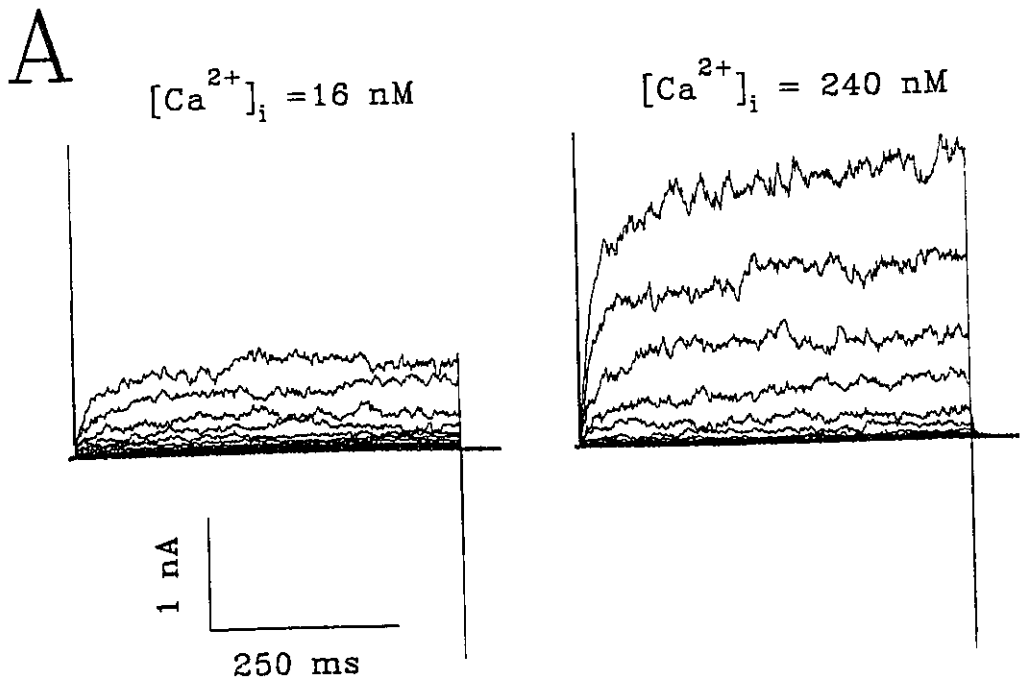
II. Single Channel Recording of Outward Currents.

Single channel outward currents were mainly studied with the outside-out isolated membrane patch configuration. After establishment of whole-cell recording, patch pipette was slowly pulled away from the cell and a piece of membrane was excised from the cell

Figure 28. Effect of $[Ca^{2+}]_i$ on Ca^{2+}-activated K^+ Current.

Panel A. Representative traces showing the effect of intracellular Ca^{2+} concentration on the outward current. Currents were recorded from a holding potential of -40 mV to test potentials ranging from -40 to +70 mV. Left panel: outward currents recorded with 16 nM free Ca^{2+} in the pipette solution. Right panel: outward currents recorded with 240 nM Ca^{2+} in the pipette solution.

Panel B. I-V curves for the outward currents recorded with 16 (○) and 240 nM (●) intracellular Ca^{2+}. Holding potential = -40 mV.


A
[Ca2+]i =16 nM
[Ca2+]i = 240 nM
1 nA
250 ms

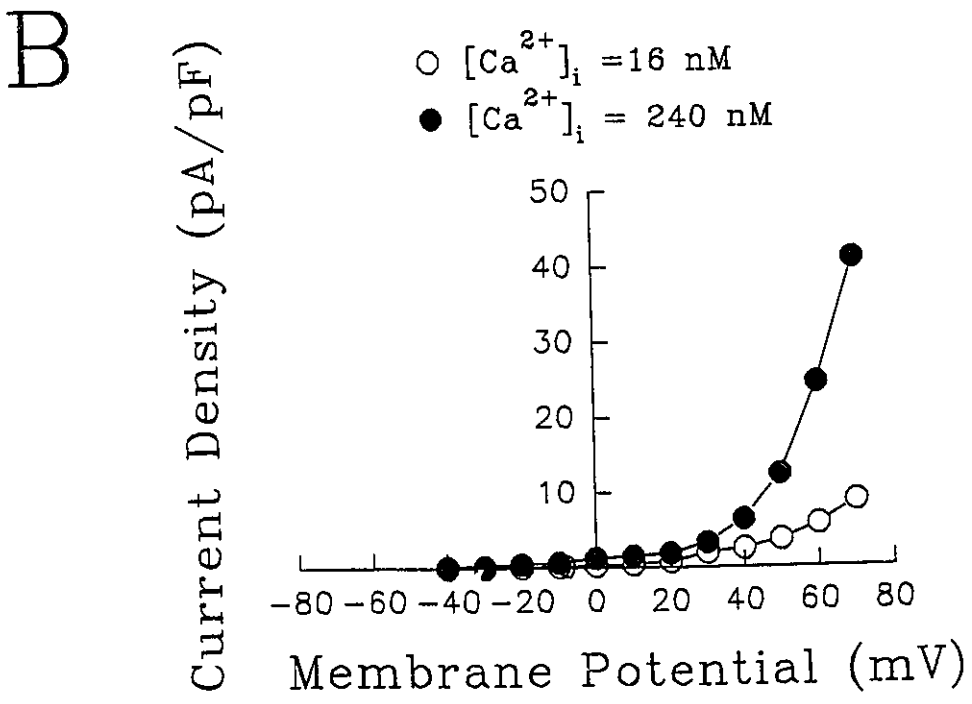
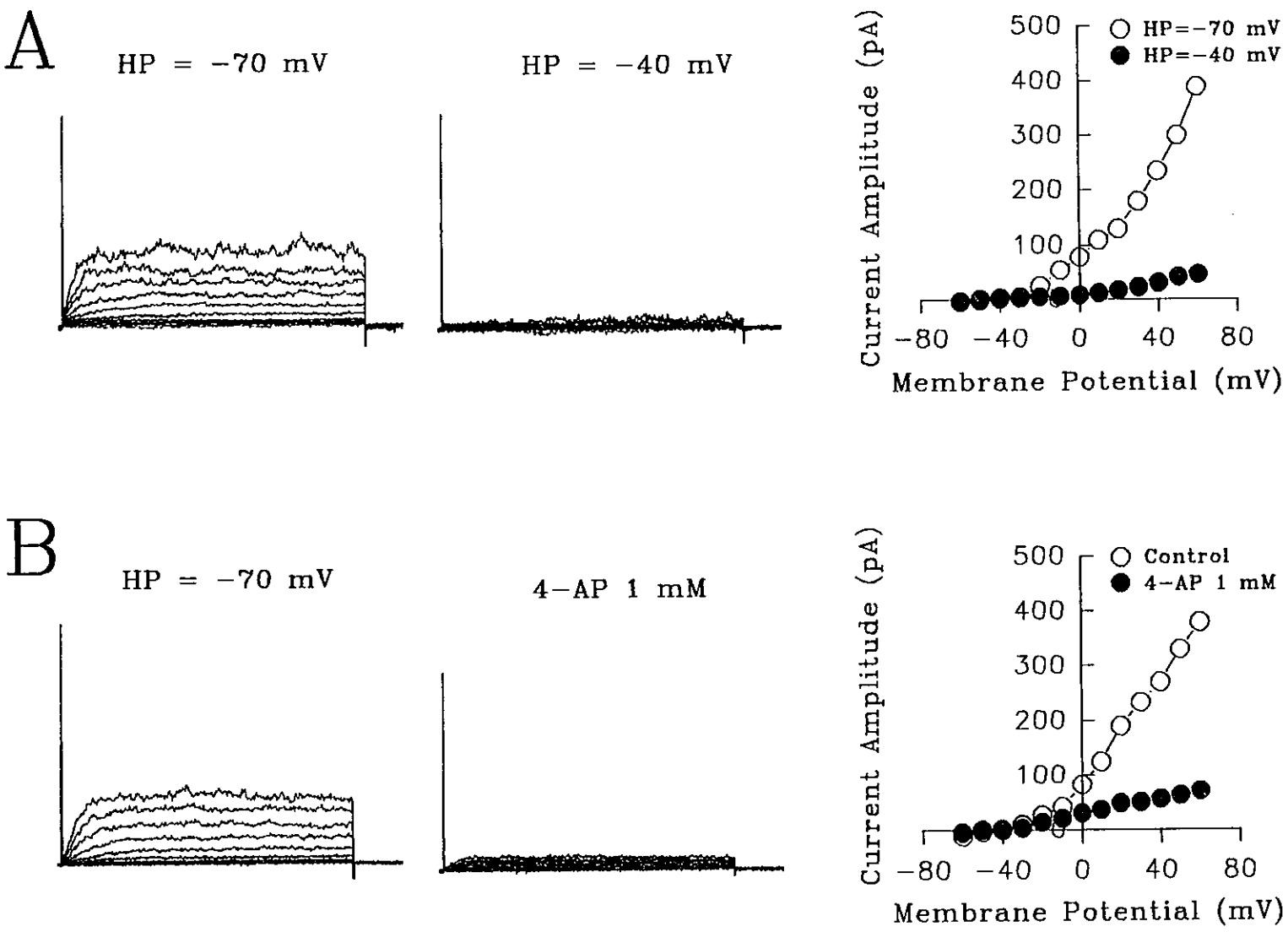

B
○ [Ca2+]i =16 nM
● [Ca2+]i = 240 nM
Current Density (pA/pF)
50
40
30
20
10
-80 -60 -40 -20 0 20 40 60 80
Membrane Potential (mV)

Figure 29. Effects of Holding Potential and 4-AP on Delayed Rectifier K^+ Current.

Panel A. Effect of low holding potential on outward current. Currents were elicited from a holding potential of -70 mV to test potentials ranging from -70 to +60 mV with 10 mV increment. CTX 150 nM was present in the bath solution to block the Ca^{2+}-activated K^+ channels. Left panel: control. Middle panel: holding potential was set to -40 mV. Right panel: I-V curves for outward currents recorded with a holding potential of -70 (○) and -40 mV (●). CTX 150 nM was present in the bathing solution throughout the experiment.

Panel B. Effect of 4-aminopyridine (4-AP) 1 mM on outward current. Currents were elicited from a holding potential of -70 mV to test potentials ranging from -70 to +60 mV with 10 mV increment. CTX 150 nM was used in the bath solution to block the Ca^{2+}-activated K^+ channels. Left panel: control. Middle panel: after 4-AP (1 mM) for 5 min. Right panel: I-V curves for outward currents recorded before (○) and after 4-AP (●). CTX 150 nM was present in the bathing solution throughout the experiment.


A
HP = −70 mV
HP = −40 mV
Current Amplitude (pA)
500
400
300
200
100
HP=−70 mV
HP=−40 mV
−80
−40
0
40
80
Membrane Potential (mV)
B
HP = −70 mV
4−AP 1 mM
Current Amplitude (pA)
500
400
300
200
100
Control
4−AP 1 mM
−80
−40
0
40
80
Membrane Potential (mV)

with the outside membrane facing the bath solution. Two types of single channel currents corresponding to K_{dr} and $K_{(Ca)}$ were observed with a holding potential of -70 mV (Bolzon et al., 1994). Similar to whole-cell recordings, K_{dr} was activated at test potentials positive to -40 mV and $K_{(Ca)}$ was activated at test potentials positive to -10 mV. The amplitude of K_{dr} was much smaller with a slope conductance around 10 pS in physiological external solution. In the present study, we concentrated on $K_{(Ca)}$. Figure 30 is an example of single channel recording of $K_{(Ca)}$ with outside-out configuration. Currents were elicited from a holding potential of -40 mV to test potentials ranging from 0 to 40 mV. The single channel event was recorded with test potential positive to 0 mV and the amplitude increased with more depolarized test potentials (Fig. 30A & B). The open probability of single channel was also voltage-dependent. With test potentials negative to +10 mV only sporadic openings were observed. While with test potentials positive to +20 mV, more frequent openings were recorded. Figure 30B shows the unitary current amplitude of $K_{(Ca)}$ at different test potentials. The current had a slope conductance around 120 pS with physiological external solution.

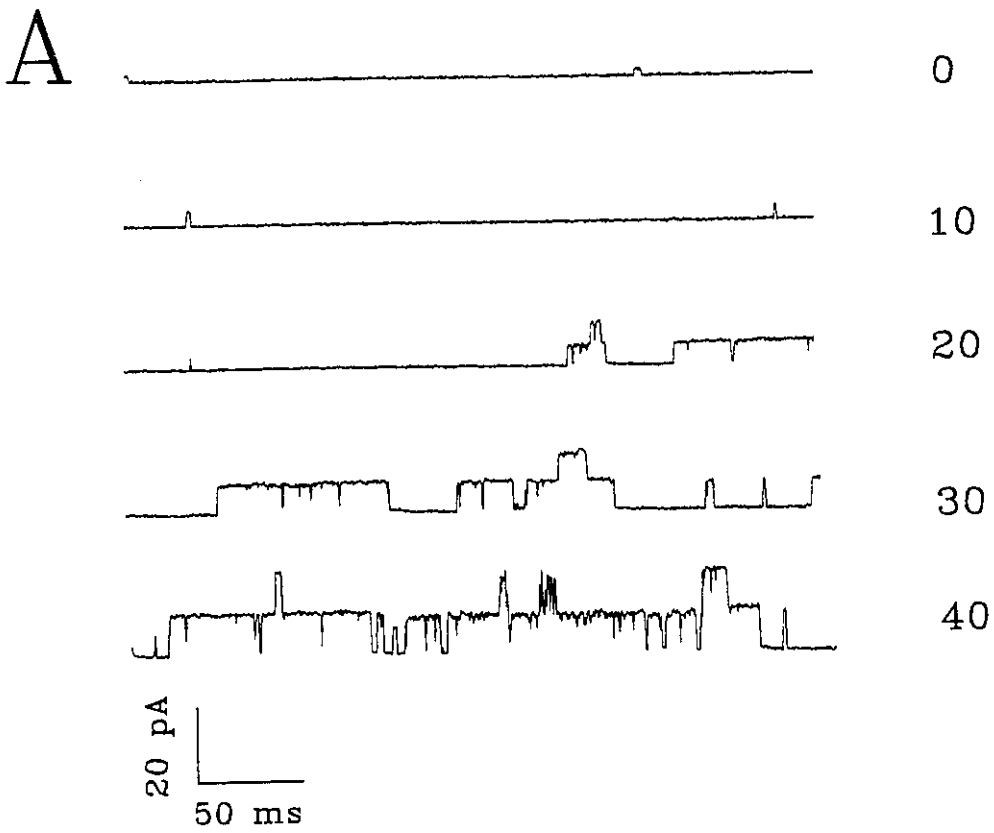
The stability of single $K_{(Ca)}$ current was studied by holding the patch membrane at +20 mV for long period of time and the open probability at different time was plotted. In most of cells, the single channel activity was very stable for at least 30 mins. Figure 31A shows the time course of single channel current of $K_{(Ca)}$ recorded with outside-out configuration. Current was elicited at a holding potential of +20 mV. The single channel activity was rather stable and no obvious change in the open probability was observed within a 30 min period (Fig. 31A). This single channel current was very

sensitive to blockade by low concentration of TEA (Fig. 31B) and CTX (Fig. 36D, PART IV). The amplitude of single channel current gradually decreased and disappeared within 1 min of perfusion with TEA 2 mM. Upon washing, the amplitude gradually increased and fully recovered within 2 min (Fig. 31B).

Figure 30. Single Channel Recording of $K_{(Ca)}$ with Outside-out Configuration.

Panel A. Voltage-dependence of single channel current recorded with outside-out membrane patch configuration in physiological external solution. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from 0 to 40 mV. Single channel event was detected with test potential positive to 0 mV. Both the current amplitude and the open probability increased at more depolarized membrane potential. The leak and capacitive currents have been subtracted by constructing templates from blank traces. Pipette solution contained 240 nM free Ca^{2+}.

Panel B. Current-voltage (I-V) relationship for single channel $K_{(Ca)}$ current recorded from outside-out membrane patches in physiological external solution. The slop conductance was about 120 pS.


A
0
10
20
30
40
20 pA
50 ms

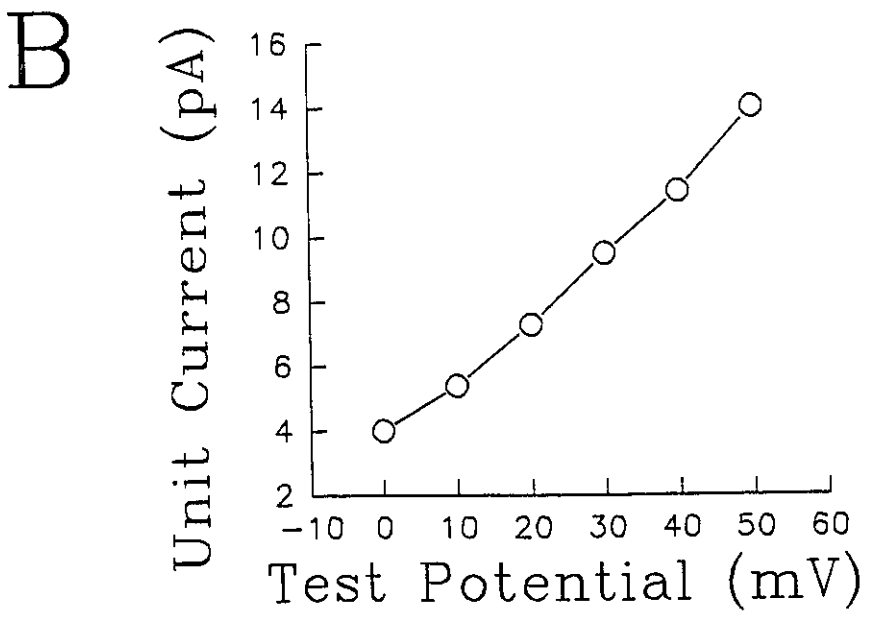
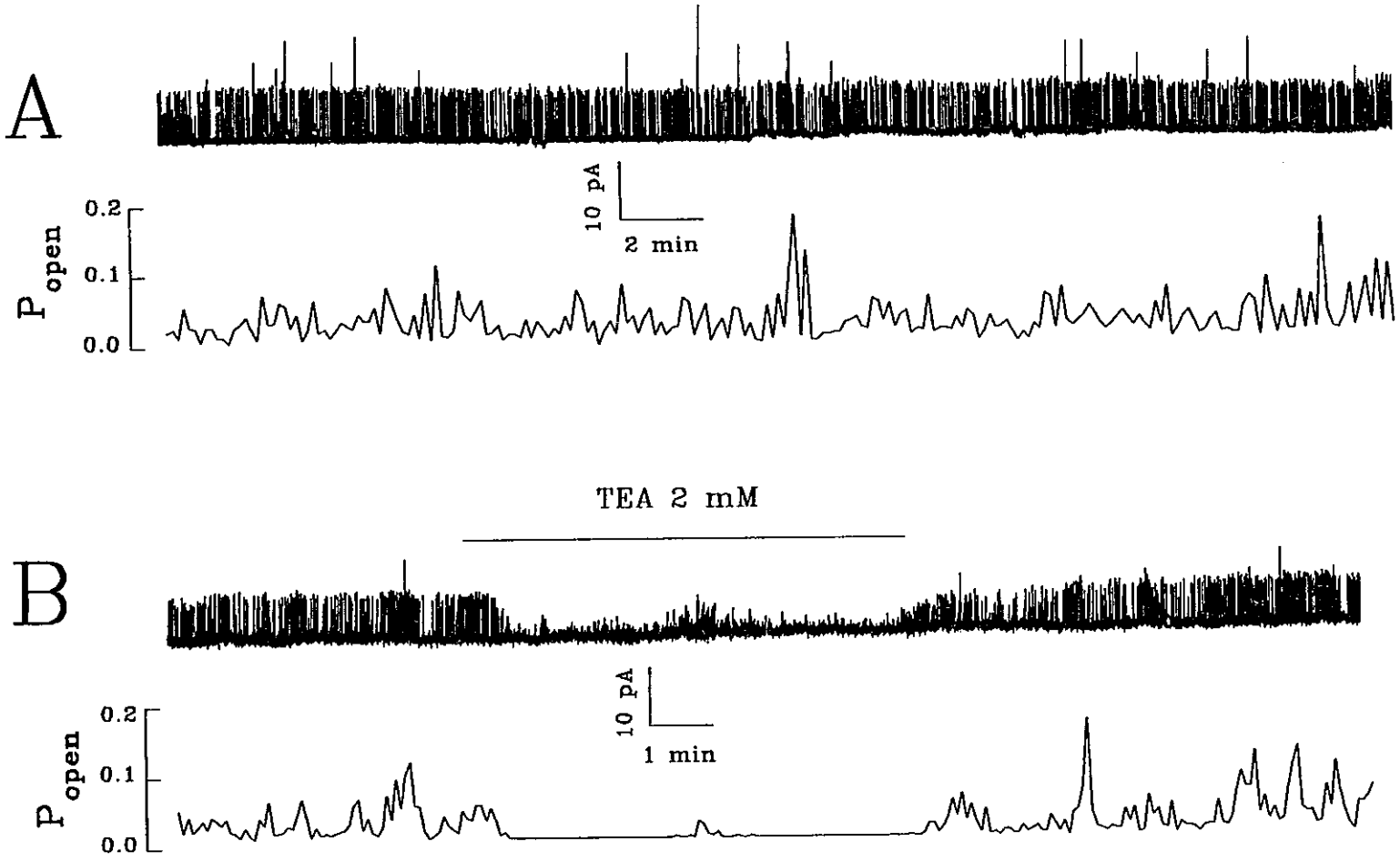

B
Unit Current (pA)
16
14
12
10
8
6
4
2
−10
0
10
20
30
40
50
60
Test Potential (mV)

Figure 31. Stability and TEA Sensitivity of $K_{(Ca)}$ Currents.

Panel A. Top trace: current was recorded from an outside-out membrane patch with a holding potential of +20 mV. Single channel activity was stable with time. Bottom trace: open probability for single channel current as calculated every 3 sec.

Panel B. Top trace: effect of TEA 2 mM on single channel activity of $K_{(Ca)}$ recorded from an outside-out membrane patch with a holding potential of +20 mV. Bottom trace: open probability for the single channel current as calculated every 2 sec and plotted against the time.


A
10 pA
2 min
P open
0.2
0.1
0.0
TEA 2 mM
B
10 pA
1 min
P open
0.2
0.1
0.0

PART IV. Effect of NPY on Outward Currents

I. Effect of NPY on Whole-cell $K_{(Ca)}$ Currents.

In the presence of nifedipine (1 μM) and with a holding potential of -40 mV, outward currents were elicited by 500 ms depolarizing pulses. NPY depressed the currents in a dose-dependent manner from 20 nM to a maximum at 200 nM (Figs. 32 and 37). As a result, the current-voltage relationships were shifted to the right with increasing concentrations of NPY. The inhibition of NPY on the outward currents was only partial, even at the highest effective concentration of 200 nM. At a test potential of +20 mV, the outward current was reduced to 48.0 ± 5.1 % of control. The effect of NPY is reversible and the currents recovered completely within 10 mins of washing (Fig. 32A). We have also tested the effect of NPY (200 nM) using 10 mM BAPTA in the pipette solution. The whole-cell currents were reduced to 49.7 ± 5.6% (n=4) of control, almost identical to the reduction observed with EGTA. These outward currents could be abolished with the addition of TEA (5 mM) or charybdotoxin (100-150 nM) (Fig. 33A) and have previously been identified as Ca^{2+}-activated K^{+}channel currents.

II. Effect of NPY on K_{dr} Currents.

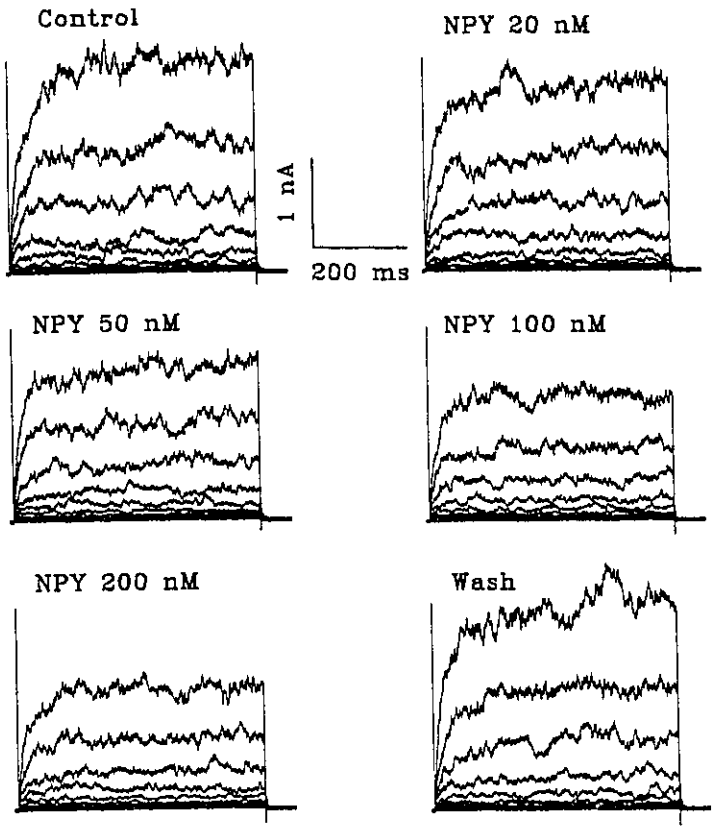
At a holding potential of -40 mV, the K_{dr} currents were inactivated (Fig. 33A). The presence of K_{dr} can be demonstrated if the holding potential was kept at -80 mV. The K_{dr} currents were insensitive to charybdotoxin (Fig. 33B) and unaffected by NPY (200 nM) (Fig. 33C). At a test potential of +20 mV, the amplitude of K_{dr} was 98.6 ± 5.8% of control (n=3, $p>0.05$) with NPY 200 nM.

Figure 32. Effect of Neuropeptide Y (NPY) on Whole-cell $K_{(Ca)}$ Currents.

Panel A. Currents were elicited by depolarizing potentials from -40 to +70 mV from a holding potential of -40 mV. The records show currents 5 mins after the introduction of NPY from 20 to 200 nM. The effect of NPY was reversible and the currents recovered fully upon washing. Capacitive currents have been truncated.

Panel B. The effect of NPY on current-voltage relationships of outward current recorded from a holding potential of -40 mV (n = 6 cells). The current amplitudes have been normalized to cell capacitance. To obtain the capacitance of each cell, a 10 mV hyperpolarizing step was applied from a holding potential of -70 mV. Cell capacitance was calculated by integrating the capacitative transient and divided by the voltage step.

A


Control
NPY 20 nM
1 nA
200 ms
NPY 50 nM
NPY 100 nM
NPY 200 nM
Wash

B

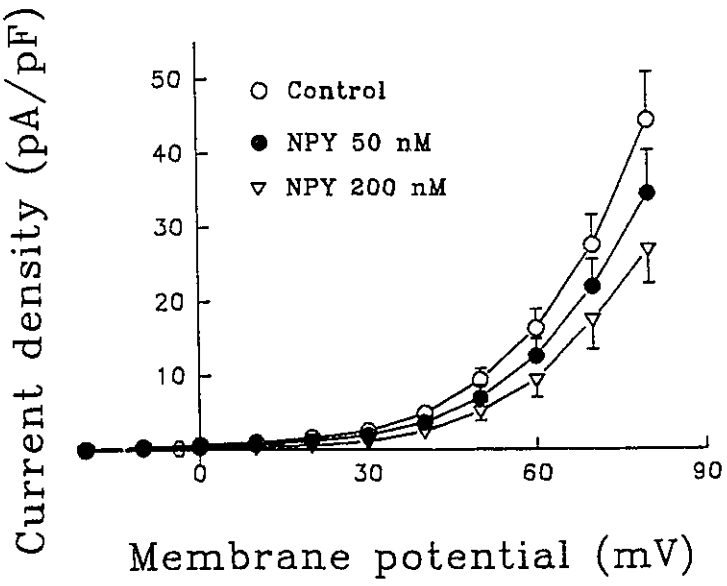
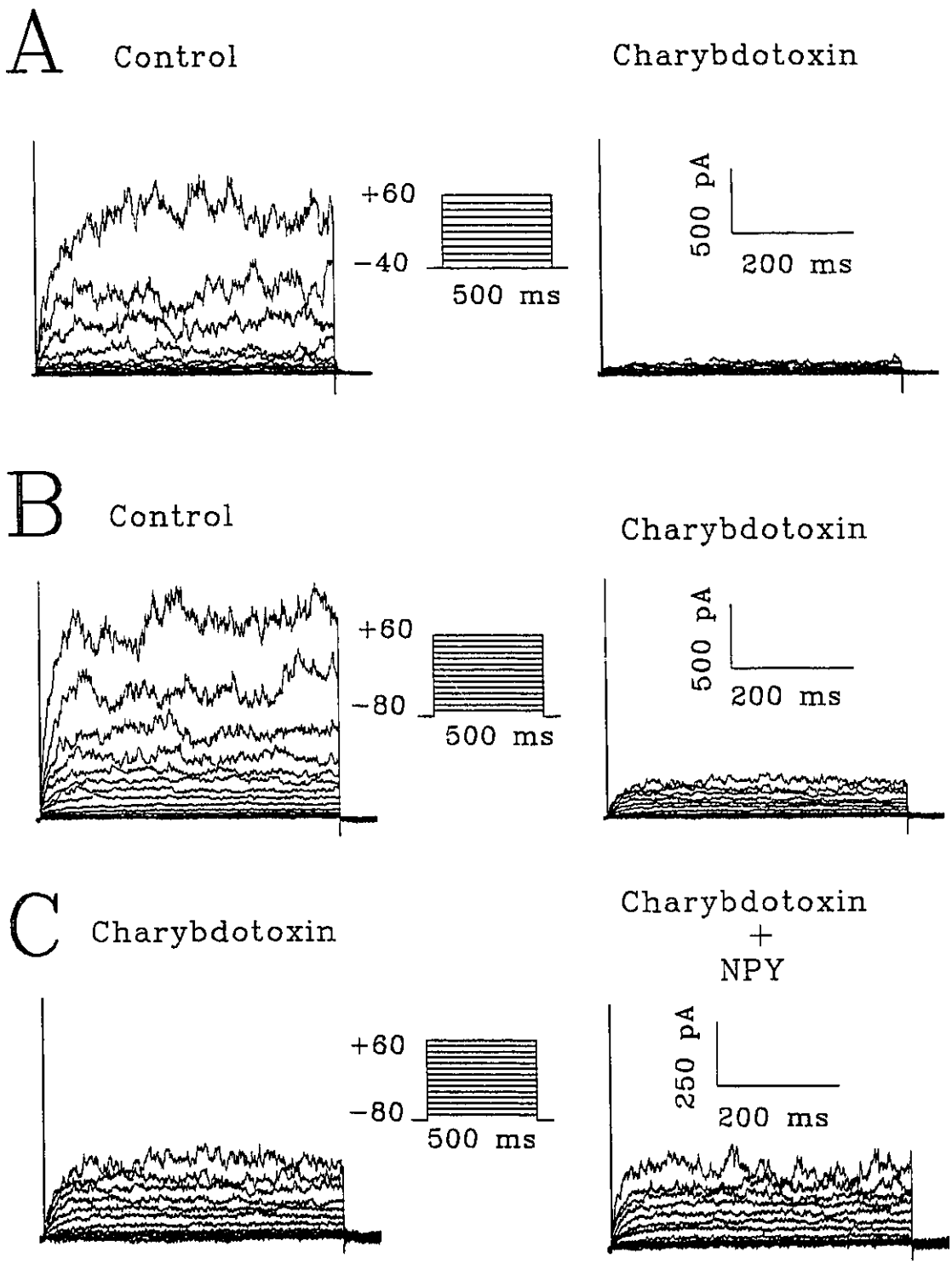

Current density (pA/pF)
50
40
30
20
10
0
Control
NPY 50 nM
NPY 200 nM
0
30
60
90
Membrane potential (mV)

Figure 33. Effects of Charybdotoxin and NPY on Outward Currents.

Panel A. At a holding potential of -40 mV, outward currents elicited by depolarizing pulses were inhibited by charybdotoxin (150 nM).

Panel B. At a holding potential of -80 mV, additional smooth charybdotoxin-insensitive currents corresponding to the delayed rectifier was elicited.

Panel C. The delayed rectifier currents were not affected by NPY (200 nM). Note change in scale.


