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UNIVERSITY OF ALBERTA

CHARACTERIZATION OF THE DELAYED RECTIFIER K CHANNEL IN RAT TAIL ARTERY VASCULAR SMOOTH MUSCLE CELLS

BY

JUN REN ©

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in partial fulfilment of the degree of

Doctor of Philosophy

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

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DEDICATION

To

My parents

De-ji Ren and Yu-kun Ma

and

My wife Bonnie H. L. Ren-Zhao

My lovely baby who is on the way

Without Whose Love and Understanding

This Thesis Would Not Have Been Written

ABSTRACT

A voltage-dependent, delayed rectifier K current (I_k) was characterized in both primary and subcultured vascular smooth muscle cells (VSMCs) from rat tail artery. The current was activated at around -40 mV and showed almost no inactivation. It was inhibited by TEA and was resistant to inhibition by 4-aminopyridine, charybdotoxin, glibenclamide and α-dendrotoxin. Neither a Ca^{2+}-activated component nor a current through Cl^- channel contributed to the outward current. Analysis also showed that K is the major ion responsible for the outward current. β-dendrotoxin inhibited I_k in both primary and subcultured VSMCs, and the inhibition was concentration-dependent. The inhibition was not dependent on either the membrane potential or the physical occupancy of the channel pore.

Under current-clamp conditions, PGE_2 depolarized the membrane of primary cultured VSMCs. Under voltage-clamp, PGE_2 suppressed I_k in a concentration-dependent manner. This effect was a receptor-mediated process inhibited by the PGE_2 receptor blocker meclofenamate. A cholera toxin-sensitive and/or pertussis toxin-insensitive G protein was responsible for the postreceptor activation of the subcellular signal transduction mechanism. Pharmacological studies on cAMP-PKA and PKC cascades were performed. Pretreatment of the cells with Rp-cAMPs, an inhibitor of PKA, almost completely abolished PGE_2-induced inhibition of I_k. Forskolin, Db-cAMP and Sp-cAMPs, activators of adenylate cyclase or PKA, mimicked the effect of PGE_2 on I_k. Maximal inhibition of the phosphodiesterase did not prevent the PGE_2-induced inhibition of I_k. Tetradecanoyl phorbol acetate (TPA), a PKC activator, also significantly suppressed I_k.

The kinase inhibitor staurosporine and prolonged exposure to TPA to down-regulate PKC, eliminated the PGE_2-induced inhibition of I_k, but had no effect on the forskolin, Db-cAMP and Sp-cAMPs-induced effect on I_k. Pretreatment of the cells with Rp-cAMPs did not significantly affect the degree of inhibition of I_k evoked by TPA. Measurement of cAMP showed that both PGE_2 and TPA produced cAMP accumulation. It was thus concluded that the modulation of I_k by PGE_2 in rat tail artery VSMCs might involve cAMP-PKA and possibly PKC pathways. Further studies have shown that PGE_2 inhibited the L-type Ca^{2+} current. Intracellular Ca^{2+} measurement with Fura-2 revealed that PGE_2 did not affect the basal $[Ca^{2+}]_i$, but potentiated the KCl-stimulated increase in $[Ca^{2+}]_i$. PGE_2 also evoked a concentration-dependent contractile response in rat tail artery helical strips in the presence of extracellular Ca^{2+}. The contractile response to PGE_2 was blocked by the kinase inhibitors staurosporine and H-7, and the PKC activators TPA and phorbol dibutyrate mimicked the contractile response caused by PGE_2. PKA inhibition with either Rp-cAMPs or HA1004 did not affect PGE_2-induced tension development. The Ca^{2+} channel antagonist nifedipine eliminated the contractile action of PGE_2. These results suggest that PGE_2 induced muscle contraction through the activation of PKC and the influx of extracellular Ca^{2+}.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
ACh	Acetylcholine
4-AP	4-aminopyridine
ATP	Adenosine 5'-triphosphate
Ba^{2+}	Barium
BK	Big-conductance Ca^{2+}-activated K chanel
BSA	Bovine serum albumin
BP	Blood pressure
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
cAMP	Cyclic adenosine 3',5'-monophosphate
cGMP	Cyclic guanosine 3',5'-monophosphate
Cl^-	Chloride
CNS	Central nervous system
CO	Cyclooxygenase
Cs	Cesium
CTX	Cholera toxin
Db-cAMP	Dibutyl adenosine 3',5'-cyclic monophosphate
DG	Diacylglycerol
DNA	Deoxyribonucleic acid
DTX	Dendrotoxin
DHP	Dihydropyridine
Dic8	sn-dioctanoylglycerol
DIDS	4,4'-Diisothiocyano-2,2'-disulfonic acid stilbene
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethylsulfoxide

EC_{50}	Median effective concentration
EGTA	Ethylene glycol bis(β-aminoethyl ether)-N,N'- tetraacetic acid
E_k	K equilibrium potential
EP	E-series prostaglandins receptor
F_{340}	Fluorescence excited at wavelength of 340 nm
F_{380}	Fluorescence excited at wavelength of 380 nm
FBS	Fetal bovine serum
Fura-2/AM	Fura-2/acetoxymethyl ester
G Protein	Guanine nucleotide binding protein
GDP	Guanosine 5'-diphosphate
GDPβS	Guanosine 5'-[β-thio] diphosphate
G_i	Inhibitory guanosine triphosphate binding protein
G_o	Other (unidentified) guanosine triphosphate binding protein
G_s	Stimulatory guanosine triphosphate binding protein
GTP	Guanosine 5'-triphosphate
H-7	1-(5-isoquinolinylsulfonyl)-2-methypiperazine
HA1004	N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxy-ethyl]-piperazine-N'-[2-ethanesulfonic acid]
12-HETE	12-Hydroxy-5,8,10,14-eicosatetraenoic acid
HPETES	Hydroperoxyeicosatetraenoic acids
HPLC	High performance liquid chromatography
5-HT	Serotonin
I_A	Transient outward K channel
I_{IR}	Inward rectifier K channel
I_k	Delayed rectifier K channel
IK	Intermediate-conductance Ca^{2+}-activated K channel
IP_3	Inositol trisphosphate
IP_4	Inositol tetrakisphosphate

I-V	Current-voltage
IBMX	3-isobutyl-1-methylxanthine
K	Potassium
K_{ACh}	ACh-gated K channel
K_{ATP}	ATP-gated K channel
K_d	Equilibrium dissociation constant
LT	Leukotriene
MCD	Mast cell degranulating (peptide)
NE	Norepinephrine
MCLK	Myosin light chain kinase
PDB	4β-Phorbol 12,13-dibutyrate
PDE	Phosphodiesterase
PG(s)	Prostaglandin(s)
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PI	Phosphatidyl inositol
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PNS	Penicillin, neomycin and streptomycin
PSS	Physiological salt solution
PTX	Pertussis toxin
R_{in}	Input resistance
R_{max}	Maximal ratio of fluorescence
R_{min}	Minimal ratio of fluorescence
R_p-cAMPs	Adenosine 3',5'-cyclic monophosphothioate R_p-isomer

RIA	Radioimmunoassay
SD	Sprague-Dawley
S_p-cAMPs	Adenosine 3',5'-cyclic monophosphothioate S_p-isomer
SHR	Spontaneous hypertensive rat
SITS	4-Acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene
SK	Small-conductance Ca^{2+}-activated K channel
SR	Sarcoplasmic reticulum
TEA	Tetraethyl ammonium chloride
TPA	Tetradecanoyl phorbol acetate
TTX	Tetrodotoxin
TX	Thromboxane
V_h	Membrane potential where half of the channels are activated/inactivated
V_m	Resting membrane potential
VSMCs	Vascular smooth muscle cells
WKY	Wistar Kyoto

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

I. Potassium (K) channels

A. Introduction

Blood vessels display a certain degree of the vascular smooth muscle tone that controls the blood flow to specific vascular beds. Regional changes in the vascular smooth muscle tone affect not only the local blood flow but also the systemic blood pressure (BP). Various factors influence a given level of the vascular smooth muscle tone. These factors may range widely from the intrinsic electrical properties of the plasma membrane of the vascular smooth muscle cells (VSMCs) to the extrinsic actions of the circulating hormones or local neurotransmitters (Longmore and Weston, 1990). Recently, attention has been focused on the role of potassium (K) channels in the modulation of the vascular smooth muscle tone. It has been well established that changes in the K conductance play an important role in the modulation of cellular excitability. Closure of the K channels prolongs the action potential duration and makes the cell more excitable in heart (Noble, 1984), central nervous system (CNS) (North, 1989) and vascular smooth muscles (Bulbring and Tomita, 1987; Standen *et al.*, 1989), whereas activation of the K channels causes the plasma membrane to repolarize or hyperpolarize, and thus, reduces the cellular excitability.

The roles of various K channels in the different cells or within the different

regions of the same cell in the control of the patterns of cellular excitability have been well characterized in neurones (Hille, 1984). In the past decade, more progress has been achieved towards the understanding of the detailed functions of the K channels in the heart (Hume *et al.*, 1990; Arena *et al.*, 1990; Yuan *et al.*, 1990a) and vascular smooth muscles (Kirber *et al.*, 1990; Longmore and Weston, 1990; Kirber *et al.*, 1992; Beech *et al.*, 1993; Kitamura and Kamouchi, 1993; Minami *et al.*, 1993a; 1993b). These have been largely credited to the improvement of the macroscopic physiological methods, particularly the emergence of the patch-clamp techniques, the refinement of the biochemical purification methods, the introduction of the lipid bilayer ion channel approaches, and the sophistication of the molecular biology assays. These approaches have greatly contributed to the development of our knowledge of the structure-function relationship of the K channels at the cellular level.

K channels are the most ubiquitous among all the ion channels found in plasma membranes (Hille, 1984; Latorre *et al.*, 1984; Cook, 1988; Rudy, 1988). Knowledge of the biochemistry, physiology, and pharmacology of K channels has exploded over the past few years. K channels are present in both excitable and non-excitable cells and play pivotal roles in membrane functions, by virtue of the fact that they establish the resting membrane potential, repolarize and hyperpolarize the cells after action potentials, and modulate repetitive firing. As a result, the K channels play an important role in regulating the level of cellular excitability. Regulation of the K channels, often voltage-gated, may be involved in not only normal cellular homeostasis but also higher functions

such as behaviour and learning (Kandel *et al.*, 1983). In particular, the K channel conductance may also be modulated by hormones, neurotransmitters and second messengers. Various kinds of K channels may be expressed in different cells, or even in different regions of the same cell. It is such diversity and prevalence of K channels that may contribute to their versatile physiological as well as pathological functions. A number of reviews on some specific aspects of K channels have appeared recently (Cook, 1988; Rudy, 1988; Pallotta and Wagoner, 1992). This chapter takes a broad view, focusing on the properties that may be distinctive between different types of the K channels, especially those found in VSMCs.

B. K channel classifications

All living cells maintain a K ion concentration gradient across the plasma membrane. The movement of K ions down their electrochemical gradients, through the K channels in the plasma membrane, is of fundamental importance for many cellular functions. The field of K channels has drawn the interest of many researchers over the past decades. Since Hodgkin and Huxley (1952) first described a voltage-dependent K current in the squid giant axon, K channels have developed into a large family, varying widely in kinetics, voltage-dependence, pharmacology, sensitivity to modulation and single-channel conductance (Cook, 1988; Rudy, 1988). However, the properties of the K channels in VSMCs have been much less well described than those in other excitable systems, e. g., the nervous system. This may be largely due to the fact that the cultured

VSMCs de-differentiate rapidly in culture. In addition, the harsh enzymatic treatments which are often necessary for the acute separation of the cells from the blood vessels, have also been considered to alter the genetic expression of the channel proteins (Chamley-Campbell *et al.*, 1981; Owens *et al.*, 1987; Yuan *et al.*, 1993a).

The diverse family members of the K channels can be identified and characterized on the basis of kinetic behaviour, voltage-dependence, ion-dependence, permeation specificity, ligand sensitivity, as well as pharmacological properties (Latorre and Miller, 1983). However, it has been shown that although there are a number of distinct K channel types, it is often difficult, and sometimes impossible, to assign every K channel into a particular type or class. This is primarily due to the fact that certain K channels possess the characteristics of more than one type or class, e. g., the muscarinic acetylcholine (ACh) receptor K channel (K_{ACh}) which is gated by both ACh and guanine nucleotide binding protein (G protein). Thus, caution needs to be taken when identifying a K channel type solely on the basis of a few, non-specific properties.

1. Voltage-dependent K channels

The voltage-dependent K channels comprise the largest and the most diversified family of ion channels. First described in the membranes of skeletal muscle and nerve axons, the voltage-dependent K channels are ubiquitously found in excitable as well as non-excitable cells. The voltage-dependent K channels consist of three different subclasses which can be distinguished mainly by the response to changes in the

membrane potential, and the pattern of activation as well as inactivation. This distinction was confirmed by their sensitivity to various pharmacological agents (Dubois, 1982) and toxins (Benoit and Dubois, 1986). Each subclass of the voltage-dependent K channels could be further divided into subtypes of channel, such as two subtypes of transient outward currents in *Drosophila* myotubes and neurones (Solc *et al.*, 1987). The subtypes of the voltage-dependent K channels can be characterized by the difference in the voltage-dependence and gating kinetics. Several excellent reviews on the voltage-dependent K channels have appeared recently (Jan and Jan, 1989; Adams and Nonner, 1990; Sokabe *et al.*, 1991; Pallotta and Wagoner, 1992; Pongs, 1992).

a. The delayed outward rectifier K channels (I_k)

This type of K channel was first recognized in the squid giant axon (Hodgkin and Huxley, 1952). It contributes to the macroscopic outward K currents in many cells. The channel has a typical single-channel conductance of 15-20 pS and activates following the membrane depolarization, with a distinct time delay. The current rarely exhibits inactivation while the depolarization is maintained. Its amplitude rises sigmoidally on activation and shows a slow inactivation with a $t_{1/2}$ of several hundreds of milliseconds to several seconds. Blockers of this current include cesium (Cs^+), barium (Ba^{2+}) and tetraethyl ammonium chloride (TEA). 4-aminopyridine (4-AP) is also sometimes effective. Several toxins from the snake, scorpion and bee venoms have also been documented to block the delayed rectifier K channel. These toxins include: α-dendrotoxin

(DTX) (Schauf, 1987), β-dendrotoxin (Benishin *et al.*, 1988; Ren *et al.*, 1994), dendrotoxin I (Benoit and Dubois, 86), β-bungarotoxin (Petersen *et al.*, 1986), crotoxin (Dreyer and Penner, 1987), and taipoxin (Dreyer and Penner, 1987) from snake venoms, charybdotoxin (Lewis and Cahalan, 1988; Brau *et al.*, 1990) from scorpion *Leiurus quinquestriatus* var. *hebraeus* venom, as well as mast cell degranulating (MCD) peptide (Stuhmer *et al.*, 1988) from bee venom. In smooth muscles, the delayed rectifier K channel opens during the repolarization and may function in the termination of the agonist-induced depolarization.

b. The transient outward K channels (I_A)

The second type of voltage-dependent K channel, which was originally described in molluscan neurons (Hagiwara *et al.*, 1961), produce transient outward currents, or "I_A" currents (Rogawski, 1985). This type of voltage-dependent K channel is also activated by membrane depolarization similar to the delayed rectifier K current, but decays rapidly even with the maintained depolarization. It has a typical single-channel conductance of 15-30 pS. I_A is sensitive to 4-AP, TEA and α-dendrotoxin (Halliwell *et al.*, 1986). It is thought to play a role in neurons that fire repetitively in response to tonic depolarization. In smooth muscles, a current with properties similar to that carried by I_A has been described in pulmonary artery (Ohya *et al.*, 1986), although the precise function in the smooth muscle is still largely unknown.

c. The inward rectifier K channels (I_{IR})

Previously described voltage-dependent K channels exhibit outward rectification, a property that is commonly seen for most K channels. The discovery of a K channel current which activates by membrane hyperpolarization beyond the K equilibrium potential (E_k) gave rise to the term "anomalous rectifier" K channel. This type of channel has characteristics mirroring that of the delayed rectifier. This I_{IR} channel passes large K currents in the inward, rather than outward, direction. The concentration gradient of K ion could affect the permeability, or the conductance of this type of the voltage-dependent K channel. Anomalous rectification was first reported in frog skeletal muscle (Katz, 1949). It has also been described in sea urchin eggs (Hagiwara and Jaffe, 1979), skeletal and cardiac muscles (Adrian, 1969; Noble, 1984), smooth muscles (Benham *et al.*, 1987) and neurones (North, 1989). The I_{IR} channel, which displays a typical single-channel conductance of about 20 pS, can be blocked by Cs^+, Ba^{2+} and intracellular application of TEA. This inward rectifying K channel is usually activated at around the resting membrane potential, suggesting it plays a role in maintaining the resting membrane potential.

2. Ca^{2+}-activated K channels

A second category of the K channel classification is known as the Ca^{2+}-activated K channels, including big- (BK), intermediate- (IK) and small- (SK) conductance K channels. This family of channels opens and closes following the change of intracellular

Ca^{2+} concentration ($[Ca^{2+}]_i$), although in some cases Ca^{2+}-activated K channels may also be voltage-dependent. The three groups of Ca^{2+}-activated K channels can be separated by their single channel conductances and their pharmacological properties.

a. The big-conductance Ca^{2+}-activated K channels (BK)

The big-conductance Ca^{2+}-activated K channels have been described in both excitable and non-excitable cells ranging from neurones (Meech, 1978), skeletal muscles (Miller *et al.*, 1985) to the cells of pancreas (Findlay *et al.*, 1985) and salivary glands (Maruyama *et al.*, 1983). The macroscopic current through BK channels is a typically large and long-lasting outward current (with single-channel conductance of 150-200 pS), which can be blocked by extracellular TEA, quinine, Ba^{2+}, charybdotoxin, and noxiustoxin from the venom of Mexican scorpion *Centruroides noxius* (Valdivia *et al.*, 1988). The BK channel exhibits a voltage-dependence even at a constant elevated level of Ca^{2+} concentration. In neurones, the Ca^{2+}-activated K current (BK) contributes to the spike repolarization and the control of the frequency of repetitive firing (Meech, 1978). In mammalian smooth muscles, such a channel may function to terminate the excitatory processes which are initiated or maintained by an increase in $[Ca^{2+}]_i$. In secretory cells, it plays an important role in controlling secretion (Petersen and Maruyama, 1984).

b. The intermediate-conductance Ca^{2+}-activated K channels (IK)

IK has been identified in red blood cells (Hamill, 1981) and neurones (Herman,

1986). It is voltage-independent and has a typical single-channel conductance of 18-50 pS. This channel could be blocked by quinine, quinidine, TEA or charybdotoxin. The channel is active at the resting membrane potential, suggesting a possible role in the maintenance of resting membrane potential.

c. The small-conductance Ca^{2+}-activated K channels (SK)

SK has a typical single-channel conductance of 10-14 pS and was first described in cultured rat skeletal muscle cells (Blatz and Magleby, 1986) and guinea-pig hepatocytes (Cook and Haylett, 1985). This channel is also activated by an increase in $[Ca^{2+}]_i$, and can be blocked by apamin and leiurotoxin I from scorpion *Leiurus quinquestriatus* venom (Castle and Strong, 1986). SK has little dependence on the membrane potential and is more sensitive to intracellular Ca^{2+} than BK. Opening of SK channels produces a sustained membrane hyperpolarization that follows the action potential in many excitable cells.