A
Control
Charybdotoxin
+60
−40
500 ms
500 pA
200 ms
B
Control
Charybdotoxin
+60
−80
500 ms
500 pA
200 ms
C
Charybdotoxin
Charybdotoxin
+
NPY
+60
−80
500 ms
250 pA
200 ms

III. Effects of NPY on Single Channel $K_{(Ca)}$ in Outside-out Patches.

Single channel activities of $K_{(Ca)}$ were recorded using outside-out membrane patches. The effect of NPY was studied by continuous recording of single channel activity at a holding potential of +20 mV (Fig. 34). After the single channel activity had stabilized for about 5 mins, NPY (200 nM) was applied to the perfusion solution. A decrease in the single channel open probability was observed after 1 min of NPY application and the effect of NPY peaked within 2-3 min. At a holding potential of +20 mV and with NPY 200 nM, single channel open probability decreased to 36 $\pm$ 3.6% ($n=4$, $p<0.01$) of control value. The inhibitory effect of NPY was completely reversible upon washout (Fig. 34). Figure 35 shows the effect of NPY on single $K_{(Ca)}$ current at different test potentials. Currents were elicited by 500 ms depolarizing pulse from a holding potential of -40 mV. Single channel events were observed with test potential positive to 0 mV. Both the amplitude and the open probability increased at more depolarized test potentials (Fig. 35A). NPY 200 nM decreased channel open probability at all test potentials without altering single channel amplitude (Fig. 35B). The threshold for channel opening was shifted toward more positive membrane potential. In the presence of NPY 200 nM, no single channel event was detected at the test potential of 0 mV. Similar effect of NPY on single channel $K_{(Ca)}$ was observed with a ramp-pulse protocol. Voltage ramp depolarization from -40 to +60 mV at a rate of 100 mV/s elicited outward currents that were dependent on the membrane potential (Fig. 36). NPY (200 nM) significantly inhibited single channel activities, resulting in a reduction of the ensemble averaged current (Fig. 36B). Our previous studies have identified these

Figure 34. Effect of NPY on Single Channel $K_{(Ca)}$ in an Outside-out Patch.

Panel A. Single channel $K_{(Ca)}$ current was recorded at a holding potential of +20 mV from an outside-out membrane patch. After single channel activity stabilized for about 5 mins, NPY was applied to the perfusion solution. A decrease in frequency of channel opening was observed within 1 min of NPY application and the effect of NPY reached the peak within 2 - 3 mins. The inhibitory effect of NPY on single channel activity was reversible upon washing.

Panel B. The open probability of single channel current in panel A was calculated every 2 sec and plotted against the time.

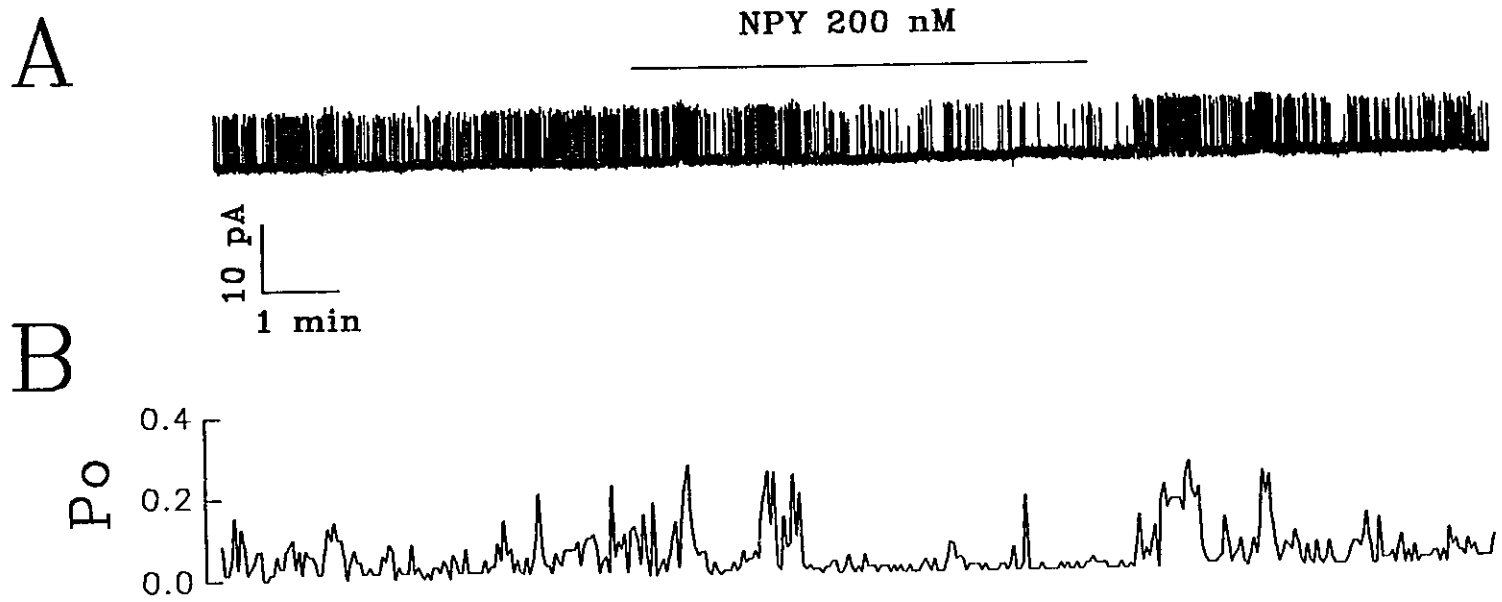

A
NPY 200 nM
10 pA
1 min
B
Po
0.4
0.2
0.0

Figure 35. Effect of NPY on Single Channel $K_{(Ca)}$ at Different Test Potentials.

Panel A. Single channel recording of outward current from a holding potential of -40 mV to test potentials from 0 to +30 mV. Both the amplitude and the frequency of channel opening increased at more depolarized potentials.

Panel B. NPY 200 nM decreased single channel activity at all test potentials.

Panel C. Recovery of single channel activity after washout of NPY for 5 min.

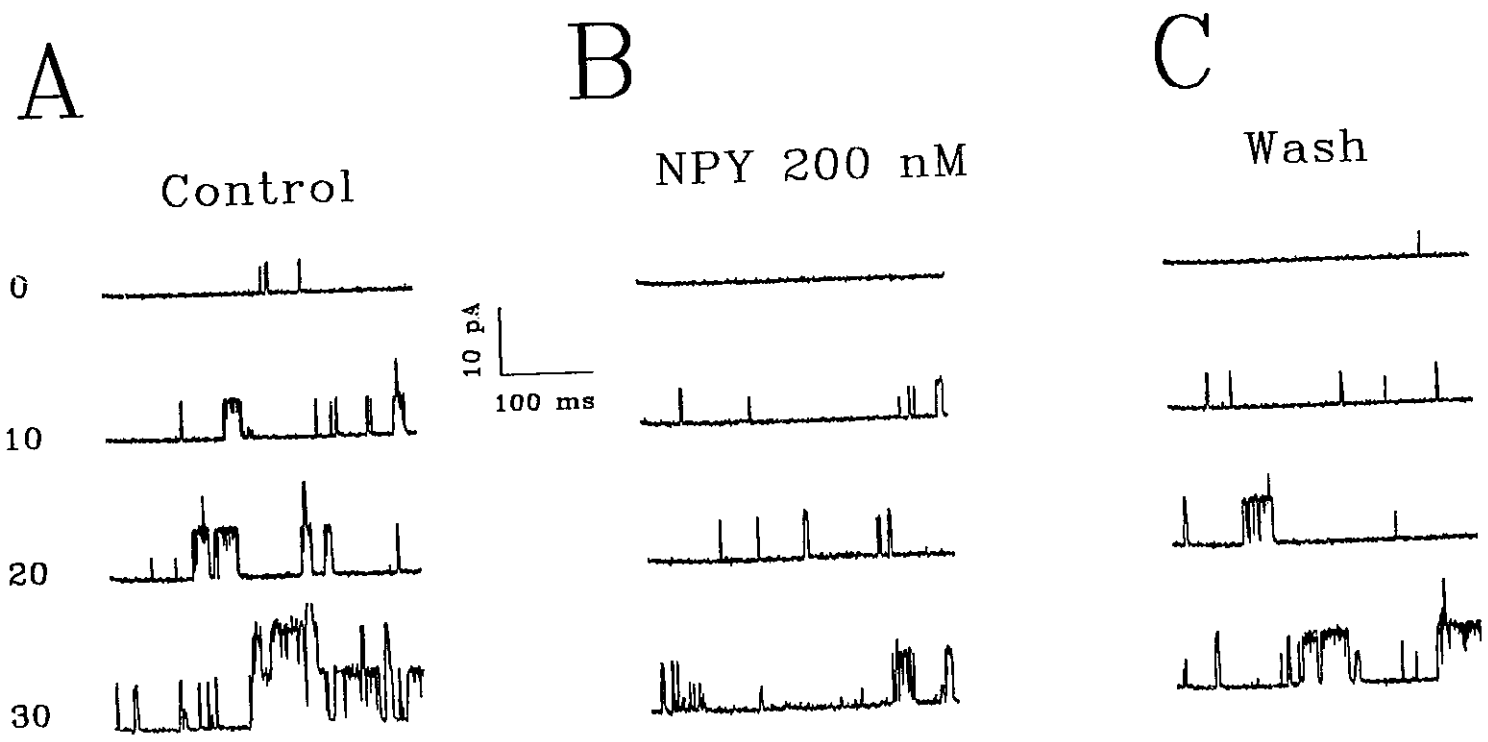
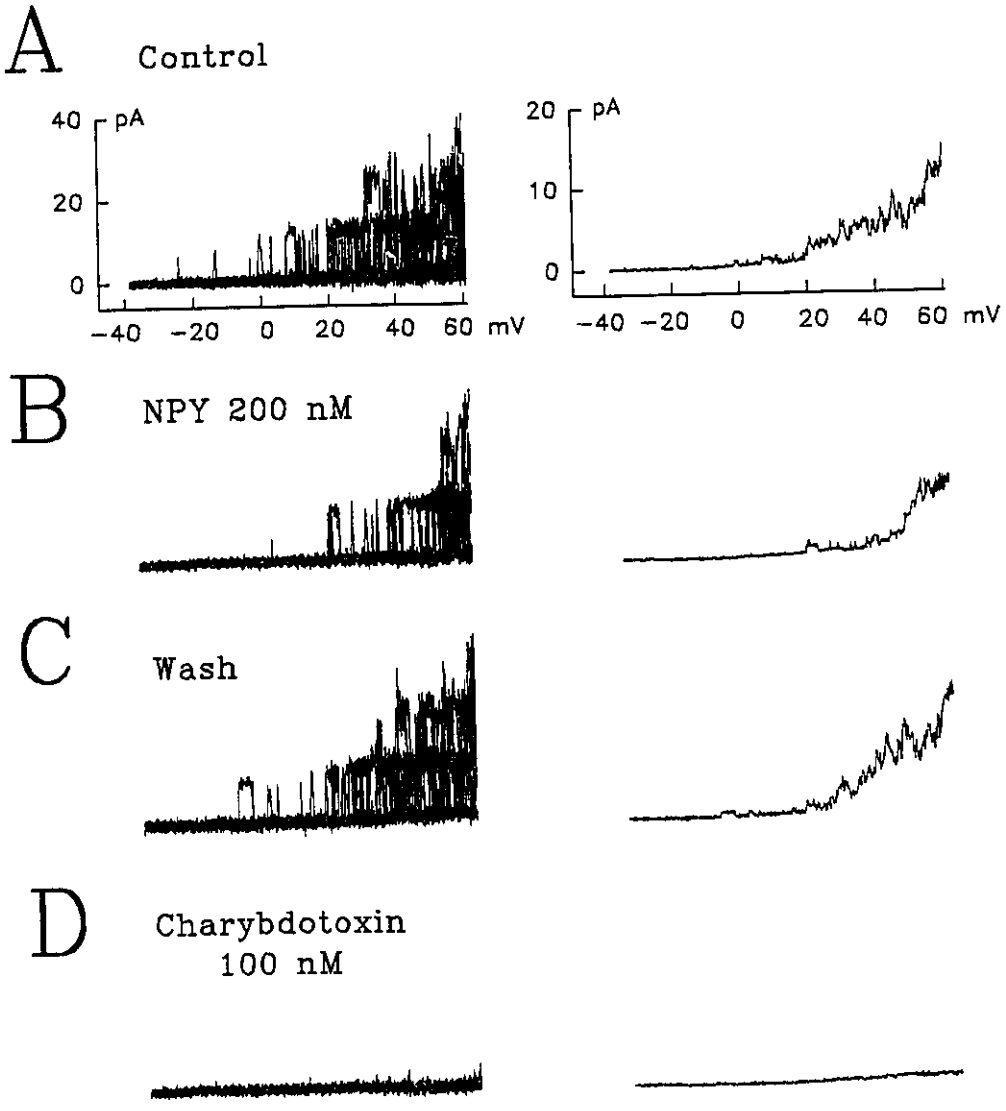

A
Control
0
10
20
30
10 pA
100 ms
B
NPY 200 nM
C
Wash

Figure 36. Effect of NPY and CTX on Single Channel Activity of $K_{(Ca)}$.

Currents were induced by voltage ramp from -40 to +60 mV at a rate of 100 mV/sec. Left panels show superimposed traces from 25 consecutive ramps. Right panels show the corresponding ensemble average current from the 25 traces. *A.* Control. *B.* In the presence of NPY (200 nM). *C.* Recovery after washing. *D.* In the presence of charybdotoxin (100 nM). Leak currents have been subtracted from a blank template.


A
Control
40
pA
20
0
−40 −20 0 20 40 60 mV
20
pA
10
0
−40 −20 0 20 40 60 mV
B
NPY 200 nM
C
Wash
D
Charybdotoxin
100 nM

channels as Ca^{2+}-activated K^+ channels and can be inhibited by charybdotoxin (100-150 nM) (Fig. 36D).

The inhibition of single channel activities by NPY was dose-dependent as demonstrated in Fig. 37A. At a holding potential of +20 mV, a decrease in the frequency of channel opening was observed at 20 nM. At a concentration of 200 nM, the probability of single channel opening was reduced significantly to 36 ± 3.6% of control ($p < 0.01$; $n = 4$; Fig. 37B). Similarly, the open probability in outside-out patches was reduced significantly to 39.1 ± 4.0% of control using 10 mM BAPTA in the pipette solution ($P<0.01$; $n=4$). However, the amplitude of the single channel currents was not altered by NPY (control = 5.8 ± 0.1 pA; NPY (200 nM) = 5.7 ± 0.2 pA; $n = 4$). The concentration-dependent inhibition of single channel open probability parallels that of the NPY effect on whole-cell currents (Fig. 37B).

Analysis of single channel activities revealed two open times of time constants 1.24 ± 0.08 ms and 8.25 ± 0.48 ms ($n = 4$). NPY (200 nM) had no significant effects on the open times (1.07 ± 0.20 ms and 6.63 ± 0.45 ms respectively). However NPY increased significantly the closed times from 0.49 ± 0.11 ms and 440.84 ± 29.07 ms to 0.58 ± 0.09 and 728.01 ± 68.07 ms respectively (Fig. 38).

IV. Effect of NPY on Single Channel $K_{(Ca)}$ in Cell-attached Patches.

To investigate whether any second messenger was involved, the NPY effect on $K_{(Ca)}$ was studied in the cell-attached configuration. If NPY modulates channel activity directly without involvement of a diffusible second messenger, application of NPY in the

Figure 37. Concentration-dependent Effect of NPY on Single Channel $K_{(Ca)}$.

Panel A. Single channel currents at different NPY concentrations were recorded at a holding potential of +20 mV.

Panel B. Bar graph showing the effect of different concentrations of NPY on single channel open probability (n = 4) and whole-cell current amplitude (n = 6) at +20 mV.

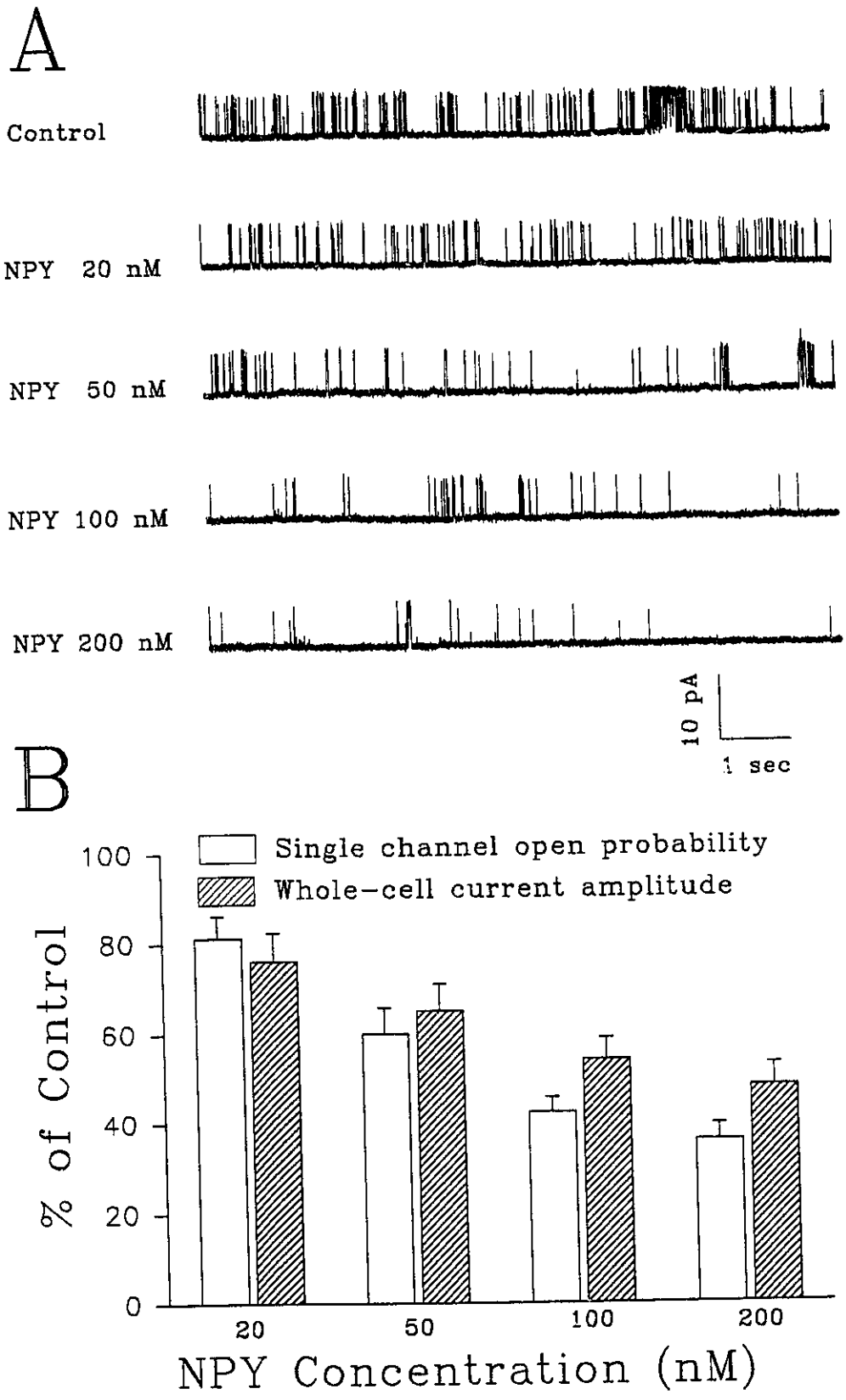
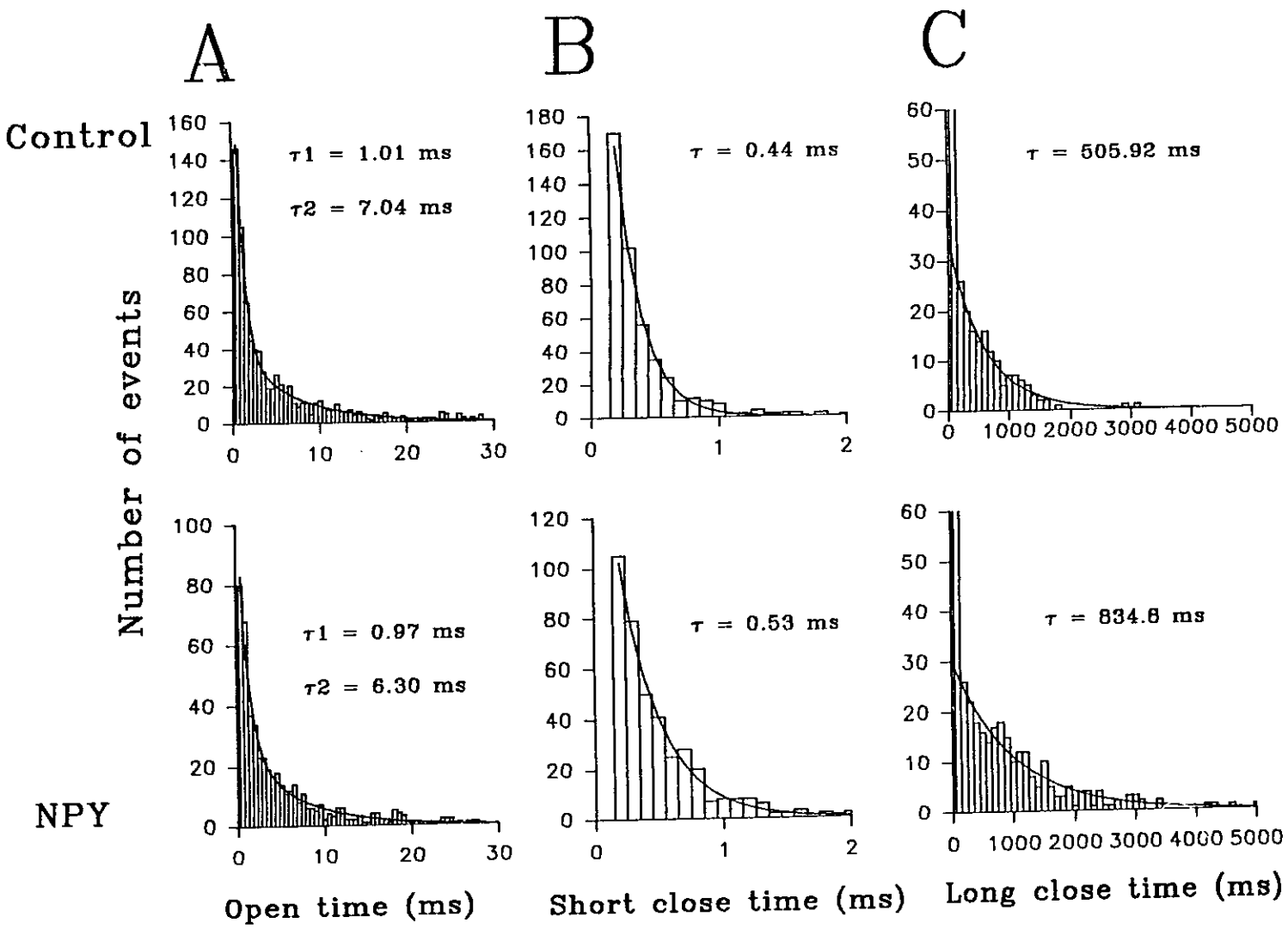

A
Control
NPY 20 nM
NPY 50 nM
NPY 100 nM
NPY 200 nM
10 pA
1 sec
B
Single channel open probability
Whole-cell current amplitude
% of Control
100
80
60
40
20
0
20
50
100
200
NPY Concentration (nM)

Figure 38. Effects of NPY on Open and Close Times of Single Channel $K_{(Ca)}$.

Panel A. Histograms showing the effect of neuropeptide Y (NPY, 200 nM) on open times of single Ca^{2+}-activated K^+ channels on an outside-out patch. The open time histograms could be fitted to two exponential with time constants of 1.01 and 7.04 ms in the control and 0.97 and 6.30 ms in NPY.

Panel B. Short close time histograms fitted to an exponential with time constants of 0.44 and 0.53 ms for control and NPY respectively.

Panel C. Long close time histograms fitted to an exponential with time constants of 505.92 and 834.80 ms for control and NPY respectively.


A
B
C
Control
NPY
Number of events
τ1 = 1.01 ms
τ2 = 7.04 ms
τ = 0.44 ms
τ = 505.92 ms
τ1 = 0.97 ms
τ2 = 6.30 ms
τ = 0.53 ms
τ = 834.6 ms
Open time (ms)
Short close time (ms)
Long close time (ms)

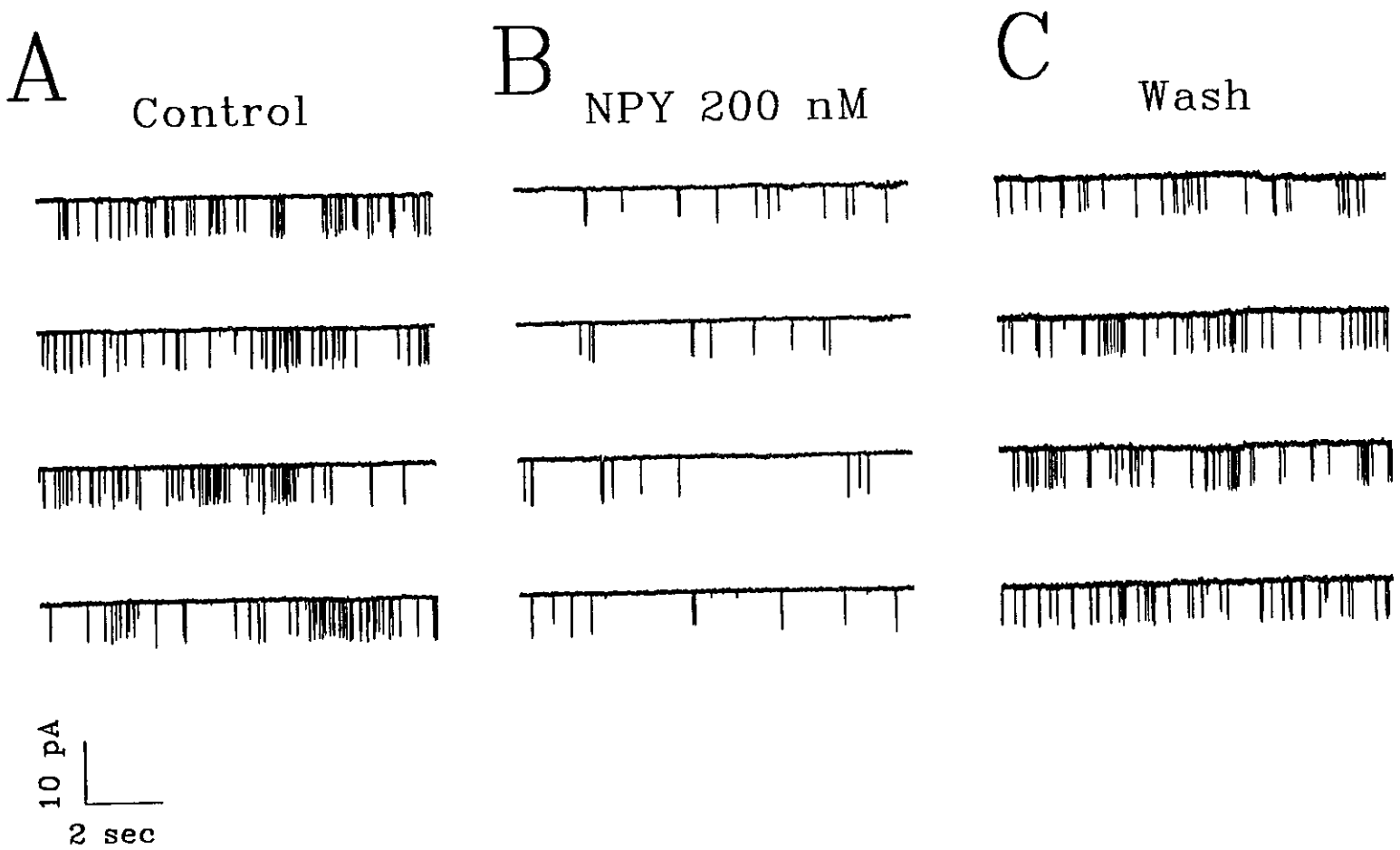
bath solution should not affect channel activity of the membrane patch within the pipette. Figure 39 shows the effect of NPY 200 nM on single channel $K_{(Ca)}$ in cell-attached recording with a holding potential of +30 mV. The cells were depolarized by high K^+ solution to zero the resting membrane potential so that the real membrane potential during voltage clamp was determined. The outward currents were displayed as downward deflections. Following a 5 min period of stable control activity, NPY was applied to the perfusion solution. Similar to outside-out recordings, NPY decreased single channel activity in cell-attached configuration. In 7 cells studied, single channel open probability decreased to 54 $\pm$ 7.3% of the control value ($p<0.05$) with NPY 200 nM. In most of the cells (5/7), a full recovery of the channel activity was observed after washout of NPY for 5 mins.

Figure 39. Effect of NPY on Single Channel $K_{(Ca)}$ in a Cell-attached Patch.

Panel A. Four consecutive traces showing the single channel $K_{(Ca)}$ current recorded from a cell-attached configuration at a holding potential of +30 mV. Single cells were depolarized by high K^+ solution to zero the resting membrane potential so that the real membrane potential during voltage clamp was determined. Downward deflection represents outward current.

Panel B. Single channel current recorded 5 mins after NPY 200 nM. The frequency of single channel opening was decreased by NPY.

Panel C. Single channel current recorded after washout of NPY for 5 mins.


A
Control
B
NPY 200 nM
C
Wash
10 pA
2 sec

PART V. ATP-dependent Regulation of $K_{(Ca)}$ Channels

I. Effect of NPY on $K_{(Ca)}$ is ATP-dependent.

To test whether ATP was essential for NPY effect and whether phosphorylation process was involved, ATP was removed from pipette solution. Stable $K_{(Ca)}$ current can be recorded in the absence of ATP in both whole-cell and outside-out recordings. Figure 40B shows the effects of NPY on whole-cell $K_{(Ca)}$ currents in the absence of ATP. Application of NPY 200 nM for 5 - 10 mins had no significant effect on the whole-cell currents. At a testing potential of +30 mV, the whole-cell current amplitude was 96.7 $\pm$ 7.4% of the control value (n=4, p>0.05, Fig. 42B). Similarly, the single channel open probability from outside-out patches was not changed by NPY in the absence of ATP. At a test potential of +30 mV, single channel open probability was 99.6 $\pm$ 12% of the control value (n=4, p>0.05) after NPY 200 nM (Fig. 41). The outward currents in the absence of ATP were still sensitive to TEA (2 mM) (Fig. 40B). Application of TEA (2 mM) for 5 min almost completely abolished the whole-cell $K_{(Ca)}$ current.

These results suggested that the effect of NPY on $K_{(Ca)}$ was ATP-dependent. ATP may act either as a ligand or as a phosphate donor in the phosphorylation process. To differentiate between these two possibilities, the effect of NPY on $K_{(Ca)}$ was studied with ATP replaced by the non-hydrolysable ATP analogue ß,γ-methylene adenosine triphosphate (AMP-PCP) in the intracellular solution. This compound has been shown to be an effective ligand for ATP receptor but it cannot be hydrolysed to serve as a phosphate donor. Figure 43A and 44A show the effect of NPY on whole-cell $K_{(Ca)}$ with ATP replaced by 1 mM AMP-PCP in the intracellular solution. As in the ATP-free

Figure 40. ATP Dependence of NPY Effect on Whole-cell $K_{(Ca)}$ Current.

Panel A. Representative traces showing the effect of NPY 200 nM on whole-cell $K_{(Ca)}$ current with ATP 1 mM in the pipette solution. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from -40 to +70 mV. NPY 200 nM reversibly inhibited whole-cell $K_{(Ca)}$ current.

Panel B. Effect of NPY and TEA on whole-cell $K_{(Ca)}$ recorded with ATP-free intracellular solution. NPY 200 nM had no effect on outward current. TEA 2 mM almost completely blocked the outward current.

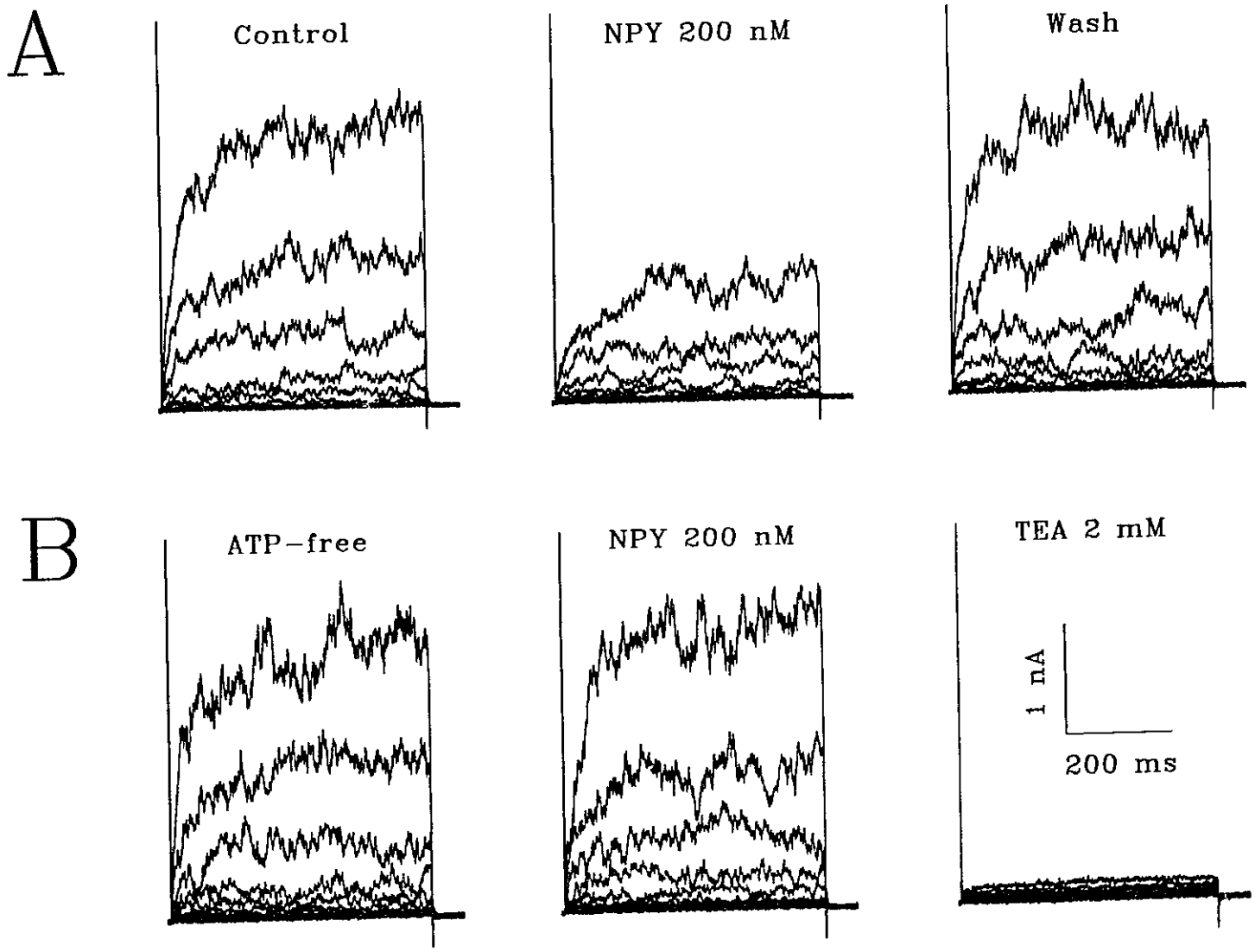

A
Control
NPY 200 nM
Wash
B
ATP−free
NPY 200 nM
TEA 2 mM
1 nA
200 ms

Figure 41. Effect of NPY on Single Channel $K_{(Ca)}$ Current in the Absence of ATP.

Panel A. Representative trace showing the effect of NPY on single channel $K_{(Ca)}$ current with ATP-free intracellular solution. Current was recorded at a holding potential of +30 mV.

Panel B. The open probability of single channel current in panel A was calculated every 2 sec and plotted against the time.

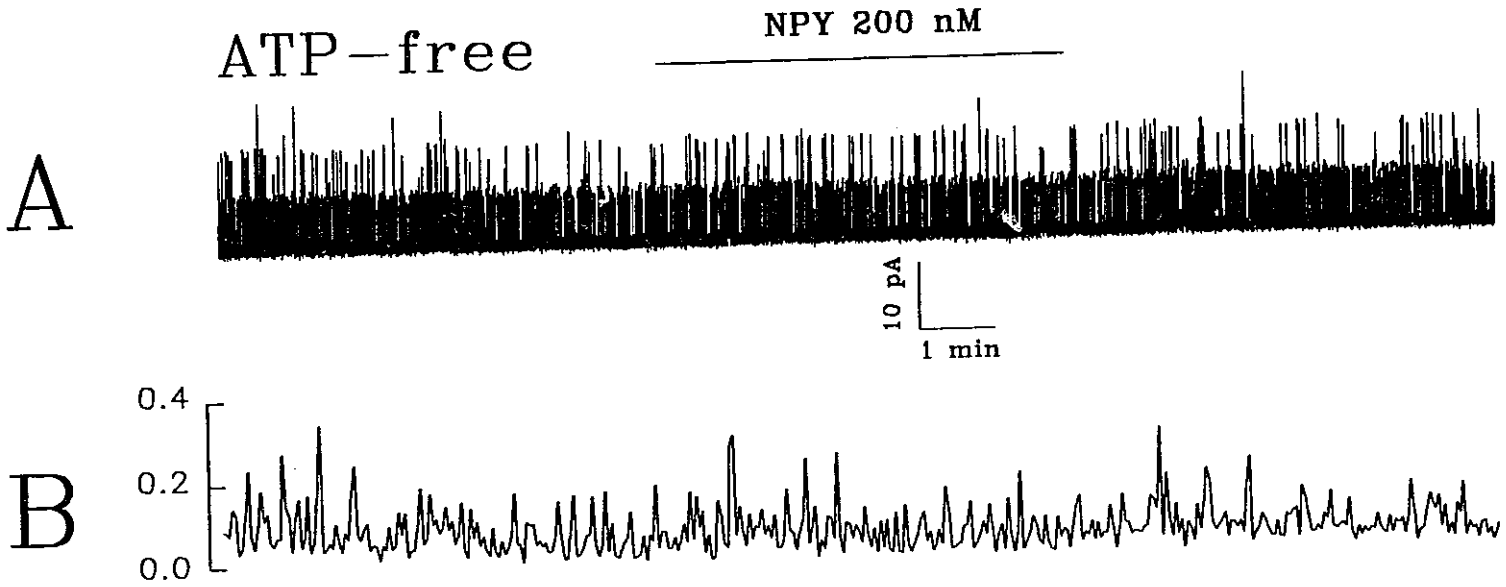
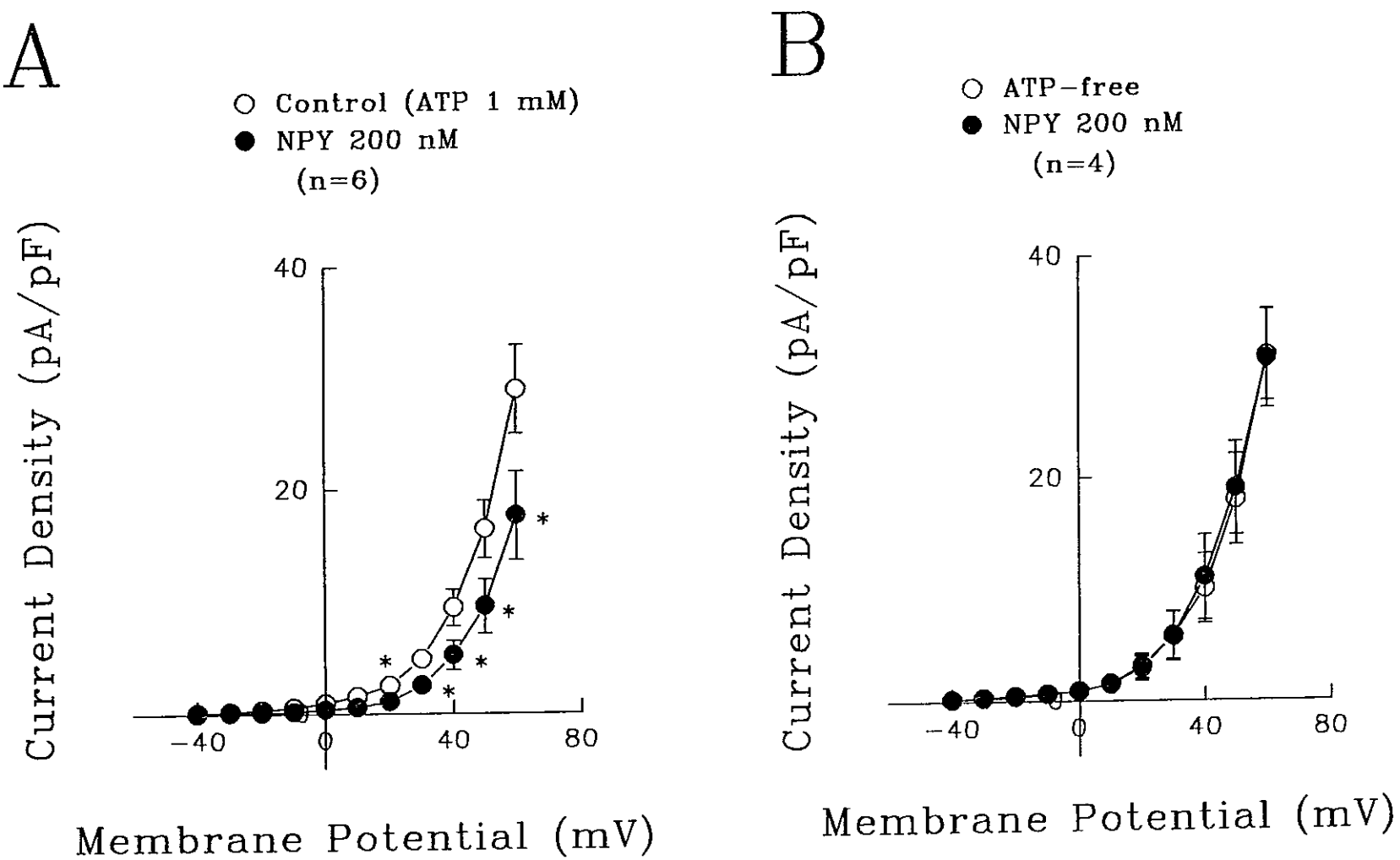

A
ATP−free
NPY 200 nM
10 pA
1 min
B
0.4
0.2
0.0

Figure 42. Effect of NPY on I-V Curves of Whole-cell $K_{(Ca)}$ Current.

Panel A. Control. I-V curves for whole-cell $K_{(Ca)}$ before and after NPY 200 nM. Currents were recorded from a holding potential of -40 mV with ATP 1 mM in the pipette solution.

Panel B. ATP-free. I-V curves for whole-cell $K_{(Ca)}$ before and after NPY 200 nM. Currents were recorded with ATP-free pipette solution.


A
Control (ATP 1 mM)
NPY 200 nM
(n=6)
Current Density (pA/pF)
40
20
−40
0
40
80
Membrane Potential (mV)
B
ATP−free
NPY 200 nM
(n=4)
Current Density (pA/pF)
40
20
−40
0
40
80
Membrane Potential (mV)

condition, NPY 200 nM had no effect on $K_{(Ca)}$. At a test potential of +30 mV, whole-cell current amplitude was 99.1 ± 3% of control value (n=3, p>0.05).

In the condition that ATP was excluded from intracellular solution, the free ATP concentration may not be exactly zero since some endogenous ATP may be trapped in the cell and the cell may still produce ATP. To ensure that ATP production was eliminated, another set of experiment was performed. In this case glucose was removed from bath solution and osmolarity was supplemented with sodium ions. ATP was replaced with equimolar non-hydrolysable ATP analogue adenylimidodiphosphate (AMP-PNP). 2-deoxyglucose (1 mM) and carbonyl cyanide-M-chlorophenyl hydrozone (CCCP) (10 μM) were also added to the intracellular solution to prevent ATP production. Figure 43B and 44B show the effect of NPY on $K_{(Ca)}$ in this "death brew" condition. Perfusion of NPY 200 nM for 10 min had no effect on $K_{(Ca)}$. In four cells studied, whole-cell current amplitude was 93.1 ± 6.5% of control value (p>0.05) at a testing potential of +30 mV (Fig. 43B, 44B).

II. Effect of NPY on $K_{(Ca)}$ in the Presence of ATP-γ-S.

The above experiments demonstrated that ATP hydrolysis was required for NPY effect indicating that phosphorylation or dephosphorylation process was involved in NPY effect on $K_{(Ca)}$. To test whether NPY effect was mediated through a dephosphorylation process, ATP-γ-S was used as replacement of ATP. It has been documented that thiophosphorylated residues in protein are poor substrates for phosphatases. If the NPY effect was due to a change in phosphatase activity, it would be expected that with

Figure 43. Effect of NPY on $K_{(Ca)}$ Current in the Presence of AMP-PCP.

Panel A. Effect of NPY 200 nM on whole-cell $K_{(Ca)}$ current with ATP replaced with the non-hydrolysable analogue ß,γ-methylene adenosine triphosphate (AMP-PCP) in the pipette solution. Currents were recorded by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials from -40 to +70 mV with 10 mV increment.

Panel B. Effect of NPY 200 nM on whole-cell $K_{(Ca)}$ current with a "death brew" solutions. The "death brew" condition was created by removal of glucose from extracellular solution and replacement of ATP with AMP-PCP in intracellular solution. Carbonyl cyanide-M-chlorophenyl hydrozone (CCCP) 10 μM and 1 mM 2-deoxyglucose were also included in the pipette solution.

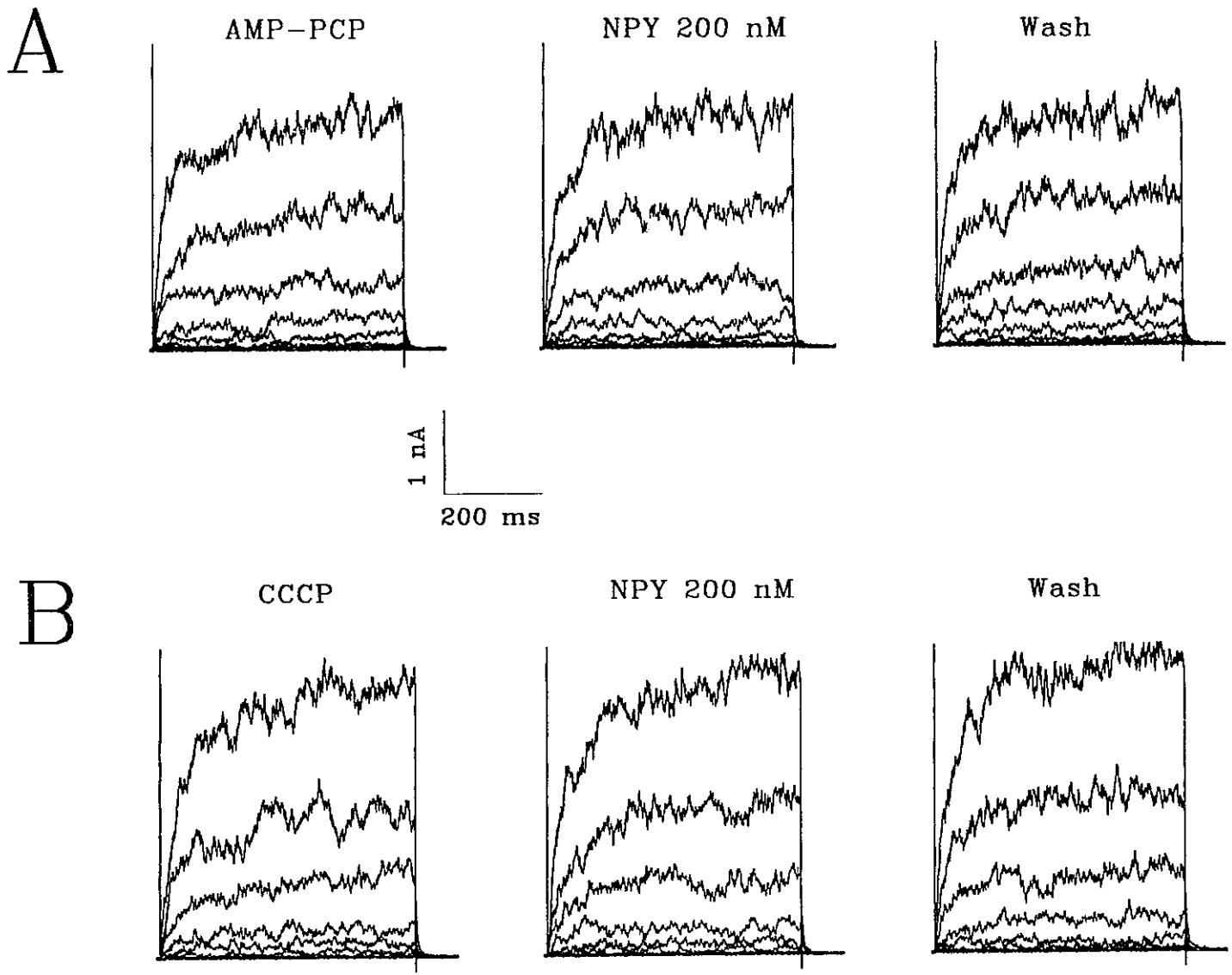
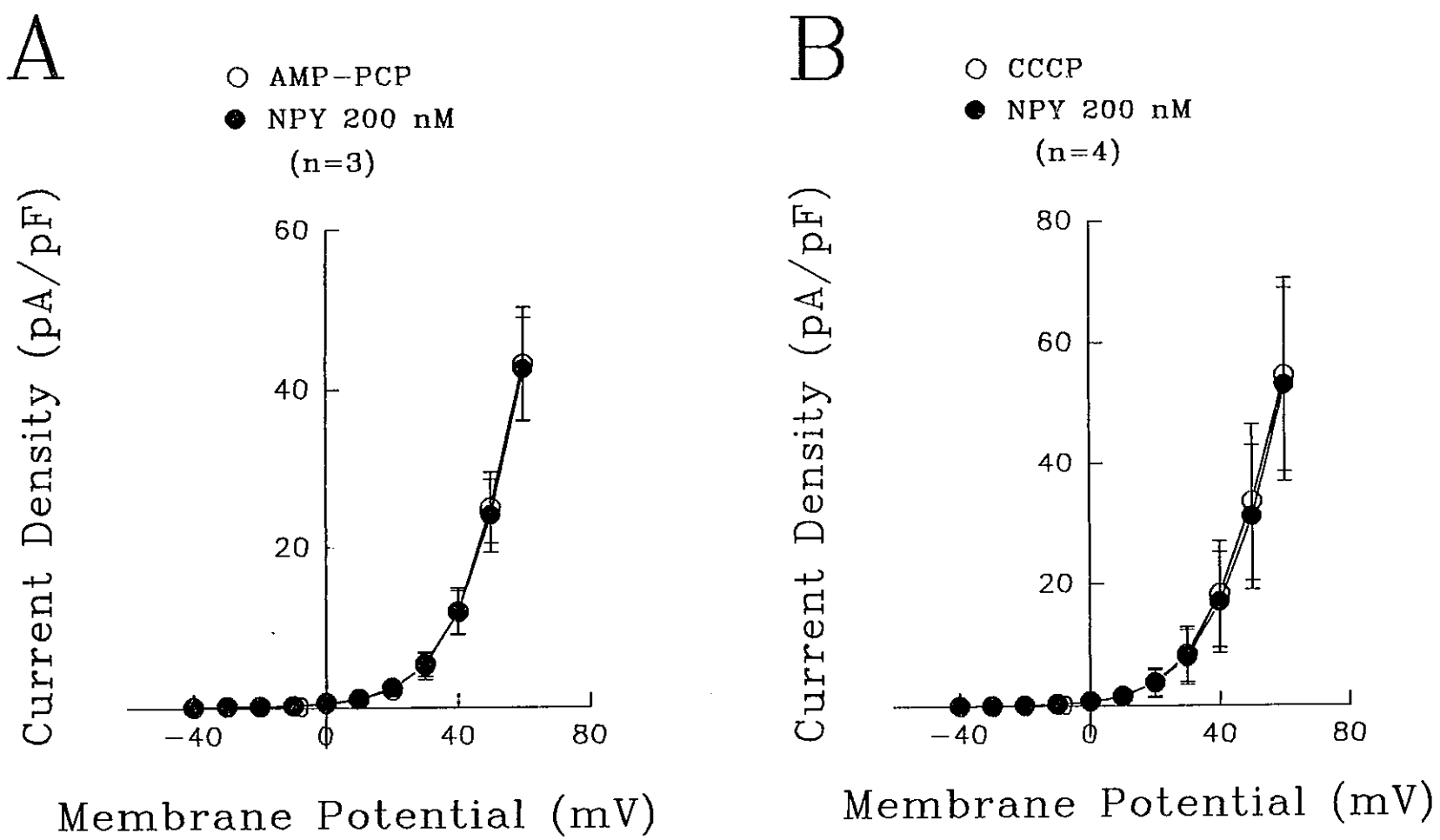

A
AMP–PCP
NPY 200 nM
Wash
1 nA
200 ms
B
CCCP
NPY 200 nM
Wash

Figure 44. NPY effect on I-V Curves in the Presence of AMP-PCP or CCCP.

Panel A. Effect of NPY 200 nM on I-V curve of whole-cell $K_{(Ca)}$ current recorded with ATP replaced with equimolar non-hydrolysable ATP analogue ß,γ-methylene adenosine triphosphate (AMP-PCP) in the pipette solution. Holding potential = -40 mV.

Panel B. Effect of NPY 200 nM on I-V curve of whole-cell $K_{(Ca)}$ current recorded with carbonyl cyanide-M-chlorophenyl hydrozone (CCCP) 10 μM in the pipette solution (see text). Holding potential = -40 mV.


A
AMP-PCP
NPY 200 nM
(n=3)
Current Density (pA/pF)
60
40
20
−40
0
40
80
Membrane Potential (mV)
B
CCCP
NPY 200 nM
(n=4)
Current Density (pA/pF)
80
60
40
20
−40
0
40
80
Membrane Potential (mV)

ATP-γ-S as phosphate donor, NPY would have no more effect on the channel. However, if the NPY effect was due to a change in kinase activity, replacing ATP by ATP-γ-S would not alter the NPY effect. Figure 45 shows the effect of NPY 200 nM on single $K_{(Ca)}$ current from an outside-out membrane patch with 1 mM ATP-γ-S in the pipette solution. Similar to the experiment with ATP in the pipette solution, NPY was able to decrease the open probability of $K_{(Ca)}$. In the presence of NPY 200 nM, the open probability was decreased to 37.2 ± 7.5% of control value ($n=4$, $p<0.05$). Unlike that with ATP in the pipette solution, the effect of NPY on $K_{(Ca)}$ was not reversible with ATP-γ-S in the pipette solution, consistent with a thiophosphorylation involvement.

III. Effect of NPY on $K_{(Ca)}$ in the Presence of PKI.

To investigate whether protein kinase A (PKA) was involved in NPY effect on $K_{(Ca)}$, protein kinase inhibitor (PKI), a specific inhibitor of cAMP-dependent protein kinase, was included in the pipette solution. Figure 46A shows an example of this experiment. Whole-cell $K_{(Ca)}$ currents were recorded with 1 μM PKI in the pipette solution. After the outward current had stabilized for at least 10 min, NPY 200 nM was applied to the bath solution. NPY decreased the amplitude of whole-cell $K_{(Ca)}$ current. At a test potential of +30 mV, current amplitude was decreased by 40.3 ± 7.4% ($n=4$, $p<0.05$). This inhibitory effect of NPY on whole-cell $K_{(Ca)}$ current was slightly smaller than the 52% inhibition of the current by NPY when PKI was not included in the pipette solution. However, no significant difference was found between these two values.

Figure 45. Effect of NPY on $K_{(Ca)}$ in the Presence of ATP-γ-S.

Panel A. Representative trace showing the effect of NPY on single channel $K_{(Ca)}$ current with ATP replaced with equimolar ATP-γ-S in the pipette solution.

Panel B. The open probability of single channel current in panel A was calculated every 2 sec and plotted against the time.

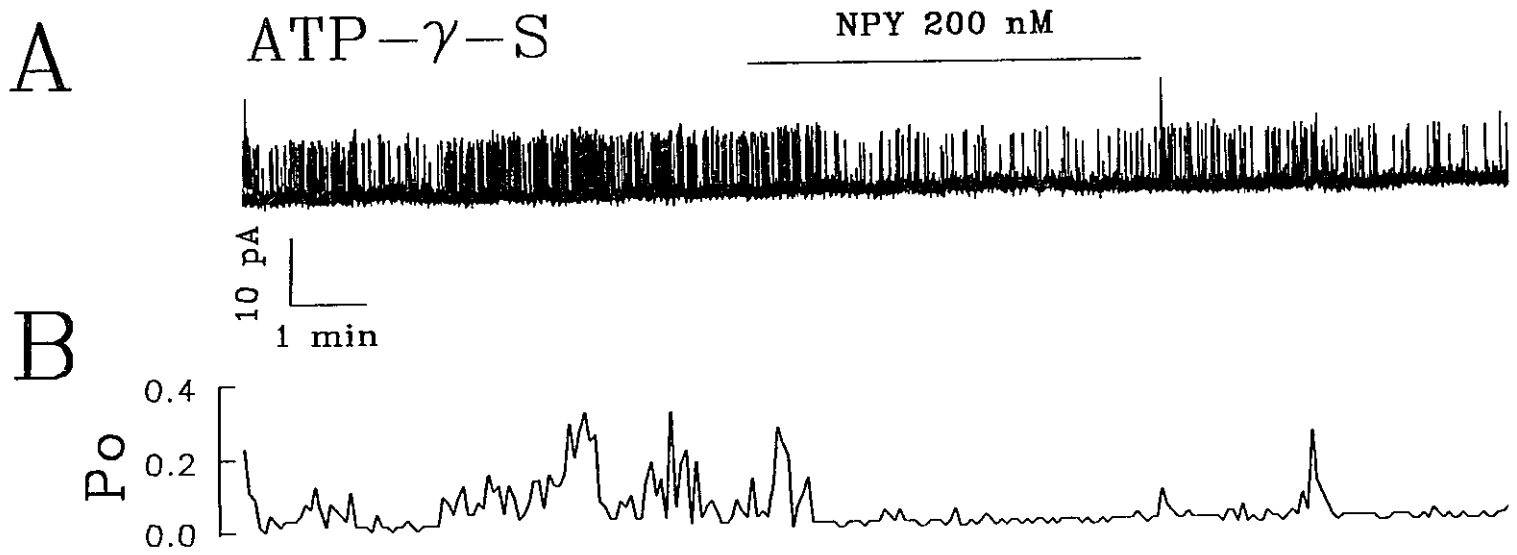
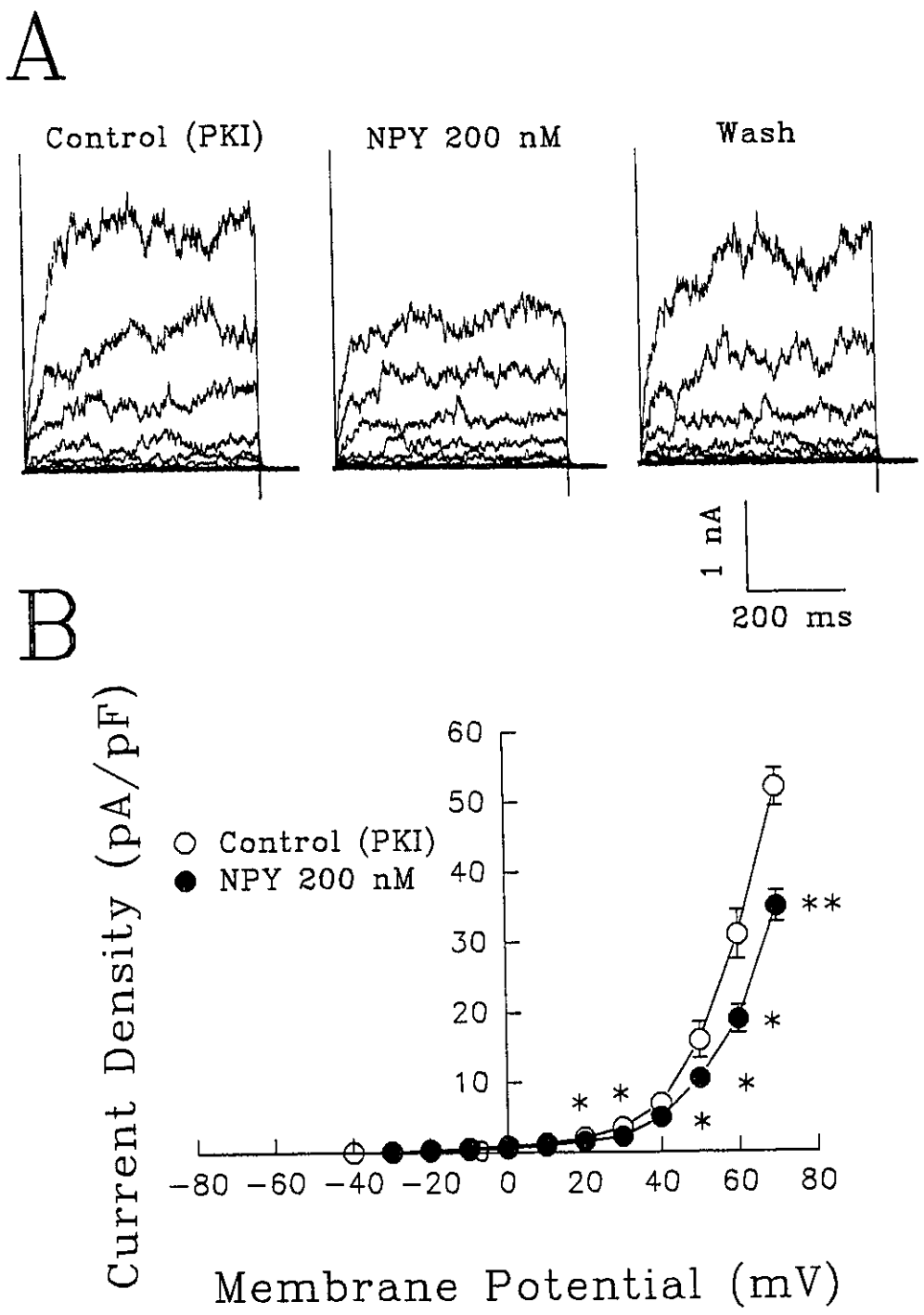

A
ATP−γ−S
NPY 200 nM
10 pA
1 min
B
Po
0.4
0.2
0.0

Figure 46. Effect of NPY on $K_{(Ca)}$ in the Presence of PKI.

Panel A. Representative traces showing the effect of NPY (200 nM) on whole-cell $K_{(Ca)}$ current recorded with protein kinase inhibitor (PKI; 1 μM) in the pipette solution. Currents were recorded from a holding potential of -40 mV to test potentials ranging from -40 to +70 mV. Introduction of PKI in the pipette solution did not affect the basal $K_{(Ca)}$ current. Left panel: control. Middle panel: after NPY 200 nM for 5 mins. Right panel: after washout of NPY for 5 mins.

Panel B. I-V curves for control (○) and after NPY (200 nM) for 5 mins (●). n = 4. * $p<0.05$; ** $p<0.01$.


A
Control (PKI)
NPY 200 nM
Wash
1 nA
200 ms
B
Current Density (pA/pF)
Control (PKI)
NPY 200 nM
60
50
40
30
20
10
−80 −60 −40 −20 0 20 40 60 80
Membrane Potential (mV)
*
**

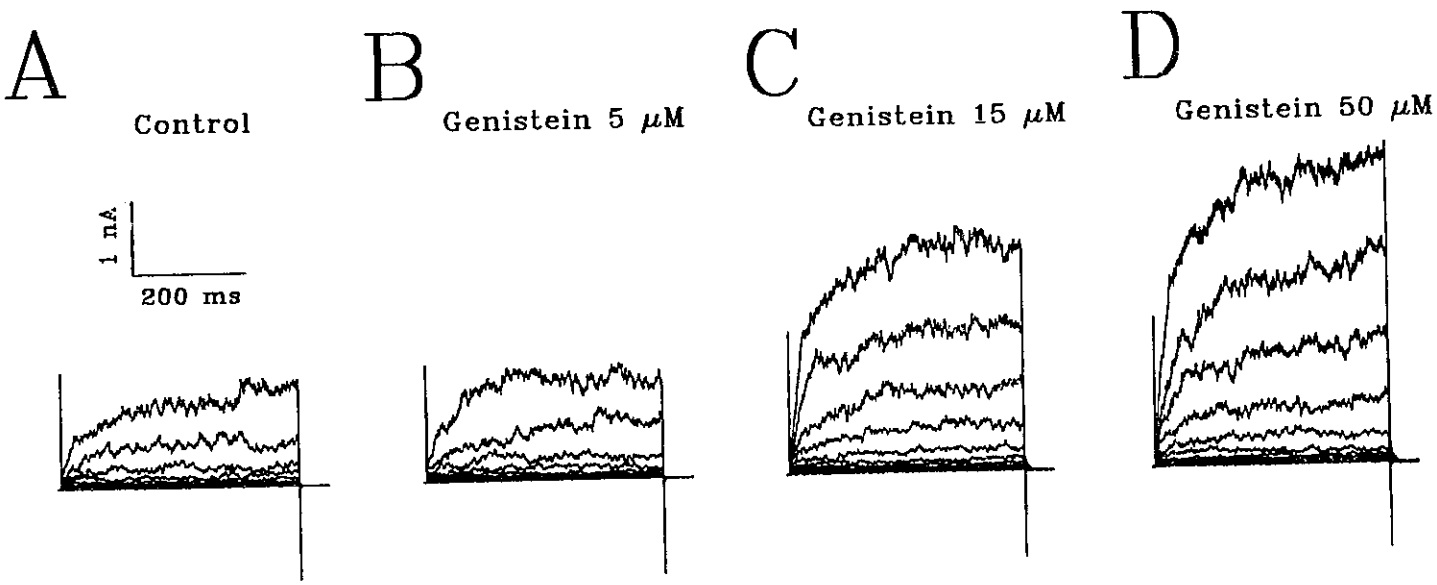
IV. Modulation of $K_{(Ca)}$ by Tyrosine Kinase Inhibitors.