3. Ligand-gated K channels

The ligand-gated K channels are a group of K channels modulated by the interaction of ligands with the receptors on the plasma membrane. Cells which have gone through enzymatic treatments or are grown in culture often fail to respond to typical agonists. Thus, relatively few members of this K channel family have so far been described. A number of hormones and neurotransmitters modify the cellular excitability

through modulation of the K channels. The nicotinic acetylcholine (ACh) receptor, a K conducting cation channels where the channel structure is physically a part of the receptor protein, is an example of this family of K channels .

a. ACh and adenosine receptor-gated K channels

One of the first observed effects of ACh, the slowing of the heart rate, can be attributed primarily to the activation of an ACh-gated K channel (K_{ACh}) in the cardiac pacemaker and atrial cell membranes. An interaction of ACh with muscarinic ACh receptors was believed to be involved in the inhibitory effects of ACh (Noble, 1984). A significant, steeply temperature-dependent delay (about 100 msec) between the receptor occupation and K_{ACh} activation was the indication that the ligand (ACh) occupation of receptors could be coupled to the K_{ACh} channel activation through a series of intracellular processes. The involvement of membrane bound guanine nucleotide binding proteins (G proteins) in the coupling process has been demonstrated, while that of cytoplasmic messengers has, however, been ruled out (Brown *et al.*, 1990).

Adenosine, a physiological metabolite, hyperpolarizes and shortens the atrial action potential like ACh. It has been suggested that adenosine increases the K conductance via a mechanism similar to that of ACh (Belardinelli and Isenberg, 1983). It was later demonstrated that both agonists activate the same K channel (Kurachi *et al.*, 1986), although different receptors were believed to be involved. A different type of K channel, designated as "K_M", was found to be inactivated by ACh, also acting through

muscarinic receptors in vertebrate neurones. This has been proposed to be responsible for the muscarinic excitation in this tissue (Brown and Adams, 1980; Brown, 1988). The "K_M" is a small voltage-dependent current. Its threshold of activation lies at potentials negative to the resting membrane potential. K_M contributes to the resting K conductance of the cell as well as the pattern of repetitive firing. Other than ACh, β-adrenoceptor agonists could also stimulate K_M (Sims *et al.*, 1988).

b. Serotonin- and FMRFamide-gated K channels

Application of serotonin (5-HT) to *Aplysia* sensory neurones closes a type of K channel, denoted as the "S" current. The closure of the "S" current was believed to be responsible for the presynaptic facilitatory effect of 5-HT in increasing the action potential duration and the neurotransmitter release (Siegelbaum *et al.*, 1982). This mechanism is thought to provide the cellular basis for behavioral sensitization, a simple form of learning (Kandel and Schwartz, 1982). In contrast, FMRFamide, a neuropeptide, opens the "S" channel, producing a slow hyperpolarization, a shortening of action potential duration as well as a decrease in the neurotransmitter release. However, the modulatory action by FMRFamide is not the mirror image of 5-HT. Single channel studies revealed 5-HT acts to decrease the number of channel openings, while FMRFamide causes an increase in channel open probability. Thus, they appear to act at different sites on the "S" channel (Volterra and Siegelbaum, 1990).

4. Second messenger and intracellular metabolite-modulated K channels

This category of K channels is actually not a true class of channels in its own right. It simply serves to describe some characteristicses of certain kinds of K channels, which could also be voltage-dependent or ligand-gated. As we know, some K channels can be modulated by hormones and neurotransmitters through a variety of second messengers (Schuster *et al.*, 1985), while others can be modulated directly by specific intracellular agents such as adenosine trisphosphate (ATP) (Stanfield, 1987), second messengers such as cyclic adenosine 3',5'-monophosphate (cAMP) (Belardetti and Siegelbaum, 1988), cyclic guanosine 3',5'-monophosphate (cGMP) (Cook and Babcock, 1993), inositol 1,4,5-trisphosphate (IP_3) (Dutar and Nicoll, 1988; Jones *et al.*, 1990; Muraki *et al.*, 1992; Hamada *et al.*, 1993) or active metabolite of IP_3 such as inositol 1,3,4,5-tetrakisphosphate (IP_4) (Berridge, 1988; Chadwick *et al.*, 1992), and G proteins (Dunlap *et al.*, 1987).

a. ATP-gated K channels (K_{ATP})

K_{ATP} is a kind of K channel which closes as the intracellular ATP concentration or the ATP:ADP ratio increases. It has raised considerable interest over the past few years since the discovery of K_{ATP} in cardiac muscles (Noma, 1983; Trube and Hescheler, 1983; 1984), skeletal muscles (Spruce *et al.*, 1987), pancreatic β-cells (Cook and Hales, 1984), and neurones (Bezanilla *et al.*, 1985). More recently, Standen *et al.* (1989) and Nelson (1993) demonstrated the existance of K_{ATP} in vascular smooth muscles. This type

of K channel has the characteristics of being in a closed state under the normal physiological condition. A decrease in the intracellular ATP concentration or the ATP:ADP ratio could stimulate the opening of the channel. The typical single-channel conductance ranges from 40 to 80 pS. This channel is considered to be a major determinant of the resting membrane potential (Cook *et al.*, 1988).

b. cAMP-gated K channels

In several tissues an increase in intracellular cAMP concentration modulates certain K currents through the activation of cAMP-dependent protein kinase (PKA). The above mentioned "S" current in *Aplysia* is also believed to be mediated by this intracellular mechanism (Schuster *et al.*, 1985). The cAMP-gated K channels have been described elsewhere in a variety of tissues (Kaczmarek and Strumwasser, 1984; Ewald *et al.*, 1985; Avenet *et al.*, 1987). More detailed description of the modulation of cAMP on K channel will be discussed in the following section.

c. IP_3-modulated K channels

IP_3 is an intracellular second messenger produced from phosphatidyl inositol (PI) hydrolysis. It is known to cause an increase of $[Ca^{2+}]_i$ through the release of Ca^{2+} from the intracellular stores (Berridge and Irvine, 1984), and thus, can lead to the activation of the Ca^{2+}-activated K channels (Trautmann and Marty, 1984). Intracellular application of IP_3 was found to activate the Ca^{2+}-activated K channels in porcine trachea smooth

muscle cells (Muraki *et al.*, 1992) and human gastric epithelial cells (Hamada *et al.*, 1993). Such IP_3-induced activation of K channels could be abolished by the IP_3 receptor antagonist heparin. Intracellular perfusion of IP_3 was also reported to activate cloned muscarinic ACh receptor-activated, Ca^{2+}-dependent K currents in transfected mouse fibroblasts (Jones *et al.*, 1990). However, K_M currents was found to be modulated by IP_3 through a Ca^{2+}-independent mechanism (Dutar and Nicoll, 1988). In some cases, the active metabolites of IP_3 such as IP_4, IP_5, IP_6 and cyclic IP_3 may be synergistic to IP_3 on the modulation of K channels (Berridge, 1988). IP_4 was shown to modulate K channels although IP_3 remained to be ineffective (Chadwick *et al.*, 1992).

d. G protein-gated K channels

G proteins are often thought to couple receptors with ion channels via intracellular second messengers. Recent studies have uncovered the existence of a class of K channels that is directly gated by G proteins, without interaction of any intracellular second messengers. The K_{ACh} of the muscarinic ACh receptor has been the best example of such a class of G protein-gated K channels (Pfaffinger *et al.*, 1985; Gilman, 1987; Brown *et al.*, 1990). G protein inhibitors, such as the non-hydrolyzable guanosine 5'-triphosphate (GTP) analogue $G_{PP}(NH)_P$, could prevent the activation of K_{ACh} (Breitwieser and Szabo, 1985). The G protein-gated K channels are referred to as the K channels that are directly gated by G protein(s). However, K channels could be also modulated by G protein(s) indirectly, through the activation of intracellular second messengers. This will be

discussed in the following section.

5. Other classes of K channels

There have been numerous reports describing the existence of other classes of K channels that are not listed above. One example is the K channel activated by intracellular Na (Kameyama *et al.*, 1984; Hartung, 1985). This channel is gated by an increase in intracellular Na concentration over 20 mM and is probably of little importance under physiological conditions. It has been speculated that it may be activated by the accumulation of Na due to the failure of Na pump. A class of K channels that could be activated by changes in cell volume (stretch-activated) was reported (Richards and Dawson, 1986). Finally, a Ca^{2+}-activated, non-specific cation current has also been characterized, although it is not likely a pure K current since K and Na ions are equally permeable, e.g. the nicotinic ACh receptor cation channel (Reuter, 1984). This channel is inward rectifying at resting potential and is considered to be responsible for the oscillatory depolarizing after-potentials.

C. Modulation of K channels by G proteins and second messenger cascades

K channels are often modulated by different hormones and neurotransmitters, leading to changes in either current amplitude or channel kinetics, or both. These alterations can then result in the regulation of the electrical properties in various cells. The modulation of K channels has been found to be closely associated with the changes

in resting membrane potential and input resistance, action potential duration, threshold, accommodation, spontaneous bursting activity as well as neurotransmitter release (Pfaffinger and Siegelbaum, 1990).

The modulatory actions of hormones and neurotransmitters on K channels are generally considered to be mediated by the interactions with two distinct receptor families. One family includes the direct channel-gating receptors, where the receptors and ion channels are one, or a part of, macromolecular protein complex (Miller, 1989). Binding of the ligand to the respective membrane receptor directly leads to the opening or closure of the channel, such as the nicotinic ACh receptor at the neuromuscular junction (Karlin, 1980; Noda *et al.*, 1983). Unlike the direct channel-gating receptors, actions of the second type of receptors involve G proteins. The receptor and the channel are distinct structures. Binding of a ligand to its receptor can not lead to a direct opening or closure (the "gating") of the channel. However, this ligand-receptor interaction is able to trigger the activation of certain G protein(s), which in turn interacts with the channel protein, leading to changes in the channel conductance, or to activation of a second messenger cascade. The muscarinic ACh receptor is an excellent example of this family (Gilman, 1987). In many cases, the G protein-coupled activation of diffusible intracellular second messengers is involved in the modulation of ion channel conductance (Kaczmarek and Levitan, 1987).

These two types of receptors, which modulate ion channel either directly or indirectly, display very different characteristics of physiological functions, which makes

it easy to separate one type from the other. The directly gated receptor normally acts on a very rapid time scale, producing conductance changes that only last a few milliseconds. The function of this receptor family is thus to provide rapid transmission. In contrast, the actions of the G protein-mediated receptor tend to be much slower, producing conductance changes that can last for seconds or even minutes. Their function is to produce long-lasting modulatory changes in channel and cellular activity. So far, most known modulations of hormones or neurotransmitters on K channels are believed to occur via the G protein-mediated family, which may or may not involve the activation of the intracellular second messenger cascades.

1. G proteins and K channels

G proteins are a large family of signal transducing proteins that couple membrane receptors to their effectors, e.g. ion channel proteins or second messenger cascades (Gilman, 1984; 1987; Stryer and Bourne, 1986; Brown *et al.*, 1990). The structure of G proteins is believed to be conserved among all members of the gene family (Pfaffinger and Siegelbaum, 1990). Each G protein is basically a heterotrimer consisting of a hydrophilic α subunit and hydrophobic β and γ subunits. The α subunit is unique to each type of G proteins, whereas the $\beta\gamma$ subunits are essentially the same for all kinds of G proteins. Upon binding of the agonist to its receptor, GTP is exchanged for guanosine 5'-diphosphate (GDP) on the α subunit. Then the α subunit dissociates from the $\beta\gamma$ subunits and becomes activated, exerting actions on the effectors. The uncoupled α

subunit remains active until the hydrolysis of GTP to GDP which leads to a reassociation of the α subunit with the $\beta\gamma$ subunits (Gilman, 1987).

The discovery of two bacterial toxins, cholera toxin (CTX) and pertussis toxin (PTX), has provided a further view of the mechanism of action of G proteins. These two toxin are known to modify different kinds of G proteins through an ADP ribosylation mechanism of the α subunit. CTX affects certain G proteins to prevent the hydrolysis of GTP (Abood *et al.*, 1982), whereas PTX acts on certain G proteins to block the interaction of the receptor with the G protein (Ui, 1984).

The actions of many hormones and neurotransmitters are usually mediated through certain G proteins. A number of hormones and neurotransmitters have been reported to either stimulate or inhibit adenylate cyclase (AC) activity through the stimulatory or inhibitory G proteins, abbreviated as G_s and G_i, respectively. Activation of phospholipase C (PLC) through an unidentified G protein (G_o) has also been confirmed. G proteins have been shown to couple receptors to K channels via intracellular second messengers such as cAMP, cGMP, IP_3, Ca^{2+}, diacyglycerol (DG), PKC, PKA, etc. However, in some cases, the receptor-effector coupling may not involve the second messenger cascades, such as the coupling of the atrial muscarinic receptor and the K_{ACh} channel, as already mentioned (Yaffinger *et al.*, 1985).

The involvement of membrane bound G proteins in modulation, either directly or indirectly, has been demonstrated by the observation that intracellular application of GTP leads to K channel activation (Brown *et al.*, 1990), and also by the fact that PTX or the

non-hydrolyzable GTP analogue $G_{PP}(NH)_P$ abolished the receptor agonist-induced activation of K channels (Breitweiser and Szabo, 1985). There has been more evidence which may lead to the conclusion of the involvement of G proteins in the modulation of K channels. These observations include: a long delay after the agonist ACh application compared to that of the nicotinic ACh receptor where the receptor and ion channel are the same physical complex, the fact that agonist occupancy is not the rate-limiting step, etc (Brown *et al.*, 1990). Studies were also carried out in membrane-delimited conditions to rule out any possible second messengers in order to verify the direct action of G proteins on K channels (Brown *et al.*, 1990; Pfaffinger and Siegelbaum, 1990).

2. Second messenger cascades and K channels

As mentioned above, in addition to the direct actions on K channels, G proteins are also proposed to influence K channel functions by activating various intracellular second messenger cascades (Kaczmarek and Levitan, 1987). Many actions (although with exceptions) of these second messenger cascades are mediated by protein phosphorylation through the activation of various protein kinases (Nairn *et al.*, 1985; Levitan, 1985; 1988). Protein phosphorylation by kinases has been one of the important mechanisms for the functional regulation of ion channels. Protein phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) are two of the best studied and characterized second messenger systems that involve K channel modulation (Krebs and Beavo, 1979; Levitzki, 1988; Walsh and Kass, 1988; Chen and Yu, 1994). Other second messengers reported

to modulate K channel activity include Ca^{2+}, IP_3, cGMP, DG and arachidonic acid (AA) (Ewald *et al.*, 1985; Belardetti and Siegelbaum, 1988; Pfaffinger and Siegelbaum, 1990; Muraki *et al.*, 1992; Cook and Babcock, 1993).

a. The cAMP-PKA cascade

The initial step in the cAMP-PKA cascade is the activation of the stimulatory G protein, G_s, through an interaction of ligand-receptor binding. The membrane receptors that are known to be coupled to G_s include the β-adrenergic receptors, 5-HT receptors, dopamine D_1 receptor as well as receptors for various peptides and prostaglandins (PGs) (Drummond, 1983; Cooper *et al.*, 1986; Kanba *et al.*, 1991; Goureau *et al.*, 1992). The activated α subunit of G_s stimulates adenylate cyclase, an integral membrane protein, to catalyse the conversion of ATP into cAMP. The cAMP then activates cAMP-dependent protein kinase (PKA) (Krebs and Beavo, 1979; Blackshear *et al.*, 1988), leading to the phosphorylation of a wide variety of substrate proteins on the serine or threonine residues (Scott and Soderling, 1992).

cAMP has been well established as an intracellular second messenger in the regulation of various physiological actions such as carbohydrate metabolism, where it modulates the activities of certain enzymes such as glycogen synthase, protein phosphotase, and glycogen phosphorylase, by causing them to be phosphorylated (Cohen, 1982). As a second messenger in cell responses to hormones and neurotransmitters, cAMP has been confirmed to be involved in the control of K channel activities in a

number of cases (Siegelbaum and Tsien, 1983; Minami et al., 1993b; Chen and Yu, 1994). Various kinds of experimental approaches have been achieved in the past decade to study the role of the cAMP-PKA cascade in modulating K channel activities, e. g., the intracellular injection of cAMP through microelectrodes, the extracellular application of membrane-permeant analogues such as dibutyryl cAMP (Db-cAMP) or 8-bromo-cAMP, and the use of phosphodiesterase inhibitors or adenylate cyclase (AC) activators (Choquet *et al.*, 1987; Soliven *et al.*, 1988; Pfaffinger and Siegelbaum, 1990).

The cAMP-dependent modulation of K channels has been widely documented (Siegelbaum and Tsien, 1983; Kaczmarek and Levitan, 1987; Levitan, 1988; Minami *et al.*, 1993b; Mori *et al.*, 1993; Chen and Yu, 1994; Harada and Iijima, 1994). cAMP and cAMP-dependent protein phosphorylation could both inhibit or enhance the conductance of certain types of K channels. Intracellular perfusion with cAMP or the adenylate cyclase activator forskolin was shown to decrease the voltage-dependent K current amplitude in murine B cells and in oligodendrocytes (Choquet *et al.*, 1987; Choquet and Korn, 1988; Soliven *et al.*, 1988). The effect of forskolin was attributed to its ability to increase intracellular cAMP. However, potentiation of Ca^{2+}-activated K channels by cAMP and cAMP-dependent mechanisms have also been reported in smooth muscle cells from canine proximal colon (Carl *et al.*, 1991), rat aorta (Sadoshima *et al.*, 1988), rabbit trachea (Kume *et al.*, 1989) and porcine coronary artery (Minami *et al.*, 1993b). The delayed rectifier K channel current from guinea pig ventricular myocytes has also been reported to be enhanced by direct activation of adenylate cyclase (Harada and Iijima,

1994). Chen and Yu (1994) observed that a cAMP analogue completely abolished the desensitization of an opioid ligand-gated K channel expressed in *Xenopus* oocyte, and thus, increased the current amplitude. The cAMP effect was believed to be mediated by PKA phosphorylation, since the PKA catalytic subunit mimicked the cAMP effect (Chen and Yu, 1994).

Although the modulation of K channels by cAMP has been commonly considered to be mediated by cAMP-dependent kinase (PKA) protein phosphorylation of the channel or associated proteins, it is possible that in some cases cAMP-dependent modulation of K channels is mediated directly by cAMP, independent of protein phosphorylation (Nakamura and Gold, 1987). In rare cases, forskolin itself could be responsible for the observed inhibition of K currents, independent of its action on cAMP accumulation (Hoshi *et al.*, 1988; Krause *et al.*, 1988). It should be pointed out that cAMP does not modulate all K currents. No effect of cAMP on voltage-dependent K channel conductance was observed in human peripheral blood T lymphocytes or human B cells and *Aplysia* neurones (Krause *et al.*, 1988; Zunkler *et al.*, 1988; Kaczmarek and Strumwasser, 1984).

In recent studies, direct evidence was provided that cAMP regulated the gene expression of a mammalian voltage-dependent K channel. The process was mediated by cAMP-dependent protein kinase (Fisch *et al.*, 1989; Grieshammer *et al.*, 1992; Mori *et al.*, 1993). This evidence has provided a new insight for the role of cAMP in the long term modulation of K channels, i.e., cAMP could modulate K channels not only by post-translational processes such as channel protein phosphorylation, but also by pre-

translational processes at the gene translational level.

b. The protein kinase C cascade

Protein kinase C, a phosphorylating enzyme stimulated by Ca^{2+} and phospholipid, is involved in the control of a wide variety of physiological processes including differentiation, tumour promotion, transmitter secretion and release, ion channel modulation, and membrane receptor functions (Nishizuka, 1986; 1988; 1994). PKC mediates cellular responses to hormones and neurotransmitters following the stimulation of certain membrane receptors. The interaction of the agonist (hormones or neurotransmitters) and the membrane receptor leads to the uncoupling of G protein subunits. The uncoupled α subunit of the G protein then activates the cytoplasmic effector, phospholipase C (PLC). The enzymatic action of PLC causes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) and the release of DG and IP_3 into the cytoplasm. DG directly activates PKC, whereas IP_3 releases Ca^{2+} from the intracellular stores such as sacroplasmic reticulum (SR) (Berridge and Irvine, 1984; Nishizuka, 1984a; 1984b).