To investigate whether protein tyrosine phosphorylation is involved in the modulation of $K_{(Ca)}$ channels in vascular smooth muscle cells, we have studied the effects of various tyrosine kinase inhibitors on $K_{(Ca)}$ channel currents with whole-cell and single channel recordings. Figure 47 shows the effects of different concentration of the tyrosine kinase inhibitor genistein on whole-cell $K_{(Ca)}$ currents. Currents were elicited by 500 ms depolarizing pulse from a holding potential of -40 mV to test potentials ranging from -40 to +60 mV. After the currents had stabilized for 10 mins, genistein was applied to the perfusion solution. Genistein significantly potentiated the whole-cell currents within 2 mins of incubation and the effect of genistein stabilized after 5 mins. The potentiation effect of genistein was dose-dependent from 1 μM to 50 μM. At a test potential of +30 mV, whole-cell current amplitude was increased by 23.5 ± 25% (n=4), 50 ± 25% (n=6), 143 ± 52% (n=8), and 227 ± 104% (n=4) in the presence of genistein 1, 5, 15 and 50 μM respectively (Fig. 47, 48). As a result, the current-voltage relationship was shifted to the left in the presence of genistein (Fig. 48A). The effect of genistein was reversible and the currents recovered completely within 10 mins of washing (Fig. 49A). The current potentiated by genistein was sensitive to the blockade by CTX (Fig. 49B).

Single channel $K_{(Ca)}$ current was studied using inside-out membrane patches by continuous recording of the channel activity at a holding potential of +30 mV. Genistein substantially increased the open probability of $K_{(Ca)}$ channels within 2 mins of application (Fig. 50A). In the presence of genistein 15 μM, the single channel open

Figure 47. Dose-dependent Effect of Genistein on Whole-cell $K_{(Ca)}$ Currents.

Representative traces showing the dose-dependent potentiating effect of tyrosine kinase inhibitor genistein on whole-cell $K_{(Ca)}$ currents. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from -40 to +60 mV. *Panel A:* control; *Panel B:* 5 mins after genistein 5 μM; *Panel C:* 5 mins after genistein 15 μM; *Panel D:* 5 mins after genistein 50 μM.


A
Control
B
Genistein 5 μM
C
Genistein 15 μM
D
Genistein 50 μM
1 nA
200 ms

probability was increased by 120 ± 31% (n=4, $p<0.01$), similar to the effect of genistein on whole-cell current amplitude. The effect of genistein on single channel activity of $K_{(Ca)}$ was reversible after washing out of the drug (Fig. 50A). The effect of genistein on single channel open probability was dose-dependent, as shown in figure 48B. At a test potential of +30 mV, single channel open probability was increased by 20 ± 19% (n=4), 72 ± 36% (n=5), 120 ± 31% (n=4), and 214 ± 109% (n=4) in the presence of 1, 5, 15, and 50 μM of genistein (Fig. 48B). Daidzein, an inactive analogue of genistein, was used as a control for genistein. No significant changes in single channel activity was observed with daidzein (15 μM, n=3) (Fig. 53A).

Lavendustin A, another inhibitor of tyrosine kinase, had a similar potentiation effect on $K_{(Ca)}$ with both whole-cell and single channel recordings (Fig. 50B, 51). At a test potential of +30 mV, whole-cell current amplitude of $K_{(Ca)}$ was increased by 139 ± 53% after perfusion of 10 μM lavendustin A for 5 mins (n=3; $p<0.05$) (Fig. 51). Single channel open probability in inside-out patches was increased by 205 ± 93% (n=5; $p<0.01$) in the presence of 10 μM lavendustin A (Fig. 50B).

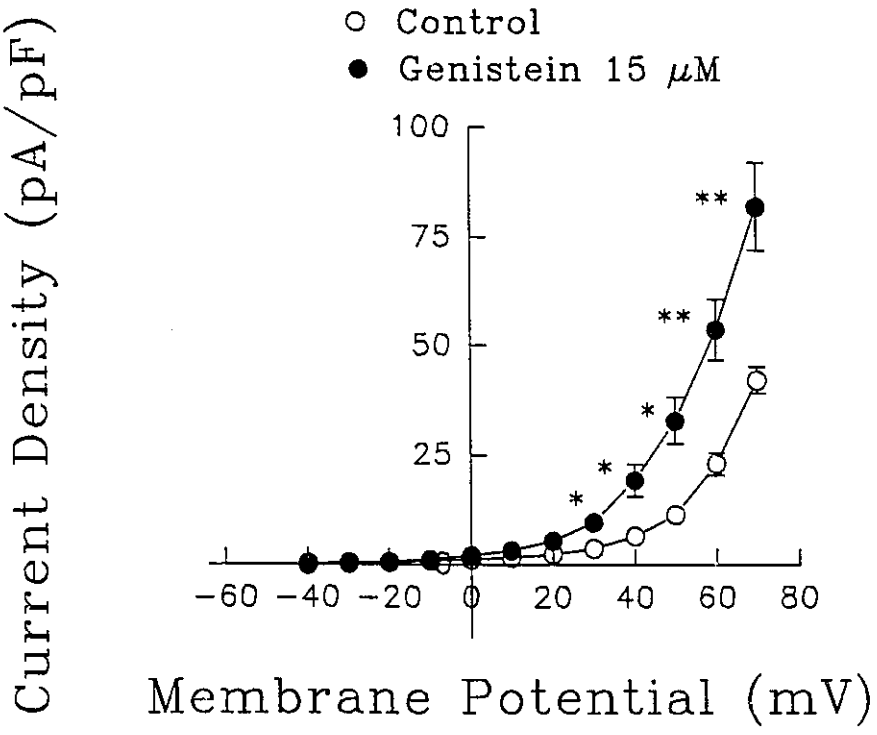
Tyrphostin A25, a specific inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase, substantially increased single channel open probability in inside-out patches (Fig. 50C). At a holding potential of +30 mV, single channel open probability increased by 841 ± 337% (n=5, $p<0.05$) after perfusion of 10 nM tyrphostin A25 for 5 mins. However, no significant effect of tyrphostin A25 was observed with whole-cell recording, even with a concentration as high as 10 μM and perfused for as long as 30 mins (not shown).

Figure 48. I-V and Dose-response Curves for Genistein.

Panel A. Graph showing the change in current-voltage (I-V) relationship of $K_{(Ca)}$ channels induced by 15 μM genistein (n=5). Whole-cell currents were elicited by depolarizing pulses from a holding potential of -40 mV. * $p<0.05$; ** $p<0.01$.

Panel B. Graph showing the dose-dependent increase in whole-cell current amplitude (○) and single channel open probability (●) of $K_{(Ca)}$ by genistein. Test potential was +30 mV. n = 4-5.

A


Control
Genistein 15 μM
Current Density (pA/pF)
100
75
50
25
−60 −40 −20 0 20 40 60 80
*
*
*
**
**
Membrane Potential (mV)

B

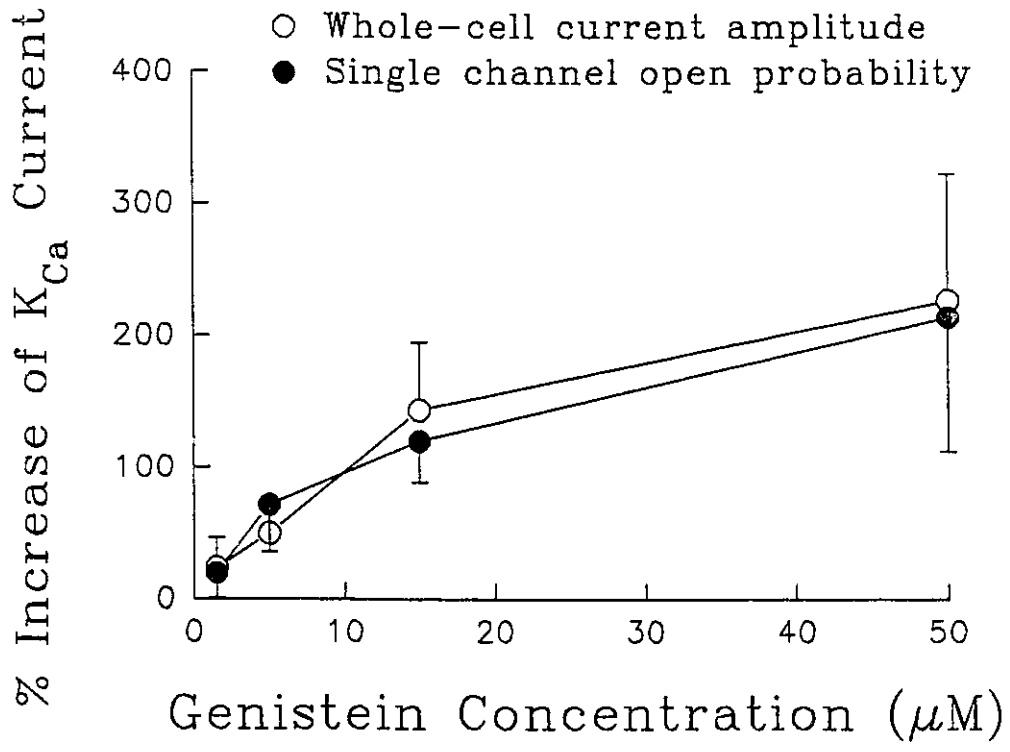

Whole-cell current amplitude
Single channel open probability
% Increase of K_{Ca} Current
400
300
200
100
0
0 10 20 30 40 50
Genistein Concentration (μM)

Figure 49. Effect of Genistein on Whole-cell $K_{(Ca)}$ Currents.

Panel A. Representative traces showing the effect of genistein 15 μM on whole-cell $K_{(Ca)}$ currents. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from -40 to +60 mV. Genistein 15 μM reversibly potentiated whole-cell $K_{(Ca)}$ currents.

Panel B. Representative traces showing the sensitivity of whole-cell currents potentiated by genistein to a specific $K_{(Ca)}$ channel blocker CTX (150 nM). Holding potential: -40 mV. Test potential: -40 to +60 mV.

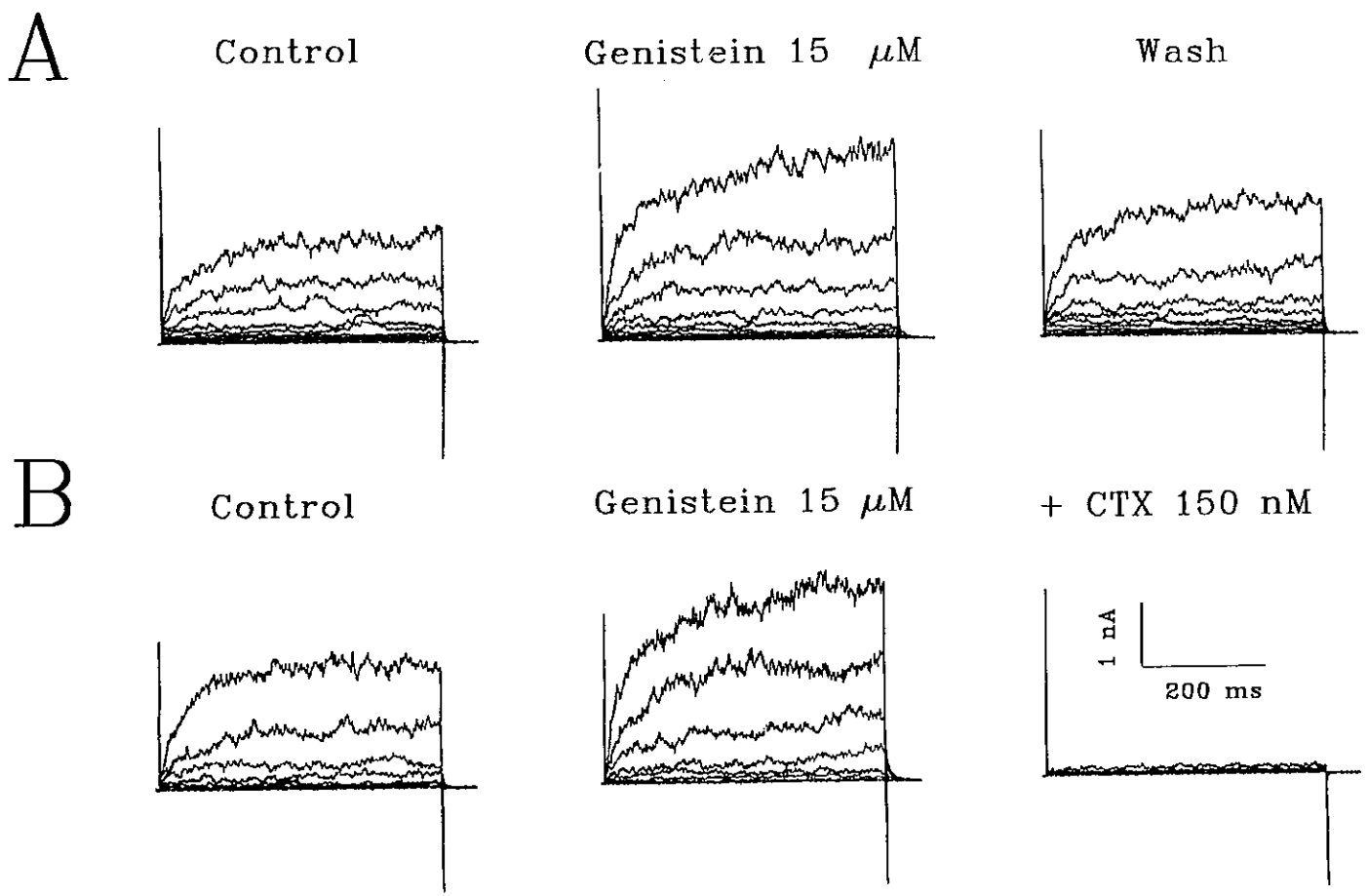
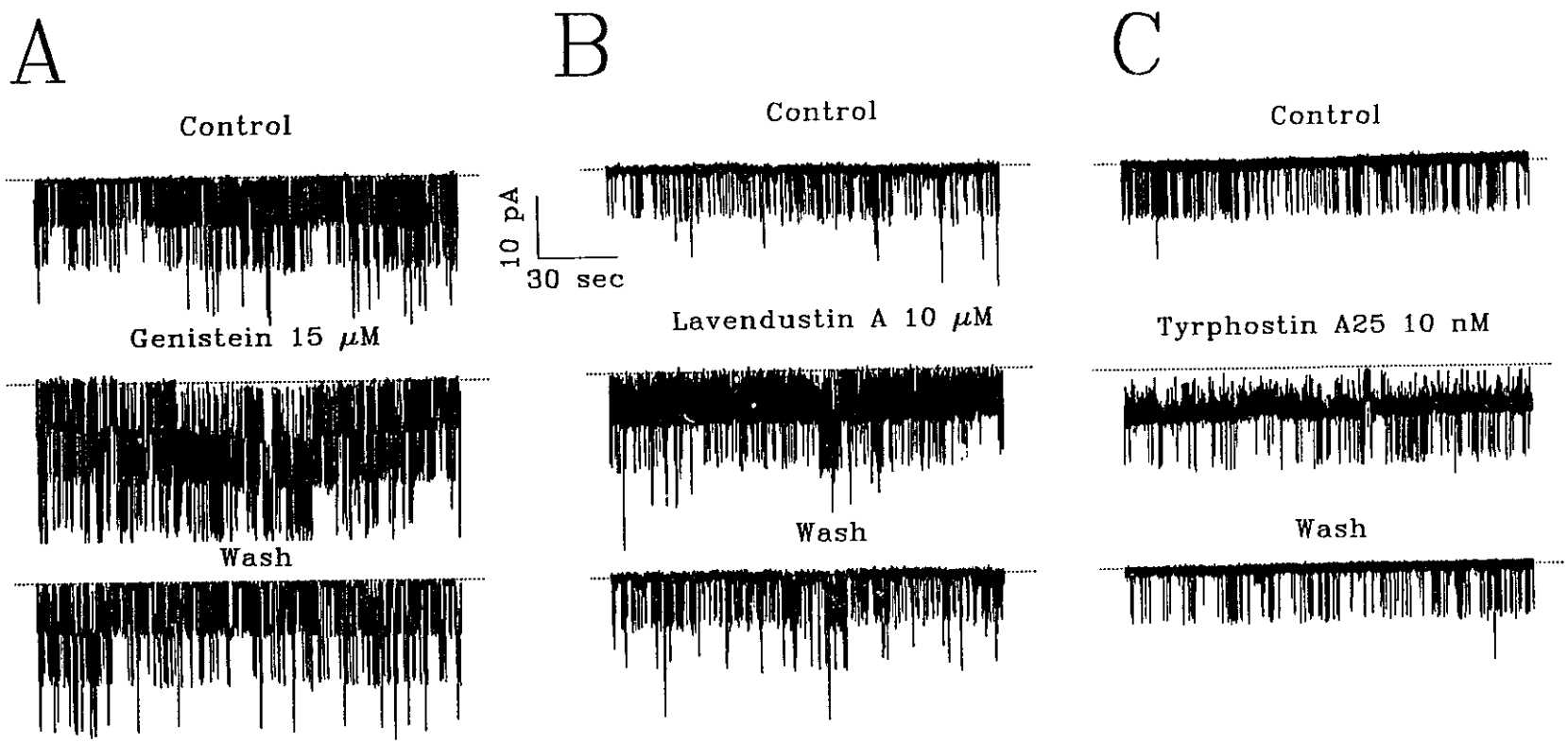

A
Control
Genistein 15 μM
Wash
B
Control
Genistein 15 μM
+ CTX 150 nM
1 nA
200 ms

Figure 50. Effects of Genistein, Lavendustin A, and Tyrphostin A25 on Single Channel $K_{(Ca)}$ Currents.

Representative traces showing the effects of tyrosine kinase inhibitors on single channel activities of $K_{(Ca)}$ channels in inside-out membrane patchs with 1 mM ATP in the bath solution. *Panel A.* Genistein 15 μM; *Panel B.* Lavendustin A 10 μM; *Panel C.* Tyrphostin A25 10 nM. Recordings were made after 5 min incubation period at a test potential of +30 mV. The responses to tyrosine kinase inhibitors were reversible upon washout for 5 min.


A
Control
Genistein 15 μM
Wash
B
Control
10 pA
30 sec
Lavendustin A 10 μM
Wash
C
Control
Tyrphostin A25 10 nM
Wash

The effect of tyrosine kinase inhibitors on $K_{(Ca)}$ channels was ATP-dependent. When ATP was omitted from the intracellular solutions, they had no effect on $K_{(Ca)}$ currents. Figure 52 shows the effect of genistein on whole-cell $K_{(Ca)}$ current in the absence of ATP in the pipette solution. After the currents had stabilized for at least 10 mins, genistein 15 μM was perfused into the bath solution. Genistein had no significant effect on the whole-cell current. At a test potential of +30 mV, whole-cell current amplitude was 97 ± 23% (n=3; $p>0.05$) of control value (Fig. 53B). Similarly, in inside-out patches, genistein 15 μM failed to increase the single channel open probability of $K_{(Ca)}$ in the absence of ATP (Fig. 53B). In four cells studied, single channel open probability was 96.8 ± 10% (n=4; $p>0.05$) of control value after 15 μM genistein.

V. Effect of NPY on $K_{(Ca)}$ in the Presence of Genistein.

To test whether tyrosine kinase(s) are involved in NPY effect on $K_{(Ca)}$ channels, NPY was applied in the presence of genistein. Figure 54 is an example of this experiment with outside-out recording. Following a 5 min stable control recording of single channel activity, genistein 10 μM was applied to the perfusion solution. Single channel open probability of $K_{(Ca)}$ was substantially increased within 1 - 2 mins of genistein application (Fig. 54A). At a test potential of +30 mV, single channel open probability was increased by 118 ± 40% (n=5; $p<0.05$) in the presence of 10 μM genistein. After the effect of genistein on single channel activity had stabilized for 5 mins, NPY 200 nM was applied on top of genistein. In this case, NPY (200 nM) failed to inhibit $K_{(Ca)}$ channel activities (Fig. 54B). In four cells studied, single channel open

Figure 51. Effect of Lavendustin A on Whole-cell $K_{(Ca)}$ Currents.

Panel A. Representative traces showing the effect of tyrosine kinase inhibitor lavendustin A 10 μM on whole-cell $K_{(Ca)}$ currents. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from -40 to +60 mV. Lavendustin 10 μM reversibly potentiated the whole-cell $K_{(Ca)}$ currents.

Panel B. I-V curves for control (○) and after lavendustin (10 μM) for 5 mins (●). n = 3. Holding potential = -40 mV.

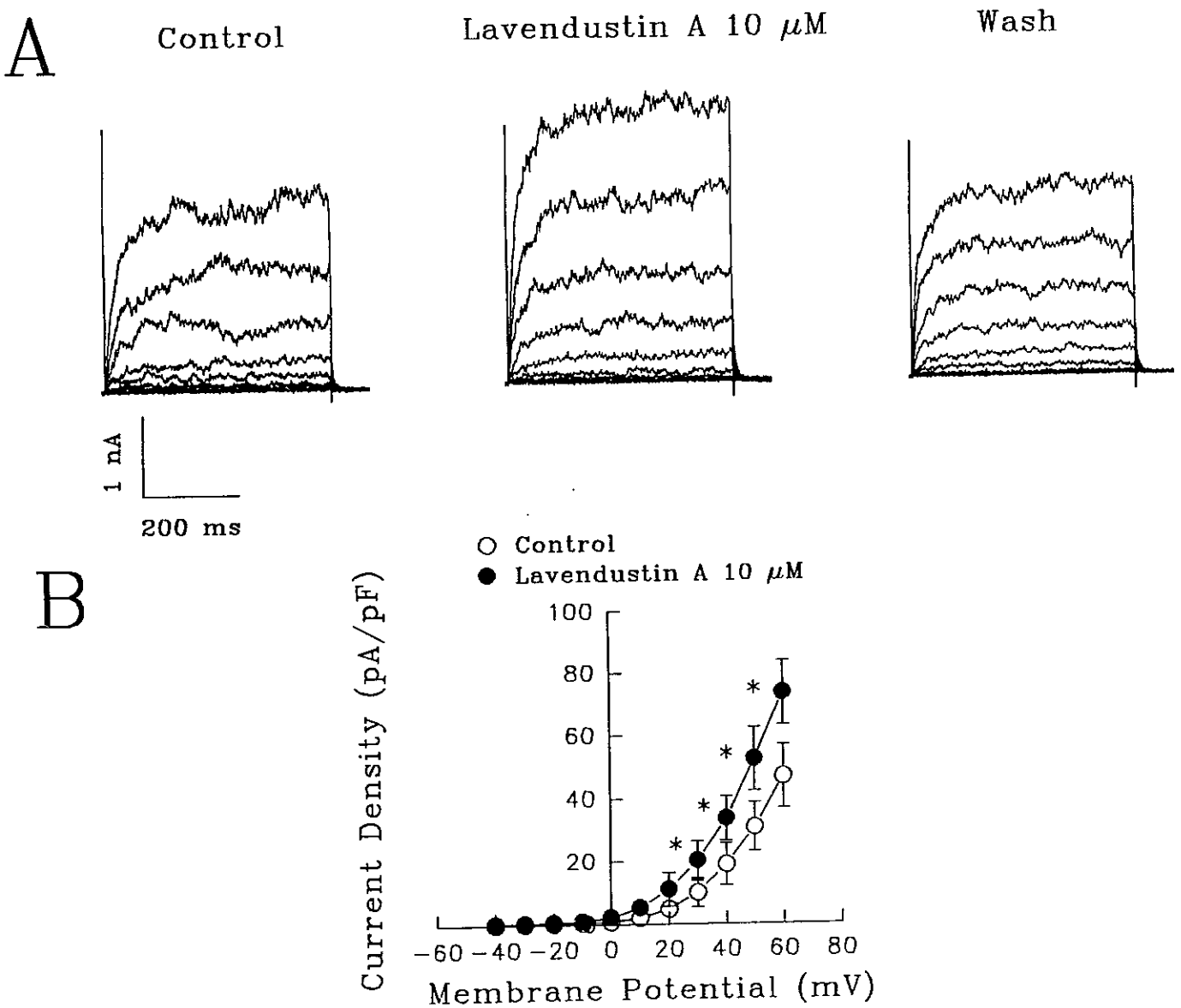

A
Control
Lavendustin A 10 μM
Wash
1 nA
200 ms
B
Control
Lavendustin A 10 μM
Current Density (pA/pF)
100
80
60
40
20
−60 −40 −20 0 20 40 60 80
Membrane Potential (mV)
*

Figure 52. Effect of Genistein on Whole-cell $K_{(Ca)}$ Currents in the Absence of ATP.

Panel A. Representative traces showing the effect of genistein 15 μM on whole-cell $K_{(Ca)}$ currents in the absence of ATP in the pipette solution. Currents were elicited by 500 ms depolarizing pulses from a holding potential of -40 mV to test potentials ranging from -40 to +60 mV. Application of genistein 15 μM for more than 10 mins had no effect on the whole-cell $K_{(Ca)}$ currents.

Panel B. I-V curves for control (○) and after genistein (15 μM) for 10 mins (●). n = 3. Holding potential = -40 mV. Pipette solution contained no ATP.

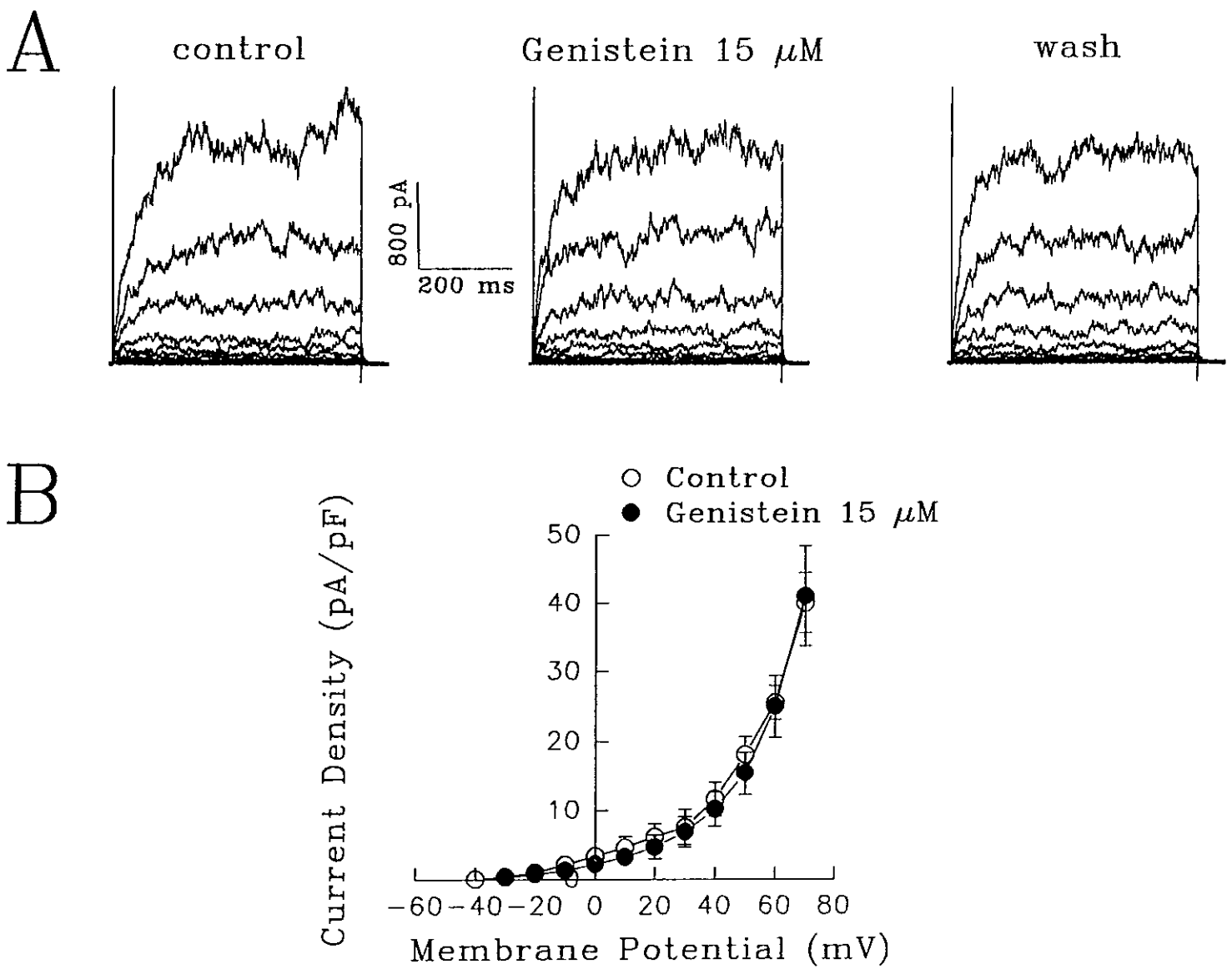
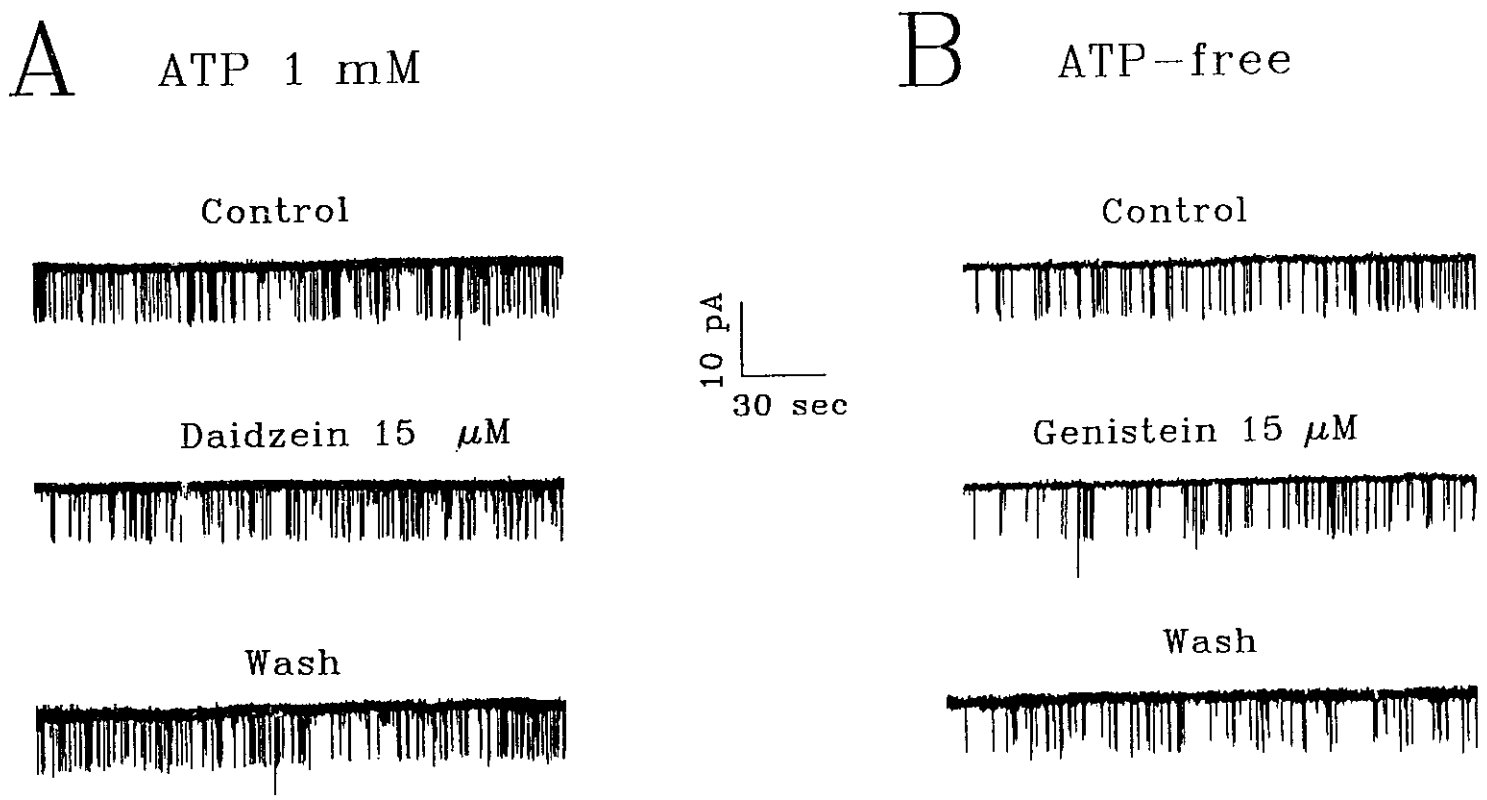

A
control
Genistein 15 μM
wash
800 pA
200 ms
B
Control
Genistein 15 μM
Current Density (pA/pF)
50
40
30
20
10
−60 −40 −20 0 20 40 60 80
Membrane Potential (mV)

Figure 53. Effect of Daidzein on Single Channel $K_{(Ca)}$ Currents.