The PKC cascade has been confirmed to be another important messenger system involved in K channel modulation (Shearman *et al.*, 1989). Phorbol esters, the tumor promotors that activate PKC, have been widely used as probes to detect the role of PKC in the regulation of ion conductance and neurotransmitter release. A number of researchers reported the regulation of K channels by phorbol ester-induced PKC

activation. Malenka *et al.* (1986) found that phorbol esters reduced Ca^{2+}-activated K currents in CA1 pyramidal cells. No blockade of Ca^{2+} entry was found which could account for the blockade of the Ca^{2+}-activated K channels. A different type of K channel, the "I_A" channel expressed in *Xenopus* oocytes with the *Shaker* H4 clone isolated from *Drosophila*, was found to be inhibited by tetradecanoyl phorbol acetate (TPA) (Moran *et al.*, 1991). Activation of PKC by treatment of oocytes with phorbol ester potentiated the desensitization of the opioid receptor-gated K channel, and thus, inhibited the current amplitude (Chen and Yu, 1994). Colby and Blaustein (1988) demonstrated that the synthetic DG analogue sn-dioctanoylglycerol (DiC8), an activator of PKC, reduced ^{86}Rb efflux in a time- and dose- dependent manner, of both delayed rectifier and transient outward voltage-dependent K components in rat brain synaptosomes, but had no effect on the Ca^{2+}-activated K component. Such inhibition could be antagonized by the PKC inhibitors H-7 and sphingosine. However, using a similar methodology but different regions of the rat brain, it was found that PKC activator 4β-phorbol 12,13-dibutyrate (PDBu) inhibited the Ca^{2+}-activated K channels, but had no effect on voltage-dependent K channels (Ren and Benishin, 1994). These observations provided evidence for a role of PKC in nerve endings. In another study, Walsh and Kass (1988) observed the activation of a delayed rectifier K channel by PDBu in cardiac ventricular myocytes. In smooth muscle cells and neurons, PKC activation by DG (or synthetic phorbol ester compounds) and intracellular Ca^{2+} has also been reported to regulate a number of K currents (Baraban *et al.*, 1985; Walsh and Kass, 1988; Lester *et al.*, 1991; Ren and

Benishin, 1994). Activation of PKC has been shown to directly inhibit different types of K channels. Lester *et al.* (1991) applied DG and arachidonic acid simultaneously to the *Hermissenda* photoreceptor, and found a reduction in voltage-dependent K current in a synergistic manner via the action of PKC.

II. Dendrotoxins

The venoms of a number of poisonous snakes of the family *Elapidae* which includes the cobras, mambas, and kraits contain numerous substances including neurotoxins, cardiotoxins, myotoxins, coagulants and enzymes (Chang, 1979). These snake toxins have long fascinated the interest of many investigators. Pharmacological studies on the properties of purified venoms on isolated tissue and cell preparations have revealed that many of these toxins were extremely potent molecules that acted, with high affinity and selectivity, on certain physiological processes. The snake toxins are, thus, of potential importance and instrumental in the elucidation of the cellular basis of certain physiological processes as pharmacological probes. The use of certain high affinity toxins as the selective blocking agents thus makes it possible to 'dissect out' various individual ion currents from cells in patch-clamp studies. These toxins, which may interfere with the function of ion channels, enable us to identify particular channel proteins following the isolation of a specific toxin receptor from the membrane. The nicotinic ACh receptor was one of the first ion channels to be characterized following the isolation of α-

bungarotoxin from *Bungarus multicinctus* venom (Haggerty and Froehner, 1981). More toxins have recently been isolated from various exotic venomous creatures, giving rise to toxins of new selectivity for different ion channels.

K channels are the most diverse group of ion channels found in excitable and non-excitable cells (Hille, 1984; Latorre *et al.*, 1984; Cook, 1988; Rudy, 1988). However, the knowledge of the structure and function of K channels was very limited compared to our knowledge of most other ion channels, such as Na, Ca^{2+} channels, and the ACh receptor. One simple reason has been that until recently, there have been no specific K channel ligands with high affinity and selectivity, such as tetrodotoxin (TTX) for Na channels, dihydropyridines (DHP) for L-type Ca^{2+} channels and α-bungarotoxin for the nicotinic ACh receptor. The development of the molecular biology of the K channel proteins, was slow until the *Shaker* K channel was cloned (Kim *et al.*, 1994; Shih and Goldin, 1994). Fortunately, the emergence of a group of mamba snake venom toxins, the dendrotoxins, which block the voltage-dependent K channels, has recently facilitated the isolation and purification of the K channels (Dolly *et al.*, 1984; Harvey and Anderson, 1985; Halliwell *et al.*, 1986; Benoit and Dubois, 1986; Stansfeld *et al.*, 1987; Benishin *et al.*, 1988; Sorensen and Blaustein, 1989; Parcej *et al.*, 1992; Foray *et al.*, 1993).

A. General properties of dendrotoxins

Barret and Harvey (1979) first described that the venom from the Eastern green mamba *Dendroaspis angusticeps* contained a peptide toxin that was able to facilitate

neuromuscular transmission. Not long after, Harvey and Karlsson (1980) separated the venom into several component fractions, one of which was dendrotoxin. It has drawn special attention for its ability to potentiate the quantal ACh release at the neuromuscular junctions. Dendrotoxin constitutes about 2.5% of the total venom protein. It is composed of 59 amino acids with a molecular weight of 7077 (Harvey and Karlsson, 1980; Joubert and Taljaard, 1980). Two other members of the dendrotoxin family, toxin I and toxin K, isolated from the venom of black mamba *Dendroaspis polylepis* (Strydom, 1972), consist of 60 and 57 amino acid residues with molecular weights of 7573 and 6542, respectively.

Dendrotoxin that was isolated from the venom of the green mamba *Dendroaspis angusticeps* (Harvey and Karlsson, 1980; Joubert and Taljaard, 1980) has been the most intensively studied member of this family. Dendrotoxin that was originally described by by Harvey and Anderson (1985) is normally referred to as α-dendrotoxin. Using the high performance liquid chromatography (HPLC) cation exchange technique, Benishin *et al.* (1988) identified three more polypeptide components of the crude venom of the green mamba *Dendroaspis angusticeps*, designated as β-, γ-, and δ-dendrotoxins. These toxins have the similar molecular weight.

The pharmacological effects, toxicities, chemical characterizations and structure-activity relations of dendrotoxins have been extensively studied (Harvey *et al.*, 1984; Harvey and Anderson, 1985). Dendrotoxins facilitate the quantal ACh release and exhibit a high degree of sequence homology with several protease inhibitors such as bovine pancreatic trypsin inhibitor. However, dendrotoxins are unable to inhibit the protease

activity of either trypsin or chymotrypsin, and are much less toxic than the whole venom itself (Harvey and Karlsson, 1982; Anderson, 1985; Dufton, 1985; Dreyer and Penner, 1987). Intravenous or intraperitoneal administration of dendrotoxins produced hyperactivity and convulsion in mice (Othman *et al.*, 1982; Silveira *et al.*, 1988). An increase in the excitability of hippocampal neurones *in vitro* has also been observed following dendrotoxin treatment (Dolly *et al.*, 1984). Application of dendrotoxins to chick biventer cervices nerve-muscle preparation or mouse phrenic nerve-hemidiaphragm was found to augment the nerve-induced twitch height (Harvey and Karlsson, 1980). Such a preparation may be used as a biological test for evaluation of the activity of dendrotoxins. The effects of the dendrotoxins have also been studied on peripheral autonomic neurotransmission (Harvey *et al.*, 1984; Anderson, 1985) in mouse and rat vas deferens (predominantly sympathetic) and in chick esophagus (parasympathetic). An augmentation of the smooth muscle contraction in response to nerve stimulation was consistently observed.

B. The inhibition of the voltage-dependent K channels by dendrotoxins

Research on the dendrotoxins has been greatly facilitated by the discovery that the excitatory effects induced by the dendrotoxins, such as the facilitation of synaptic transmission at the neuromuscular junction, were attributed to the inhibition of certain voltage-dependent K channels (Dolly *et al.*, 1984). Both transient and delayed rectifier K channels have been documented to be inhibited by different dendrotoxins (Dolly *et al.*,

1984; Halliwell *et al.*, 1986; Penner *et al.*, 1986).

1. The inhibition of fast-inactivating voltage-dependent K channels

The α-dendrotoxin from the green mamba snake venom was found to affect the fast-inactivating, voltage-dependent, 4-AP-sensitive K channels that are related to the control of cellular excitability and transmitter release. α-dendrotoxin was found to block a fast-inactivating, voltage-dependent K current in hippocampal CA1 neurones, while leaving other components of K channels untouched (Dolly *et al.*, 1984; Halliwell *et al.*, 1986). In myelinated frog nerve fibres the toxin prolonged the nerve action potential duration (Weller *et al.*, 1985). At relatively high concentrations (>100 nM), α-dendrotoxin caused a selective (70%) block of the fast-inactivating outward "I_A" current. The toxin had no effect on the Ca^{2+}-activated K current, mixed Na/K currents and non-inactivating "K_M" currents (Halliwell *et al.*, 1986). The increase of nerve-induced twitch height and of quantal ACh release at neuromuscular junctions brought by α-dendrotoxin could thus probably be ascribed to the inhibition of K permeability which prolongs presynaptic depolarization and thereby increases the Ca^{2+} influx (Harvey and Karlsson, 1980; 1982; Gandolfo *et al.*, 1989).

By using the ^{86}Rb efflux from rat brain synaptosomes as an assay, Benishin *et al.* (1988) found that two polypeptide components, α- and δ-dendrotoxins (10-100 nM), blocked preferentially the 4-AP-sensitive, fast-inactivating component of ^{86}Rb efflux. However, the other two polypeptide components, β- and γ-dendrotoxins, failed to exert

any action on this component of ^{86}Rb efflux.

2. The inhibition of non-inactivating voltage-dependent K channels

Further studies have revealed that the non-inactivating, delayed rectifier K channel could be another target of dendrotoxin actions. In guinea pig dorsal root ganglion cells, α-dendrotoxin irreversibly blocked the delayed non-inactivating, voltage-dependent K current whereas the fast-inactivating K current was not affected (Penner *et al.*, 1986). This is in contrast to the observation in hippocampal neurones as well as the frog node of Ranvier. Under voltage-clamp conditions, α-dendrotoxin blocked the delayed rectifier K current at nanomolar concentrations whereas it did not affect the leakage current nor the Na current (Weller *et al.*, 1985).

In the studies of the action of dendrotoxins on isolated visceral sensory neurones in rat nodose ganglion, Stansfeld *et al.* (1986; 1987) observed that α-dendrotoxin inhibited the outward non-inactivating voltage-dependent K current by 50%. The dendrotoxin-sensitive current in dorsal root ganglia was recently found to have a maximum single-channel conductance of between 5-10 pS in a physiological K gradient (Stansfeld and Feltz, 1988). Blockade of the current appeared to be via a direct action on the K channel. About 30 to 80% of the delayed rectifier K current is dendrotoxin-sensitive, which supported the notion that different subtypes of delayed rectifier K channels may exist. As already demonstrated by Dubois (1981), the delayed rectifier K current in frog nerve fibres was composed of at least three kinetically distinct

components (Dubois, 1982). Using dendrotoxin I from the black mamba, Benoit and Dubois (1986) showed that one such component of non-inactivating voltage-dependent K currents was selectively and potently blocked by the toxin (IC_{50}=0.4 nM). The same specific action was also obtained with α-dendrotoxin from the green mamba, but it was about 20 times less potent (Brau *et al.*, 1990). The dendrotoxin-sensitive K current has a typical feature of being activated at the membrane potential of around -60 mV.

Dorsal root ganglion cells of guinea pig exhibit three different types of K current, a transient outward current, a Ca^{2+}-activated current and a delayed non-inactivating outward current. Using single electrode voltage-clamp on hippocampal pyramidal cells, bath application of α-dendrotoxin selectively blocked (about 50%) the non-inactivating K current, leaving the transient outward and the Ca^{2+}-activated currents unaffected. The inhibitory action of dendrotoxin could not be reversed upon wash out (Penner *et al.*, 1986). At higher concentrations, up to 1.4 μM, the toxin did not further reduce the delayed outward K current, indicating there are indeed two distinct sets of the voltage-dependent, delayed rectifier K channels. Kinetic studies also demonstrated that the current-voltage relationships were quite different between dendrotoxin-sensitive and -insensitive components. Using the ^{86}Rb efflux technique, Benishin *et al.* (1988) found that β- and γ-dendrotoxins were selective for the non-inactivating voltage-dependent K channels. Experiments performed with other K channel blockers revealed that dendrotoxin could affect the K channels that are more specific for 3,4-diaminopyridine than for TEA (Dreyer and Penner, 1987; Dreyer, 1990).

In giant axons of the marine annelid *Myxicola*, α-dendrotoxin inhibited the delayed rectifier K current completely with an IC_{50} of 150 nM. Interestingly, the toxin showed equal potency when applied from either side of the membrane (Schauf, 1987). In contrast, Penner *et al.* (1986) found α-dendrotoxin to be effective only by applying it from the outside.

3. Properties of the dendrotoxin-sensitive voltage-dependent K channels

The specific blockade of the voltage-dependent K channels by α-dendrotoxin has been intensively studied over the past decade. The α-dendrotoxin-sensitive, voltage-dependent K channels were almost exclusively found in neurones. Very little has been reported on K channels in other tissues. The dendrotoxin-sensitive K channels were normally activated [illegible] membrane potentials between -70 and -30 mV, with single channel conductances of 5-20 pS (Penner *et al.*, 1986; Brau *et al.*, 1990; Dreyer, 1990). These channels showed a higher sensitivity to aminopyridines than TEA. Recent molecular biology investigations have indicated the dendrotoxin-sensitive K channels are indeed a family of voltage-dependent K channels.

C. The association of the dendrotoxin receptor with voltage-dependent K channels

The search for high affinity and selective ligands for the identification and purification of different classes of K channels underlies the importance of the studies of

dendrotoxins. In a recent review (Dolly *et al.*, 1994), the relationship of dendrotoxin receptor and voltage-dependent K channel molecular structure was extensively discussed. α-dendrotoxin has so far been the most important member of the dendrotoxin family that has been studied in an attempt to identify, characterize and purify the voltage-dependent K channel proteins. Using ^{125}I-labelled α-dendrotoxin, the presence of high-affinity acceptor proteins for α-dendrotoxin in rat brain synaptosomes (Mehraban *et al.*, 1984; Black *et al.*, 1986; Sorensen and Blaustein, 1989), bovine brain (Scott *et al.*, 1990; 1994a; 1994b) and chick brain (Black and Dolly, 1986) has been shown. The binding to these acceptors that are thought to be associated with K channel proteins was inhibited by homologous unlabelled polypeptides isolated from mamba snake venom.

α-dendrotoxin and dendrotoxin I completely inhibited the binding of radiolabelled β-bungarotoxin to synaptic membrane fractions of rat and chick brain (Othman *et al.*, 1982; Rehm and Betz, 1984; Stansfeld *et al.*, 1987; Schmidt *et al.*, 1988), whereas β-bungarotoxin was relatively inefficient in inhibiting ^{125}I-dendrotoxin binding to its high-affinity acceptors. These observations suggested the possible existence of at least two subtypes of acceptor proteins for dendrotoxins in synaptic membrane that could be distinguishable by β-bungarotoxin (Black *et al.*, 1986; Black and Dolly, 1986; Stansfeld *et al.*, 1987).

Binding studies using other radiolabelled dendrotoxins, such as β-dendrotoxin (Sorensen and Blaustein, 1989) and δ-dendrotoxin (Rehm *et al.*, 1988), have also been carried out. Acceptors for both α- and β-dendrotoxins were solubilized from rat brain

synaptosomal membrane (Sorensen and Blaustein, 1989). The toxin-binding and pharmacological properties were preserved in the solubilized acceptor proteins. Although the two dendrotoxins exhibited some specificity in blocking different types of voltage-dependent K channels (Benishin *et al.*, 1988), their characteristics of binding were found to be very similar. Both solubilized acceptors had similar molecular weights of 65 kDalton. These data suggested a structural homology in the acceptor proteins (Sorensen and Blaustein, 1989).

Further investigations showed that the dendrotoxin-sensitive K channel is likely to be an oligomeric protein complex of 450 kDaltons (Black *et al.*, 1988) consisting of tetramer polypeptide chains of 76 kDalton and 38 kDalton (Rehm and Lazdunski, 1988; Parcej *et al.*, 1992). The amino terminal sequence of the 76 kDalton subunit was homologous to that of a voltage-dependent K channel ($K_{V1.2}$) α subunit cloned from rat and bovine brain. Interestingly, the K channel produced when this α subunit was expressed in *Xenopus* oocytes was highly sensitive to α-dendrotoxin (Pongs, 1992; Reid *et al.*, 1992). These findings have provided convincing evidence that dendrotoxin acceptors are a family of voltage-dependent K channels, and that dendrotoxins are feasible for use in the study of the molecular structure of the voltage-dependent K channels.

III. Prostaglandin E_2 (PGE_2)

A. Nomenclature, biosynthesis and metabolism of prostaglandins

The biological activity of prostaglandins was first described in the early 1930s (Kurzrok and Lieb, 1930; Van Euler, 1935). However, not until 1960s did the research of prostaglandins really get on track. During the last decades, numerous studies have shown that prostaglandins are associated with diverse biological functions. Recently, the improvement of the bioassay techniques and the commercial availability of certain prostaglandins as well as their analogues have further expanded the knowledge of this family of natural occurring substances.

1. Nomenclature of prostaglandins

Prostaglandins are derived from arachidonic acid, which is released from the membrane phospholipids by phospholipases (McGiff, 1981; Needleman *et al.*, 1986). Prostaglandins are also continously degraded by enzymes in extracellular fluids. They are essentially unsaturated fatty acids with a 20-carbon skeleton and consisting of a cyclopentane ring with two side chains. Classifications can be made to divide PGs into series one, two and three (e. g., PGE_1, PGE_2, PGE_3), based on the degree of unsaturation, i. e. the number of double bonds in the side chains. The prostaglandins of the two series, such as PGE_2 and $PGF_{2\alpha}$, have been considered to be biologically the most important prostanoids (Greeley *et al.*, 1986; Coleman *et al.*, 1994). The additional subscripts α and β, as in $PGF_{2\alpha}$, indicate the stereometric orientation of the -OH group attached at carbon site 9. Nine classes of prostaglandins have so far been discovered, and named in the alphabetic order (A to I). The newest member was prostacyclin (PGI_2).

2. Biosynthesis of prostaglandins

Linoleic acid is an unsaturated 18-carbon fatty acid with two double bonds. It is present in food sources and serves as the most important precursor of the prostaglandins. Linoleic acid could undergo further desaturation and elongation of its side chain into the 20-carbon arachidonic acid. Arachidonic acid, which is usually incorporated into the phospholipid fraction of the cell membranes, has generally been considered to be the final substrate for enzymatic synthesis, or release of the prostaglandins. However, the biosynthesis of prostaglandins does not occur with the precursor remaining esterified in the phospholipids. Certain phospholipases need to be activated to release arachidonic acid from the membrane phospholipids. Different enzymatic mechanisms have been postulated for the liberation of arachidonic acid from the membrane phospholipids. The major enzyme involved in this procedure is phospholipase A_2 (PLA_2) (Lapetina, 1982). However, evidence has been accumulating that arachidonic acid can also be released by the synergistic action of phospholipase C and DG lipase (Irvine, 1982). Firstly, PLC promotes the breakdown of phosphoinositides and generates DG as well as IP_3. Secondly, DG is phosphorylated by DG kinase to form phosphatidic acid. Finally, phosphatidic acid is able to serve as a substrate for phosphatidic acid-specific phospholipase A_2 or DG lipase and release arachidonic acid (Billah *et al.*, 1981; Prescott and Majerus, 1983). In some cases, triglycerides may also be able to release arachidonic acid in tissues such as renal medullary interstitial cells (Bojesen, 1974).

The relative contribution of above mentioned pathways to prostaglandin synthesis

in different tissues is complex and still not clear. Although various hormones and neurotransmitters have been reported to cause the activation of more than one phospholipase (e. g., PLA_2 and PLC), they seem to selectively use only one pathway, to librate arachidonic acid for prostaglandin synthesis (Silvka and Insel, 1987). Once arachidonic acid is released from the membra[illegible]pholipid, it is ready to serve as the substrate for at least two enzymatic systems, i. e., the cyclooxygenase (CO) pathway and the lipoxygenase pathway. A third pathway, the cytochrome P-450-dependent epoxygenase oxygenation metabolism of arachidonic acid, has also been elucidated (Capdevila *et al.*, 1982).

Cyclooxygenase is present in many cells and transforms arachidonic acid into the prostaglandin endoperoxides PGG_2 and PGH_2. These endoperoxides are quite unstable in aqueous solution and isomerize spontaneously or enzymatically, by specific isomerases, into the stable prostaglandins, such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane B_2 (TXB_2) (Moncada and Higgs, 1986; Gresele *et al.*, 1991). These products can exert diverse and important biological actions (Horton, 1969; Moncada and Higgs, 1986; Curtis-Prior, 1988). Lipoxygenase, on the other hand, was initially described in platelets but later also found in leucocytes and other tissues. This enzyme inserts a hydroperoxy group (-OOH) at different positions of arachidonic acid. The insertion results in the formation of hydroperoxyeicosatetraenoic acids (HPETES), e. g., 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Lipoxygenase can also transform arachidonic acid into the more recently discovered leukotrienes (LTs), e. g., LTC_4 or

LTD_4 that appear to be the constituents of the "slow-reacting substance of anaphylaxis" (Samuelsson, 1983).

3. Metabolism of prostaglandins

Prostaglandins or other arachidonic acid metabolites are never stored in the cell. They are synthesized and released on demand *de novo*. Prostaglandins are very rapidly and effectively removed by the lung and metabolized by the 15-hydroxyprostaglandin dehydrogenase and $\Delta 13$ reductase into inactive metabolites, which are then excreted by the kidney (Terragno and Terragno, 1979). This efficacious removal makes the arterial concentration of these substances very low, so that they can only be detected with sophisticated techniques such as radioimmunoassay and gas chromatography-mass spectrometry (Curtis-Prior, 1988).

B. General actions of prostaglandins

The biological significance of naturally-occurring active substances is often difficult to establish. This is the case for prostaglandins, although accumulating evidence has suggested many important biological roles for this ever-growing family. As already mentioned, arachidonic acid, the naturally-occurring polyunsaturated fatty acid, can be metabolized by cyclooxygenase to produce prostaglandins, prostacyclin and thromboxanes. Alternatively, arachidonic acid can be metabolized by lipoxygenase to produce hydroxy fatty acids and LTs. The release of arachidonic acid from the membrane

phospholipids has been considered as the initial step in the mechanism of action of some hormones and neurotransmitters (Samuelsson, 1981).

Prostaglandins are synthesized in response to various kinds of stimuli and are known to mediate numerous physiological functions. But the situation may be far more complicated than that of the hormones produced in the specific organs, as the removal of an endocrine gland would easily provide the information about its functional role. Recently, progress has been made on the synthetic antagonists of prostaglandins, which have proven to be of great value in studying the function of prostaglandins. Prostaglandins exert diverse biological effects on various kinds of tissues and systems. In the central and peripheral nervous systems, prostaglandins are involved in thermoregulation (Milton and Wendlandt, 1970; Ueno *et al.*, 1982a), induction of sleep (Ueno *et al.*, 1982b; 1983; 1985), nociception (Ohkubo *et al.*, 1983; Horiguchi *et al.*, 1986), anticonvulsive effects (Forestman *et al.*, 1982), and release of neurotransmitters (McGee *et al.*, 1978; Higashida *et al.*, 1984; Deckers *et al.*, 1989; Serio and Daniel, 1989; Ito *et al.*, 1991b). In the reproductive system, prostaglandins participate in the transport of sperm (Goldblatt, 1935), occurrence of menstruation (Pickles, 1967; Horton, 1969) and the induction of parturition (Challis and Olson, 1987), etc. Prostaglandins have also been documented to play critical roles in hemostasis, distribution of blood flow, platelet aggregation, smooth muscle contraction and relaxation, regulation of gastrointestinal function, chemotactic effects on polymorphonuclear leukocytes, inflammation, and modification of the immune system (Curtis-Prior, 1988; Ren and

Wang, 1988; Sellio and Daniel, 1989; Choquet *et al.*, 1990; Vezza *et al.*, 1993).

The best characterized mode of action of prostaglandins and other eicosanoid arachidonate metabolites, following their release from the cells in which they are produced, is by acting on the membrane surface receptors (Samuelsson *et al.*, 1978; Moncada *et al.*, 1985; Halushka *et al.*, 1989; Smith, 1989). Different types, as well as subtypes, of prostaglandin receptors have been elucidated, such as four for PGEs (designated as EP_1, EP_2, EP_3, EP_4). EP receptors have been reported to mediate a wide range of biological actions, such as contraction and relaxation of smooth muscle, stimulation and inhibition of neurotransmitter release, inhibition of gastric acid secretion, inhibition of immunoglobulin expression, *etc.* (Coleman *et al.*, 1994). Different EP receptors have been considered to interact with different cytosolic second messenger systems (Goureau *et al.*, 1992; Smith *et al.*, 1992; Coleman *et al.*, 1994). The EP_1 receptor has been reported to be responsible for the PGE_2-induced smooth muscle contraction, by increasing the intracellular Ca^{2+} concentration (Funk *et al.*, 1993; Watabe *et al.*, 1993). Activation of the EP_2 receptor, on the other hand, is coupled to an increase of intracellular cAMP and smooth muscle relaxation (Coleman *et al.*, 1994). The most ubiquitous EP receptor, EP_3 receptor has been found to be coupled to different second messenger systems, leading to either inhibition of autonomic neurotransmitter release or smooth muscle contraction (Coleman *et al.*, 1994). So far, there is very little information concerning the EP_4 receptor.

Prostaglandins elevate the intracellular levels of cAMP and cGMP in many tissues

(Gilman and Nirenberg, 1971; Hamprecht and Schultz, 1973; Molnar *et al.*, 1987; Hagel-Bradway *et al.*, 1991; Coleman *et al.*, 1994). They are also able to activate the PLC-PKC second messenger cascade (Choquet *et al.*, 1990; Hebert *et al.*, 1990; Vezza *et al.*, 1993). Lipoxygenase metabolites have also been found to activate guanylate cyclase through a G protein mediated mechanism (Snider *et al.*, 1984). The lipophilic prostaglandins and other eicosanoid arachidonate metabolites could also act as second messengers within cells, and may modulate mRNA transcription and gene expression in the nucleus (Curtis-Prior, 1988).

C. Actions of PGE_2 on the cardiovascular system

Prostaglandins participate in the regulation of cardiovascular activities by exerting local actions on the blood vessels (McGiff and Vane, 1975). Local vascular smooth muscle tone is subject to the continuous influence of endogenous prostaglandins. It has been postulated that prostaglandins are capable of affecting local vascular smooth muscle tone in many tissues, either directly or through modulation of vascular response to hormones, neurotransmitters, and neuronal activity (Curtis-Prior, 1988). Many actions of eicosanoids have been considered to be executed by this cyclooxygenase product. PGE_2 has been considered to be both a physiological and a pathological mediator in a variety of cardiovascular disorders such as myocardial ischemia and infarction, thrombosis, and hypertension (Cannon, 1984; Vanhoutte and Houston, 1985; Greeley *et al.*, 1986; Jaiswal *et al.*, 1992; Vezza *et al.*, 1993). Like most of the eicosanoids and

their metabolites, PGE_2 was found to be highly concentrated in the vasculature associated with its respective functions. The major sources of PGE_2 in the cardiovascular system include cardiac myocytes, endothelial cells, VSMCs, leukocytes and platelets. Recent investigations of PGE_2 have largely focused on its subcellular mechanisms of action, such as the interactions with ion channels, second messenger cascades and transmitter release.

PGE_2 exerts significant haemodynamic actions in the control of blood pressure in normotensive and hypertensive subjects (Camu *et al.*, 1992; Dorn *et al.*, 1992; Ando *et al.*, 1993; Jaiswal *et al.*, 1993). It has been suggested that abnormal metabolism of the arachidonic acid-cyclooxygenase system may contribute to the development of hypertension, either directly (by altering the balance between vasodilator and vasoconstrictor prostanoids) or indirectly, through an interaction with the renin-angiotensin system (Schror, 1992).

Prostaglandins induce both vasodilation and vasoconstriction. PGE_2, similar to PGI_2, can function as a potent vasodilator in many vascular beds and is probably one of the most important physiological modulators of vascular smooth muscle tone (Greeley *et al.*, 1986). PGE_2 decreases the blood pressure together with a concomitant increase in cardiac output as well as a reduction in systemic vascular resistance secondary to peripheral vasodilation. In addition, vasodilation within the splanchnic, pulmonary and coronary vascular beds has been observed, with increased blood flow to these areas. Further, PGE_2 appears to possess myogenic properties required of a modulator of pressor hormones not possessed by PGI_2 (Herman *et al.*, 1978). The vasodilating effect of PGE_2,

however, is believed to be organ-specific. Secher and colleagues (1982) noted PGE_2 induced a 31% decrease in the systemic blood pressure and a 33% decrease in the systemic vascular resistance with a 36% increase in the cardiac output in pregnant women undergoing suction abortion. Intravenous PGE_2 also had significant vasodilator activity in canine coronary vascular beds (Hyman *et al.*, 1978). Perfused rabbit mesenteric blood vessels have been shown to release PGE_2 when treated with angiotensin I, angiotension II, arachidonic acid and bradykinin (Blumberg *et al.*, 1977). The addition of indomethacin, a CO inhibitor, abolished the ability of the vessels to release PGE_2 in response to angiotensin II, inhibited vessel dilation and increased the mesenteric perfusion pressure. Subsequent administration of PGE_2 caused a profound vasodilation. The endogenous prostaglandin biosynthesis in the coronary vasculature of the isolated rabbit heart was increased under the conditions of elevated oxygen tension but was decreased during anoxia. These findings are consistent with the requirement of oxygen for cyclooxygenase activity (Needleman *et al.*, 1975).

Similar to thromboxane A_2 (TXA_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2 is a powerful vasoconstrictor in a variety of vascular beds, such as coronary arteries and renal vasculature, and has been implicated in the pathogenesis of hypertension and vasospasmic disorders affecting cardiac or cerebral blood flow (Armstrong *et al.*, 1976; Camu *et al.*, 1992; Dorn *et al.*, 1992). PGE_2 constricts bovine and human coronary artery strips. Recently, through the use of radioligand binding to cultured VSMCs, specific receptors for the vasoconstricting prostaglandins such as PGH_2, PGE_2, $PGF_{2\alpha}$

have been identified (Hanasaki and Arita, 1989; Dorn, 1991; Coleman *et al.*, 1994). Several mechanisms have been postulated for the vasoconstricting action of PGE_2, such as the cross-reactivity with $PGF_{2\alpha}$ or TXA_2 receptors (Dorn *et al.*, 1992), including the activation of certain second messenger pathways (Camu *et al.*, 1992; Goureau *et al.*, 1992), and the sensitization of myosin light chain kinase (Gong *et al.*, 1992).

Since local synthesis of PGE_2 appears to be an important mechanism controlling the local vascular smooth muscle tone and blood flow in microcirculation, it is believed to play a key role in both the pathogenesis and the treatment of cardiovascular diseases. PGE_2 may act on vascular smooth muscle as a modulator of adrenergic neurotransmission, and play an important role in the regulation of vasomotor tone in the systemic vascular beds. Disturbance of the cyclooxygenase pathway could alter blood pressure by causing an imbalance in prostaglandin production, especially PGE_2, and has been reported to aggravate hypertension in human subjects (Martin *et al.*, 1981). Renal PGE_2 could contribute to the development of hypertension in the rat with genetic hypertension, as an increase in renal vascular resistance produced by PGE_2 might be an initiating factor in the pathogenesis of the disease (Armstrong *et al.*, 1976). However, as a vasodilator, low urinar levels of PGE_2 have been reported in essential hypertension, and particularly in low-renin hypertension (Tan *et al.*, 1978; Rathaus *et al.*, 1983). A deficiency of PG was also demonstrated in patients with Gordon's syndrome, a rare syndrome of hypertension characterized by excessive sodium reabsorption resulting in the volume expansion (Klemm *et al.*, 1991).

D. PGE_2 and ion channel modulation

PGE_2 exerts diverse actions in a variety of cells and tissues. However, the cellular basis of its action, such as changes in membrane electrical activity, have been sparsely documented. PGE_2, released from the cell membrane as a local hormone, may regulate membrane ion channels. This section will discuss the action of PGE_2 on two kinds of ion channels, the Ca^{2+} channels and the K^+ channels.

1. PGE_2 and Ca^{2+} channels

PGE_2 can regulate, or perhaps activate the Ca^{2+} channels in bovine adrenal chromaffin cells, Madin-Darby canine kidney cells and rat brain synaptosomes (Yokohama *et al.*, 1988; Aboolian *et al.*, 1989; Negishi *et al.*, 1989; Kandasamy and Hunt, 1990; Ito *et al.*, 1991a; Mochizuki-Oda *et al.*, 1991). As an extracellular local hormone, PGE_2 has the capacity of regulating Ca^{2+} channels. In bovine adrenal chromaffin cells, PGE_2 stimulates Ca^{2+} entry and phosphoinositide hydrolysis, which is dependent on the extracellular Ca^{2+}, and is accompanied by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Yokohama *et al.*, 1988). Using Fura-2, PGE_2 was shown to induce an initial transient increase in $[Ca^{2+}]_i$ followed by a sustained elevation in bovine adrenal chromaffin cells, dependent upon the extracellular Ca^{2+} (Ito *et al.*, 1991a). However, an alternative explanation of these results could be that the Ca^{2+} release from the intracellular stores was somehow dependent on the extracellular Ca^{2+} entry. To get a better view of the effect of PGE_2 on the Ca^{2+} channels, the PGE_2

receptor-operated Ca^{2+} channel in bovine adrenal chromaffin cells was studied using electrophysiological techniques (Mochizuki-Oda *et al.*, 1991). Under both whole-cell and cell-attached patch-clamp conditions, PGE_2 evoked an inward Ca^{2+} current. This observation has provided direct evidence for the activation of Ca^{2+} channels by PGE_2. In a different study using ^{45}Ca uptake assay, PGE_2 was found to enhance Ca^{2+} influx through the L-type voltage-dependent Ca^{2+} channels in rat brain synaptosomes (Kandasamy and Hunt, 1990). There has been a suggestion that eicosanoids may modulate Ca^{2+} currents via PKC activation (Keyser and Alger, 1990). However, it is not clear if this is also true for PGE_2.

2. PGE_2 and K channels

To date, there have been few reports on the regulation of K channels by PGE_2 and other eicosanoid metabolites. Bastin and colleagues (1990) observed that PGE_2 inhibited a K current in human lymphocytes (Jurkat T cell line). This effect was attributed to an increase in cytosolic cAMP concentration and a subsequent phosphorylation of the K channel protein by cAMP-dependent protein kinase (PKA). Fowler *et al.* (1985) reported that PGE_2 blocked a Ca^{2+}-activated K component, independent of effects on Ca^{2+} influx, in rabbit visceral neurons. Using the ^{86}Rb efflux technique, it was found that PGE_2 inhibited a Ca^{2+}-activated K channel in rat brain synaptosomes (Ren and Benishin, 1994). However, a recent study using similar methodology showed that PGE_2 promoted the opening of the ligand- (e. g., purinergic, muscarinic and opioid) gated K channels

(Zoltay and Cooper, 1994). There are a number of reports suggesting the role of other eicosanoids in the modulation of K channels. At certain identified neurones of the marine snail *Aplysia*, eicosanoids were implicated in the activation mechanism of a K current ("S" current), which contributes to the inhibition of electrically evoked transmitter release, or presynaptic inhibition (Piomelli *et al.*, 1987a; 1987b). Eicosanoids were also shown to directly activate K channels in excised patches from heart and smooth muscle cells (Kim *et al.*, 1989; Ordway *et al.*, 1989). Several other observations of eicosanoid-mediated modulation of K currents have been seen, implicating a role of lipooxygenase metabolites in the regulation of muscarinic receptor-gated K channels (Kim *et al.*, 1989; Kurachi *et al.*, 1989).

E. PGE_2 coupling of signal transduction systems

Considerable experimental evidence has accumulated suggesting that PGE_2, like many hormones and neurotransmitters, effectively regulates diverse biological activities via interaction with specific membrane surface receptors and intracellular second messenger systems. The diverse physiological and pathological actions of PGE_2, such as smooth muscle contraction and relaxation (Greeley *et al.*, 1986; Dorn *et al.*, 1992), immunoregulation (Ren and Wang, 1988), inflammation (Zurier, 1988), platelet aggregation (Vezza *et al.*, 1993), intracellular Ca^{2+} mobilization (Molnar *et al.*, 1987), cAMP formation (Molnar *et al.*, 1987), and ion channel modulation (Bastin *et al.*, 1990; Ren and Benishin, 1994) were attributed to the activation of the intracellular signal

transduction systems. The two most important are the cAMP-PKA and the PKC pathways (Goureau *et al.*, 1992). PGE_2 has been shown to stimulate adenylate cyclase activity in various types of cells. More recently, its activation of phospholipase C has also been documented in fibroblasts (Yamashita and Takai, 1987), osteoblasts (Farndale *et al.*, 1988), adrenal chromaffin cells (Yokohama *et al.*, 1988), astrocytes (Kitanaka *et al.*, 1991) and human platelets (Vezza *et al.*, 1993), *etc.* Other than these two signal transduction pathways, PGE_2 has also been reported to activate phospholipase D (PLD) (Wu *et al.*, 1992).

PGE_2 has been demonstrated to exert its actions through cytosolic second messengers, which include diverse molecules as cAMP, Ca^{2+}, IP_3 and DG. Second messengers such as cAMP and DG activate specific protein kinases, i.e., PKA and PKC, respectively (Scott and Soderling, 1992), and are known to be the essential second messenger pathways for various physiological processes (Ho *et al.*, 1988a; Di Marzo *et al.*, 1991; Nishizuka, 1994), such as modulating the activity of a large number of enzymes, membrane ion channels as well as structural proteins. Many K channels are believed to be modulated by various vasoactive substances, through the intracellular second messengers (Pfaffinger and Siegelbaum, 1990).

1. PGE_2 and the cAMP-PKA cascade

Intracellular cAMP concentration is dependent upon the activity of adenylate cyclase, which catalyses the conversion of ATP to cAMP, and phosphodiesterase (PDE),

which degrades cAMP to 5'-AMP. Adenylate cyclase activity can be regulated by the binding of agonists to membrane receptors, which are coupled to adenylate cyclase by at least two regulatory G proteins, G_s and G_i. Many biological actions of PGE_2 involve the activation of cAMP-PKA second messenger cascade. PGE_2 has been well documented to increase the cAMP level in many types of cells via stimulation of adenylate cyclase (Gilman *et al.*, 1971; Hamprecht *et al.*, 1973; Molnar *et al.*, 1987; Hagel-Bradway *et al.*, 1991; Coleman *et al.*, 1994).