Panel A. Representative traces showing the lack of effect of daidzein (15 μM) on single channel activities of $K_{(Ca)}$ channels in inside-out patch with ATP in the bath solution. Currents were recorded at a test potential of +30 mV. Recordings were made after 5 min incubation period.

Panel B. Representative traces showing the lack of effect of genistein (15 μM) on single channel activities of $K_{(Ca)}$ channels in inside-out patch when ATP was omitted from the bath solution. Currents were recorded at a test potential of +30 mV. Recordings were made after 5 min incubation with genistein.

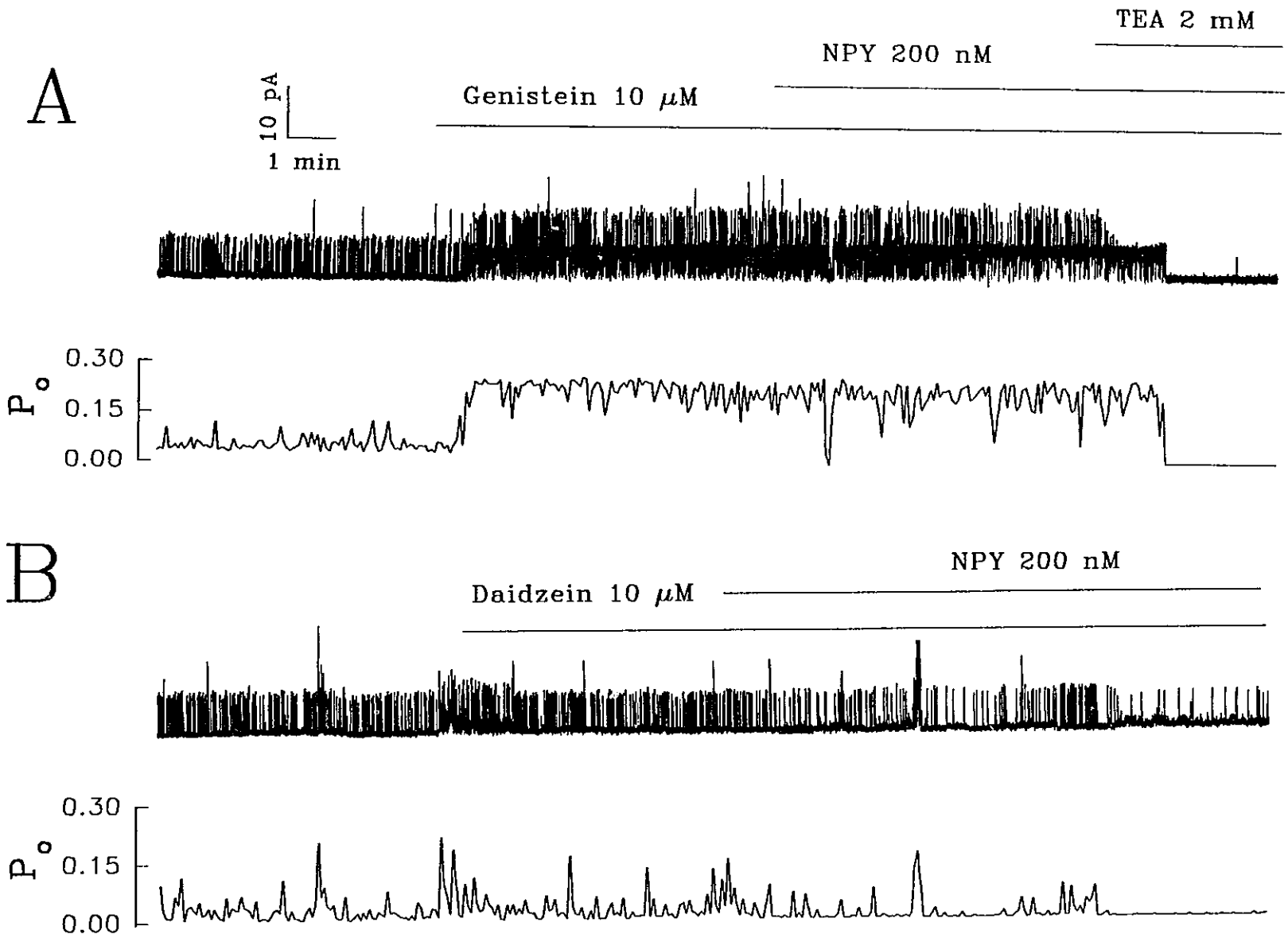

A
ATP 1 mM
Control
Daidzein 15 μM
Wash
10 pA
30 sec
B
ATP-free
Control
Genistein 15 μM
Wash

probability after NPY was 97.5 ± 2% ($p > 0.05$) of that in genistein 10 μM. Application of TEA 2 mM completely blocked the single channel activity within 1 - 2 mins (Fig. 54A). Unlike genistein, its inactive analogue daidzein (10 μM) had no significant effect on single channel activities of $K_{(Ca)}$ channels. The open probability of $K_{(Ca)}$ channel remained unchanged at 98.3 ± 3.2% (n = 3) of control in the presence of daidzein. Daidzein also did not prevent the inhibitory action of NPY on $K_{(Ca)}$ channels (Fig. 54B). In the presence of daidzein, NPY (200 nM) significantly reduced the open probability to 48.5 ± 8.6% (n = 3).

Figure 54. Effect of NPY on Single Channel $K_{(Ca)}$ in the Presence of Genistein or Daidzein.

Panel A. Top trace: single channel trace showing the effect of NPY in the presence of tyrosine kinase inhibitor genistein (10 μM). Current was recorded at a holding potential of +30 mV from an outside-out membrane patch. Bottom trace: the open probability of single channel current in panel A was calculated every 4 sec and plotted against the time.

Panel B. Top trace: single channel trace showing the effect of NPY in the presence of daidzein (10 μM). Current was recorded at a holding potential of +30 mV from an outside-out membrane patch. Bottom trace: the open probability of single channel current in panel A was calculated every 4 sec and plotted against the time.


A
TEA 2 mM
NPY 200 nM
Genistein 10 μM
10 pA
1 min
P_o
0.30
0.15
0.00
B
NPY 200 nM
Daidzein 10 μM
P_o
0.30
0.15
0.00

DISCUSSION

I. Effect of NPY on I_{Ca}

NPY potentiates contraction to various vasopressor agents and to stimulation of perivascular nerves (Cheung, 1991; Pernow et al., 1986). In the present studies, single smooth muscle cells from the rat tail artery were used. In the rat tail artery, NPY has been shown to potentiate the vasoconstriction induced by KCl, α,ß-methylene ATP, and NA (Small et al., 1992). Similar potentiating effect of NPY was also observed in isolated single smooth muscle cells (Small et al., 1992). Studies using NPY analogues showed that the potentiation of vasoconstriction by NPY in rat tail artery is mediated exclusively by postsynaptic Y1 receptor (Tschopl et al., 1993). Thus [Leu^{31},Pro^{34}]-NPY, a Y1 receptor selective peptide, produced similar potentiation of phenylephrine-induced vasoconstriction as NPY with a EC_{50} of 20-46 nM. However, NPY_{13-36}, a selective Y2 receptor agonist, was inactive at concentration as high as 3 μM (Tschopl et al., 1993). Therefore, it is likely that the effect of NPY observed in the present study is also mediated by Y1 receptor.

One important feature of the NPY effect is that it potentiates only the component of the contraction sensitive to Ca^{2+}-channel blockers (Andriantsitohaina & Stoclet, 1988; Cheung, 1991; Pernow et al., 1986; Small et al., 1992). Therefore, an action of NPY on Ca^{2+}-channels in vascular smooth muscles is implicated. In the present study, using voltage-clamp recordings, we demonstrated for the first time an enhancement of inward currents through nifedipine-sensitive Ca^{2+}-channels by NPY.

One problem with recording Ca^{2+}-channel currents with a conventional whole-cell recording technique is current run-down. This has been suggested to be due to wash-out of endogenous cytosolic components necessary to maintain Ca^{2+}-channel activity by the solution in the recording pipette (Kameyama et al., 1988; Belles et al., 1988; Romanin et al., 1991). Studies carried by Belles et al. in guinea-pig ventricular myocytes suggested that run-down of the Ca^{2+} current was caused by a rise in intracellular Ca^{2+} concentration and a loss of high energy compounds such as ATP. A loss of high buffering capacity Ca^{2+}-binding protein such as calmodulin together with an impaired capability of extrusion or sequestration of Ca^{2+} may be the reason for the rise in the Ca^{2+} concentration (Belles et al., 1988). Rise in the Ca^{2+} will activate the protease(s) and hence the enzymatic hydrolysis of proteins. Studies by Romanin et al. (1991) demonstrated that a loss of endogenous protease inhibitor and nucleotides is largely responsible for run-down of the Ca^{2+} currents in guinea-pig ventricular myocytes, therefore a combined application of both protease inhibitors (leupeptin, pepstatin A or calpastatin) and neucleotides (ATP + GTP) resulted in a significant stabilization of Ca^{2+} channel activity (Romanin et al., 1991). In the present study we employed perforated-patch recording to slow current run-down. The small channels formed by nystatin have a diameter of about 0.8 nm, making it impossible for large molecular mass compounds to leak out (Horn & Marty, 1988). With this technique, the inward current remained at the same level for at least 40 min, thereby allowing meaningful comparison of the effects of NPY with control in the same cell.

NPY affects Ca^{2+}-channels in a variety of tissues. In neurons, NPY inhibits N-

type Ca^{2+}-channels selectively and has no effect on L-type channels (Hirning et al., 1990; Wiley et al., 1990). It has been suggested by Hirning et al. that, in myenteric neurons, NPY directly modulates the N-type Ca^{2+} channels without involvement of an easily diffusible second messenger (Hirning et al., 1990). In ventricular myocytes, both stimulatory and inhibitory effect of NPY on Ca^{2+} currents were reported (Bryant et al., 1991; Millar et al., 1991). In guinea-pig ventricular myocytes, NPY in high micromolar concentrations reduces L-type Ca^{2+}-currents (Bryant et al., 1991). However, in rat ventricular myocytes, NPY at a concentration of 100 nM stimulates verapamil-sensitive slow inward current (I_{si}) (Miller et al., 1991). In the present study, NPY was found to enhance Ca^{2+}-channel currents in the same concentration range as its potentiating effect on vasoconstriction. Similar to its effect on vasoconstriction, the effects of NPY on Ca^{2+}-channel currents were inhibited by nifedipine, a specific blocker of L-type Ca^{2+}-channels. NPY was also ineffective after the inward currents were blocked by Cd^{2+}, a non-specific blocker of Ca^{2+}-channels. The currents potentiated by NPY had the characteristics of those mediated by L-type Ca^{2+}-channels. The potentiation of inward currents by NPY is achieved by both an increase in current density and a shift of the activation curve to less positive potentials so that there is a greater potentiation with weak depolarization of the cell. This correlates well with studies showing a shift in the dose-response relationship to lower concentrations of potassium required to initiate depolarization-induced contraction (Andriantsitohaina & Stoclet, 1988).

The time course of action of NPY on the Ca^{2+}-channel currents is slow. This is consistent with in vitro studies showing slow development of contraction and membrane

depolarization responses to NPY, requiring more than 5 min to reach the peak (Neild, 1987). The slow time course may indicate intermediary cellular processes after activation of the NPY receptors. The cellular mechanism of NPY is not well understood. In vascular smooth muscle, NPY is a potent inhibitor of cyclic AMP accumulation and it does not stimulate formation of inositol phosphates (Lundberg et al., 1988, Reynolds & Yokota, 1988). The vasoconstrictive effect of NPY was attenuated by pertussis toxin, suggesting regulation by GTP-binding proteins (Morris, 1991). Participation of G-proteins in NPY receptor regulation has also been implicated in neuronal (Hirning et al., 1990; Wiley et al, 1990) and cardiac cells (Bryant et al., 1991). Further experiments are required to determine if modulation of vascular Ca^{2+}-channel activity by NPY is mediated by a similar mechanism.

II. Possible Mechanism Underlying NPY Effect on Ca^{2+}-Channels.

Dihydropyridine Ca^{2+}-channel agonist Bay K 8644 has been shown to potentiate L-type Ca^{2+}-channel current in various preparations including neurons, cardiac cells, and smooth muscle cells (Fox et al., 1987; Nowycky et al., 1985b; Droogmans & Callewaert, 1986). Its effects on L-type calcium channels can be summarized as: (1) increase in current amplitude; (2) shift in both activation and inactivation curves toward more negative membrane potentials; (3) shift of I-V curve toward more negative membrane potential. (4) acceleration of time-dependent inactivation; and (5) slow-down the decay of tail current. The effects of NPY on calcium channels are similar to Bay K 8644 in: (1) increase in current amplitude; (2) shift in activation curve toward more negative

membrane potential; (3) shift of I-V curve toward more negative membrane potential. However, there are many differences between NPY and Bay K 8644 effects on Ca^{2+} channels. First, the potentiating effect of NPY on Ca^{2+} current is much smaller compared to Bay 8644. At a test potential of +10 mV, the maximum potentiation of current amplitude by NPY was only about 40%, compared to a 300% increase by Bay K 8644. The shift in activation and I-V curves by NPY was also smaller compared to that induced by Bay K 8644. A maximum of 6 mV shift in activation curve was produced by NPY, compared to a 20 mV shift in activation curve produced by Bay K 8644. Second, unlike Bay K 8644, NPY had no effect on inactivation curve and the time-dependent inactivation of Ca^{2+} current.

To further identify the possible mechanism underlying NPY effect on L-type Ca^{2+}-channels, the interactions between NPY and Bay K 8644 on Ca^{2+} channels were studied in the following conditions: (1) Bay K 8644 was applied in the presence of maximal concentration (200 nM) of NPY. (2) NPY was applied in the presence of maximal concentration (2 μM) of Bay K 8644. (3) NPY was applied in the presence of low concentration of Bay K 8644 (2 nM). One possibility is that NPY may share the same mechanism as Bay K 8644 in modulating the Ca^{2+}-channels: Bay K 8644 being a full agonist and NPY a partial agonist. If this is the case, NPY in the presence of maximal concentration of Bay K 8644 would decrease or have no further effect on Ca^{2+} currents. This was not the case as the combined effect of NPY and Bay K 8644 was much large than that of Bay K 8644 alone. A second possibility is that NPY may modulate a new type of Ca^{2+}-channels different from that modulated by Bay K 8644.

If this is the case, the effects of NPY and Bay K 8644 would be independent, and an **additive** effect between NPY and Bay K 8644 would be expected. This is also unlikely since the relationship between NPY and Bay K 8644 is not simply additive. Rather, there is a **synergistic** effect between these two agents with the combined effect greater than the sum of NPY and Bay K 8644. At a test potential of +10 mV, NPY 200 nM alone produced a 42% increase in inward current amplitude. Bay K 8644 2 μM alone produced a 300% increase in the current amplitude. When Bay K 8644 2 μM was applied on top of NPY 200 nM, a total of 409% increase in current amplitude was recorded. Similarly, when NPY 200 nM was applied on top of Bay K 8644 μM, a total of 383% increase in inward current amplitude was observed. The potentiation effect between NPY and Bay K 8644 was clearly demonstrated when subtracting the inward currents recorded after NPY by that before NPY with or without Bay K 8644. At a test potential of 0 mV, NPY 200 nM alone induced additional current density of only 0.67 pA/pF. While in the presence of 2 μM Bay K 8644, it induced additional current density of 2.1 pA/pF. These observations suggest that NPY may modulate the same population of Ca^{2+}-channels as Bay K 8644 but through a different mechanism. Similar observation had been shown for isoproterenol on Ca^{2+} channels in cardiac cells. Studies by Tiaho et al. (1990) demonstrated that in rat ventricular myocytes, isoproterenol or Bay K 8644 alone substantially increased the amplitude of Ca^{2+} current. When isoproeterenol was applied on top of maximal concentration of Bay K 8644, an additional increase in the current amplitude was observed. The effect of isoproterenol can be mimicked by forskolin or internally perfused cAMP, indicating that the effect of isoproterenol was mediated through

cAMP-dependent protein kinase pathway. It was therefore suggested that cAMP-dependent phosphorylation of serine or threonine residues on Ca^{2+} channels can modulate the effect of dihydropyridine agonist on Ca^{2+} currents (Tiaho et al., 1990).

Similar to other neurotransmitters (Trautwein & Hescheler, 1990; Tsien et al., 1988), the effect of NPY on Ca^{2+} channels may involve intermediary G-protein and diffusible second messengers. One possible candidate is adenosine 3',5' cyclic monophosphate - dependent protein kinase (cAMP-PKA) pathway. This second messenger pathway has been shown to be involved in the modulation of L-type Ca^{2+}-channels in many different preparations including vascular smooth muscle cells. A down-regulation of L-type Ca^{2+}-channels in vascular smooth muscle cells through activation of cAMP-PKA pathway has been reported by several investigators (Droogmans et al., 1987; Bkaily et al., 1988; Xiong et al., 1994a), although an upregulation has also been reported recently (Marks et al., 1990; Fukumitsu et al., 1990). The inhibition of Ca^{2+}-channel currents by cAMP-PKA has been used to explain the vasodilating effect of ß-adrenergic stimulation by isoproterenol (Sperelakis & Ohya, 1991). Since stimulation of cAMP-PKA pathway causes a down-regulation of Ca^{2+} channels, one can expect that a decrease in intracellular cAMP concentration would cause an upregulation of the channels. NPY has been shown to inhibit cAMP accumulation in many cell types including vascular smooth muscles (Reynolds & Yokota, 1988; Mihara et al., 1989). This inhibition of cAMP-accumulation may possibly interpret its potentiating effect on L-type Ca^{2+}-channels. However, it is important to note that NPY only inhibits agonist (such as isoproterenol, forskolin) stimulated cAMP accumulation and has no effect on the

basal level of cAMP concentration (Zhu et al., 1992; Lundberg et al., 1988; Mihara et al., 1989). This inhibition on cAMP accumulation is not consistent with its potentiation effect on Ca^{2+}-channels which was observed in the present study in the absence of any agonist.

Guanosine 3',5'cyclic monophosphate - dependent protein kinase (cGMP-PKG) pathway has also been shown to be involved in the modulation Ca^{2+}-channels in cardiac cells and vascular smooth muscle cells (Levi et al., 1994; Blatter & Wier, 1994). In vascular smooth muscle cells, increase of cGMP concentration and activation of PKG has been reported to decrease L-type Ca^{2+}-channel current (Blatter & Wier, 1994; Bkaily et al., 1988; Sperelakis & Ohya, 1990). Effect of NPY on cGMP content has been studied by Lobaugh and Blackshear (1990a) in vascular smooth muscle cells. No detectable change in cellular cGMP content by NPY was observed. Therefore, it is not likely that NPY effect on Ca^{2+}-channels is through a change of intracellular cGMP concentration.

Similar to cAMP-PKA and cGMP-PKG pathways, phosphorylation through protein kinase C (PKC) pathway has been shown to modulate L-type Ca^{2+}-channel activities in vascular smooth muscle cells. It was generally believed that stimulation of PKC leads to a potentiation of Ca^{2+} currents (Sperelakis & Ohya, 1991). PKC pathway has been shown to be involved in the modulation of L-type of Ca^{2+}-channels in vascular smooth muscle cells from the rat portal veins by noradrenaline (Mironneau, 1991). Although NPY stimulates protein kinase C in murine macrophages (De la Fuente et al., 1993), it does not stimulate this enzyme in vascular smooth muscle (Lobaugh & Blackshear, 1990).

Therefore it is also unlikely that PKC pathway is involved in the NPY effect on L-type Ca^{2+}-channels.

Recently, signal transduction through protein tyrosine kinase (PTK) pathway has brought much attention. In addition to its important role in regulating cell growth, proliferation and differentiation (Ullrich & Schlessinger, 1990), protein tyrosine phosphorylation has been shown to participate in the regulation of many types of ion channels including L-type Ca^{2+}-channel in vascular smooth muscle cells (Wijetunge et al., 1992). Our study has shown that NPY effect on $K_{(Ca)}$ channel is mediated by this pathway (see below). It may be possible that protein tyrosine phosphorylation is also involved in the effect of NPY on Ca^{2+}-channels.

III. Effect of NPY on $K_{(Ca)}$.

In vascular smooth muscle cells, Ca^{2+}-activated K^+ channels have a very important role in regulating excitability. They have been shown to regulate myogenic tone in pressurized cerebral arteries (Brayden & Nelson, 1992). Charybdotoxin induced vasoconstriction in these vessels by depolarization of the membrane. It was proposed that activation of the Ca^{2+}-activated K^+-channels by Ca^{2+} influx through voltage-dependent Ca^{2+} channels may serve as a negative feedback pathway to regulate membrane depolarization and thereby Ca^{2+} entry and vasoconstriction (Brayden & Nelson, 1992).

Recent studies indicate that there is a close interaction between Ca^{2+} and Ca^{2+}-activated K^+ channels. These channels may be colocalized so that the latter can be activated by local increase in Ca^{2+} (Gola & Crest, 1993). The Ca^{2+}-activated K+

channels would then in turn regulate the duration of the action potential and therefore the amount of Ca^{2+} entering the cell. Clearly one efficient means of modulating cell excitability would be to potentiate the currents of one of the channels and inhibit the other. In chromaffine cells, opioid peptides inhibit secretion by potentiating Ca^{2+}-activated K^+ currents and inhibiting Ca^{2+} Currents (Twitchell & Rane, 1993). A similar mechanism may be operative with somatostatin on GH_4C_1 cells (White et al., 1991).

At a similar concentration range that potentiated Ca^{2+} channel currents, we found that NPY reduced whole-cell Ca^{2+}-activated K^+-channel currents without affecting the delayed rectifier K^+-channels. Single channel recordings showed that the inhibitory effect of NPY was the result of a decrease in channel open probability due to increases in channel closed times. Relative to TEA and charybdotoxin, NPY is a rather weak inhibitor of Ca^{2+}-activated K^+ channels. It is also a weak potentiator of Ca^{2+} channel currents, compared to Bay K 8644. However, NPY may be very effective in potentiating vasoconstriction by its synergistic effects on these two types of ion channels. Inhibition of the Ca^{2+}-activated K^+ channels, coupled to potentiation of the Ca^{2+} current, would promote membrane depolarization and/or prolong the duration of the action potential, thereby further increasing the influx of Ca^{2+}.

Under physiological conditions, NPY is co-released with ATP and noradrenaline from perivascular sympathetic nerves. NPY by itself has no or only a very weak constrictive effect on most peripheral blood vessels. However, it potentiates significantly the contraction to ATP and the calcium antagonist-sensitive component of the

noradrenaline response (Cheung, 1991; Lundberg et al., 1989). The purinergic component of the neural response is mediated by the excitatory junction potential and an action potential is triggered when the depolarization reaches a certain threshold (Cheung, 1982). A fast phasic contraction is elicited by the action potential (Cheung, 1984). The action potential and the resulting contraction are potentiated by Bay K 8644 and inhibited by nifedipine (Cheung & MacKay, 1986). NPY is most efficient in potentiating this purinergic component of the neurally-mediated vasoconstriction (Cheung, 1991). This could by achieved by enhancing Ca^{2+} entry during the action potential and prolonging the duration of the action potential through its synergistic action on Ca^{2+} and Ca^{2+}-activated K^+ Channels.

IV. Possible Cellular Mechanisms Underlying NPY Effect on $K_{(Ca)}$.

(i). Involvement of Phosphorylation Process.

It is well established that protein phosphorylation can significantly alter the function of ion channels (Kaczmarek & Levitan, 1987). The phosphorylation event can dramatically change the electrical properties of nerve and muscle cells (Siegelbaum, 1994). Many of the modulatory effects of neurotransmitters and hormones acting through receptors have now been shown to be mediated by protein phosphorylation process. For example, isoproterenol modulates cardiac L-type Ca^{2+} channels and smooth muscle $K_{(Ca)}$ channels by protein phosphorylation through protein kinase A pathway (Sperelakis & Ohya, 1991; Kume et al., 1989; Sadoshima, 1988); Noradrenaline modulates vascular smooth muscle cell Ca^{2+} channels by protein phosphorylation through protein kinase C

pathway (Mironneau, 1991); It has also been suggested that atrial natriuretic factor (ANF) modulates $K_{(Ca)}$ channels in vascular smooth muscle cell by protein phosphorylation through cGMP-dependent protein kinase pathway (Kubo et al., 1994).

Although it was generally assumed that the kinase reaction alone was the key regulatory process for determining the level of phosphorylated proteins and that the dephosphorylation reaction was an unregulated event, recent observations have suggested a regulatory role for the protein phosphatase in the determination of overall level of phosphorylated proteins and hence the activities of the ion channels (Lau & Baylink, 1993). In rat pituitary tumour cells, somatostatin has been shown to stimulate $K_{(Ca)}$ channels through a dephosphorylation of a cAMP-dependent phosphorylation site on the channel protein or a closely associated regulatory molecule (White et al., 1991). Similarly, natriuretic peptide was found to stimulate $K_{(Ca)}$ channels in the same cell through a cGMP-dependent dephosphorylation process (White et al., 1993).

To know whether the protein phosphorylation or dephosphorylation process is involved in the effect of NPY on $K_{(Ca)}$ channels, we first removed the ATP from the pipette solution. Stable $K_{(Ca)}$ currents can be recorded in the absence of ATP in both whole-cell and outside-out recordings, indicating that ATP is not required for the basal activity of $K_{(Ca)}$ channels. However, the inhibitory effect of NPY on both whole-cell and single channel $K_{(Ca)}$ currents was abolished in the absence of ATP. This result indicates that the effect of NPY on $K_{(Ca)}$ channels is ATP-dependent. To further identify whether ATP is required as a ligand or as a phosphate donor in the phosphorylation process, we studied the NPY effect on $K_{(Ca)}$ channels with ATP replaced by its non-

hydrolysable analogue ß, γ-methylene adenosine triphosphate (AMP-PCP) in the pipette solution. This compound has been shown to be an effective ligand for ATP receptor but it cannot be hydrolysed to serve as a phosphate donor (Bielefeldt & Jackson, 1994). As in the ATP-free condition, NPY had no effect on $K_{(Ca)}$ channels in the presence of AMP-PCP. These experiments demonstrate that ATP hydrolysis is required for NPY effect indicating that protein phosphorylation or dephosphorylation process is involved in the effect of NPY on $K_{(Ca)}$ channels. To further identify whether NPY effect was mediated through a change in kinase(s) or phosphatase(s) activity, ATP-γ-S was used as replacement of ATP in the pipette solution. It has been documented that thiophosphorylated residues in protein are poor substrates for phosphatases (Esguerra et al., 1994). If the NPY effect was due to a change in phosphatase activity, it would be expected that with ATP-γ-S as a phosphate donor, NPY would have no effect on $K_{(Ca)}$ channels. However, if the NPY effect was due to a change in kinase activity, replacing ATP with ATP-γ-S would not alter the NPY effect. In the present study, a similar inhibitory effect of NPY on $K_{(Ca)}$ channels was observed when ATP was replaced by equimolar ATP-γ-S and the inhibition was maintained even when NPY was washed out. This result indicates that the effect of NPY on $K_{(Ca)}$ channels was mediated through a change in protein kinase(s) activity.

(ii). Second Messenger Involvement of NPY Effect.

Agonists can act directly on the receptor-channel complex or indirectly through diffusible, intracellular second messengers (Hille, 1984). NPY was able to decrease the

$K_{(Ca)}$ channel activity in cell-attached patches when applied in the bath solution. Since the gigaohm seal between a patch pipette and a membrane forms a lateral diffusion barrier (Hamill et al., 1981), it is not likely that NPY can diffuse to the interior of the patch pipette and affect the channel directly from the outside of the cell membrane. Rather, it is likely that a diffusible second messenger was involved in NPY effect on $K_{(Ca)}$ channels. This is in contrast to the inhibitory effect of NPY on N-type Ca^{2+} channels in neurons (Hirning et al., 1990). In cultured mesenteric neurons from neonatal Sprague-Dawley rats, NPY was shown to inhibit N-type Ca^{2+}-channels in whole-cell recordings. However, it had no effect on the single channel current in cell-attached recording when applied in the bath solution (Hirning et al., 1990). These results may suggest that different mechanisms are involved in the inhibitory effect of NPY on $K_{(Ca)}$ channels in vascular smooth muscle cells and N-type Ca^{2+} channels in neurons.

Ca^{2+} is an important intracellular second messenger which mediates many cellular responses (Hille, 1984). The activities of $K_{(Ca)}$ channels also depend on free Ca^{2+} concentration. Alteration of intracellular Ca^{2+} concentration can change both the open probability and the threshold of the activation of $K_{(Ca)}$ channels (Latorre & Miller., 1983; Hille, 1984). An increase of Ca^{2+} concentration causes an increase in open probability and a shift in the threshold of activation toward less positive membrane potential. On the other hand, a decrease in Ca^{2+} causes a decrease in the open probability and a shift in the threshold of activation toward more positive membrane potential (Bolzon et al., 1993). NPY decreases the single channel open probability of $K_{(Ca)}$ and shifts the threshold of activation toward more positive potential. These effects

could be explained by a decrease in intracellular Ca^{2+} concentration. However, in both whole-cell and outside-out recordings, intracellular Ca^{2+} was buffered using 5 mM EGTA and the Ca^{2+} entry through L-type channels was blocked by nifedipine (1 μM). Moreover, a similar effect of NPY on $K_{(Ca)}$ was recorded when 10 mM BAPTA was used in the pipette solution. Therefore it is unlikely that the effect of NPY on $K_{(Ca)}$ was caused by a change in the intracellular Ca^{2+} concentration.