Diverse biological actions of PGE_2 have been reported to be mediated by PGE_2-induced cAMP accumulation. These actions include stimulation of bone resorption and osteoblast activity (Raisz and Koolemans-Beynen, 1974; Arkins and Martin, 1977; Chambers *et al.*, 1984; Conaway *et al.*, 1986), stimulation of collagen and DNA synthesis (Chyun and Raisz, 1984), uterine smooth muscle contraction (Molnar *et al.*, 1987), cell differentiation and mutation (Ballard *et al.*, 1991), neuronal excitability (Kanba *et al.*, 1991), inhibition of gastric acid secretion (Reeves *et al.*, 1988) and lipolysis (Strong *et al.*, 1992).

As already mentioned, ion channels can be regulated by cAMP and cAMP-dependent protein phosphorylation. An increase in cAMP level was shown to influence the membrane ion currents. Addition of PKA activators and inhibitors directly into the cells and were found to influence membrane ion channel conductance (Levitan, 1985; 1988). These experiments demonstrated that phosphorylation of the membrane ion channel proteins, by cAMP-dependent protein kinase (PKA) could lead to the modulation

of ion channels. However, very few reports concerning the direct action of cAMP on ion channels were seen. Ling *et al.* (1994) described that PGE_2 activated clusters of apical chloride (Cl^-) channels in principal cells. The activation was via a cAMP-dependent pathway. The inhibition of K channel conductance by PGE_2 in human T lymphocytes and cultured arterial smooth muscle cells was attributed to the accumulation of cytosolic cAMP and a subsequent phosphorylation of K channels by PKA (Bastin *et al.*, 1990; Dixon *et al.*, 1990). The modulation of K channels by cAMP has been discussed earlier in this thesis.

Although coupling of the PGE_2 receptors to the cAMP-PKA pathway, through both G_s and G_i, is one possible explanation for for the actions of PGE_2, other potential second messenger mediated mechanisms of action of PGE_2 are equally important and should not be ignored.

2. PGE_2 and the PLC-PKC cascade

Studies have demonstrated that PGE_2 can stimulate PLC and the phosphoinositide hydrolysis, resulting in the formation of DG and IP_3, which lead to the activation of PKC and elevation of $[Ca^{2+}]_i$, respectively (Sonnenburg and Smith, 1988; Yamaguchi *et al.*, 1988; Yokohama *et al.*, 1988; Aboolian *et al.*, 1989; Hayaishi, 1989). PGE_2-induced IP_3 generation triggered the release of intracellular Ca^{2+} from cytoplasmic stores such as SR in smooth muscles. The increase in $[Ca^{2+}]_i$ can thus turn on numerous biological actions such as ion channel activation (Ito *et al.*, 1991a). However, this will not be further

discussed in this thesis.

Many actions of PGE_2 have been credited to the activation of the PLC-PKC second messenger pathway. PGE_2 is capable of amplifying the platelet aggregation response to stimuli by facilitating the activation of PKC (Vezza *et al.*, 1993). Activation of the PLC-PKC cascade has also been shown to mediate the response of PGE_2 in bone metabolism (Yamaguchi *et al.*, 1988; Kozawa *et al.*, 1992), Ca^{2+} uptake and cAMP production (Hagel-Bradway *et al.*, 1991), transmitter release (Yokohama *et al.*, 1988; Negishi *et al.*, 1989), immunosuppression (Piau *et al.*, 1989), gene expression (Aksamit *et al.*, 1993), salt and water transport (Hebert *et al.*, 1990), muscle contraction (Heaslip and Sickels, 1989), gastric acid secretion (Choquet *et al.*, 1990), and ion channel conductance (Ren and Benishin, 1994).

The idea that PGE_2 and other eicosanoid metabolites are capable of modulating K channel conductance via a novel PKC activation mechanism came from the work on Ca^{2+} channels, as mentioned earlier (Keyser and Alger, 1990). There has been very limited evidence suggesting that PGE_2 could modulate K channels through the activation of PKC. A Ca^{2+}-activated K channel in rat brain synaptosomes was inhibited by PGE_2 through PKC activation (Ren and Benishin, 1994).

PKC is often associated with the membrane phospholipids. It has been proposed that a translocation of PKC from the cytoplasm to the cell membrane could be the key step for direct PGE_2 activation in the presence of Ca^{2+} (El-Fakahany *et al.*, 1988). PGE_2 might be able to act through its specific surface receptor to activate PKC (Scherer and

Breitwieser, 1990). Similar to the cAMP-PKA system, the PLC-PKC cascade is another major signalling system that certain kinds of G proteins (which are PTX-sensitive) may be involved in, to couple the PGE_2 receptor to PLC (Gilman, 1987). However, PGE_2 has also been reported to activate PKC through a PTX-insensitive G protein (Negishi *et al.*, 1989; 1990) or directly at sites distal to DG and IP_3 generation (Piau *et al.*, 1989).

3. Cross-talk between the two signal transduction pathways

Cross-talk between signal transduction pathways may play a critical role in integrating multiple signal controls into an unitary cellular response. Interactions between the cAMP-PKA and the PKC cascades have been well described, including in smooth muscles (Nishizuka, 1984a; Berridge, 1987; Yamaguchi *et al.*, 1988; Di Marzo *et al.*, 1991), and was recently postulated in the mechanism of PGE_2 action, such as in osteoblast-like cells (Kawase *et al.*, 1991; Kozawa *et al.*, 1992), neuroblastoma cells (Kanba *et al.*, 1991), and gastric parietal cells (Choquet *et al.*, 1990).

The modulation of the cAMP-PKA signal transduction pathway by PKC activation is complex. PKC activation may either augment or attenuate the agonist-induced cAMP accumulation, depending on the cell species, and the types and duration of phorbol ester treatments. The diversity in cellular regulation may also be related to the number of PKC isoenzymes present in the cells which may be differentially activated by agonists such as phorbol esters.

Activation of PKC has been documented to promote cAMP accumulation,

although the sites of action in the cAMP-PKA pathway are still not clear. PGE_2 could activate PKC through a PTX-sensitive as well as -insensitive G protein, or at sites distal to phosphoinositide hydrolysis (Negishi *et al.*, 1989; Piau *et al.*, 1989; Kitanaka *et al.*, 1991). TPA was reported to potentiate the PGE_2-induced cAMP accumulation in fetal rat osteoblasts. The action was probably due to the potentiation of AC. Pretreatment the cells with PTX did not prevent such action (Bos *et al.*, 1991). Regulation of progesterone production in monkey luteal cells by PGE_2 has also been reported to involve both PKC activation and cAMP accumulation (Michael *et al.*, 1993).

The notion that PKC may play a direct role in mediating adenylate cyclase activity has been suggested for many different tissues (Katada *et al.*, 1985; Nagshineh *et al.*, 1986; Yoshimasa *et al.*, 1987; Gusovsky and Gutkind, 1991; Hagel-Bradway *et al.*, 1991; Eikvar *et al.*, 1993). PKC has been shown to have a dual action, providing either positive feed-forward or negative feedback control (Mukhopadhyay and Schumader, 1985; Rebois and Patel, 1985; Kawase *et al.*, 1991) on cAMP accumulation, depending on the cell types and the experimental conditions. Substantial progress has been made recently towards characterizing the "cross-talk" mechanism between these two messenger systems in a variety of systems (Yoshimasa *et al.*, 1987; Abdel-Latif, 1991; Houslay, 1991; Morimoto and Koshland, 1994).

V. Objectives

K channels play an important role in the control of excitability of VSMCs. Suppression of K channels has been shown to lead to membrane depolarization and spontaneous action potential activity in many arteries. The single VSMCs isolated from the rat tail artery are an ideal model for investigation of small peripheral resistance vessels. The present study focuses on the isolation and identification of a voltage-dependent, delayed rectifier K channel current, and the characterization of the channel current with β-dendrotoxin, in VSMCs from rat tail artery. The effect of a vasoactive substance, PGE_2, on the K channel conductance in VSMCs, was studied. To elucidate the mechanism of action of PGE_2, pharmacological profiles of receptor, G protein and intracellular second messenger cascades were extensively studied. Further experiments were carried out to define the relationship between PGE_2, tension and intracellular Ca^{2+} in smooth muscle strips as well as in isolated cells, using tension measurements and analysis of Fura-2 fluorescence.

There are four major objectives in this thesis:

1. Characterization and identification of the voltage-dependent, delayed outward rectifier K channel currents (I_k) in VSMCs from rat tail artery;
2. Characterization of the action of β-dendrotoxin on I_k in VSMCs from rat tail artery;
3. Investigation of the effect of PGE_2 on I_k in VSMCs from rat tail artery and the intracellular messenger cascade(s) mediating the action;

4. Study of the action of PGE_2 on isometric tension development in smooth muscle helical strips from rat tail artery, and the subcellular mechanism(s) involved.

CHAPTER 2

MATERIALS AND METHODS

In this chapter, all the methods used to carry out the thesis work will be described. These will not be repeated in the following chapters.

I. Preparation of VSMCs

VSMCs were isolated from rat tail artery as reported previously (Ren *et al.*, 1994). The tail arteries from 12-14 week male Sprague-Dawley (SD) rats (100-200 g) were excised and the connective tissue was removed. The arteries were then opened and cut into small pieces, and incubated in Hank's balanced salt solution (HBSS) (Gibco) at 4°C for 30 min. The tissue was treated with enzyme solution I (collagenase/dispase 1.5 mg/ml, elastase 0.5 mg/ml, trypsin inhibitor 1 mg/ml and bovine serum albumin 2 mg/ml) for 45 min at 37°C. The tissue was further digested with enzyme solution II (collagenase 1 mg/ml, trypsin inhibitor 0.5 mg/ml and bovine serum albumin 2 mg/ml) for 45 min at 37°C. The tissue was then mechanically dispersed and the harvested cells were plated onto petri dishes. The calcium concentration was stepwise increased to 2 mM. The cells were stored for 4 hours at 4°C in Dulbecco's Modified Eagle medium (DMEM) containing 1% PNS (penicillin, neomycin and streptomycin) without fetal bovine serum (FBS) but containing insulin (0.8 U/ml, Sigma). The solution was then

replaced by DMEM containing 10% FBS to facilitate the attachment of the cells. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Both primary cultured and subcultured cells were used in the study. The recordings were made 8-36 hours after isolating. In the studies of subcultured cells, confluent cells were treated with trypsin (1 mg/ml) in HBSS and resuspended in DMEM. The cell suspension was then centrifuged and the pellet was washed with HBSS before being replated in DMEM on petri dashes. Cells from both primary culture and subculture (passage 8-12) were spherical or oval in shape and the diameter was about 20-30 μM when observed with an inverted microscope (Nikon Diaphot, Nikon, Tokyo, Japan).

II. Patch-Clamp

A. Voltage-Clamp

The whole-cell variation of the patch-clamp technique (Hamill *et al.*, 1981) was used in order to apply voltage-clamp methods to measure the outward current through voltage-dependent K channels. The experiments were performed at room temperature (20-22 °C) using an Axopatch 1-C (Axon Instruments, Foster City, CA, USA).

1. Electrodes

The patch pipettes were pulled from thin-walled borosilicate glass capillaries (1.2 mm outer diameter and 0.9 mm inner diameter, FHS, Brunswick, ME, USA) on a two-stage electrode puller (Narishige Scientific Instrument, Lab MF-83, Tokyo, Japan). After

fired polishing, the tips of electrodes had resistances ranging between 4-8 MΩ, when filled with the pipette solution (see below).

2. Solutions

a. K channel recording solutions

The normal physiological salt solution (PSS) was used as the bath solution, containing (mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 N-[2-hydroxy-ethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES), 10 glucose, pH adjusted to 7.4 with NaOH, with osmolarity adjusted to 320 mOsM with sucrose. Tetraethyl ammonium (TEA) containing PSS was prepared from normal PSS by replacing NaCl to an equimolar amount of TEA chloride (TEA-Cl). Two pipette solutions with high and low concentrations of the Ca^{2+} chelator ethylene glycol bis(β-aminoethyl ether)-N, N'-tetraacetic acid (EGTA) were used. The low free Ca^{2+} pipette solution contained (mM): 115 K aspartate, 25 KCl, 2 $MgCl_2$, 11 EGTA, 10 HEPES ($[Ca^{2+}]_i < 10^{-8}$ M). A second pipette solution designated as the high free Ca^{2+} pipette solution contained (mM): 135 KCl, 2.5 $MgCl_2$, 10 HEPES, 2 adenosine 5-triphosphate disodium salt (Na_2ATP), 0.2 EGTA ($[Ca^{2+}]_i$ of about 10^{-6} M). The pH value of both pipette solutions was adjusted to 7.4 with NaOH, with an osmolarity of 320 mOsM adjusted with sucrose. Note that the KCl concentration was reduced in order to compensate for the increased osmolarity contributed by the elevated EGTA concentration.

b. Ca^{2+} channel recording solutions

The ionic composition of the extracellular medium for measuring Ca^{2+} channels was (mM): 20 Ba^{2+}, 105 Tris, 5 KCl, 5 $CsCl_2$, $0.5x10^{-3}$ TTX, 20 HEPES, 20 Glucose, pH adjusted to 7.4 with NaOH. The osmolarity was adjusted to 320 mOsM with sucrose. The pipette solution contained (mM): 70 Cs_2-aspartate, 2 ATP-Na_2, 5 K succinate, 5 K pyruvate, 5 $MgCl_2$, 5 phosphocreatin-Na_2, 15 HEPES, 25 Glucose, 10 EGTA. The pH was adjusted to 7.4 with NaOH. The osmolarity was adjusted to 320 mOsM with sucrose.

3. Experimental Procedures

Spherical cells with a clear border were usually chosen for study. The cells selected were not attached to other cells, and were round or oval in shape without any processes. The dishes with the cells attached were mounted onto the stage of an inverted phase-contrast microscope (Nikon Diaphot, Nikon, Tokyo, Japan). The liquid junction potentials between the bath solution and the pipette solutions were balanced by applying a D.C. offset voltage until the recorded current reading became zero. Connection of the the pipette and the bath was made via a low resistance agar bridge. The pipette tips were manipulated with a hydraulic three dimensional oil driven micromanipulator (Narishige, Tokyo, Japan). The pipette was pressed against the surface of the cell membrane and a mild suction was applied. Once a membrane-pipette seal with a high resistance (over 2 GΩ) was formed, a gentle suction was applied to break the membrane patch and access

was gained to the interior of the cell. The successful rupture of the patch was usually accompanied by an increase in the noise level and a large capacitance-charging current transient in response to the 10 mV command. All current records were filtered with a 4 pole Bessel filter set at a corner frequency of 5 kHz (Axon Instruments, Foster City, CA, USA). Electrical responses were monitored on an oscilloscope (Nicolet 310, Nicolet Instrument Co., Madison, WI, USA). The data were digitized and stored on floppy disks. All experiments were performed at room temperature (20-22°C).

4. Compensation of Leakage Current and Series Resistance

Leakage subtraction was achieved with the P/N protocol in pClamp software. The linear leakage conductance was estimated by applying two hyperpolarizing subpulses from the holding potential (-80 mV). It was assumed that these hyperpolarizing subpulses could not generate any active currents, and thus, any currents recorded were solely due to the passive leakage conductance. All current measurements were corrected by substracting the linear leakage current. The error of voltage drop caused by the series resistance could be significant when measuring the large, whole cell current. It could be partially compensated by adding an extra voltage signal to the command voltage. However, in most cases, the product of the series resistance and the peak outward current was less than 2 mV. Thus, the series resistance compensation was not activated. If the product was more than 2 mV, the series resistance compensation was activated using the Axopatch 1-C electronics, although it was usually impossible to compensate completely.

5. Membrane Current Measurements

The outward currents were activated by depolarizing the cells from a holding potential of -80 mV. The pulse protocol was generated using pClamp software and a Zenith personal computer (Zenith 386, Vancouver, Canada). Data analysis was carried out using pClamp software. The membrane current was always measured as the peak outward current (leakage corrected) unless otherwise indicated.

6. Capacitance Current and Space-Clamp Factor

The capacitive current transients caused by the charge and discharge of the electrons in the circuit can be partially reduced by minimizing the length of the pipette electrodes, and the connection [illegible] voltage input. These transients normally settled down within several hundred [illegible] ms in most of the VSMCs, which was fast enough to be separated from the outward K currents. The space-clamp factor is usually related to the spatial control of the voltage command. It is usually present to a certain extent in cells with irregular processes or large size. The space-clamp factor could be estimated under the experimental conditions by the following criteria (Liu, 1992): the speed of the decay of capacitive current (Byerly and Hagiwara, 1982); the dependence of the peak current amplitude on membrane voltage; keeping the time to the peak outward current independent of conditioning depolarizing pulses (Ogata *et al.*, 1989). Cells were discarded if they were not satisfactory with these criteria.

7. Administration of Drugs

Drugs were added directly to the bath. The currents were recorded 10 min later. The duration of depolarization test pulse was 250 msec unless otherwise indicated. In order to determine if the effect of PGE_2 on the K channels occurred via the intracellular second messenger system, several activators and inhibitors of PKA and PKC were used. Ethanol or dimethylsulfoxide (DMSO) was used as the vehicle for the water insoluble drugs. The final concentration of either ethanol or DMSO was less than 0.5%, and did not have a discernible effect on the K currents.

B. Current-clamp

The membrane potential was recorded by use of a glass micropipette connected to patch-clamp amplifier used in the current-clamp mode (model, Axopatch 1-C; Axon Instrument, Foster City, CA, USA) and was monitored with an oscilloscope (model, Nicolet 310, Japan). Measurements were made at room temperature (20-22°C).

III. Intracellular Ca^{2+} determination

Intracellular Ca^{2+} concentrations were measured using the Fura-2 technique. VSMCs (monolayer) grown on glass coverslips were incubated with 5 μM Fura-2/AM (acetoxymethyl ester) for 60 min in dark at room temperature according to the method of Verhage *et al.* (1988). After incubation the dye-loaded VSMCs were washed three times with physiological salt solution (PSS) containing (mM): NaCl 145, KCl 5, $MgCl_2$

2, $CaCl_2$ 2, glucose 10, $NaH_2PO_4 H_2O$ 0.5 and HEPES 10. The glass coverslips were then transferred to a Sykes-Moore perfusion chamber. The intracellular Ca^{2+} fluorescence measurements were performed at room temperature, using a spectrofluorometer (Spex, Edison, NJ, USA). Excitation of Fura-2 loaded VSMCs was at the wavelengths of 340 and 380 nM and emissions at 510 nM were recorded. The ratio of the fluorescence excited at 340 nM (F_{340}) divided by that excited at 380 nM (F_{380}) was calculated, and converted to $[Ca^{2+}]_i$ using the equation $[Ca^{2+}]_i = K_D[(R-R_{min})/(R_{max}-R)]\beta$, as described by Grynkiewicz *et al.* (1985). R is the measured F_{340}/F_{380} ratio. R_{min} and R_{max} were taken after addition of 5 mM EGTA (zero Ca^{2+}) and 10 μM ionomycin (Ca^{2+} saturation), respectively. β is the ratio of emission intensities at 380 nM excitation in these two sets of conditions. The K_D is the effective dissociation constant for Fura-2+Ca^{2+} which is assumed to be 224 nM (Grynkiewicz *et al.*, 1985). Depolarization was achieved by direct addition of 30 mM KCl into the chamber. Addition of a similar concentration of NaCl produced no change in $[Ca^{2+}]_i$, suggesting that the osmotic effects of this KCl challenge were not significant.

IV. Rat tail artery tension measurement

The method used for tension measurement was described previously (Pang *et al.*, 1985). Male SD rats weighing 250-300 g were anaesthetized with pentobarbital (50 mg/ml) before removal of the tail artery. The artery was rinsed in Krebs-Henseleit solution (KHS) which was oxygenated with 95% O_2-5% CO_2, and cleaned of adherent

connective tissue. Each vessel was then cut helically to yield strips of approximately 2 cm in length. These were mounted in KHS in a 10-ml Sawyer-Bartlestone organ bath chamber (37°C) also aerated with 95% O_2-5% CO_2. The development of isometric force was monitored using a Grass FT.03 force displacement of transducer and recorded on a Grass 79D polygraph. Each tissue was allowed to equilibrate under a basal tension of 0.7 g for 30-60 min in normal KHS prior to further experimentation. To allow inter-tissue comparisons of contractile activity, a reference contraction to 60 mM KCl was obtained in each tissue. The strips were then washed with KHS three times and allowed to return to base line resting tension. The detailed experiments will be described in Chapter 6 of this thesis. To study the contractile mechanisms that do not depend on extracellular Ca^{2+}, the artery strips were washed repeatedly with and equilibrated for a minimum of 20 min in a Ca^{2+}-free buffer containing 1 mM EGTA. Previous study (Shan, 1993) has shown that this procedure effectively removed the extracellular Ca^{2+}. The artery strips washed in this manner are neither contracted by an agent which depends on extracellular Ca^{2+} (KCl) nor relaxed by agents that block Ca^{2+} entry (such as nifedipine and verapermil).

V. cAMP measurement

VSMCs were exposed to the designated drugs for 20 min. The experimental medium was then removed from each well and replaced with 300 μl acetic acid (5 mM). After freezing and thawing three times, the contents of each well were transferred to 2-

ml Eppendorf tubes. The cell lysates were then boiled for 5 min. The lysate was used for the determination of cAMP by radioimmunoassay (RIA) (Harper and Brooker, 1975).

VI. List of chemicals and the sources

Antiserum for cAMP: from Dr. A. K. Ho, University of Alberta, Edmonton, Canada

4-AP: Sigma Chemical Co., St. Louis, MO, USA

BSA (Fatty Acid Free): Sigma Chemical Co., St. Louis, MO, USA

$CaCl_2$: Sigma Chemical Co., St. Louis, MO, USA

cAMP[^{125}I]: Bionetics Research Lab., Rockville, MD, USA

$CdCl_2$: Sigma Chemical Co., St. Louis, MO, USA

Charybdotoxin: Research Biochemicals Inc. Natick, MA, USA

Cholera toxin (a&b subunits): Sigma Chemical Co., St. Louis, MO, USA

Collagenase (Type II): Sigma Chemical Co., St. Louis, MO, USA

Collagenase/Dispase: Boehringer Mannheim Biochemica, Montreal, Canada

CsCl: Sigma Chemical Co., St. Louis, MO, USA

Db-cAMP: Biolog Life Science Institute, La Jolla, CA, USA

DiDS: Sigma Chemical Co., St. Louis, MO, USA

DMEM: Gibco., Grand Island, NY, USA

DMSO: Sigma Chemical Co., St. Louis, MO, USA

α- & β-DTXs: prepared according to the method (Benishin *et al.*, 1988) from *Dendroaspis angusticeps* venom, Latoxan, Rosans, France

EGTA: Sigma Chemical Co., St. Louis, MO, USA

Elastase (type I): Sigma Chemical Co., St. Louis, MO, USA

FBS: Gibco., Grand Island, NY, USA

Fura-2/AM: Molecular Probes, Inc. Eugene, OR, USA

GDPβS: Boehringer Mannheim GmbH, Mannheim, Germany

Glibenclamide: Research Biochemicals Inc. Natick, MA, USA

H-7: Seikagaku America Inc. St. Petersburg, FL, USA

HA1004: Seikagaku America Inc. St. Petersburg, FL, USA

HBSS: Gibco., Grand Island, NY, USA

HEPES: Fisher Scientific, Fair Lawn, NJ, USA

IBMX: Sigma Chemical Co., St. Louis, MO, USA

KCl: BDH Inc., Toronto, Canada

$LaCl_3$: Sigma Chemical Co., St. Louis, MO, USA

$MgCl_2$: Sigma Chemical Co., St. Louis, MO, USA

NaCl: BDH Inc., Toronto, Canada

NaH_2PO_4: BDH Inc., Toronto, Canada

NE: Sigma Chemical Co., St. Louis, MO, USA

4β-PDBu: Sigma Chemical Co., St. Louis, MO, USA

Pertussis toxin: Sigma Chemical Co., St. Louis, MO, USA

PGE_2: Sigma Chemical Co., St. Louis, MO, USA

PGI_2: Sigma Chemical Co., St. Louis, MO, USA

PNS: Gibco., Grand Island, NY, USA

Rp-cAMPs: Biolog Life Sciences Institute, La Jolla, CA, USA

SITS: Sigma Chemical Co., St. Louis, MO, USA

Sodium meclofenamate: Sigma Chemical Co. St. Louis, MO, USA

Sp-cAMPs: Biolog Life Sciences Institute, La Jolla, CA, USA

Staurosporine: Upstate Biologicals Inc. Lake Placid, NY, USA

[illegible]: Sigma Chemical Co., St. Louis, MO, USA

[illegible]A: Sigma Chemical Co., St. Louis, MO, USA

Trypsin Inhibitor (Type I): Sigma Chemical Co., St. Louis, MO, USA

VII. Statistics

The data were expressed as M $\pm$ S.E.. Tests of significance were performed using analysis of variance in conjunction with the Newman-Keuls multiple range test. P values less than .05 were accepted as being significant.

In patch-clamp studies, all voltage-current (I-V) plots were generated using the peak value of the outward current (leakage corrected). The concentration-response curves were fitted to a Michaelis-Menton equation (Hill's function: $I=I_{max}/[1+(k/x)^n]$) using a Marquart-Levenberg nonlinear least squares algorithm. In this equation, I_{max} is the maximal response, and k is the half-maximal response concentration, x is the testing concentration, n stands for the slope or the power factor. The currents after addition of drugs were normalized to the control responses. The concentration at which half-maximal

inhibition occurred (EC_{50}) was calculated from the fitted equation. The steady-state inactivation curve (Boltzmann equation) was fitted to the data using the same algorithm. The V_h (membrane potential at which 1/2 of the channels are inactivated) and the slope factor were calculated from the fitted curve.

CHAPTER 3

CHARACTERIZATION OF VOLTAGE-DEPENDENT K CHANNELS IN RAT TAIL ARTERY VASCULAR SMOOTH MUSCLE CELLS

I. Introduction

Ion channels, especially Ca^{2+} and K channels, which are present in the cell membrane, play critical roles in determining the excitability of smooth muscle cells. Almost all smooth muscle cells can respond to excitatory stimuli with membrane depolarization or the generation of action potentials when depolarization has reached threshold. Closure of K channels contributes to the membrane depolarization, action potential prolongation, and spontaneous activities of many tissues. Since methods for the isolation of single smooth muscle cells have been developed (Bagby *et al.*, 1971), the distribution, structure, and function of various K channels in VSMCs have been explored. Almost every type of K current that exists in neuronal membranes has also been found and characterized in isolated VSMCs. It appears that most VSMCs exhibit at least two types of K currents: one that is voltage-dependent, including the delayed rectifier (I_k), the transient outward (I_A), and the inward anomalous rectifer (I_{IR}) (Rogawski, 1985; Rudy, 1988; Bolzon, 1992; Quayle *et al.*, 1993); and one that is Ca^{2+}-

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Footnote: Some parts of this chapter have been published (Ren *et al.*, J. Pharmacol. Exp. Ther. 269: 209-214, 1994)

activated, with circumstantial voltage-dependence. Big-conductance Ca^{2+}-activated K currents (designated as BK channels) appear to be ubiquitous (Benham *et al.*, 1986; Hume and Leblanc, 1989; Beech and Bolton, 1990). A small-conductance (SK) Ca^{2+}-activated K channel has also been reported (Inoue *et al.*, 1985). Other types of K channels such as ATP-gated K channels (K_{ATP}) have been observed (Okabe *et al.*, 1987; Benham *et al.*,1986; 1987; Standen *et al.*, 1989). Like K channels in other tissues, the K channels in VSMCs have been considered to participate in the control of diverse vascular activities, especially the control of vascular smooth muscle tone (Benham and Bolton, 1986; Benham *et al.*, 1986; Bolton and Lim, 1989, Hume and Leblanc, 1989; Longmore and Weston, 1990; Zholos *et al.*, 1991; Nelson, 1993). The characterization of K channels in different tissues and vascular beds may help us to understand the subcellular electrical activities of local vascular smooth muscle function, since they are believed to be essential for local vascular smooth muscle tone. The rat tail artery is used as a model of small resistance arteries, and these small arteries, which are usually innervated by the autonomic nervous system, are known to play a major role in the regulation of peripheral resistance, and, ultimately, blood pressure (Myers *et al.*, 1985; Folkow, 1991). Different methods for the isolation of the VSMCs, either cultured or non-cultured, from rat tail artery have been described and modified recently (Wang *et al.*, 1989; Furspan, 1992; Bolzon *et al.*, 1993; Evans and Kennedy, 1994; Ken *et al.*, 1994). These VSMCs have then been used as a model of the peripheral resistance vessels for the investigation of various kinds of vasoactive agents (Pang *et al.*, 1990; Wang *et*

al., 1991; Furspan, 1992; Shan *et al.*, 1993; Earle and Triggle, 1994; Ren *et al.*, 1994; Shan *et al.*, 1994). The first-half of the present chapter is aimed at characterizing a K channel current in cultured VSMCs from rat tail artery.

The isolation and purification of K channels has been greatly hindered by the lack of specific high affinity pharmacological ligands for the channel proteins. Traditional K channel blockers such as TEA, 4-AP, and quinine are non-specific and can not be used for isolation studies. Several toxins have been isolated from bee or scorpion, and are specific antagonists for certain types of K channels, such as apamin which acts on SK, and charybdotoxin which acts on BK channels. Recent studies have indicated that certain vertebrate polypeptide toxins, the "dendrotoxins", from the venom of elapid snakes of the genus *Dendroaspis*, may be potent and selective antagonists for the voltage-dependent K channels (Dolly *et al.*, 1984; Harvey and Anderson, 1985; Halliwell *et al.*, 1986; Benishin *et al.*, 1988; Blaustein *et al.*, 1988; Muniz *et al.*, 1992; Tauc *et al.*, 1993). The well known excitatory action of toxins from these snake venom in facilitating transmitter release from nerve terminals can be accounted for by the selective inhibition of voltage-dependent K channels (Harvey and Anderson, 1985). Most of the knowledge about the effects of dendrotoxins is confined to the nervous system. In mammalian central neurons, α-dendrotoxin from the venom of the Eastern green mamba *Dendroaspis angusticeps* was found to selectively inhibit a fast-inactivating voltage-dependent K current, which corresponds to the I_A current (Dolly *et al.*, 1984; Halliwell *et al.*, 1986). α-Dendrotoxin blocked a slowly inactivating or non-inactivating voltage-dependent K current in

mammalian peripheral neurons, which corresponds to the "delayed rectifier" (Stansfeld *et al.*, 1987; Penner *et al.*, 1987). Stansfeld *et al.* (1986) reported that nanomolar concentrations of α-dendrotoxin could induce repetitive firing in rat visceral sensory neurons by inhibiting a slowly inactivating K current. Further characterization of the venom of *Dendroaspis angusticeps* resulted in the identification of four distinct peptide toxins, α-, β-, γ-, and δ- dendrotoxins, which have been shown to block different presynaptic voltage-dependent K channels in nerve endings (Benishin *et al.*, 1988). Sequence analyses have shown high structural homologies between pairs of toxins, α- and δ-, and β- and γ-. The inter-pair structural differences may reflect the differential actions of the two pairs of toxins, since α- and δ- dendrotoxins preferentially blocked the fast-inactivating 4-AP-sensitive component of the ^{86}Rb efflux attributed to the "I_A" channel, in rat brain synaptosomes, whereas β- and γ- dendrotoxins preferentially blocked the phencyclidine-sensitive non-inactivating component, which may be attributed to the "I_k" channel. Toxin sensitivity studies have revealed that the dendrotoxins are ineffective in inhibiting Ca^{2+}-activated K channels (Tauc *et al.*, 1993). Most studies have focused on the characterization of α-dendrotoxin (Sorensen *et al.*, 1990; Reid *et al.*, 1992; Parcej *et al.*, 1992; Muñoz *et al.*, 1992; Scott *et al.*, 1994a,b). There are few studies on the other dendrotoxins from the green mamba (*Dendroaspis angusticeps*), such as β-dendrotoxin. However, the dendrotoxins have the potential of being developed as valuable pharmacological probes for voltage-dependent K channels (Benishin *et al.*, 1988). The second half of the present chapter constitutes an attempt to characterize the

effects of β- and α-dendrotoxins on the voltage-dependent outward K currents in rat tail artery VSMCs.

II. Experimental design

The outward K currents present in both primary and subcultured VSMCs isolated from rat tail artery were described, using the protocol described in chapter 2. This was followed by a detailed characterization of the predominant outward K channel current.

Two different pipette solutions, with low and high intracellular $[Ca^{2+}]_i$ were used to characterize the outward K channel currents. These solutions were achieved by using high (11 mM) and low (0.2 mM) concentrations of the Ca^{2+} chelator EGTA, respectively (Hu *et al.*, 1991). Pharmacological profiles of the outward K channel current were determined to establish the identity of this outward K channel current and to rule out possible contributions from other sources of outward currents. Pharmacological blocking agents used included the K channel blockers TEA, 4-AP, charybdotoxin (Miller *et al.*, 1985), and glibenclamide, the Ca^{2+} channel antagonists nifedipine, La^{3+}, Cd^{2+}, and also the chloride channel blockers 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) and 4-acetamino-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) (Lewis *et al.*, 1993; McEwan *et al.*, 1993). The instantaneous tail currents were analysed to determine the reversal potential (equilibrium potential) and the major permeable ion through the channel. A K selectivity study was also carried out to compare the relationship between equilibrium potential and extracellular K concentration. Deactivation kinetics of the

outward K current was also analyzed.

Further characterization of the delayed rectifier voltage-dependent K channel current was performed with β-dendrotoxin, which has been considered to be a potential specific high affinity voltage-dependent K channel binding ligand. Experiments on the concentration-dependence of the response, steady-state-inactivation and use-dependence of inhibition were conducted to explore the mechanism of action for β-dendrotoxin on the delayed rectifier K channel. The effect of α-dendrotoxin was also examined to compare the specificity of different species of dendrotoxins.

III. Results

A. Morphology and passive membrane properties of freshly dispersed VSMCs from rat tail artery

Enzymatic dissociation yielded spherical or ovoid-shaped VSMCs 10-30 μm in diameter. Dispersed cells attached onto the petri dish during the first 6 hrs in culture. Progressive changes in cell morphology from spherical or ovoid to elongated and spindle-shaped then occurred. Some cells reached the latter morphological stage within 1 day, but most did so after 2-3 days of culture. The subcultured VSMCs did not exhibit significant morphological difference from the primary cells. Almost all the cells (>90%) obtained with the procedure described in the previous chapter were VSMCs as determined by the α-actin staining study (Wang *et al.*, 1989). Considering that irregular

shape and size of the cells may alter the space-clamp, only those spherical or ovoid cells were chosen for the patch-clamp study. The cells were used within 72 hrs of culturing. The cells were electrically quiescent with a resting membrane potential (V_m) of -45.0 $\pm$ 1.7 mV (M $\pm$ S.E., N=6). The input resistance (R_m) of these cells, estimated with a 10 mV test pulse, was 668.4 $\pm$ 56.5 MΩ (N=12). The cells could not be stimulated to fire action potentials by intense depolarization with normal PSS in thirteen cells tested.

B. The delayed rectifier outward K current in VSMCs with low intracellular free $[Ca^{2+}]_i$

In order to separate the outward current components which were not Ca^{2+}-sensitive, a pipette solution containing 11 mM EGTA, was used in an attempt to minimize the activation of the Ca^{2+} rents. From a holding potential of -80 mV, test pulses to more po erated a voltage-dependent, delayed rectifier outward K curre ltured and subcultured VSMC. Fig. 3-1 shows the outward c ; I-V relationship from a VSMC (primary culture). This cur ed at -45 to -40 mV and showed almost no inactivation. There was no significant decline in the current amplitude for at least 60 min. Very similar responses such as the activation voltage were also seen in subcultured VSMCs.

C. Pharmacological profiles of the delayed rectifier outward K current

1. Sensitivity to the K channel blockers TEA, 4-AP, charybdotoxin and glibenclamide

External application of TEA (10 mM), a quaternary ammonium ion that is known to block delayed rectifier K channels, inhibited the outward current by approximately 35% to 45% in both primary cultured and subcultured VSMCs. Fig. 3-2 shows the suppression of the outward current by TEA. At membrane potentials between -50 mV and +80 mV, the percentage of the inhibition of the outward current was relatively constant at 35% to 45%, as shown by the I-V relationship (Fig. 3-2). This suggests that there is not much voltage-dependence of the TEA-induced inhibition on the outward current at this voltage range. The I-V relationship also indicated that no shifts in the activation voltage occurred after TEA application. At a higher concentration (100 mM), TEA inhibited the current by 73.5 $\pm$ 10.4% (N=7), as shown in Fig. 3-3. The effect of TEA was reversible upon washout. 4-AP, charybdotoxin and glibenclamide, three other pharmacological blockers for transient outward "I_A" current, Ca^{2+}-activated K current, and ATP-gated K current, respectively, were used to examine the outward K current. As shown in Fig. 3-4, the delayed rectifier outward K currents were completely resistant to 4-AP (5 mM), charybdotoxin (1 μM) and glibenclamide (100 μM). These results suggested that the outward current was most likely conducted through a delayed rectifier K channel, and not that of transient outward, Ca^{2+}-activated or ATP-gated K channels.

2. Sensitivity to Ca^{2+}-channel blockers nifedipine, La^{3+} and Cd^{2+}

In some experiments, Ca^{2+} channel antagonists were used to test the possible contributions of any Ca^{2+}-sensitive components such as Ca^{2+}-activated K channels. Fig. 3-5 shows that nifedipine (1 μM), La^{3+} (1 mM) and Cd^{2+} (300 μM) did not have any noticeable effect on the outward current. This suggested that Ca^{2+}-activated components are not likely to contribute to the outward current.

3. Sensitivity to chloride channel blockers DIDS and SITS

The distribution of Cl^- across the membrane, as described in Chapter 2, raised the possibility that part of the outward current may be due to the inward movement of the Cl^- ions. However, when the chloride channel antagonists DIDS (100 μM) and SITS (1 mM) were applied to the bath, there was no effect on the delayed rectifier K current (Fig. 3-6), suggesting that the contribution of Cl^- channels under these conditions was minimal.

D. Kinetic analysis of the outward current

1. Reversal potential and K selectivity

In order to determine the reversal potential of this outward current, the instantaneous tail currents were measured using the pulse protocol shown in Fig. 3-7A. The value of the measured reversal potential was -80.8 $\pm$ 2.5 mV (N=5), which was similar to the calculated K equilibrium potential of -83.9 mV. To test the selectivity of

the outward current for K ion, the extracellular K concentration was elevated from 5 mM to 10 mM, while the Cl gradient remained unchanged. Figure 3-8 shows that the reversal potential was dependent on the K gradient. The reversal potential shifted towards more positive voltages when the external K concentration was increased to 10 mM. The K equilibrium potential under these conditions was calculated to be -66.5 mV. These results indicated the outward channel current is selective for K ions.