Adenosine 3',5'-cyclic monophosphate (cAMP) is another important second messenger. Several ion channels including Ca^{2+}, K^+, and Cl^- channels have been shown to be modulated by cAMP-dependent phosphorylation (Ewald et al., 1985; Hartzell et al., 1991). Activation of cAMP-dependent protein kinase has been shown to upregulate $K_{(Ca)}$ channel activity in canine colon smooth muscle (Carl et al., 1991), tracheal myocytes (Kume et al., 1989), and vascular smooth muscle cells (Sadoshima et al., 1988). This upregulation of $K_{(Ca)}$ channel activity by cAMP-dependent protein kinase can partially explain the vasorelaxation effect of ß-receptor agonists which increase cellular cAMP production (Sadoshima et al., 1988). Since activation of cAMP-PKA pathway upregulates the $K_{(Ca)}$ channel activity, one would expect that a decrease in intracellular cAMP concentration would have an opposite effect: down-regulation of $K_{(Ca)}$ channel activity. NPY has been shown to inhibit adenylyl cyclase activity and decrease intracellular cAMP accumulation in vascular smooth muscle cells (Reynolds & Yokota, 1988; Mihara et al., 1989). It is therefore interesting to know whether this inhibitory effect on cAMP accumulation is responsible for the NPY effect on $K_{(Ca)}$ channels. To test this possibility, we have used the specific peptide inhibitor of cAMP-

dependent protein kinase (PKI_{5-24}) in the present studies. PKI has been shown to block cAMP-dependent protein kinase with an inhibition constant of 0.3 nM (Cheng et al., 1986). At concentrations of 10 nM or 100 nM, it has been shown to be effective in blocking cAMP-PKA mediated responses (Bielefeldt & Jackson, 1994; Sculptoreanu et al., 1993). In the present study, we used 1 μM PKI in the pipette solution. This concentration of PKI should be high enough to completely block cAMP-PKA mediated responses. With 1 μM PKI in the pipette solution, NPY's ability to inhibit $K_{(Ca)}$ channel activity was not affected. These results suggest that the effect of NPY on $K_{(Ca)}$ channels was not mediated through cAMP-dependent protein kinase pathway.

cGMP-PKG pathway has also been shown to be involved in the modulation of $K_{(Ca)}$ channels in vascular smooth muscle cells. In aortic smooth muscle cells, nitroglycerin, cGMP and its primary metabolite cGMP have been shown to increase the open probability of $K_{(Ca)}$-channels (Williams et al., 1988; Fujino et al., 1991; Kubo et al., 1994). Modulation of $K_{(Ca)}$ channels through cGMP pathway can partially explain the vasodilation effect of nitrovasodilators and atrial natriuretic factor (ANF) (Kubo et al., 1994). However, this pathway is not likely involved in NPY effect on $K_{(Ca)}$ channels since NPY had no detectable effect on cGMP content in vascular smooth muscle cells (Lobaugh & Blackshear, 1990).

Recent study by Minami et al. (1993) has shown that PKC pathway is also involved in the modulation of $K_{(Ca)}$ channels in vascular smooth muscle cells. In cultured porcine coronary artery smooth muscle cells, bath application of phorbol 12-myristate 13-acetate (PMA) or 1-oleoyl-2-acetylglycerol (OAG), the C-kinase activators,

significantly decrease the open probability of $K_{(Ca)}$ channel in cell-attached patches. This effect can be reversed by subsequent application of staurosporine, a C-kinase inhibitor. In inside-out patches, bath application of the C-kinase fraction in the presence of ATP and PMA markedly inhibited the $K_{(Ca)}$ activity. These results indicate that activation of PKC down-regulates the $K_{(Ca)}$ channel and may cause membrane depolarization and vasoconstrition (Minami et al., 1993). Since NPY did not affect the PKC activity in vascular smooth muscle cells (Lobaugh & Blackshear, 1990), such mechanism is not likely involved in the NPY effect on $K_{(Ca)}$ channels in the present studies.

iii. Modulation of $K_{(Ca)}$ by Tyrosine Kinase Inhibitor.

Although regulation of ion channels by phosphrylation of serine or threonine residues is a well recognized process (Carl et al., 1991; Chung et al., 1991), the role of tyrosine phosphorylation in the regulation of ion channels has remained largely unexplored until recently. Huang et al. (1993) co-expressed a cloned delayed-rectifier K^+ (RAK) channel from neuron with m1 muscarinic acetylcholine receptor in Xenopus oocytes. They found that stimulation of the m1 receptor with carbachol reduced the amplitude of RAK current by 90%. This effect of carbachol depends on both the mobilization of internal Ca^{2+} and the stimulation of PKC. Surprisingly, the effects of Ca^{2+} and PKC appear to be mediated, at least in part, by activation of tyrosine kinase and direct tyrosine phosphorylation of the RAK channel. Thus, the tyrosine kinase inhibitor genistein largely blocked the inhibitory actions of carbachol on the K^+ channel.

In Aplysia bag cell neurons, Wilson et al. (1993) demonstrated that tyrosine

phosphorylation rapidly modified a voltage-gated cation channel. Application of T-cell protein tyrosine phosphatase (PTPs) caused the cation channel to switch from a bursting mode to a continuously active mode. Moveover, the endogenous PTPs can be regulated by protein kinase A. Phosphorylation of endogenous PTPs on serine or threonine residues by cAMP-dependent protein kinase activated PTPs and subsequently modulates the channel activity.

Modulations of ion channels in vascular smooth muscle cells by tyrosine kinase have also been demonstrated recently. Wijetunge et al. (1992) demonstrated that tyrosine kinase is possibly involved in the regulation of Ca^{2+} channels. Using whole-cell voltage-clamp, calcium channel currents in single smooth muscle cells from rabbit ear artery were recorded. Tyrosine kinase inhibitors tyrphostin 23 and genistein produced a dose-dependent inhibition of Ca^{2+} channel current amplitude without obvious changes in activation and inactivation. Daidzein, an inactive analogue of genistein, had little effect on this current, consistent with an action of these agents at a tyrosine kinase(s).

Studies by Minami et al. (1994) have shown that a non-selective cation channel in vascular smooth muscle cells is also regulated by tyrosine kinase. In primary cultured porcine coronary smooth muscle cells, genistein activated a non-selective channel in cell-attached recording, while its inactive form daidzein had no effect. This channel discriminated poorly between Na^+ and K^+, and also transported Ca^{2+}. Staurosporine, a non-specific protein kinase inhibitor, also activated this channel. While H-7, an inhibitor of protein serine/threonine kinase had no effect. These results suggest that the activity of the non-selective cation channel is negatively regulated by tyrosine kinase

activity (Minami et al., 1994).

In the present study, we have studied the effects of tyrosine kinase inhibitors genistein, tyrphostin A25 and lavendustin A on $K_{(Ca)}$ currents in single vascular smooth muscle cells from the rat tail artery. Our results show that both the whole-cell current amplitude and the single channel open probability of $K_{(Ca)}$ channels were substantially potentiated in the presence of these inhibitors, suggesting that tyrosine kinase(s) are involved in the modulation of $K_{(Ca)}$ in vascular smooth muscle cells.

Genistein and tyrphostin have been reported to inhibit several tyrosine kinases including EGF receptor, v-src (Akiyama et al., 1987) and v-abl (Geissler et al., 1990). At a concentration as high as 100 μM, these agents were reported to be selective inhibitors of tyrosine kinases and did not affect the activity of other kinases such as cAMP-dependent protein kinase (PKA) or protein kinase C (PKC) (Akiyama et al., 1987; Yaish et al., 1988).

One alternative possibility is that these agents modulate the channel activity through a mechanism other than inhibition of tyrosine kinase, such as a direct inhibitory effect on $K_{(Ca)}$ channels. This possibity seems unlikely since the potentiation effect was abolished by removal of ATP. Moreover, daidzein, an inactive analogue of genistein, had no effect on $K_{(Ca)}$-channels.

Genistein and Lavendustein A had a similar potentiation effect on $K_{(Ca)}$ channels in both whole-cell and inside-out recordings, indicating that these two agents can easily pass through cell membrane. In contrast, tyrphostin A25 was effective only in inside-out patches and had little effect on $K_{(Ca)}$ channels with whole-cell recording. This may

suggest that tyrphostin A25 has difficulty to pass through the cell membrane. Similar problem has been reported by Lee et al. (1993) which showed that a prolonged incubation time (16 hours) with tyrphostin was required to inhibit protein tyrosine phosphorylation in intact human foreskin fibroblast cells.

iv. Involvement of Tyrosine Kinase Pathway in NPY Effect on $K_{(Ca)}$ Channels.

Other vasoconstrictive neuropeptides such as angiotension II and endothelin have also been shown to inhibit $K_{(Ca)}$ channel activity in vascular smooth muscle cells (Toro et al., 1990; Hu et al., 1991). Study by Toro et al. demonstrated that angiotension II, with a $k_{1/2}$ of 58 nM, inhibited coronary $K_{(Ca)}$ channels incorporated into lipid bilayer. Since the effect was observed in the absence of GTP, Mg^{2+} or ATP, a direct effect of angiotension II on the channel was suggested (Toro et al, 1990). Hu et al. reported that endothelin, at a concentration of 10 - 100 nM, inhibited $K_{(Ca)}$ channels in porcine coronary smooth muscle cells. In this case, a change in the intracellular Ca^{2+} concentration was proposed for the effect of endothelin on the channel (Hu et al., 1991). In the present studies, we demonstrated that NPY, with a similar concentration range as angiotension II and endothelin, inhibited the $K_{(Ca)}$ channel activity in vascular smooth muscle cells from the rat tail artery. However, unlike angiotension II and endothelin, a protein tyrosine phosphorylation process was involved in the effect of NPY on $K_{(Ca)}$ channels.

A tyrosine kinase pathway was probably involved in NPY's effect on $K_{(Ca)}$ channels as the action of NPY was blocked by pre-application of genistein but not

affected by its inactive form daidzein.

Two types of PTKs have been documented: receptor PTKs and non-receptor PTKs. Receptor PTKs include receptors for many growth factors such as EGF, PDGF, CSF and insulin (Ullrich & Schlessinger, 1990). These receptors contain an extracellular ligand binding domain, a membrane spanning domain and a cytoplasmic tyrosine kinase catalytic domain (Ullrich & Schlessinger, 1990). Binding of growth factors to their receptors activates PTK through a conformation change in the receptor. Activation of PTK first causes autophosphorylation of the receptor and subsequently phosphorylates other cellular proteins. In contrast to these receptor PTKs, so called non-receptor PTKs can function at various location within the cell. For example, members of the src family of PTKs can be associated with the inner face of the plasma membrane by a process dependent on specific amino-terminal amino acids as well as a myristic acid residue (Resh, 1990; Shattil & Brugge, 1991). Here, they are strategically placed to relay signals from transmembrane receptors that have no intrinsic kinase activity to cytoplasmic or cytoskeletal proteins that participate in downstream signalling events. Which type of PTKs is possibly mediating NPY effects? Amino acid analysis and hydrophobicity profiles of NPY receptors have shown that NPY receptors contain seven putative hydrophobic transmembrane spanning regions characteristic of receptors coupled to G-protein. No cytoplasmic tyrosine kinase domain was found in NPY receptors (Rimland et al., 1991; Larhammar et al., 1992). This may suggest that NPY effect on $K_{(Ca)}$ is mediated via a non-receptor PTK (for example $pp60^{src}$) which could be coupled, either directly or indirectly, to an NPY receptor.

Tyrosine kinase has been reported to be involved in the smooth muscle contraction induced by neurotransmitters, vasoconstrictive peptides or growth factors. Studies by Di Salvo et al. (1993) demonstrated that contraction of canine carotid artery smooth muscle induced by norepinephrine, phenylephrine or carbachol were markdly (>80%) inhibited by tyrosine kinase inhibitors tyrphostin, genistein and geldanomycin. In contrast, only slight inhibition (20%) occurred in contraction elicited by high K^+ (100 mM)-induced depolarization. Moreover, in ß-escin permeabilized vascular preparations, contraction induced by a direct Ca^{2+}-mediated activation of the contractile apparatus was not affected by tyrosine kinase inhibitor. It is suggested that tyrosine kinase activity is more important for pharmacomechanical coupling (receptor-mediated contraction) than for either electromechanical coupling (depolarization-mediated contraction) or direct activation of the contractile apparatus with Ca^{2+}. In the porcine coronary artery, a tyrosine kinase pathway also appears to be involved in the contractile actions of vasopressin and $PGF_{2\alpha}$ (Laniyonu et al., 1992). In guinea-pig gastric smooth muscle, agiotension II induced contraction of longitudinal muscle strips was completely blocked by genistein and tyrphostin (Yang et al., 1993). Similarly, contraction of gastric smooth muscle by epidemal growth factor-urogastrone, transforming growth factor-a were blocked by tyrosine kinase inhibitors (Yang et al., 1992). Further experiments are required to see whether the vasoconstrictive effect of NPY can also be blocked by the tyrosine kinase inhibitors.

It is not understood how the protein tyrosine phosphorylation is involved in the smooth muscle contraction. One possible mechanism is a direct tyrosine phosphorylation

of myosine light chain (MLC). It has been shown that upon activation of tyrosine kinase, the p20 protein of MLC, which is not phosphorylated in the resting state, becomes heavily phosphorylated (Rendu et al., 1992). Since serine-threonine phosphorylation of MLC in smooth muscle initiates contraction and dephosphorylation of MLC is associated with smooth muscle relaxation (Kamm and Stull, 1985), it was postulated that PTK induced MLC phosphorylation may contribute additionally to contraction (Sauro & Thomas, 1993). In the present studies, we demonstrated that tyrosine phosphorylation is involved in the modulation of $K_{(Ca)}$ channel activity in single vascular smooth muscle cells. Potentiation of $K_{(Ca)}$ channel activity by tyrosine kinase inhibitors suggests that phosphorylation through tyrosine kinase down-regulates the channel activity. This effect will prolong the depolarization of the cell membrane and hence increase ca^{2+} entry through the Ca^{2+} channels. Increase in Ca^{2+} concentration will activate MLC-kinase which in turn catalyzes the serine and threonine phosphorylation of MLC and hence promote the muscle contraction.

Direct biochemical studies have demonstrated that several neurotransmitters or vasoconstrictive peptides stimulate tyrosine phosphorylation of cellular proteins in vascular smooth muscle cells. Studies by Tsuda et al. (1991) showed that, in cultured rat aortic smooth muscle cells, angiotension II induced tyrosine phosphorylation of at least 9 proteins with molecular masses of 190, 117, 105, 82, 77, 73, 45, and 40 kDa in a time- and dose-dependent manners. Vasopressin, norepinephrine and 5-hydroxytryptamine induced the tyrosine phosphorylation of the same set of proteins as angiotension II. Further experiments are required to know whether NPY promotes

tyrosine phosphorylation of cellular proteins in vascular smooth muscle cells.

It is not clear how tyrosine kinase is involved in NPY effect on $K_{(Ca)}$ channels. One possibility is that binding of NPY to its receptor activates a non-receptor tyrosine kinase which is associated with plasma membrane. Activation of tyrosine kinase will alter the production of a unknown messenger which in turn modulates the channel activity. In this case, tyrosine phosphorylation is only an intermediary step and a direct tyrosine phosphorylation on channel or closely associated protein is not involved. The other possibility is that NPY first alters the production of a second messenger and in turn activates a non-receptor tyrosine kinase which is associated with $K_{(Ca)}$ channel or closely associated protein. In this case, a direct tyrosine phosphorylation on the channel protein is involved, as has been shown for carbachol induced inhibition of a cloned delayed rectifier K^+ channel (Huang et al., 1993).

v. G-Protein Involvement.

Activation of receptor-linked guanosine nucleotide-binding (G) proteins is a key event in the modulation of cellular activity by neurohumoral agents (Gilman, 1987). For example, the enhancement of calcium current that underlies ß-adrenergic stimulation of cardiac pacemaker and contractile activity requires the coupling of surface ß-adenoreceptors to the adenylate cyclase cascade by receptor-associated stimulatory G protein G_s (Gilman, 1987; Rodbell, 1980). Recently, it is proposed that G protein, in addition to being an intermediate messenger in signal transduction through cytoplasmic second messengers (for example cAMP), may also function as a membrane-delimited,

direct ion channel regulator (Pelzer et al., 1990)). It has been shown that isoproterenol can modulate cardiac Ca^{2+} channels through both direct, membrane-delimited and indirect, protein phosphorylation pathways (Pelzer et al., 1990). Similarly, a membrane-delimited G-protein pathway has also been shown to be involved in the effect of acetylcholine on inwardly rectifying K^+-channel in atrial cells (Brown & Birnbaumer, 1990), 5-HT modulation of a neuronal K^+ channel (Van Dongen et al., 1988), and somatostatin activation of inwardly rectifying K^+ channel in GH_3 anterior pituitary cells (Yamashita et al., 1987). NPY receptors belong to G-protein coupled superfamily. The vasoconstrictive effect of NPY was attenuated by pertussis toxin, suggesting regulation by GTP-binding proteins (Morris, 1991). Participation of G-proteins in NPY receptor regulation has also been implicated in neuronal (Hirning et al., 1990; Wiley et al, 1990) and cardiac cells (Bryant et al., 1991). It is not clear whether G-protein is also involved in the modulation of $K_{(Ca)}$ channel in the present study. Although the effect of NPY was observed without adding GTP in the intracellular solution, the possibility that some endogenous GTP may be trapped in the membrane cannot be excluded, as has been suggested by Scornik & Toro (1992). The effect of NPY on $K_{(Ca)}$ channels can be completely abolished by blocking phosphorylation pathways, therefore it is not likely that a direct channel regulation by G-protein is involved.

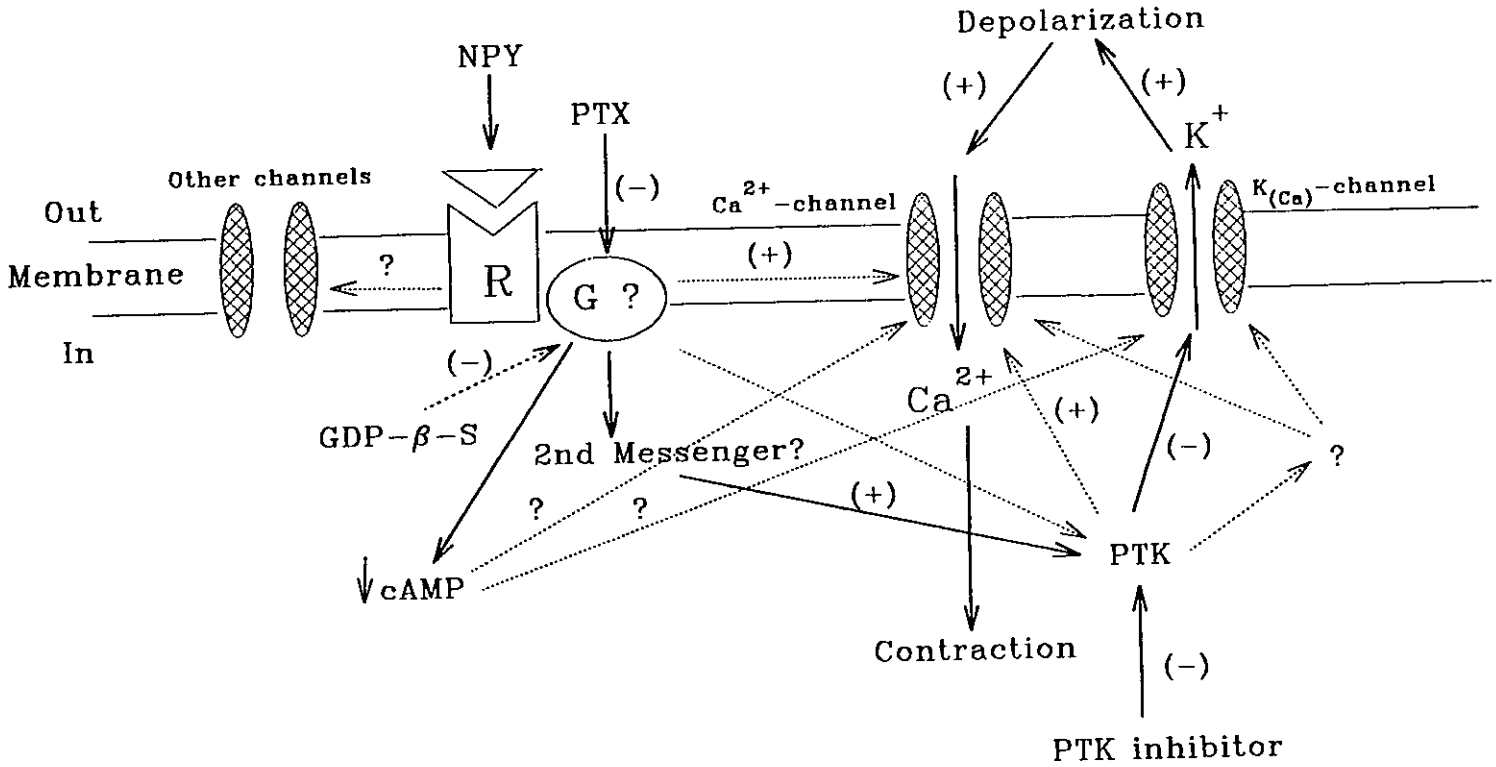
CONCLUSION

The present studies demonstrated for the first time an enhancement of Ca^{2+}-channel current in vascular smooth muscle cells by NPY at physiological concentrations. The potentiation was due to an increase in current amplitude and a shift in threshold of activation. The mechanism underlying NPY effect on Ca^{2+}-channel is different from those of dihydropyridine agonist Bay K 8644, possibly involving protein phosphorylation. At the same concentration range, NPY inhibits $K_{(Ca)}$-channel activity. This effect of NPY involves a diffusible second messenger and phosphorylation process. We also demonstrated for the first time an involvement of tyrosine kinase pathway in the modulation of $K_{(Ca)}$-channels in vascular smooth muscle cells. Our results suggest that NPY effect on $K_{(Ca)}$-channels may be mediated by this pathway.

Possible signal transduction pathways linking NPY to Ca^{2+} and $K_{(Ca)}$-channels are illustrated in the following diagram: Stimulation of NPY receptor leads to production of an unidentified second messenger. The second messenger then activates one or more non-receptor tyrosine kinases which act on the channels. In addition to causing a direct tyrosine phosphorylation on the channels, it is possible that tyrosine kinase may regulate other kinases, which in turn act on the channels. NPY may also modulate Ca^{2+} and other (?) channels through a membrane delimited direct pathway. NPY promotes vasoconstriction by increasing influx of Ca^{2+} through Ca^{2+}-channels. Inhibition of $K_{(Ca)}$ helps to maintain the membrane potential at a depolarized state for the activation of Ca^{2+}-channels.

Figure 55. Schematic Diagram Showing the Possible Signal Transduction Pathways Involved in Vasoconstrictive Effect of NPY

Stimulation of NPY receptor leads to production of an unidentified second messenger. The second messenger then activates one or more non-receptor tyrosine kinases which act on the channels. In addition to causing a direct tyrosine phosphorylation on the channels, it is possible that tyrosine kinase may regulate other kinases, which in turn act on the channels. NPY may or may not modulate Ca^{2+}-channel through a membrane delimited direct pathway in VSM. G, G-protein; R, NPY receptor; PTK, protein tyrosine kinase; PTX, pertussis toxin; (+), upregulation; (-), down-regulation; ·····→ , possible pathway.


Depolarization
NPY
(+)
(+)
PTX
K+
Other channels
(−)
Ca2+−channel
K(Ca)−channel
Out
Membrane
In
?
R
G ?
(+)
(−)
Ca2+
(+)
GDP−β−S
2nd Messenger?
(−)
?
? ?
(+)
PTK
cAMP
Contraction
(−)
PTK inhibitor

FUTURE STUDY

1. Is Second Messenger Involved in NPY Effect on Ca^{2+}-Channels ?

To know whether a diffusible second messenger is involved in the effect of NPY on Ca^{2+}-channels, cell-attached recording will be used to record single channel current from L-type Ca^{2+}-channels. If the single channel activity in the recording pipette is potentiated by bath application of NPY, it would indicate that a diffusible second messenger is involved in NPY effect on Ca^{2+}-channels.

2. Is G-protein Involved in NPY effects on Ca^{2+}- and $K_{(Ca)}$-Channels ?

To know whether GTP-binding protein is involved in the effect of NPY on Ca^{2+}- and $K_{(Ca)}$-channels, single cells will be incubated with pertussis toxin for 4 - 8 hours and the effect of NPY on the Ca^{2+}- and $K_{(Ca)}$-channels will be studied thereafter. If the effect of NPY is blocked by this pretreatment, it would indicate an involvement of G-protein. Similarly, GTP-γ-S and GTP-ß-S will also be used to test this possibility.

3. Is the Phosphorylation Process Involved in NPY Effect on Ca^{2+}-Channels ?

The possibility that protein phosphorylation or dephosphorylation process is involved in NPY effect on Ca^{2+}-channels will be studied by using protein kinase inhibitor (such as H7, staurosporine, genistein, tyrphostin) as well as proteinphosphatase inhibitor (such as okadaic acid, vanadate).

REFERENCES

Aaronson, P.I., C.D. Benham, T.B. Bolton, P. Hess, R.J. Lang, and R.W. Tsien. (1986). Two types of single channel and whole-cell calcium and barium currents in single smooth muscle cells of rabbit ear artery and the effects of noradrenaline. *J. Physiol.* 377:36p.

Abel, P.W., and C. Han. (1989). Effects of neuropeptide Y on contraction, relaxation, and membrane potential of rabbit cerebral arteries. *J. Cardiovas. Pharmacol.* 13:52-63.

Adrian, R.H. (1969). Rectification in muscle membrane. *Prog. Biophy. Mol. Bio.* 19:340-369.

Aizawa, Y., M. Murata, M. Hayashi, T. Funazaki, S. Ito, and A. Shibata. (1985). Vasoconstrictor effect of neuropeptide Y (NPY) on canine coronary artery. *Jpn. Circ. J.* 49:584-588.

Aizawa, Y., M. Satoh, and M. Aizawa. (1987). Potency and receptors involved in coronary vasoconstriction caused by neuropeptide Y (NPY). *Jpn. Heart J.* 28:891-898.

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases.

J. Bio. Chem. 262:5592-5595.

Alexander, R.W., T.A. Brock, M.A. Gimbrone Jr., and S.E. Rittenhouse. (1985). Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension* 7:447-451.

Allen, J.M., P.M.M. Bircham, A.V. Edwards, K. Tatemoto, and S.R. Bloom. (1983). Neuropeptide Y (NPY) reduces myocardial perfusion and inhibits the force of contraction of the isolated perfused rabbit heart. *Regul. Peptides* 6:247-53.

Allen, J.M., F. Schon, J.C. Yeats, J.S. Kelly, and S.R. Bloom. (1986a). Effect of reserpine, phenoxybenzamine and cold stress on the neuropeptide Y content of the rat peripheral nervous system. *Neurosci.* 19:1251-1254.

Allen, J.M., P. Gjorstrup, J.A. Bjorkman, T. Abrahamsson, and S.R. Bloom. (1986b). Studies on cardiac distribution and function of neuropeptide Y. *Acta Physiol. Scand.* 126:405-411.

Allen, J.M., J. Rodrigo, D.J. Kerle, et al. (1990). Neuropeptide Y (NPY)-containing nerves in mammalian ureter. *Urology* 35:81-6

Allen, J.M., J. Rodrigo, J.C. Yeats, A.P. Savage AP, J.M. Polak, and S.R. Bloom.

(1984). Vascular distribution of neuropeptide Y (NPY) and effect on blood pressure. *Clin. Exp. Theory & Pract.* 6:1879-1882.

Amedee, T., W.A. Large, and Q. Wang. (1990). Characteristics of chloride currents activated by noradrenaline in rabbit ear artery cells. *J. Physiol.* 428:501-516.

Anderson, K.D. and A. Reiner. (1990). Distribution and relative abundance of neurons in the pigeon forebrain containing somatostatin, neuropeptide Y or both. *J. Comp. Neurol.* 299(3):261-282.

Andriantsitohaina, R., P. Andre, and J. C. Stoclet. (1990). Pertussis toxin abolishes the effect of neuropeptide Y on rat resistance arteriole contraction. *Am. J. Physiol.* 259:H1427-H1432.

Andriantsitohaina, R., and J. C. Stoclet. (1988). Potentiation by neuropeptide Y of vasoconstriction in rat resistance arteries. *Br. J. Pharmacol.* 95: 419-428.

Aoki, C., and V.M. Pickel. (1990). Neuropeptide Y in cortex and striatum. Ultrastructural distribution and coexistence with classical neurotransmitters and neuropeptides. *Ann. NY. Acad. Sci.* 611:186-205.

Armstrong, C.M. (1975). Potassium pores of nerve and muscle membrane. In:

Membrane: A Series of Advances, G. Eisenman (ed.), Dekker, New York. pp.325-358.

Asano, M., K. Masuzawa-Ito, T. Matsuda, Y. Suzuki, H. Oyama, M. Shibuya, and K. Sugita. (1993). Functional role of charybdotoxin-sensitive K^+ channels in the resting state of cerebral, coronary and mesenteric arteries of the dog. *J. Pharmacol. Exp. Ther.* **267**:1277-1285.

Ashcroft, F.M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Ann. Rev. Neusci.* **11**:97-118.

Balasubramanium, A., I. Grupp, M.A. Matlib, R. Benza, R.L. Jackson, J.E. Fischer, and G. Grupp. (1988). Comparison of the effects of neuropeptide Y (NPY) and 4-norleucine-NPY on isolated perfused rat hearts: effects of NPY on atrial and ventricular stripes of rat heart and on rabbit mitochondria. *Regul. Pept.* **21**:289-292.

Barrett, J.N., E.F. Barrett, and L.B. Dribin. (1981). Calcium dependent slow potassium conductance in rat skeletal myotubes. *Developmental Bio.* **82**:258-266.

Barrett, J.N., K.L. Magleby, and B.S. Pallotta. (1982). Properties of single calcium activated potassium channels in cultured rat muscle. *J. Physiol.* **331**:211-230.

Beam, K.G., and C.M. Knudson. (1988). Calcium currents in embryonic and neonatal

mammalian skeletal muscle. *J. Gen. Physiol.* 91:781-798.

Bean, P.B., (1989). Classes of calcium channels in vertebrate cells. *Ann. Rev. Physiol.* **51**:367-384.

Beech, D.J., and T.B. Bolton. (1989a). Two components of potassium current activated by depolarization of single smooth muscle cells from the rabbit portal vein. *J. Physiol.* **418**:293-309.

Beech, D.J. and T.B. Bolton. (1989b). A voltage-dependent outward current with fast kinetics in single smooth muscle cells isolated from rabbit portal vein. *J. Physiol.* **412**:397-414.

Beech, D.J. and T.B. Bolton. (1989c). Properties of cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *J. Physiol.* **98**:851-864.

Belles, B., C.O. Malecot, J. Hescheler, and W. Trautwein. (1988). "Run-down" of the Ca current during long whole-cell recordings in guinea pig heart cells: role of phosphorylation and intracellular calcium. *Pflugers Arch.* **411**:353-360.

Benham, C.D., T.B. Bolton, and R.J. Lang. (1984). Membrane potential and voltage

clamp recording from single smooth muscle cells of rabbit jejunum. *J. Physiol.* 353:p67.

Benham, C.D., T.B. Bolton, R.J. Lang, and T. Takewaki. (1986). Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea pig mesenteric artery. *J. Physiol.* 371:45-67.

Benham, C.D., P. Hess, and R.W. Tsien. (1987). Two types of calcium channels in single smooth muscle cells of rabbit ear artery studied with whole-cell and single channel recording. *Circ. Res.* 61(suppl. 1):110-116.

Biel, M., R. Hullin, S. Freundner, D. Singer, N. Dascal, V. Flockerzi, and F. Hofmann. (1991). Tissue specific expression of high voltage dihydropyridine sensitive L-type calcium channels. *Eur. J. Biochem.* 200:81-88.

Bielefeldt, D., and M.B. Jackson. 1994. Phosphorylation and dephosphorylation modulate a Ca^{2+}-activated K^+ channel in rat peptidergic nerve terminals. *J. Physiol.* 475:241-254.

Bilder, G.E., J.A. Krawiec, K. McVety, A. Gazit, C. Gilon, R. Lyall, A. Zilberstein, A. Levitzki, M.H. Perrone, and A.B. Schreiber. (1991). Tyrphostins inhibit PDGF-induced DNA synthesis and associated early events in smooth muscle cells. *Am. J. Physiol.* 260:C721-C730.

Bkaily, G., T. Yamamoto, M. peyrow, A. sulptoreanu, D. Jacques, and N. Sperelakis. (1988). Macroscopic Ca^{2+}, Na^{+}, and K^{+} currents in single heart and aortic cells. *Mol. Cell. Biochem.* **80**:59-72.

Bkaily, G. (1990). Bethanidine, nitroprusside and atrial natriuretic factor open a cGMP-sensitive K^{+} channel in aortic muscle. *Prog. Clin. Biol. Res.* **327**:507-515.

Blatter, L.A., and W.G. Wier. (1994). Nitric oxide decrease $[Ca^{2+}]i$ in vascular smooth muscle by inhibition of the calcium current. *Cell Calcium.* **15**:122-131.

Blatz, A.L., K.L. Magelby. (1983). Single voltage-dependent chloride-selective channels of large conductance in cultured rat muscle. *Biophy. J.* **43**:237-241.

Blatz, A.L., K.L. Magelby. (1987). Calcium-activated potassium channels. *Trends. Neurosci.* **10**:463-467.

Bleakman, V.P. Bindokas, and R.J. Miller. (1993). Neuropeptide Y and pancreatic polypeptide reduce calcium currents in acutely dissociated neurons from adult rat superior cervical ganglia. *J. Pharmacol. Exp. Ther.* **265**:903-909.