2. Tail current analysis of channel deactivation

Tail current analysis showed that the current deactivated as a single exponential function (Fig. 3-7), indicating there may be a single component contributing to the outward current. However, the possible existence of two or more components with very similar kinetic profiles could not be ruled out.

E. Outward K currents in VSMCs with high intracellular free $[Ca^{2+}]_i$

When the pipettes were filled with low (0.2 mM) EGTA solution (Hu *et al.*, 1991) and with 2 mM Ca^{2+} in the bath, stepwise depolarization of cells (primary culture) from a holding potential of -80 mV elicited a slowly activating outward current (Fig. 3-9), which demonstrated little inactivation. The currents were activated at a membrane potential of approximately -45 mV, which was close to that observed for the I_k current obtained in VSMCs with low intracellular $[Ca^{2+}]_i$ (11 mM EGTA) (Fig. 3-2). It showed some "noisy" fluctuations of the current records, which is characteristic for the Ca^{2+}-

activated K channels. Further pharmacological studies revealed that this outward current was inhibited by about 60% when 10 mM TEA was applied to the bath solution (Fig. 3-9). The L-type Ca^{2+} channel blocker nifedipine (1 μM) inhibited the outward current amplitude by about 20% (Fig. 3-10), suggesting that a portion of the outward current was likely to include a Ca^{2+}-activated component, i. e. the Ca^{2+}-activated K channel current. The activation of this Ca^{2+}-activated K current was secondary to the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channels (mostly L-type in VSMCs from rat tail artery, Wang, 1991). The I-V relationship of the macroscopic current was different from that of the I_k current in VSMCs with low $[Ca^{2+}]_i$ in the pipette solution (Fig. 3-2). A small plateau appeared in the voltage range of about -10 to +40 mV, which was sensitive to 10 mM TEA. This result suggested the possible existence of a second, Ca^{2+}-activated component, other than I_k, in the macroscopic current under the high pipette $[Ca^{2+}]_i$ experimental conditions.

F. Characterization of the delayed rectifier K current (I_k) with β-dendrotoxin

1. Effect of β-DTX on I_k

Figure 3-11 shows the inhibition of I_k by 100 nM β-DTX. The original records (leakage corrected) before and after β-DTX as well as the corresponding I-V relationships are shown in Fig. 3-11. β-DTX caused a significant reduction in the current amplitude, indicating that it is a delayed rectifier K channel antagonist. The concentration-dependence of the inhibition of I_k by β-DTX for both primary and

subcultured VSMCs is shown in Fig. 3-12. The peak amplitude of I_k measured at the test potential of +30 mV was normalized to its respective control and plotted as a function of the concentration of β-DTX. The concentration response curves were fitted to a Michaelis-Menton equation using a Marquart-Levenberg nonlinear least-squares algorithm. After addition of β-DTX, the currents were normalized to the control response. The suppression of I_k became larger as the concentration of β-DTX increased in both types of cells. β-DTX produced a slightly greater inhibition in primary cultured cells. A concentration of 1 μM β-DTX inhibited I_k by 45% in primary cultured cells compared with 35% in subcultured cells. The concentration for half-maximal inhibition (EC_{50}) of I_k by β-DTX was 51 nM in primary cultured cells and 71 nM in subcultured cells (calculated from the fitted curve). The effect of β-DTX on I_k was reversible upon washout, recovering essentially to the control level.

2. Effect of β-DTX on the I_k steady-state inactivation curve

The previous experiments suggested that β-DTX suppressed I_k. To determine whether this inhibition was also mediated by altering the state of the channel, the steady-state inactivation curves in the presence or absence of β-DTX were compared. The membrane potentials were adjusted to different levels before the application of a test pulse to +30 mV. The outward currents were normalized to the value of the peak outward current activated from the most negative membrane potential (-110 mV). In the case of the control, the currents were reduced in amplitude at less negative holding

potentials and were completely inactivated at around +10 mV. When β-DTX was in the bath, neither the slope nor the potential at which half the channels are inactivated (V_h) was significantly altered (Fig. 3-13). These data suggested that the inhibitory effect of β-DTX on I_k did not depend on whether the channel was inactivated.

3. Effect of β-DTX on use-dependent inhibition

The degree of I_k block could be influenced by the frequency and duration of the repetitive depolarization during a series of test pulses. Thus, eight consecutive pulses to +30 mV were applied at different intervals from a holding potential of -80 mV. The duration of each pulse was 250 msec. The peak amplitude of each train of pulses was measured and normalized to the first pulse of each series. The current decreased in amplitude during repetitive stimulation with interpulse intervals of 400 and 300 msec, and remained at a constant level from the third pulse onward. In the presence of β-DTX, the degree of decrease in current amplitude was not significantly different from the control level ($p > 0.05$, Fig. 3-14), suggesting that the inhibition of I_k by β-DTX was not use-dependent.

G. Effect of α-dendrotoxin on I_k

There was little or no change in the current amplitude after the VSMCs were exposed to α-DTX (200 nM) for 10 min (Fig. 3-15), suggesting that α-DTX did not inhibit I_k in VSMCs over the concentration range tested. α-DTX has, however, been

shown to inhibit I_k in mammalian peripheral neurones (Stansfeld *et al.*, 1987).

IV. Discussion

A. Characterization of the outward current in rat tail artery VSMCs

Most VSMCs exhibit two types of K channels, one voltage-dependent, one Ca^{2+}-activated (Longmore and Weston, 1990). The outward currents displayed a distinctive voltage-dependence in both low and high $[Ca^{2+}]_i$ VSMCs. However, DHP Ca^{2+} channel blocker nifedipine reduced the macroscopic outward current amplitude in high $[Ca^{2+}]_i$ VSMCs (with 0.2 mM EGTA in the pipette) by 20%, indicating a possible contribution of the Ca^{2+}-activated component(s), i.e., the Ca^{2+}-activated K channel(s). The activation of this Ca^{2+}-activated K channel(s), thus, appeared to be secondary to the entry of extracellular Ca^{2+}.

Following enzymatic dissociation, the freshly dispersed VSMCs were kept at 37°C in a humidified atmosphere composed of 95%O_2 and 5%CO_2. The cells usually started to attach to the bottom of the petri dish within the first 6 hrs of culture. Addition of fetal bovine serum (FBS) after the second hour of culture to facilitate the settling of the cells. Morphological analysis revealed that upon fresh dissociation the cells were spherical or ovoid with a relatively clear membrane surface. They became elongated or spindle-shaped after 2-3 days in culture. If this culture was continued for more than 3 days, the cells would normally lose their elongated-shape and were likely to interact with each

other or adhere together, as reported previously (Chamley-Campbell *et al.*, 1979). The cultured cells could display the characteristic "hill and valley" morphology (Chamley-Campbell *et al.*, 1979) when they reached confluency. They also contracted in response to contact with the pipette tip and some chemical stimuli such as norepinephrine. Resting membrane potentials of -38 mV and -52 mV have been reported in smooth muscle cells of rat tail artery (Bryant *et al.*, 1985; Bolzon *et al.*, 1993). These values were close to those obtained in the present study (-45.0 $\pm$ 1.7 mV). The result from the attempt to generate action potential is consistent with the finding that no action potential could be elicited in rat tail artery VSMCs (Longmore and Weston, 1990).

The present study described a distinctive voltage-dependent K current in cultured VSMCs. When cells were dialysed with an intracellular solution containing 11 mM EGTA, the dominant current was found to be solely a voltage-dependent delayed rectifier K current (I_k). A very similar voltage-dependent, Ca^{2+}-independent K current was described in acutely dissociated, non-cultured, rat tail artery smooth muscle cells (Evans and Kennedy, 1994). In this study, single VSMCs were isolated from tail arteries of Wistar-Kyoto (WKY) rats (note that the Sprague-Dawley (SD) strain of rats were used in the present study), by papain digestion followed by acute dissociation. From a holding potential of -60 mV and with 5 mM EGTA in the pipette, a voltage-dependent, the delayed rectifier K current was generated from the VSMCs. The existence of such a voltage-dependent, delayed rectifier K current as the major K current has also been confirmed in other vascular and intestinal smooth muscle cells (Shimada and Somlyo,

1992; Smirnov and Aaronson, 1992) in both cultured (Bolton *et al.*, 1985, 1986; Beech and Bolton, 1990; Smirnov and Aaronson, 1992) and enzymatically dissociated non-cultured conditions (Bolton *et al.*, 1985; Inoue *et al.*, 1985). The identification of voltage-dependent K channels supported the notion that the cultured arterial and venous cells from the rat tail respond mainly to electrical stimuli (Toro *et al.*, 1986).

As already mentioned, most VSMCs exhibit both voltage-dependent and Ca^{2+}-activated K channels. However, in the present experiments when cells were buffered with a low concentration of EGTA to achieve high $[Ca^{2+}]_i$, only 20% of the macroscopic current could be attributed to a Ca^{2+}-activated component(s), i. e., the Ca^{2+}-activated K channel. These data indicated that the contribution of Ca^{2+}-activated K channel to the degree of cellular excitability in VSMCs from rat tail artery might be relatively minor. This hypothesis presented here is in accordance with Toro's observation (Toro *et al.*, 1986) that the voltage-dependent K current is responsible for the macroscopic outward rectification under the conditions of both low and high $[Ca^{2+}]_i$ pipette solutions. The outward K currents were activated at approximately the same range of membrane potential (-45 to -40 mV) as that reported by Toro *et al.* (1986) (-35 mV). The holding potential was chosen to be -80 mV, as it exhibited the next highest degree of channel activation with little effect on membrane stability (compared to higher degrees of activation found at -110 to -90 mV which induced membrane instability).

TEA has been recognized for many years as an agent that blocks voltage- and Ca^{2+}-dependent K channels. It blocks the outward K current at millimolar concentrations

and has been widely used to discrimate different types of K channels based on the TEA-sensitivity (Rudy, 1988). TEA (100 mM) inhibited the outward current in rat tail VSMCs by 73.5 $\pm$ 10.4% (N=7). This suggests that I_k was possibly composed of two components with very similar kinetics but different sensitivity to TEA. This notion (two components with similar kinetics but different TEA sensitivity) was supported by a previous study in K channels expressed in *Xenopus* oocytes (e *et al.*, 1990). Evans and Kennedy (1994) also reported that the outward K cu generated from acutely dissociated rat tail artery VSMCs could be dissected into two components based on sensitivity to TEA. However, it is very difficult to separate these subtypes under the current experimental conditions. It has been reported elsewhere that TEA could only reduce the amplitude of I_k when applied from the external side of the membrane (Ives *et al.*, 1978). The bath application and quick recovery of the current amplitude upon washout of TEA implied that TEA would most likely act on the external side of the membrane. The inhibition by TEA did not display a voltage-dependence, indicating that the site of action of TEA lies out of the electrical field of the channel pore. However, it was suggested that the blockade of the voltage-dependent K channel by external application of TEA could be voltage-dependent in some cases (Latorre and Miller, 1983).

Pharmacological profiles showed that the outward K current was resistant to 4-AP, charybdotoxin and glibenclamide. These properties provided further evidence for the identification of the outward current. 4-AP is a potent inhibitor of the transient outward K current, although it also has a weak inhibitory effect on the delayed rectifier K channel

at higher concentrations . Evans and Kennedy (1994) found that the outward K current from acutely dissociated rat tail artery VSMCs was partially sensitive (inhibited by 50%) to 10 mM 4-AP. However, a fast, transient "I_A" current was not present. The discrepancy in 4-AP sensitivity in the outward K currents was considered to be due to the difference in the culture and non-culture procedures of the VSMCs, as well as the genetic strains of the rats. Since TEA has been reported to block the big-conductance Ca^{2+}-activated K channel, regardless of the side of application (Yellen, 1984; 1987; Latorre *et al.*, 1989), the addition to the external medium of the putative Ca^{2+}-activated K channel blocker charybdotoxin would thus help to determine any possible contamination of the voltage-dependent K channels. The existance of ATP-gated K channels has been documented in vascular smooth muscles such as rat and rabbit mesenteric arteries, as well as the papain digested non-cultured VSMCs from rat tail artery (Standen *et al.*, 1989; Furspan, 1992). Glibenclamide, at a concentration higher of 100 μM, should eliminate any possible K_{ATP} activation. The resistance of the outward currents to these three K blockers indicated that neither transient outward, nor Ca^{2+}-activated nor ATP-gated K channels were likely to contribute to the macroscopic outward currents in VSMCs from rat tail artery.

The non-responsiveness of the delayed rectifier K current to the Ca^{2+} channel antagonists nifedipine, La^{3+} and Cd^{2+} in VSMCs bathed by a Ca^{2+} buffered solution (with 11 mM EGTA in the pipette) further supported the hypothesis that there were no Ca^{2+}-activated components under these conditions, such as the Ca^{2+}-activated K channels

involved in the outward rectification. Two types of voltage-dependent Ca^{2+} channels with predominately L- and some T- type components, have been described previously in the same preparation (Wang *et al.*, 1989; Wang, 1991).

Because of the Cl^- concentration gradient across the membrane, as described in Chapter 2, it was reasonable to consider the contribution of Cl^- conductance to the outward current. The most extensively studied Cl^- channel has been the outward rectifier (Solc and Wine, 1991; Widdicombe and Wine, 1991). This channel could be activated by membrane depolarization and was kinetically similar to the delayed rectifier K channels. However, the kinetics and amplitude of the outward current generated from the rat tail artery VSMCs were not affected by the Cl^- channel blockers, DIDS and SITS, thus excluding a Cl^- channel current from the outward rectification. Measurement of the reversal potential with a constant Cl^- gradient further indicated that K^+, not Cl^-, was the primary conducting ion that passes through this channel.

External K is necessary not only for the K gradient, but also for maintaining the channel in a state in which it can readily respond to intracellular Ca^{2+} or extracellular K. External K at a concentration higher than 5 mM (the "physiological" extracellular concentration) would reduce the chemical driving force for K, therefore resulting in a shift of the reversal potential to the positive or less negative direction. The K selectivity study shown in Fig. 3-8 again supported the notion of K as the primary permeable ion.

It is worthwhile to point out that during the course of this work, Bolzon *et al.* (1993) described similar studies on VSMCs from rat tail artery. However, a 4-AP-

sensitive, TEA-resistant, delayed rectifier and a TEA- sensitive, high threshold Ca^{2+}-activated K current were reported. The delayed rectifier was activated at around -30 mV whereas the Ca^{2+}-activated K current was activated at +30 mV. Several factors may account for these discrepancies: (1). The genetic strain of animal. In Bolzon's study, Wistar rats from Charles River were used, while in the present study, SD rats were used. (2). The isolation procedure. Bolzon and colleagues used collagenase and papain in a one-step enzymatic perfusion procedure (Bolzon and Cheung, 1989), whereas a two-step (see method in Chapter 2) procedure was employed in the present study. (3). The type of cells used. Non-cultured VSMCs within 3 hrs of isolation were used in Bolzon's study. In the present investigation, only cultured or subcultured VSMCs were used. There is, however, always some uncertainity as to what extent enzymatic treatment and cell culture could affect the channel properties.

B. Characterization of the delayed rectifier K channel with β-DTX

Dendrotoxins are a homologous group of 57 to 61 amino acid, single-chained polypeptides found in snake (*Dendroaspis angusticeps*) venom along with convulsant activity (Dolly *et al.*, 1984). Some dendrotoxins have been found to facilitate transmitter release at peripheral and central neurones due to the highly specific blockade of certain types of the voltage-dependent K channels (Dolly *et al.*, 1987). Fractionation of green mamba venom by size exclusion chromatography and cation exchange HPLC yields four 7000-dalton polypeptides, α β-, γ-, and δ- dendrotoxins (Benishin *et al.*, 1988), that

block different synaptosomal voltage-dependent K channels. Sequence analysis indicated that the N- terminal rather than the C- terminal regions are possibly responsible for the K channel blocking activity. There are almost no sequence homologies at the N- terminal portion between α- and δ- DTXs, and β- and γ- DTXs. The finding of structurally similar peptide toxins with preferential activities toward different populations of K channels raises the notion of developing these toxins into useful probes for the voltage-dependent K channel structure, providing us with the tools to distinguish different voltage-dependent K channels.

The present study has characterized the direct action of β-DTX on the I_k current in rat tail artery VSMCs using the whole-cell version of the patch-clamp technique. With very similar kinetics and pharmacology, the K current from the subcultured VSMCs appeared to retain the characteristics from the ancestor primary cultured VSMCs. β-DTX inhibited the I_k current. This inhibition was concentration dependent with similar EC_{50} values in these two types of cells. Primary cultured cells showed slightly higher sensitivity to β-DTX. The EC_{50} values for inhibition by β-DTX (51 and 71 nM for primary and subcultured VSMCs, respectively) were comparable to the K_d for binding of 36 nM as reported previously (Sorensen and Blaustein, 1989). The inhibition was reversible upon washout. Furthermore, the inhibition showed no membrane potential or use dependence. α-DTX produced no response indicating that the DTXs exhibited some specificity in VSMCs. These studies of the effect of β-DTX on I_k strongly suggested that β-DTX is a voltage-dependent K channel antagonist with a structure different from that

of other known K channel antagonists.

It has been suggested that α- and β-DTXs may be valuable ligands for the identification, purification, and characterization of certain voltage-dependent K channel proteins. Acceptors of both toxins were solubilized from rat brain synaptosomal membranes (Sorensen and Blaustein, 1989). The toxin-binding and pharmacological properties were preserved in the soluble acceptor proteins, but the affinities were lower. Monoclonal antibodies have been raised against the voltage-dependent K channels using purified α-DTX acceptor protein (Scott *et al.*, 1990; Muniz *et al.*, 1992; Scott *et al.*, 1994b). Coimmunoprecipitation of α- and β- DTXs provided further evidence that both acceptors were tightly associated constituents of the voltage-dependent K channel protein complexes. Although the two DTXs showed some specificity, preferentially blocking different voltage-dependent K channels (Benishin *et al.*, 1988), the characteristics of their binding to synaptosomal membranes using iodinated ^{125}I-α-DTX and ^{125}I-β-DTX were found to be very similar (Sorensen and Blaustein, 1989). Both toxins had comparable pH and cation dependence and the radiolabelled binding affinities (K_d=0.7 and 36 nM for α- and β-DTX, respectively) were inhibited in a similar fashion by the four homologous unlabeled DTXs (α-, β-, γ-, δ-). These results implied that the tissue might have a similar number of binding sites for β-DTX and for α-DTX. However, Sorensen and Blaustein (1989) reported that the density of β-DTX-binding sites was 4 to 5 times greater than the density of α-DTX-binding sites. Both iodinated DTXs covalently labelled polypeptide acceptors of approximately the same molecular weight (65 kdaltons),

implying that both toxins bound to distinct but structurally similar channel acceptors. This also supported the notion that α-DTX acceptors, estimated to have a molecular weight of 270 kdaltons (Sorensen and Blaustein, 1989), are likely to be composed of tetramers. This is consist with the proposed voltage dependent K channel structure (Stevens, 1987; Parcej *et al.*, 1992). Although these studies are at an early stage of development, it may be possible to purify the acceptor protein for β-DTX, determine the molecular weight and raise monoclonal or polyclonal antibodies against this type of voltage-dependent K channel.