Bleakman, D., W.F. Colmers, A. Fournier, and R.J. Miller. (1991). Neuropeptide Y inhibits Ca^{2+} influx into cultured dorsal root ganglion neurons of the rat via a Y2

receptor. *Br. J. Pharmacol.* **103**:1781-1789.

Bolton, T.B., R.J. Lang, and T. Takewaki. (1984). Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea pig anterior mesenteric artery. *J. Physiol.* **351**:549-572.

Bolzon, B.J., and D.W. Cheung. (1989). Isolation and characterization of single vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension* **14**:137-144.

Bolzon, B.J., Z. Xiong, and D.W. Cheung. (1993). Membrane rectification in single smooth muscle cells from the rat tail artery. *Pflugers Arch.* **425**:482-490.

Bonev, A.D., and M.T. Nelson. (1993). ATP-sensitive potassium channels in smooth muscle cells from guinea pig urinary bladder. *Am. J. Physiol.* **264**:C1190-C1200.

Bonn, V. (1990). NPY-like immunoreactivity in the brain of the teleost Tinca tinca (Cyprinidae). *J. Fur. Hirnforshung* **31(3)**:323-330.

Bons, N., N. Mestre, A. Petter, J.M. Danger, G. Pelletier, and H. Vaudry. (1990). Localization and characterization of neuropeptide Y in the brain of Microcebus murinus (Primate, Lemurian). *J. Comp. Neurol.* **298**(3):343-361.

Bosse, E., R. Bottlender, T. Kleppisch, J. Hescheler, A. Welling, F. Hofmann, and V. Flockerzi. (1992). Stable and functional expression of the calcium channel α_1 subunit from smooth muscle in somatic cell lines. *EMBO J.* 11:2033-2038.

Brayden, J.E., and M.T. Nelson. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256**:532-535.

Brown, A.M., and L. Birnbaumer. (1990). Ionic channels and their regulation by G protein subunit. *Annu. Rev. Physiol.* **52**:197-213.

Bryant, S.M., K.O. Ryder, and G. Hart. (1991). Effects of neuropeptide Y on cell length and membrane currents in isolated guinea pig ventricular myocytes. *Circ. Res.* **69**:1106-1113.

Byrne, N.G., and W.A. Large. (1988). Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. *J. Physiol.* **404**:557-573.

Caffrey, J.M., I.R. Josephson, and A.M. Brown. (1986). Calcium channels of amphibian stomach and mammalian aorta smooth muscles. *Biophy. J.* **49**:1237-1242.

Carl, A., J.L. Kenyon, D. Uemura, N. Fusetani, and K.M. Sanders. (1991). Regulation of Ca^{2+}-activated K^+ channels by protein kinase A and phosphatase inhibitors. *Am. J.*

Physiol. **261**:C387-C392

Catteral, W.A. (1988). Structure and function of voltage-sensitive ion channels. *Science.* **242**:50-61.

Chang, F., H.G. Yu, and I.S. Cohen. (1993). Actions of vasoactive intestinal peptide and neuropeptide Y on the pacemaker current in canine Purkinje fibers. *Circ. Res.* **74**:157-162.

Chen, X., and T.C. Westfall. (1994). Modulation of intracellular calcium transients and dopamine release by neuropeptide Y in PC-12 cells. *Am. J. Physiol.* **266**:C784-C793.

Cheng, H.C., B.E. Kemp, R.B. Pearson, A.J. Smith, L. Misconi, S.M. van Patten and D.A. Walsh. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Bio. Chem.* **261**:989-992.

Cheung, D.W. (1982). Two components in the cellular response of rat tail arteries to nerve stimulation. *J. Physiol.* **328**:461-468.

Cheung, D.W. (1984). Neural regulation of electrical and mechanical activities of the rat tail artery. *Pflugers Arch.* **400**:335-337.

Cheung, D.W. (1991). Neuropeptide Y potentiates specifically the purinergic component of the neural responses in the guinea pig saphenous artery. *Circ. Res.* **68**:1401-1407.

Cheung, D.W., and R. Dukkipati. (1991). An electrophysiological study on the effect of neuropeptide Y on sympathetic neurotransmission in guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.* **257**:979-983.

Cheung, D.W, and M.J. MacKay. (1986). The effects of Bay K 8644 and nifedipine on the neural responses of the rabbit ear artery. *Br. J. Pharmacol.* **88**:363-368.

Chronwall, B.M., T.N. Chase and O'Donohue. (1984). Coexistence of neuropeptide Y and somatostatin in rat and human cortical and rat hypothalamus neurons. *Neurosci. Lett.* **52(3)**:213-217.

Chung, S., P.H. Reinhart, B.L. Martin, D. Brautigan, and I.B. Levitan. (1991). Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* **253**:560-562.

Clapp, L.H., and A.M. Gurney. (1991). Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. *Pflugers Arch.* **418**:462-470.

Clapp, L.H., and A.M. Gurney. (1992). ATP-sensitive K^+ channels regulate resting

potential of pulmonary arterial smooth muscle cells. *Am. J. Physiol.* **262(31)**:H916-H920.

Clapp, F.L., A. Gurney, and M. Evans. (1994). K_{ATP} channels: Fact or artefact? *Cardiovas. Res.* **28**:742-744.

Clarke JC, Benjamin N, Carkin S, Webb D, Maseri U, Davies G. (1991). Interaction of neuropeptide Y and the sympathetic nervous system in vascular control in man. *Circulation* **83**:774-777.

Colmers, W.F., and D. Bleakman. (1994). Effects of neuropeptide Y on the electrical properties of neurons. *Trends Neurosci.* **17(9)**:373-379.

Connor, J.A. and C.F. Stevens. (1971). Voltage-clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol.* **213**:21-30.

Cook, D.L., and C.N. Hales. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic cells. *Nature* **311**:271-273.

Corr, L.A., J.A. Aberdeen, P. Milner, J. Lincoln and G. Burnstock. (1990). Sympathetic and nonsympathetic neuropeptide Y-containing nerves in the rat myocardium and coronary arteries. *Circ. Res.* **66(6)**:1602-1609.

Corrette, B.J., H. Repp, F. Dreyer, and J.R. Schwarz. (1991). Two types of fast K^+ channels in rat myelinated nerve fibres and their sensitivity to dendrotoxin. *Pflugers Arch.* **418**:408-416.

Cottechia, S., L.M. Lee-Lundberg, P.O. Hagen, R.J. Lefkowitz, and M.G. Caron. (1985). Phorbol ester effects on alpha 1-adrenoceptor binding and phosphatidylinositol metabolism in cultured vascular smooth muscle cells. *Life. Sci.* **37**:2389-2398.

Coulombe, A., H. Duclohier, E. Coraboeuf, and N. Touzet. (1987). Single chloride permeable channels of large conductance in cultured cardiac cells of newborn rats. *Eur. Biophy. J.* **14**:155-162.

Daly, R.N., and J.P. Hieble. (1987). Neuropeptide Y modulates adrenergic neurotransmission by an endothelium-dependent mechanism. *Eur. J. Pharmacol.* **138**:445-446.

Danger, J.M., M.C. Tonon, B.G. Jenks, S. St. Pierre, J.C. Martel, A. Fasolo, B. Breton, R. Quirion, G. Pelletier and H. Vaudry. (1990). Neuropeptide Y: localization in the central nervous system and neuroendocrine functions. *Fund. Clin. Pharmacol.* **4(3)**:307-340.

Daniels, A.J., E.R. Lazarowski, J.E. Matthews, and E.G. Lapetina. (1989).

Neuropeptide Y mobilizes intracellular Ca^{2+} and increases inositol phosphate production in human erythroleukemia cells. *Biochem. Biophy. Res. Commun.* **165**:1138-1144.

Daut, J., W. Maier-Rudolph, N. van Beckerath, G. Mehrke, K. Gunther, and L. Goedel-Meinen. (1990). Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. *Science* **247**:1341-1344.

De la Fuente, M., I. Bernaez, M. Del Rio, and A. Hernanz. (1993). Stimulation of murine peritonea macrophage functions by neuropeptide Y and peptide YY. Involvement of prptein kinase C. *Immunology* **80(2)**:259-265.

Desilets, M., S.P. Driska, and C.M. Baumgarten. (1989). Current fluctuations and oscillations in smooth muscle cells from hog carotid artery. *Circ. Res.* **56**:708-722.

Di Salvo, J., D. Gifford, and A. Kokkinakis. (1988). $pp60^{c\text{-}src}$ kinase activity in bovine coronary extracts is stimulated by ATP. *Biochem. Biophy. Res. Commun.* **153**:388-394.

Di Salvo, J., A. Steusloff, L. Semenchuk, S. Satoh, K. Kolquist, and G. Pfitzer. (1993). Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. *Biochem. Biophy. Res. Commun.* **190**:968-974.

Downing, J.R., B.L. Margolis, A. Zilberstein, R.A. Ashmun, A. Ullrich, C.J. Sherr, and J. Schlessinger. (1989). Phospholipase C-γ, a substrate for PDGF receptor kinase, is not phosphorylated on tyrosine residue during the mitogenic response to CSF-1. *EMBO J.* **8**:3345-3350.

Droogmans, G., L. Raeymaekers, and R. Casteels. (1977). Electro- and pharmacomechenical coupling in the smooth muscle cells of the rabbit ear artery. *J. Gen. Physiol.* **70**:129-148.

Droogmans G., and G. Callewaert. (1986). Ca^{2+}-channel current and its modification by the dihydropyridine agonist Bay K 8644 in isolated smooth muscle cells. *Pflugers Arch.* **406**:259-265.

Droogmans G., I. Declerck, and R. Casteels. (1987). Effects of adrenergic agonists on Ca^{2+}-channel currents in single vascular smooth muscle cells. *Plfugers Arch.* **409**:7-12.

Dubois, J.M. (1981). Evidence for the existence of three types of potassium channels in the frog Ranvier node membrane. *J. Physiol.* **318**:297-316.

Duchatelle-Gourdon, I., A.A. Lagrutta, and H.C. Hartzell. (1991). Effects of Mg^{2+} on basal and ß-adrenergic-stimulated delayed rectifier potassium current in frog atrial myocytes. *J. Physiol.* **435**:333-347.

Dumont, Y., A. Fournier, S. St-Pierre, T.W. Schwartz, and R. Quirion. (1990). Differential distribution of neuropeptide Y1 and Y2 receptors in the rat brain. *Eur. J. Pharmacol.* **191**:501-503.

Edvinsson, L., P. Emson, J. McCulloch, K. Tatemoto, and R. Uddman. (1983). Neuropeptide Y: cerebrovascular innervation and vasomotor effects in the cat. *Neurosci. Lett.* **43**:79-84.

Edvinsson, L., P.C. Emson, J. McCulloch, K. Tatemoto, and R. Uddman. (1984a). Neuropeptide Y: immunocytochemical localization and effect upon feline pial arteries and veins in vitro and in situ. *Acta Physiol. Scand.* **122**:155-63.

Edvinsson, L., E. Ekblad, R. Hakanson, and C. Wahlestedt. (1984b). Neuropeptide Y potentiates the effects of various vasoconstrictor agents on rabbit blood vessels. *Br. J. Pharmacol.* **83**:519-25

Edvinsson, L., J.R. Copeland, P.C. Emson, J. McCulloch, and R. Uddman. (1987). Nerve fibres containing neuropeptide Y in the cerebrovascular bed: immunocytochemistry, radioimmunoassay, and vasomotor effects. *J. Cereb. Blood. Flow Metab.* **7**:45-57.

Edvinsson, L., R. Hakanson, C. Wahlestedt, and R. Uddman. (1987b). Effects of neuropeptide Y on the cardiovascular system. *Trends Pharmacol. Sci.* **8**:231-235.

Edwards, F.R., G.D. Hirst, and G.D. Silverberg. (1988). Inward rectification in rat cerebral arterioles: Involvement of potassium ions in autoregulation. *J. Physiol.* **404**:455-466.

Edwards, F.R., and G.D.S. Hirst. (1988). Inward rectification in submucosal arterioles of guinea pig ileum. *J. Physiol.* **404**:437-454.

Edwards, G., T. Ibbotson, and A.H. Weston. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifing the gating properties of the delayed rectifier. *Br. J. Pharmacol.* **110**:1037-1048.

Edwards, G., and A.H. Weston. (1994). K_{ATP}-fact or artefact? New thoughts on the mode of action of the potassium channel openers. *Cardiovas. Res.* **28**:735-737.

Ekblad, E., L. Edvinsson, C. Wahlestedt, R. Uddman, R. Hakanson, and F. Sundler. (1984). Neuropeptide Y co-exists in perivascular nerve fibres. *Regul. Peptides* **8**:225-35.

Erlinge, D., H. Yoo, L. Edvinsson, D.J. Reis, and C. Wahlestedt. (1993). Mitogenic effects of ATP on vascular smooth muscle cells vs. other growth factors and sympathetic

cotransmitters. *Am. J. Physiol.* 265:H1089-H1097.

Erne, P., and K. Hermsmeyer. (1988). Intracellular Ca^{2+} release in vascular muscle cells by caffeine, ryanodine, norepinephrine, and neuropeptide Y. *J. Cardiovas. Pharmacol.* 12 (suppl. 5):S85-S91.

Esguerra, M., J. Wang, C.D. Foster, J.P. Adelman, R.A. North, and I.B. Levitan. (1994). Cloned Ca^{2+}-dependent K^+ channel modulated by a functionally associated protein kinase. *Nature* 369:563-565.

Evans, A.M., L.H. Clapp, and A.M. Gurney. (1994). Augmentation by intracellular ATP of the delayed rectifier current independently of the glibenclamide-sensitive K-current in rabbit arterial myocytes. *Br. J. Pharmacol.* 111(4):972-974.

Ewald, D.A., I. H. Pang, P.C. Sternweis, and R.J. Miller. (1989). Differential G protein-mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons in vitro. *Neuron* 2:1185-1193.

Ewald, D.A., A. Wiliams and I.B. Levitan. (1985). Modulation of single Ca^{2+}-dependent K^+ channel activity by protein phosphorylation. *Nature* 315:503-506.

Fabiato, A. (1988). Computer programs for calculating total from specified free or free

from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods in Enzymology* **157**:378-417.

Fish, R.D., G. Sperti, W.S. Colucci, and D.E. Clapham. (1988). Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. *Circ. Res.* **62**:1049-1052.

Force, T., J.M. Kyriakis, J. Avruch, and J.V. Bonventre. (1991). Endothelin, vasopressin, and angiotensin II enhance tyrosine phosphorylation by protein kinase C-dependent and -independent pathways in glomerular mesangial cells. *J. Bio. Chem.* **266**:6650-6656.

Foucart S, Bleakman D, Bindokas VP, Miller RJ. (1993). Neuropeptide Y and pancreatic polypeptide reduce calcium currents in acutely dissociated neurons from adult rat superior cervical ganglia. *J. Pharmacol. Exp. Ther.* **265**:903-909.

Fox, A.P., M.C. Nowycky, and R.W. Tsien. (1987). Single-channel recording of three types of calcium channels in chick sensory neurons. *J. Physiol.* **394**:173-200.

Franco-Cereceda, A., J.M. Lundberg and C. Dahlof. (1985). Neuropeptide Y and sympathetic control of heart contractility and coronary vascular tone. *Acta Physiol. Scand.* **124**:361-369.

Fredholm, B., I. Jansen, and L. Edvinssom. (1985). Neuropeptide Y is a potent inhibitor of cyclic AMP accumulation in feline cerebral blood vessels. *Acta. Physiol. Scand.* 124:467-469.

Fujino, K., S. Nakaya, T. Wakatsuki, Y. Miyoshi, Y. Nakaya, H. Mori, and I. Inoue. (1991). Effects of nitroglycerin on ATP-induced Ca^{2+}-mobilization, Ca^{2+}-activated K^+-channels and contraction of cultured smooth muscle cells of porcine coronary artery. *J. Pharmacol. Exp. Ther.* 256:371-377.

Fukumitsu, T., H. Hayashi, H. Tokuno, and T. Tomita. (1990). Increase in calcium channel current by ß-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery. *Br. J. Pharmacol.* 100:593-599.

Furness, J.B., M. Costa, P.C. Emson, R. Hakanson, E. Moghimzadeh, F. Sundler, I.L. Tayler, and R.E. Chance. (1983). Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. *Cell & Tissue Res.* 234:71-92.

Furspan, P.B., and R.C. Webb. (1993). Decreased ATP-sensitivity of a K^+ channel and enhanced vascular smooth muscle relaxation in genetically hypertensive rats. *J. Hypertens* 11:1067-72.

Geissler, J.F., P. Traxler, U. Regenass, B.J. Murray, J.L. Roesel, T. Meyer, E. McGlynn, A. Storni, and N.B. Lydon. (1990). Thiazolidine-diones. Biochemical and biological activity of a novel class of tyrosine protein kinase inhibitors. *J. Bio. Chem.* **265**:22255-22261.

Gibson, S.J., J.M. Polak, J.M. Allen, T.E. Adrian, J.S. Kelly, and S.R. Bloom. (1984). The distribution and origin of a novel brain peptide, neuropeptide Y, in the spinal cord of several mammals. *J. Comp. Neurol.* **227(1)**:78-91.

Giles, W.R., and A.C.G. van Ginneken. (1985). A transient outward current in isolated cells from the crista terminalis of rabbit heart. *J. Physiol.* **368**:243-264.

Gilman, A.G. (1987). G protein: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615-649.

Glover, I., D.J. Barlow, J.E. Pitts, S.P. Wood, I.J. Tickle, T. Blundell, K. Tatemoto, J.R. Kimmel, A. Wollmer, W. Strassburger, and Y.S. Zhang. (1984). Conformation studies on the pancreatic polypeptide hormone family. *Eur. J. Biochem.* **142**:379-385.

Glover, I., I. Haneef, J. Pitts, S. Wood, D. Moss, I. Tickle, and T. Blundell. (1983). Conformational flexibility in a small globular hormone: x-ray analysis of avian pancreatic polypeptide at 0.98-A resolution. *Biopolymers* **22(1)**:12984-12988.

Gola, M., and M. Crest. (1993). Colocalization of active KCa Channels and Ca^{2+} channels within Ca^{2+} domains in helix neurons. *Neuron* **10**:689-699.

Gray, T.S., and J.E. Morley. (1986). Neuropeptide Y: Anatomical distribution and possible function in mammalian nervous system. *Life Sci.* **38(5)**:389-401.

Grundemar, L., and R. Hakanson. (1994). Neuropeptide Y effector systems: perspectives for drug development. *Trends Pharmacol. Sci.* **15**:153-158.

Grunditz, T., R. Hakanson, C. Rerup, F. Sundler, and R. Uddman. (1984). Neuropeptide Y in the thyroid gland: neuronal localization and enhancement of stimulated thyroid hormone secretion. *Endocrinology* **115**:1537-42.

Gulbenkian, S., N. Costa-Andrade, J. Wharton et al. (1990). Immunocytochemical localization of neuropeptide Y and its C-terminal flanking peptide in the human heart. *Ann. NY. Acad. Sci.* **611**:429-31.

Haggblad, J., and B.B. Fredholm. (1987). Adenosine and neuropeptide Y enhance alpha 1 -adrenoceptor-induced accumulation of inositol phosphates and attenuate forskolin-induced accumulation of cyclic AMP in rat vas deferens. *Neurosci. Lett.* **82(2)**:211-216.

Hagiwara, N., H. Irisawa, and M. Kameyama. (1988). Contribution of two types of

calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J. Physiol.* 359:233-253.

Halliwell, J.V., I.B. Othman, A. Pelchen-Matthews, and J.O. Dolly. (1986) Central action of dendrotoxin: selective reduction of a transient K conductance in hippocampus and binding to localized acceptors. *Proc. Natl. Acad. Sci.* USA 83(2):493-497.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85-100.

Hamilton, T.C., S.W. Weir, and A.H. Weston. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical activity in rat portal vein. *Br. J. Phamacol.* 88:103-111.

Han, C., X.A. Wang, R.R. Fiscuss, J.A. Gu, and J.K. McDonald. (1989). Changes in cardiac neuropeptide Y after experimental myocardial infarction. *Neurosci. Lett.* 104:141-6.

Harder, D.R., and N. Sperelakis. (1979). Membrane electrical properties of vascular smooth muscle from the guinea pig superior mesenteric artery. *Pflugers Arch.* 378:111-119.

Hartzell, H.C., P.F. Mery, R. Fischmeister, and G. Szabo. (1991). Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylaton. *Nature* 351:573-576.

Heinrich, D., M. Reinecke, J.F.H. Gauwerky, and W.G. Forssmann. (1987). Immunohistochemical and biological evidence for a neuromodulator function of neuropeptide Y in the human oviduct. *Arch. Gynecol. Obster.* 241:127-32.

Hieble, J.P., J.G. Duesler, and R.N. Daly. (1989). Effects of neuropeptide Y on the response of isolated blood vessels to norepinephrine and sympathetic field stimulation. *J. Pharmacol. Exp. Ther.* 250:523-528.

Hille, B. (1984). Ionic channels of excitable membranes. Sunderland, MA: Sinauer, p. 117-147.

Hirning, L.D., A.P. Fox, and R.J. Miller. (1990). Inhibition of calcium currents in cultured myenteric neurons by neuropeptide Y: evidence for direct receptor/channel coupling. *Brain Res.* 532:120-130.

Hodgkin, A.L., and A.F. Huxley. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* 117:500-544.

Horie, M., H. Irisawa, and A. Noma. (1987). Voltage-dependent magnesium block of adenosine triphosphate sensitive potassium channel in guinea pig ventricular cells. *J. Physiol.* **387**:251-272.

Horn, R., and A. Marty. (1988). Muscarinic activation of ion current measured by a new whole-cell recording method. *J. Gen. Physiol.* **92**:145-159.

Hosey, M.M., and M. Lazdunski. (1988). Calcium channels: molecular pharmacology, structure, and regulation. *J. Membr. Biol.* **104**:81-105.

Hu, S.L., H.S. Kim, and A.Y. Jeng. (1991). Dual action of endothelin-1 on the Ca^{2+}-activated K^+ channels in smooth muscle cells of porcine coronary artery. *Eur. J. Pharmacol.* **194**:31-36.

Huang, X.Y., A.D. Morielli, and E.G. Peralta. (1993). Tyrosine kinase-dependent suppression of a potassium channel by the G-protein-coupled m1 muscarinic acetylcholine receptor. *Cell* **75**:1145-1156.

Hume, J.R., and N. Leblanc. (1989). Macroscopic K^+ currents in single smooth muscle cells of the rabbit portal vein. *J. Physiol.* **413**:49-73.

Hunter, T., and J.A. Cooper. (1985). Protein-tyrosine kinases. *Ann. Rev. Physiol.*

54:897-930.

Imaizuma, Y., K. Muraki, and M. Watanabe. (1990). Characteristics of transient outward currents in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol.* **427**:301-324.

Isenberg, G., and V. Klockner. (1986). Elementary currents through single Ca^{2+}-activated potassium channels (smooth muscle cells isolated from trachea or urinary bladder). *Pflugers Arch.* **405**:62-68.

Ishihara, K T. Mitsuiye, A. Noma, and M. Takano. (1989). The Mg^{2+} block and intrinsic gating underlying inward rectification of the K^{+} current in guinea pig cardiac myocytes. *J. Physiol.* **419**:297-320.

Itoh, H., and K. Lederis. (1987). Contraction of rat thoracic aorta strips induced by phorbol 12-myristate 13-acetate. *Am. J. Physiol.* **252**:C244-C249

Itoh, Y., H. Suzuki, and H. Kuriyama. (1987). Effects of sodium nitroprusside in smooth muscle cells of rabbit pulmonary artery and portal vein. *J. Pharmacol. Exp. Ther.* **207**:1022-1301.

Itoi, K., T. Mouri, K. Takahashi, S. Sasaki, Y. Imai, and K. Yoshinaga. (1986).

Synergistic pressor action of neuropeptide Y and norepinephrine in conscious rats. *J. Hypertension* **Suppl.4**:S247-S250.

Johnson, B.D., Scheuer, T, and W. Catterall. (1994). Voltage-dependent potentiation of L-type Ca^{2+} channels in skeletal muscle cells requires anchored cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 91:11492-11496.

Kaczmarek, L.K., and I.B. Levitan. (1987). *Neuromodulation. The biochemical control of neuronal excitability,* Oxford: Oxford Univ. Press. 286pp.

Kameyama, M., J. Hescheler, F. Hofmann, and W. Trautwein. (1986). Modulation of Ca current during the phosphorylation cycle in the guinea-pig heart. *Pflugers Arch.* **407**:123-128.

Kameyama, M., A. Kameyama, T. Nakayama, and M. Kaibara. (1988). Tissue extract recorves cardiac calcium channels from "run-down". *Pflugers Arch.* **412**:328-330.

Kamm, K.E., and J.T. Stull. (1985). The function of myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* 25:593-620.

Kassis, S., M. Olasmaa, L. Terenius, and P.H. Fishman. (1987). Neuropeptide Y inhibits cardiac adenylate cyclase through a pertussis toxin-sensitive G protein. *J. Bio. Chem.*

262(8):3429-3431.

Klapstein, G.J., and W.F. Colmers. (1992). 4-aminopyridine and low Ca^{2+} differentiate presynaptic inhibition mediated by neuropeptide Y, baclofen and 2-chloroadenosine in rat hippocampal CA1 in vitro. *Br. J. Pharmacol.* **105(2)**:470-474.

Kokubun, S., A. Saigusa, and T. Tamura. (1991). Blockade of Cl channels by organic and inorganic blockers in vascular smooth muscle cells. *Pflugers Arch.* **418**:204-213.

Komaru, T., K. Ashikawa, H. Kanatsuka, N. Sekiguchi, T. Suzuki and T. Takishima. (1990). Neuropeptide Y modulates vasoconstriction in coronary microvessels in the beating canine heart. *Circ. Res.* **67**:1142-1151.

Kong, J.Y., A.K. Thureson-Klein, and R.L. Klein. (1990). Are NPY and enkephalin costored in the same noradrenergic neurons and vesicles? *Peptides* **11(3)**:565-575.

Kubo, M., Y. Nakaya, S. Matsuoka, K. Saito, Y. Kuroda. (1994). Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle cells. *Circ. Res.* **74**:471-476.

Kume, H., A. Takai, H. Tokuno, and T. Tomita. (1989). Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature*

341:152-154.

Kuriyama, H., K. Ohshima, and Y. Sakamoto. (1971). The membrane properties of the smooth muscle of the guinea pig portal vein in isotonic and hypertonic solutions. *J. Physiol.* **217**:179-199.

Lang, D.G. (1989). Identification of the major membrane currents in freshly dispersed single smooth muscle cells of guinea-pig ureter. *J. Physiol.* **412**:375-395.

Lang, D.G., and A.K. Ritchie. (1990). Tetraethylammonium blockade of apamin-sensitive and insensitive Ca^{2+}-activated K^+ channels in a pituitary cell line. *J. Physiol.* **425**:117-132.

Laniyonu, AA., M. Saifeddine, S.G. Yang, and M.D. Hollenberg. (1992). *FASEB J.* **6**:A1862.

Larhammar, D., A.G. Blomqvist, F. Yee, E. Jazin, H. Yoo, and C. Wahlestedt. (1992). Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Bio. Chem.* **267**:10935-10938.

Latorre, R., and C. Miller. (1983). Conduction and selectivity in potassium channel. *J. Membr. Biol.* **71**:11-30.

Lau, K.H., and D.J. Baylink. (1993). Phosphotyrosyl protein phosphatases:potential regulators of cell proliferation and differentiation. *Critic. Rev. Oncogen.* **4(4)**:451-457.

Lee, K.M., K. Toscas, and M.L. Villereal. (1993). Inhibition of bradykinin and thapsigargin induced Ca^{2+} entry by tyrosine kinase inhibitors. *J. Bio. Chem.* **268**:9945-9948.

Lee, Y., S. Shiosaka, P.G. Emson, J.F. Powell, A.D. Smith, and M. Tohyama. (1985). Neuropeptide Y-like immunoreactive structures in the rat stomach with special reference to the noradrenaline neuron system. *Gastroenterology* **89**:118-26.

Leeb-Lundberg, L.M.F., and X.H. Song. (1991). Bradykinin and Bombesin rapidly stimulate tyrosine phosphorylation of a 120-KDa group of proteins in Swiss 3T3 cells. *J. Bio. Chem.* **266**:7746-7749.

Levi, R.C., G. Alloatti, and R. Fischeister. (1989). Cyclic GMP regulates the Ca-channel current in guinea pig ventricular myocytes. *Pflugers Arch.* **413**:685-687.

Levi, R.C., G. Alloatti, C. Penna, and M.P. Gallo. (1994). Guanylate-cyclase-mediated inhibition of cardiac I_{Ca} by carbachol and sodium nitroprusside. *Pflugers Arch.* **426**:419-426.

Levitzki, A., and C. Gilon. (1991). Tyrphostins as molecular tolls and potential antiproliferative drugs. *Trends Pharmacol. Sci.* **12**:171-174.

Llinas, R., M. Sugimori, J.W. Lin, and B. Chertsey. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funel-web spider poison. *Proc. Natl. Acad. Sci. USA.* **86**:1689-1693.

Lobaugh, LA., and P.J. Blackshear. (1990). Neuropeptide Y binding and inhibition of cAMP accumulation in human neuroepithelioma cells. *Am. J. Physiol.* **258**:C913-C922.

Lobaugh, LA., and P.J. Blackshear. (1990a). Neuropeptide Y stimulation of myosin light chain phosphorylation in cultured aortic smooth muscle cells. *J. Bio. Chem.* **265**:18393-18399.

Loesch, A., K.I. Maynard, and G. Burnstock. (1992). Calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity in endothelial cells after long-term stimulation of perivascular nerves. *Neurosci.* **48**:723-6.

Lundberg, J.M., L. Terenius, and T. Hokfelt. (1982). Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. *Acta. Physiol. Scand.* **116**:477-80

Lundberg, J.M., and K. Tatemoto. (1982). Pancreatic polypeptide family (APP, BPP, NPY and PYY) in relation to sympathetic vasoconstriction resistant to alpha-adrenoceptor blockade. *Acta. Physiol. Scand.* **116**:393-402.

Lundberg, J.M., L. Terenius, T. Hokfelt, and M. Goldstein. (1983). High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. *Neurosci. Lett.* **42**:167-72

Lundberg, J.M., A. Anggard, E. Theodorsson-Norheim, and J. Pernow. (1984). Guanethidine-sensitive release of neuropeptide Y-like immunoreactivity in the cat spleen by sympathetic nerve stimulation. *Neurosci. Lett.* **52**:175-180.

Lundberg, J.M., A. Hemsen, O. Larsson, A. Rudehill, A. Saria, and B. Fredholm. (1988). Neuropeptide Y receptor in pig spleen: binding charateristics, reduction of cAMP formation and calcium antagonist inhibition of vasoconstriction. *Eur. J. Pharmacol.* **145**:21-29.

Lundberg, J.M., J. Pernow, and J.S. Lacroix. (1989). Neuropeptide Y: sympathetic cotransmitter and modulator? *News Physiol. Sci.* **4**:13-17.

Lundy, P.M., and R. Frew. (1991). The effect of neuropeptide Y on voltage-sensitive Ca^{2+} channels. *Eur. J. Pharmacol.* **192**:439-441.

Maclean, M.R., and J.C. McGrath. (1990). Effects of pre-contraction with endothelin-1 on α_2-adrenoceptor and (endothelin-dependent) neuropeptide Y-mediated contractions in the isolated vascular bed of the rat tail. *Br. J. Pharmacol.* **101**:205-211.

Margolis, B., S.G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger. (1989). EGF induces phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* **57**:1101-1107.

Marks, T.N., G.R. Dubyak, and S.W. Jones. (1990). Calcium currents in the A7r5 smooth muscle-derived cell line. *Pflugers Arch.* **417**:433-439.

Martel, J.C., A. Fournier, S. St. Pierre, and R. Quirion. (1990). Quantitative autoradiographic distribution of [^{125}I] Bolton-Hunter neuropeptide Y receptor binding sites in rat brain. Comparison with [^{125}I] peptide YY receptor sites. *Neurosci.* **36(1)**:255-283.

Martling, C.R., R. Matran, K. Alving, T. Hokfelt, and J.M. Lundberg. (1990). Innervation of lower airways and neuropetide effect on bronchial and vascular tone in the pig. *Cell & Tissue Res.* **260**:223-233.

Maturi, M.F., R. Green, and E. Speir. (1989). Neuropeptide Y. A peptide found in human coronary arteries constricts primarily small arteries to produce myocardial

ischemia in dog. *J. Clin. Invest.* **83**:1217-1224.

McAuley, M.A., I.M. Macrae, R. Farmer, J.L. Reid. (1991). Effect of neuropeptide Y on FSK, alpha$_2$- and beta-adrenoceptor-regulated cAMP levels in the rat brain slices. *Peptides* **12**:407-412.

McCann, J.D., and M.J. Welsh. (1986). Calcium-activated potassium channels in canine airway smooth muscle. *J. Physiol.* **372**:113-127.

McDermott, B.J., B.C. Miller, and H.M. Piper. (1993). Cardiovascular effects of neuropeptide Y:receptor interactions and cellular mechanisms. *Cardiovas. Res.* **27**:893-905.

Merkel, L.A., L.M. Rivera, D.J. Colussi, and M.H. Perrone. (1993). Inhibition of EGF-induced vasoconstriction in isolated rabbit aortic rings with the tyrosine kinase inhibitor RG50864. *Biochem. Biophy. Res. Commun.* **192(3)**:1319-1326.

Mery, P.F., S.M. Lohmann, U. Walter, and R. Fischmeister. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acsd. Sci. USA.* **88**:1197-1201.

Michel, M.C., and H.J. Motulsky. (1990). He 90481: A competitive nonpeptidergic

antagonist at neuropeptide Y receptors. *Ann. N.Y. Acad. Sci.* **611**:392.

Mihara, S., Y. Shigeri, and M. Fujimoto. (1989). Neuropeptide Y-induced intracellular Ca^{2+} increase in vascular smooth muscle cells. *FEBS Lett.* **259**:79-82.

Millar, B.C., H.M. Piper, and B.J. McDermott. (1988). The antiadrenergic effect of neuropeptide Y on the ventricular cardiomyocyte. *Naunyn-Schmiedebergs Arch. Pharmacol.* **338**:426-429.

Millar, B.C., T. Weis, and H.M. Piper. (1991). Positive and negative contractile effects of neuropeptide Y on ventricular cardiomyocytes. *Am. J. Physiol.* **261**:H1727-1733.

Miller, R.J., and Fox, A.P. (1990). Voltage-sensitive calcium channels. in *Intracellular calcium regulation,* p97-138. Alan R. Liss, Inc.

Miller, C., E. Moczydowski, R. Latorre, and M. Phillips. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from skeletal muscle. *Nature* **313**:316-318.

Minami, K., K. Fukuzawa, and Y. Nakaya. (1993). Protine kinase C inhibits the Ca^{2+}-activated K^+ channel of cultured porcine coronary artery smooth muscle cells. *Biochem. Biophy. Res. Comm.* **190**:263-269.

Minami, K., K. Fukuzawa, and I. Inoue. (1994). Regulation of a non-selective cation channel of cultured porcine coronary artery smooth muscle cells by tyrosine kinase. *Pflugers Arch.* 426:254-257.

Minson, R.B., R.J. McRitchie, and J.P. Chalmers. (1987). Effects of neuropeptide Y on left ventricular function in the conscious rabbit. *Clin. Exp. Pharmacol. Physiol.* 14:263-266

Minson, R.B., R.J. McRitchie, and J.P. Chalmers. (1989). Effects of neuropeptide Y on the heart and circulation of the conscious rabbit. *J. Cardiovas. Pharmacol.* 14(5):699-706.

Minson, R.B., R.J. McRitchie, and J.P. Chalmers. (1990). Effects of neuropeptide Y on baroreflex control of heart rate and myocardial contractility in conscious rabbits. *Clin. Exp. Pharmacol. Physiol.* 17:39-49.

Mironneau, J. (1991). Noradrenaline modulation on ionic channels in vascular smooth muscle cells, in: Sperelakis N, Kuriyama H (eds): *Ion channels of vascular smooth muscle cells and endothelial cells.* Elsevier, New York, pp47-54.

Miyoshi, H and Y. Nakaya. (1993). Activation of ATP-sensitive K^+ channels by cyclic AMP-dependent protein kinase in cultured smooth muscle cells of porcine coronary

artery. *Biochem. Biophy. Res. Commun.* **193(1)**:240-247.

Mormede, P., V. Castagne, J.M. Rivet, R. Gaillard, and R. Corder. (1990). Involvement of neuropeptide Y in neuroendocrine stress responses. Central and peripheral Studies. *J. Neural. Trans.* **29** (Suppl):65-75.

Morris, J.C. (1991). Pertussis toxin attenuates postsynaptic action of neuropeptide Y on the guinea pig uterine artery. *Eur. J. Pharmacol.* **203**:275-81.

Motulsky, H.J., and M.C. Michel. (1988). Neuropeptide Y mobilizes Ca^{2+} and inhibits adenylate cyclase in human erythroleukemia cells. *Am. J. Physiol.* **255**:E880-E885.

Myers, A.K., M.Y. Farhat, G.H. Shen, W. Debinski, C. Wahlestedt, and Z. Zukowska-Grojec. (1990). Platelets as a source and site of action of neuropeptide Y. *Ann. NY. Acad. Sci.* **611**:408-11.

Nastainczyk, N., A. Rohrkasten, M. Sieber, and F. Hofmann. (1987). Phosphorylation of the purified receptor for calcium channel blockers by cAMP kinase and protein kinase C. *Eur. J. Biochem.* **169**:137-142.

Neild, T.O. (1987). Action of neuropeptide Y on innervated and denervated rat tail arteries. *J. Physiol (Lond).* **386**:19-30.

Nilsson, S.F.E. (1991). Neuropeptide Y (NPY): vasoconstrictor in the eye, brain and other tissue in the rabbit. *Acta. Physiol. Scand.* **141**:455-467.

Nishibe, S., M.I. Wahl, P.B. Wedegaertner, J.J. Kim, S.G. Rhee, and G. Carpenter. (1990). Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* **87**:424-428.

Noack, T., G. Edwards, P. Deitmer, and A.H. Weston. (1992). Potassium channel modulation in rat portal vein by ATP depletion: a comparison with the effects of levcromaklim (BRL 38227). *Br. J. Pharmacol.* **107**:945-955.

Noble, D. (1984). The surprising heart: A review of recent progress in cardiac electrophysiology. *J. Physiol.* **353**:156-159.

Noma, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature* **305**:147-148.

Nowycky, M.C., A.P. Fox, and R.W. Tsien. (1985a). Three types of neuronal calcium channels with different calcium agonist sensitivity. *Nature* **316**:440-443.

Nowycky, M.C., A.P. Fox, and R.W. Tsien. (1985b). Long-openig mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay

K8644. *Proc. Natl. Acad. Sci. USA.* **82**:2178-82.

Ohya, Y., K. Terada, K. Kitamura, and H. Kuriyama. (1986). Membrane currents recorded from a fragment of rabbit intestinal smooth muscle cells. *Am. J. Physiol.* **251**:C335-C346.

Okabe, K., K. Kitamura, and H. Kuriyama. (1987). Features of 4-aminopyridine sensitive outward current observed in single smooth muscle cells from the rabbit pulmonary artery. *Pflugers Arch.* **409**:561-568.

Oshita, M., S. Kigoshi, and I. Muramatsu. (1989). Selective potentiation of extracellular Ca^{2+}-dependent contraction by neuropeptide Y in rabbit mesenteric arteries. *Gen. Pharmacol.* **20**:363-367.

Pacaud, P., G. Loirand, J.L. Lavie, C. Mironneau, and J. Mironneau. (1989). Calcium activated chloride current in rat vascular smooth muscle cells in short term primary culture. *Pflugers Arch.* **413**:629-636.

Pammer, C., T. Gorcs, and M. Palkovits. (1990). Peptidergic innervation of the locus coeruleus cells in the human brain. *Brain Res.* **515**:247-255.

Pataky, D.M., S.B. Curtis, and A.M. Buchan. (1990). The colocalization of

neuropeptides in the submucosa of the small intestine of normal Wistar and non-diabetic BB rats. *Neurosci.* **36**:247-254.

Pavenstadt, H., S. Lindeman, V. Lindeman, M. Spath, K. Kunzelmann, and R. Greger. (1991). Potassium conductance of smooth muscle cells from rabbit aorta in primary culture. *Pflugers Arch.* **419**:57-68.

Pelzer, S., Y.M. Shuba, T. Asai, J. Codina, L. Birnbaumer, T.F. McDonald, and D. Pelzer. (1990). Membrane-delimited stimulation of heart cell calcium current by ß-adrenergic signal-transducing G_s protein. *Am. J. Physiol.* **259**:H264-H267.

Penner, R., M. Peterson, F.K. Pierau, and F. Dreyer. (1986). Dendrotoxin: a selective blocker of a non-inactivating potassium current in guinea pig dorsal ganglion neurons. *Pflugers Arch.* **407**:365-369.

Perney, T.M., and R.J. Miller. (1989). Two different G-proteins mediate neuropeptide Y and bradykinin-stimulated phospholipid breakdown in cultured rat sensory neurons. *J. Bio. Chem.* **264**:7317-7327.

Pernow, J., J.M. Lundberg, and K.L. Kaijser. (1987). Vasoconstrictor effects in vivo and plasm disappearance rate of neuropeptide Y in man. *Life Sci.* **40**:47-54.

Pernow, J., and J.M. Lundberg. (1988). Neuropeptide Y induces potent contraction of arterial vascular smooth muscle via an endothelium-independent mechanism. *Acta. Physiol. Scand.* **134**:157-158.

Pernow, J. (1989). Actions of constrictor (NPY and endothelin) and dilator (substance P, CGRP and VIP) peptides on pig splenic and human skeletal muscle arteries: involvement of the endothelium. *Br. J. Pharmacol.* **97(3)**:983-989.

Pernow, J., A. Saria, and J.M. Lundberg. (1986). Mechanisms underlying pre- and postjunctional effects of neuropeptide Y in sympathetic vascular control. *Acta. Physiol. Scand.* **126**:239-249.

Piper, H.M., S.C. Millar, and B.J. McDermott. (1989). The negative ionotropic effect of neuropeptide Y on the ventricular cardiomyocyte. *Nauyn-Schmiedebergs Arch. Pharmacol.* **340**:333-337.

Plummer, M.R., A. Rittenhouse, M. Kanevsky, and P. Hess. (1991). Neurotransmitter modulation of calcium channels in rat sympathetic neurons. *J. Neurosci.* **11(8)**:2239-2248.

Polte, T.R., A.J. Naftilan, and S.K. Hanks. (1994). Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotension II. *J. Cell Biochem.* **55**:106-119.

Quayle, J.M., and N.B. Standen. (1994). K_{ATP} channels in vascular smooth muscle. *Cardiovas. Res.* **28**:797-804.

Quayle, J.M., A.D. Bonev, J.E. Brayden, and M.T. Nelson. (1994). Calcitonin gene-related peptide activates ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase A. *J. Physiol.* **475**:9-13.

Rendu, F., A. Eldor, F. Grelac, C. Bachelot, A. Gazit, C. Gilon, S. Levy-Toledand, and A. Levitzki. (1992). Inhibition of platelet activation by tyrosine kinase inhibitors. *Biochem. Pharmacol.* **44**:881-888.

Resh, M.D. (1994). Myristylation and palmitylation of src family members: the fats of the matter. *Cell* **76**:411-413.

Reuss, S., E.C. Hurlbut, J.C. Speh, and R.Y. Moore. (1990). Neuropeptide localization in telencephalic and diencephalic structures of the ground squirrel brain. *Am. J. Anat.* **188(2)**:163-174.

Reuter, H., C.F. Stevens, R.W. Tsien, and G. Yellen. (1982). Properties of single calcium channels in cultured cardiac cells. *Nature* **297**:501-504.

Revington, M.L., and D.I. McCloskey. (1990). Sympathetic-parasympathetic interactions

at the heart, possible involving neuropeptide Y, in anaesthetized dogs. *J. Physiol.* **428**:359-370.

Reynolds, E.E., and S. Yokota. (1988). Neuropeptide Y receptor-effector coupling mechanisms in cultured vascular smooth muscle cells. *Biochem. Biophy. Res. Comm.* **151**:919-925.

Ribalet, B., S. Ciani, and G.T. Eddlestone. (1989). ATP mediates both activation and inhibition of K_{ATP} channel activity via cAMP-dependent protein kinase in insulin-secreting cell lines. *J. Gen. Physiol.* **94**:693-717.

Rigel, D.F., I.L. Grupp, A. Balasubruamaniam, and G. Grupp. (1989). Contractile effects of cardiac neuropeptides in isolated canine atrial and ventricular muscles. *Am. J. Physiol.* **257**:H1082-1087.

Rimland, J., W. Xin, P. Sweetnam, K. Saijoh, E.J. Nestler, and R.S. Duman. (1991). Sequence and expression of a neuropeptide Y receptor cDNA. *Mol. Pharmacol.* **40**:869-875.

Robitaille, R., M.L. Garcia, G.J. Kaczorowski, and M.P. Charlton. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* **11**:645-655.

Rocha-Singh, K., N. Honbo, and J.S. Joel. (1988). Neuropeptide Y modulates stimulatory and inhibitory adenylate cyclase-linked pathways in cultured neonatal rat ventricular myoctes. *Circulation* **78** (suppl II): 559.

Rodbell, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**:17-22.

Rohrkasten, A., H. Meyer, W. Nastainczyk, M. Sieler, and F. Hofmann. (1988). cAMP-dependent protein kinase rapidly phosphorylates Serine-687 of the skeletal muscle receptor for calcium channel blockers. *J. Bio. Chem.* **263**:15325-15329.

Romanin, C., P. Grosswagen, and H. Schindler. (1991). Calpastatin and nucleotides stabilize cardiac calcium channel activity in excised patches. *Pflugers Arch.* **418**:86-92.

Romey, G., and M. Lazdunski. (1984). The coexistence in rat muscle cells of two distinct classes of Ca^{2+}-dependent K^{+} channels with different pharmacological properties and different physiological functions. *Biochem. Biophy. Res. Comm.* **118**:669-674.

Rudehill, A., A. Sollevi, A. Franco-Cereceda, and J.M. Lundberg. (1986). Neuropeptide Y (NPY) and the pig heart: release and coronary vasoconstrictor effects. *Peptides* **7**:821-826.

Ruth, P., A. Rohrkasten, M. Biel, E. Bosse, S. Regulla, H.E. Meyer, V. Flockerzi, and F. Hofmann. (1989). Primary structure of the ß subunit of the dihydropyridine snsitive calcium channel from skeletal muscle. *Science* **245**:1115-1118.

Sadoshima, J., N. Akaike, H. Kanaide, and M. Nakamura. (1988). Cycli AMP modulates Ca-activated K channel in culturesd smooth muscle cells of rat aortas. *Am. J. Physiol.* **255**:H754-H759.

Salin, P., L. Kerkerian, and A. Nieoullon. (1990). Expression of neuropeptide Y immunoreactivity in the rat nucleus accumens is under the influence of the dopaminergic mesencephalic pathway. Exp. *Brain Res.* **81(2)**:363-371.

Sauro, M., and B. Thomas. (1993). Tyrphostin attenuates platelet-derived growth factor-induced contraction in aortic smooth muscle through inhibition of protein tyrosine kinase(s). *J. Pharmacol. Exp. Ther.* **267**:1119-1125.

Saxena, V.K., P.P. de Deyn, A.A. Schoups, E.P. Coen, and W.P. de Potter. (1989). Relationship between external calcium concentration and noradrenaline- and neuropeptide Y-evoked release from perfused dog spleen. *Brain Res.* **486**:310-315.

Schofield, G.G., and S.R. Ikeda. (1988). Neuropeptide Y blocks a calcium currents in C cells of bullfrog sympathetic ganglia. *Eur. J. Pharmacol.* **151**:131-134.

Schwartz, T.W., J. Fuhlendorff, L.L. Kjems, M.S. Kristensen, M. Vervelede, M. O'Hare, J.L. Krstenansky, and B. Bjurnholm. (1990). Signal epitopes in the three-dimensional structure of neuropeptide Y. Interaction with Y1, Y2, and pancreatic polypeptide receptors. *Ann. NY. Acad. Sci.* **611**:35-47.

Scornik, F.S., and L. Toro. (1992). U46619, a thromboxane A2 agonist, inhibits K_{Ca} channel activity from pig coronary artery. *Am. J. Physiol.* **262**:C708-C713.

Sculptoreanu, A., T. Scheuer, and W. Catterall. (1993). Voltage-dependent potentiation of L-type Ca^{2+} channels due to phosphorylation by cAMP-dependent protein kinase. *Nature* **364**:240-243.

Shatti, S.J., and J.S. Brugge. (1991). Protein tyrosine phosphorylation and the adhesive functions of platelets. *Current Opin. Cell Biol.* 3:869-879.

Sheriff, S., D.F. Rigel, J.E. Fischer, and A. Balasubramaniam. (1990). Interaction of ^{125}I-neuropeptide Y with cardiac membrane. *Neuropeptides* **15(3)**:157-60.

Shigeri, Y., ang M. Fujimoto. (1994). Y2 receptors for neuropeptide Y are coupled to three intracellular signal transduction pathways in a human neuroblastoma. *J. Bio. Chem.* **269**:8842-8848.

Siegelbaum, S. (1994). Ion channel control by tyrosine phosphorylation. *Current Opin. Biol.* **4**:242.

Singer, D., M. Biel, I. Lotan, V. Flockerzi, F. Hofmann, and N. Dascal. (1991). The role of the subunits in the function of the calcium channel. *Science* **253**:1553-1557.

Small, D.L., B.J. Bolzon, and D.W. Cheung. (1992). Endothelium-independent potentiating effects of neuropeptide Y in the rat tail artery. *Eur. J. Pharmacol.* **210**:131-136.

Spedding, M., and R. Paoletti. (1992). Classification of calcium channels and calcium antagonists: progress report. *Cardiovas. Drugs Ther.* **6**:35-39.

Sperelakis, N., and Y. Ohya. (1990). Cyclic nucleotide regulation of Ca^{2+} slow channels and neurotransmitter release in vascular muscle. *Prog. Clin. Biol. Res.* **327**:277-298.

Sperelakis, N., and Y. Ohya. (1991). Regulation of calcium slow channels in vascular smooth muscle cells, in: Sperelakis N, Kuriyama H (eds): *Ion channels of vascular smooth muscle cells and endothelial cells.* Elsevier, New York, pp 27-38.

Standen, N.B., J.M. Quayle, N.W. Davies, J.E. Brayden, Y. Huang, and M.T. Nelson. (1990). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial

smooth muscle. *Science* **245**:177-180.

Sternini, C., and N. Bercha. (1985). Distribution and colocalization of neuropeptide Y and tyrosine hydroxylase-like immunoreactivity in the guinea pig heart. *Cell & Tissue Res.* **241**:93-102.

Stjernquist, M., P. Emson, C. Owman, N.O. Sjoberg, and F. Sundler. (1983). Neuropeptide Y in the female reproductive trace of the rat. Distribution of nerve fibres and motor effects. *Neurosci. Lett.* **39**:279-284.

Sturek, M., and K. Hermsmeyer. (1986). Calcium and sodium channels in spontaneously contracting vascular smooth muscle cells. *Science* **233**:475-478.

Sundler, F., E. Moghimzadeh, R. Hakanson, M. Ekelund, and P. Emson. (1983). Nerve fibres in the gut and pancreas of the rat displaying neuropeptide Y immunoreactivity. Intrinsic and extrinsic origin. *Cell & Tissue Res.* **230**:487-493.

Suzuki, N., J.E. Hardebo, J. Kahrstrom, and C. Owman. (1990). Neuropeptide Y co-exists with vasoactive intestinal polypeptide and acetylcholine in parasympathetic cerebrovascular nerves originating in the sphenopalatine, otic, and internal carotid ganglia of the rat. *Neurosci.* **36**:507-519.

Suzuki, Y., M. Shibuya, I. Ikegaki, S.I. Satoh, M. Takayasu, and T. Asano. (1988). Effects of neuropeptide Y on canine cerebral circulation. *Eur. J. Pharmacol.* **146**:271-277.

Taniguchi, J., K.I. Furukawa, M. Shigekawa. (1993). Maxi K^+ channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscle cells. *Pflugers Arch.* **423**:167-172.

Tatemoto, K. (1982). Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. USA* **79 (18)**:5485-5489.

Tatemoto, K., M. Carlquist, and V. Mutt. (1982). Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **296**:659-660.

Tessel, R.D., D.W. Miller, G.A. Misse, D. Xu, and M.B. Doughty. (1993). Characterization of vascular postsynaptic neuropeptide Y receptor function and regulation. 1. NPY-induced constriction in isolated rat femoral artery ring is mediated by both Y1 and Y2 receptors:evidence from benextramine protection studies. *J. Pharmacol. Exp. Ther.* **265**:172-177.

Tiaho, F., S. Richard, P. lory, J.M. Nerbonne, and J. Nargeot. (1990). Cyclic-AMP-

dependent phosphorylation modulates the stereospecific activation of cardiac Ca channels by Bay K 8644. *Pflugers Arch.* 417:58-66.

Tohse, N. (1990). Calcium sensitive delayed rectifier potassium current in guinea pig ventricular cells. *Am. J. Physiol.* **258(22)**:H1200-H1207.

Toro, L., and E. Stefani. (1987). Ca^{2+} and K^{+} currents in cultured vascular smooth muscle. *Pflugers Arch.* **408**:417-419.

Toro, L., and F. Scornik. (1991). Modulation of Ca-activated K channels from coronary smooth muscle. in *Ion Channel of Vascular Smooth Muscle Cells and Endothelial Cells.* Editted by Sperelakis and Kuriyama. Elsevier Publishing Company Inc. p.111-124.

Toro, L., M. Amador, and E. Stefani (1990). ANG II inhibits calcium-activated potassium channels from coronary smooth muscle in lipid bilayers. *Am. J. Physiol.* **258**:H912-H915.

Toth, P.T., V.P. Bindokas, D. Bleakman, W.F. Colmers, and R.J. Miller. (1993). Mechanism of presynaptic inhibition by neupeptide Y at sympathetic nerve terminals. *Nature* **364**:634-638.

Trautwein, W., A. Cavalie, V. Flockerzi, F. Hoffmann, and Pelzer, D. (1987).

Modulation of calcium channel function by phosphorylation in guinea-pig ventricular cells and phosphorylation bilayer membrane. *Circ. Res.* **61(suppl.1)**:17-23.

Trautwein, W, and J. Hescheler. (1990). Regulation of cardiac L-type calcium current by phosphorylation and G-proteins. *Ann. Rev. Physiol.* 52:257-274.

Tschopl, M., R.C. Miller, J. Pelton, J.C. Stoclet and B. Bucher. (1993). Vasoconstrictor effects of various neuropeptide Y analogues on the rat tail artery in the presence of phenylephrine. *Br. J. Pharmacol.* 110:1098-1104.

Tseng, G.J., D. Robertson, T. Light, J.R. Atkinson, and R.M. Robertson. (1988). Neuropeptide Y is a vasoconstrictor of human coronary arteries. *Am. J. Med. Sci.* **296**:11-16.

Tsien, R.W., D. Lipscombe, D.V. Madison, K.R. Bley, and A.P. Fox. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* 11:431-437.

Tsuda, T., Y. Kawahara, K. Shii, M. Koide, Y. Ishida, and M. Yokoyama. (1991). Vasoconstrictor-induced protein-tyrosine phosphorylation in cultured vascular smooth muscle cells. *FEBS.* 285:44-48.

Tuana, B.S., B.J. Murphy, and Y. Qu. (1988). The purified Ca^{2+} antagonist receptor from skeletal muscle:subunit composition, photoaffinity labelling and endogenous protein kinase activity. *Mol. Cell. Biochem.* **80**:133-143.

Tuana, B.S., and B.J. Murphy. (1990). Biochemical analysis of L-type calcium channels from skeletal and cardiac muscle. *Can. J. Physiol. Pharmacol.* **68**:1482-1488.

Tuor, U.I., P. Kelly, L. Edvinsson, and J. McCulloch. (1990). Neuropeptide Y and the cerebral circulation. *J. Cereb. Blood Flow. Metab.* **10**:591-601.

Twitchell, W.A., and S.G. Rane. (1993). Opioid peptide modulation of Ca^{2+}-dependent K^+ And voltage-activated Ca^{2+} currents in bovine adrenal chromaffin cells. *Neuron* **10**:701-709.

Uddman, R., E. Ekblad, L. Edvinsson, and F. Sunder. (1985). Neuropeptide-like immunoreactivity in perivascular nerves of guinea-pig. *Regul. Peptides* **10**:243--57.

Ullrich, A., and J. Schlessinger. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**:203-212.

Vallejo, M., and S.L. Lightman. (1986). Pressor effects of centrally administered neuropeptide Y in rats: role of sympathetic nervous system and vasopressin. *Life Sci.*

18:1859-1866.

Van Dongen, A.M.J., J. Codina, J. Olate, R. Mattera, and O.R. Joh. (1988). Newly identified brain potassium channels gated by the guanine nucleotide binding protein Go. *Scince* 242:1433-1437.

Van Helden, D.F. (1988). An α-adrenoceptor-mediated chloride conductance in mesenteric veins of the guinea-pig. *J. Physiol.* 401:489-501.

Varadi, G., P. Lory, D. Schultz, M. Varadi, and A. Schwartz. (1991). Acceleration of activation by the ß subunit of the skeletal muscle calciun channel. *Nature* 352:159-162.

Volk, K.A., J.J. Matsuda, and E.F. Shibata. (1991). A voltage-dependent potassium current in rabbit coronary artery smooth muscle cells. *J. Physiol.* 439:751-768.

Wahlestedt, C., L. Edvinsson, E. Ekblad, and R. Hakanson. (1987). Neuropeptide Y potentiates noradrenaline evoked vasoconstriction: mode of action. *J. Pharmacol. Exp. Ther.* 234:735-741.

Wahlestedt, C., L. Grundemar, R. Hakanson, M. Heilig, G.H. Shen, Z. Zukowska-Grojec, and D.J. Reis. (1990). Neuropeptide Y receptor subtypes, Y1 and Y2. *Ann. NY. Acad. Sci.* 611:7-26.

Wahlestedt, C., S. Regunathan. and D.J. Reis. (1992). Identification of cultured cells selectively expressing Y1, Y2, or Y3 type receptors for neuropeptide Y/ peptide YY. *Life Sci.* **50**:PL7-PL12.

Walker, P., E. Grouzmann, M. Burnier, B. Waeber. (1991). The role of neuropeptide Y in cardiovascular regulation. *Trends Pharmacol. sci.* 12:111-115.

Walsh, K., and R.S. Kass. (1988). Regulation of a heart potassium channel by protein kinase A and C. Science 242:67-69.

Wang, Q., and W.A. Large. (1991). Modulation of noradrenaline-induced membrane currents by papaverine in rabbit vascular smooth muscle cells. *J. Physiol.* **439**:501-512.

Wei, X., E. Perez-Reyes, A.E. Lacerda, G. Schuster, A.M. Brown, and L. Birnbaumer. (1991). Heterologous regulation of the cardiac Ca^{2+} channel alpha 1 subunit by skeletal muscle beta and gamma subunits. Implications for the structure of cardiac L-type Ca^{2+} channels. *J. Bio. Chem.* **266**:21943-21947.

Westfall, T.C., S. Carpentier, X. Chen, M.C. Beinfeld, L. Naes, and M.J. Meldrum. (1987). Prejunctional and postjunctional effects of neuropeptide Y at the noradrenergic neuroeffector junction of the perfused mesenteric artery bed of the rat. *J. Cardiovasc. Pharmacol.* **10**:716-22.

Westfall, T.C., J. Martin, X.L. Chen, A. Ciarleglio, S. Carpentier, K. Henderson, M. Knuepfer, M. Beinfeld, and L. Naes. (1988). Cardiovascular effects and modulation of noradrenergic neurotransmission following central and peripheral administration of neuropeptide Y. *Synapse* 2:299-307.

Westlind-Danielsson, A., S. Andell, S. Abens, and T. Bartfai. (1988). Neuropeptide Y and peptide YY inhibit adenylate cyclase activity in the rat striatum. *Acta. Physiol. Scand.* 132:425-430.

Wharton,J., and S. Gulbenkian. (1989). Peptides in the mammlian cardiovascular system. *Experentia* 56:292-316.

Wharton, J., J.M. Polak, and L. Gordon, N.R. Banner, D.R. Springall, M. Rose, A. Khagani, J. Wallwork, and M.H. Yacoub. (1990). Immunohistochemical demonstration of human cardiac innervation before and after transplantation. *Circ. Res.* 66:900-12.

White, R.E., A. Schonbrunn, and D.L. Armnstrong. (1991). Somatostatin stimulates Ca^{2+}-activated K^{+} channels through protein dephosphorylation. *Nature* 351:570-573.

White, R.E., A.B. Lee, A.D. Shcherbatko, T.M. Lincoln, A. Schonbrunn,and D.L. Armnstrong. (1993). Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature* 361:263-266.

Wijetunge, S., C. Aalkjaer, M. Schachter, and A. Hughes. (1992). Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. *Biochem. Biophy. Res. Commun.* 189:1620-1625.

Wiley, J.W., R.A. Gross, Y. Lu, and R.L. MacDonald. (1990). Neuropeptide Y reduces calcium current and inhibits acetylcholine release in nodose neurons via a pertussis toxin-sensitive mechanism. *J. Neurophysiol.* 63:1499-1507.

Williams, D.L., G.M. Katz, L.R. Contancin, and J.P. Reuben. (1988). Guanosine 5'-monophosphate modulates gating of high conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. *Proc. Natl. Acad. Sci.* USA. 85:9360-9364.

Wilson, G., and L. Kaczmarek. (1993). Mode-switching of a voltage-gated cation channel is mediated by a protein kinase A regulated tyrosine phosphatase. *Nature* 366:433-436.

Xiong, Z., B.J. Bolzon, and D.W. Cheung. (1993). Neuropeptide Y potentiates calcium-channel currents in single vascular smooth muscle cells. *Pflugers Arch* 423:504-510.

Xiong, Z.L., N. Sperelakis, C. Fenoglio-Preiser. (1994a). Regulation of L-type calcium channels by cyclic nucleotides and phosphorylation in smooth muscle cells from rabbit portal vein. *J. Vasc. Res.* 31:271-279.

Xiong, Z.L., N. Sperelakis, C. Fenoglio-Preiser. (1994b). Isoproterenol modulates the calcium channels through two different mechanisms in smooth muscle cells from rabbit portal vein. *Pflugers Arch.* **428**:105-113.

Yamashita, N., I. Kojima, N. Shibuya, and E. Ogata. (1987). Pertussis toxin inhibits somatostatin-induced K^+ conductance in human pituitary cells. *Am. J. Physiol.* **253**:E28-32.

Yaish, P., A. Gazit, C. Gilon, and A. Levitzki. (1988). Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science* **242**:933-935.

Yang, S.G., M. Saifddine, and M.D. Hollenberg. (1992). Tyrosine kinase inhibitors and the contractile action of epidermal growth factor-urogastrone and other agonists in gastric smooth muscle. *Can. J. Physiol. Pharmacol.* **70**:85-93.

Yang, S.G., M. Saifeddine, A. Laniyonu, and M.D. Hollenberg. (1993). Distinct signal transduction pathway for angiotension II in guinea-pig gastric smooth muscle: differential blockade by indomethacin and tyrosine kinase inhibitors. *J. Pharm. Exp. Ther.* **264**:958-966.

Yatani, A., C.L. Seidel, J. Allen and A.M. Brown. (1987). Whole-cell and single channel currents of isolated smooth muscle cells from saphenous vein. *Circ. Res.* **60**:523-

533.

Yokoyama, R., T. Inokuchi, H. Satoh, T. Kusaba, K. Yamamoto, and K. Ando. (1990). Distribution of tyrosine hydroxylase (TH)-like, neuropeptide Y (NPY)-like immunoreactivity and acetylcholinesterase (AchE)-positive nerve fibres in the prostate gland of the monkey (Macacus fuscatus). *Kurume Med. J.* **37(1)**:1-8.

Zhu, J., W. Li, M.L. Toews, and T.D. Hexum. (1992). Neuropeptide Y inhibits forskolin-stimulated adenylate cyclase in bovine adrenal chromaffin cells via a pertussis toxin-sensitive process. *J. Pharmacol. Exp. Ther.* **263**:1479-1486.

Zidichouski, J.A., H. Chen, and P.A. Smith. (1990). Neuropeptide Y activates inwardly-rectifying K^+-channels in C-cells of amphibian sympathetic ganglia. *Neurosci. Lett.* **117**:123-128.

Zukowska-Grojec, Z., E.S. Marks, and M. Haass. (1987). Neuropeptide Y is a potent vasoconstrictor and cardiodepressant in rat. *Am. J. Physiol.* **253**:H1234-H1239.