Conclusion

The studies described in this chapter described for the first time a distinct voltage-dependent, delayed outward rectifier K channel current (I_k) in cultured VSMCs from rat tail artery and provided an insight into the pharmacological profiles of the delayed rectifier K current. It was also the first time that β-dendrotoxin, a potential pharmacological tool for the isolation and purification of the voltage-dependent K channel protein, was used to for an in depth characterization of a voltage-dependent K current. This will provide a very useful model for peripheral resistance vessels to determine the role of the voltage-dependent K channels in controlling the vascular smooth muscle tone in response to various vasoactive agents. However, the fact that β-DTX could not completely block the delayed rectifier K channel indicated the possible existance of one or several classes of β-DTX-insensitive voltage-dependent K channels. Further studies

employing single-channel recording techniques would help to answer the question. Finally, caution needs to be taken in extrapolating the electrophysiological evidence from the single cells to the intact tissue, especially with prolonged cell culture which might induce changes or mutations in the phenotype of the K channel proteins, and thus, affect the expression of K channels (Owens *et al.*, 1987; Yuan *et al.*, 1993a).

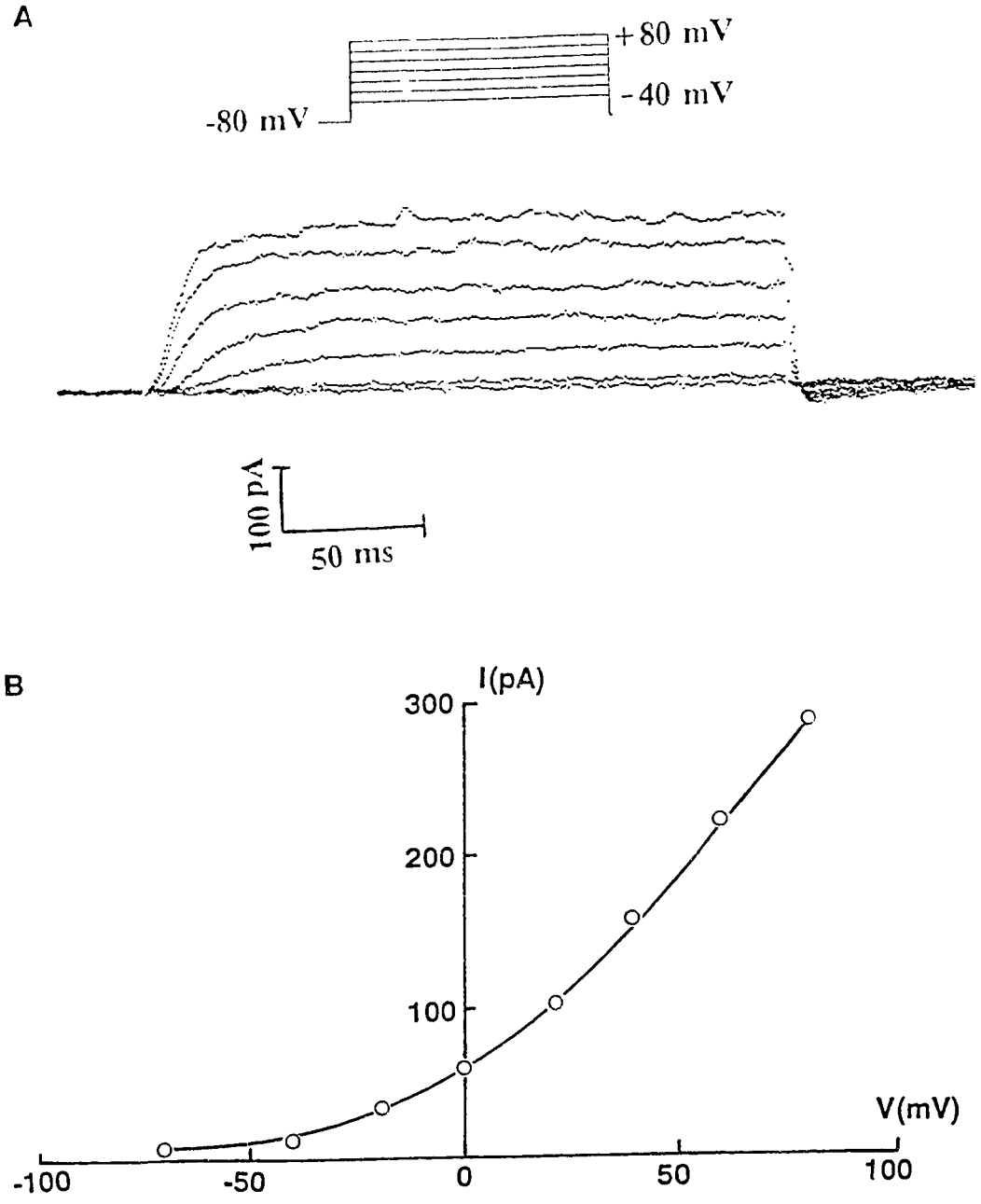

Figure 3-1. The delayed outward rectifier K current in a primary cultured VSMC. The pipette solution contained 11 mM EGTA (low intracellular $[Ca^{2+}]_i$). The current was activated by depolarizing the cell from a holding potential of -80 mV to various test potentials. A. The original outward current records (leakage corrected) and the voltage-clamp pulse protocol. B. The corresponding I-V relationship.

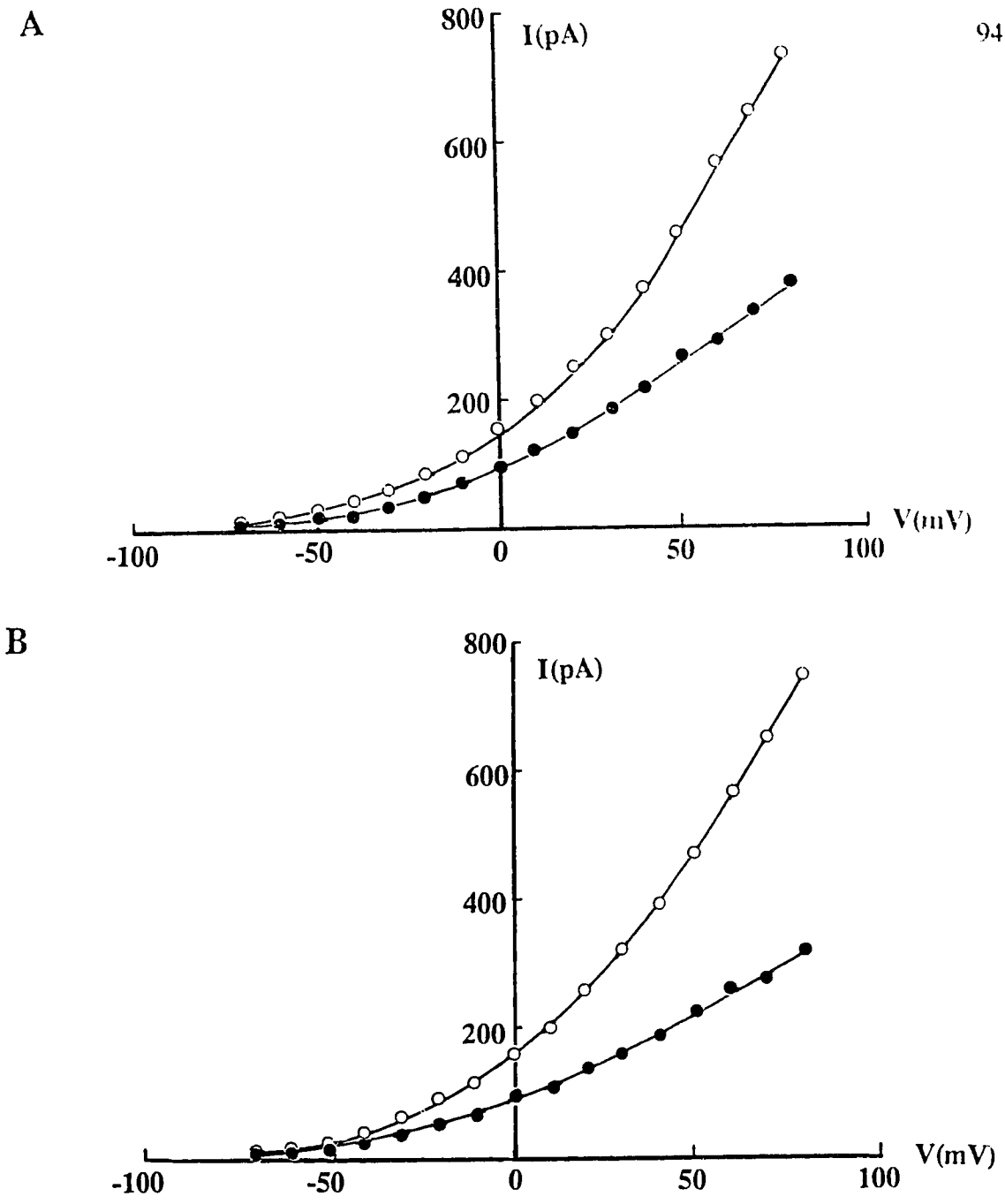

Figure 3-2. Effects of TEA on I_k in primary (A) and subcultured (B) VSMCs, with high pipette EGTA (11 mM). TEA (10 mM, filled circles) was added to the bath solution and the current was recorded after 10 min, for various potentials and compared with corresponding control record (open circles). I-V relationships were shown both in the case of control experiments (open circles) and for cells exposed to TEA (filled circles).

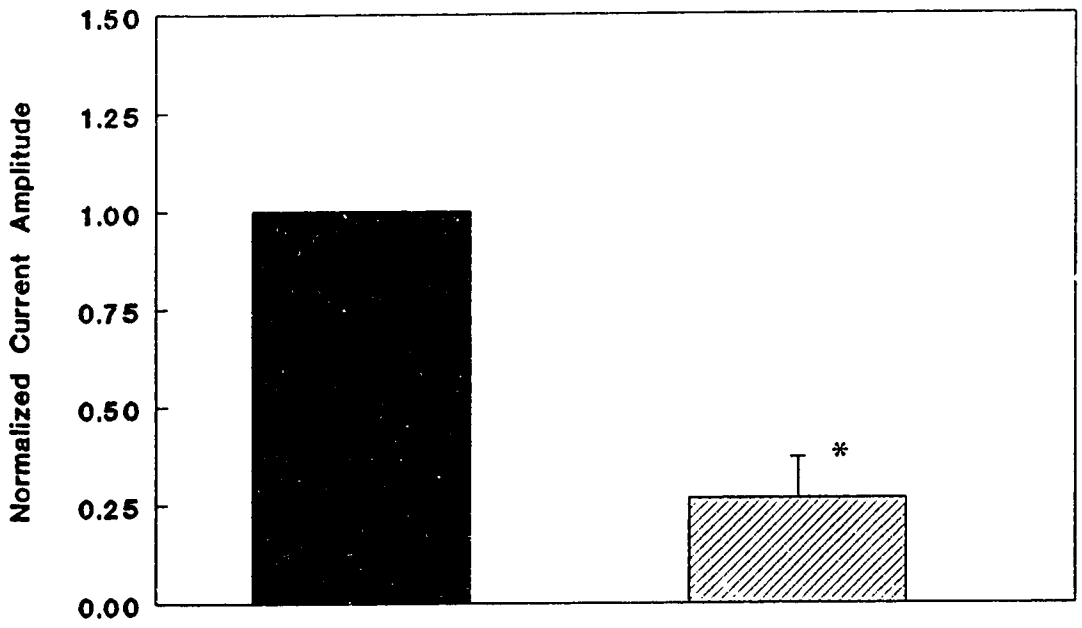

Figure 3-3. Effect of TEA on I_k in primary cultured VSMCs. The cells were treated with 100 mM TEA (N=7, bath substitution) for 10 min (cross-hatched bar). Current amplitudes were measured at a test potential of +30 mV and were normalized to the corresponding control (prior to the addition of the antagonist). *$p<0.01$ compared to control (solid bar).

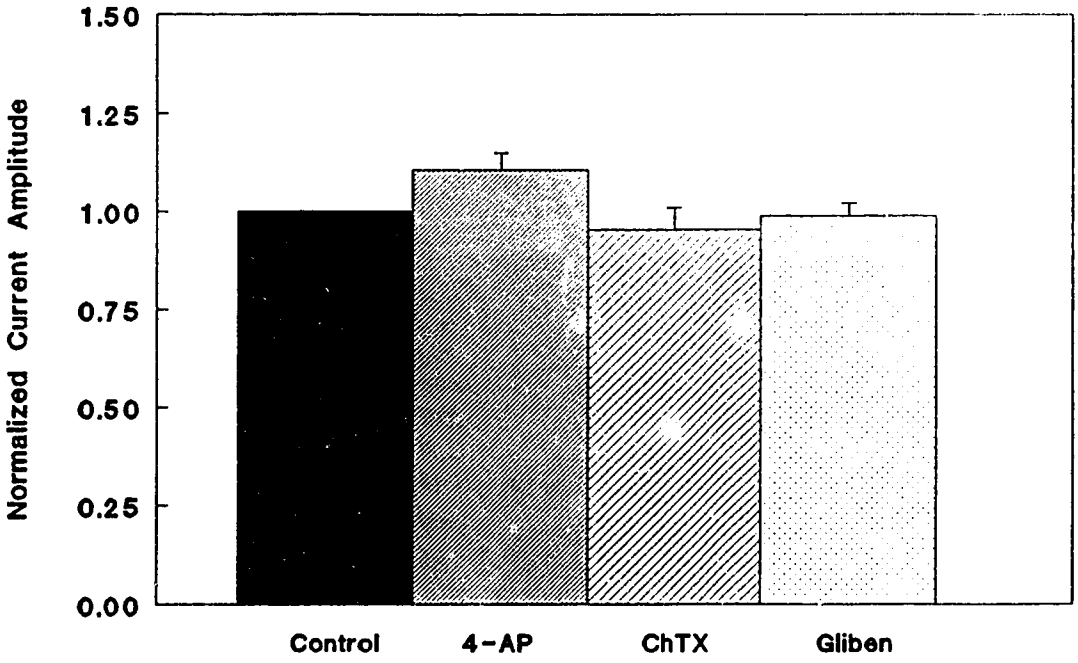

Figure 3-4. Effects of 4-AP, charybdotoxin and glibenclamide on I_k in primary cultured VSMCs. Current amplitudes to a test pulse of +30 mV were recorded before and 10 min after the administration of 4-AP (5 mM), charybdotoxin (ChTX, 1 μM) or glibenclamide (Gliben, 100 μM). The current amplitudes after pharmacological treatment (cross-hatched or stipled bars) were normalized to the values prior to the addition of drugs (solid bar). $p>0.05$ compared to control. N=4 for each drug.

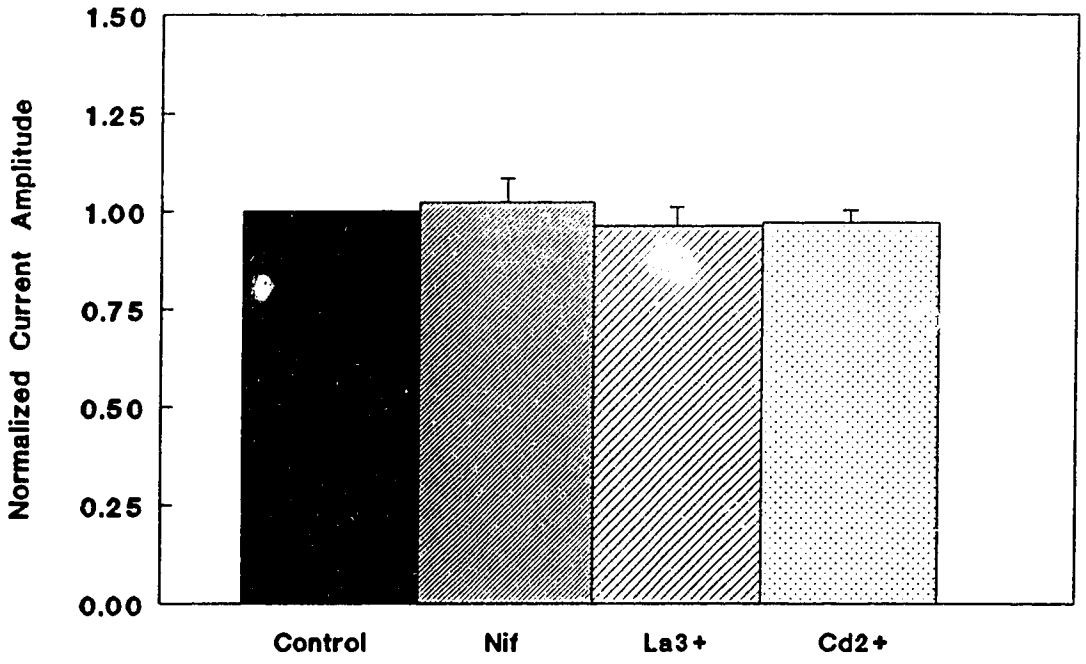

Figure 3-5. Effects of nifedipine, La^{3+} and Cd^{2+} on I_k in primary cultured VSMCs. Cells were treated with 1 μM nifedipine (Nif, N=5), 1 mM La^{3+} (N=4) or 300 μM Cd^{2+} (N=5) for 10 min (cross-hatched or stipled bars). Current amplitudes were measured at a test pulse of +30 mV and were normalized to the corresponding control values (current before the addition of the antagonists). $p > 0.05$ compared with control currents (solid bar).

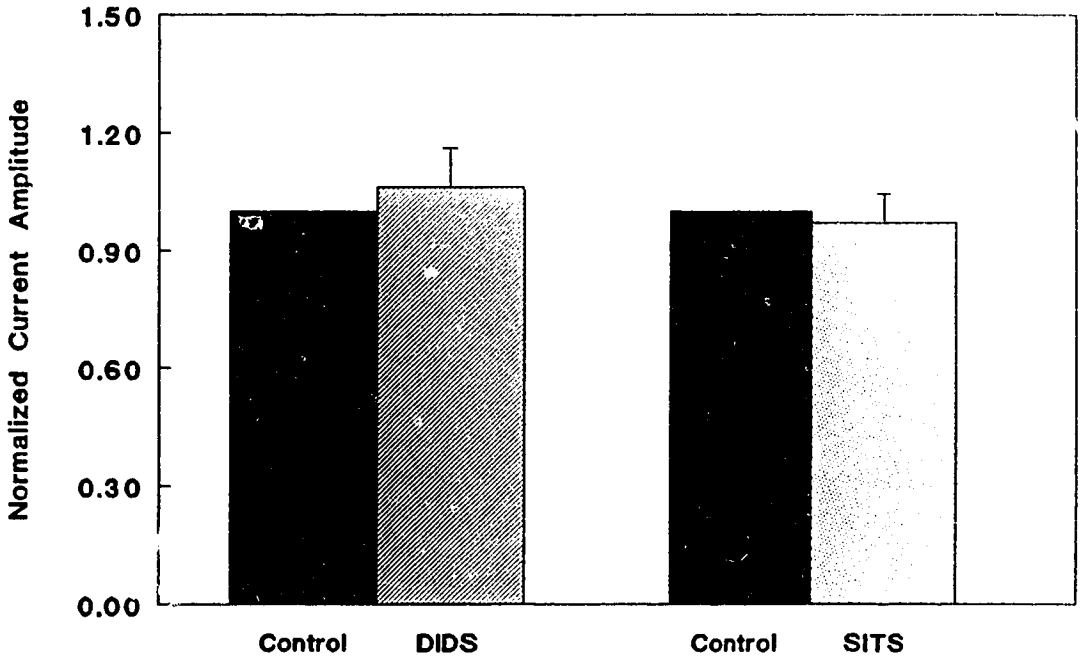

Figure 3-6. Effects of Cl^- channel blockers DIDS and SITS on I_k in primary cultured VSMCs. Cells were treated with 100 μM DIDS (N=6) or 1 mM SITS (N=7) for 10 min. Current amplitudes were measured at a test potential of +30 mV and were normalized to the corresponding control values (current before the addition of the antagonists). $p > 0.05$ compared with the control (solid bars).

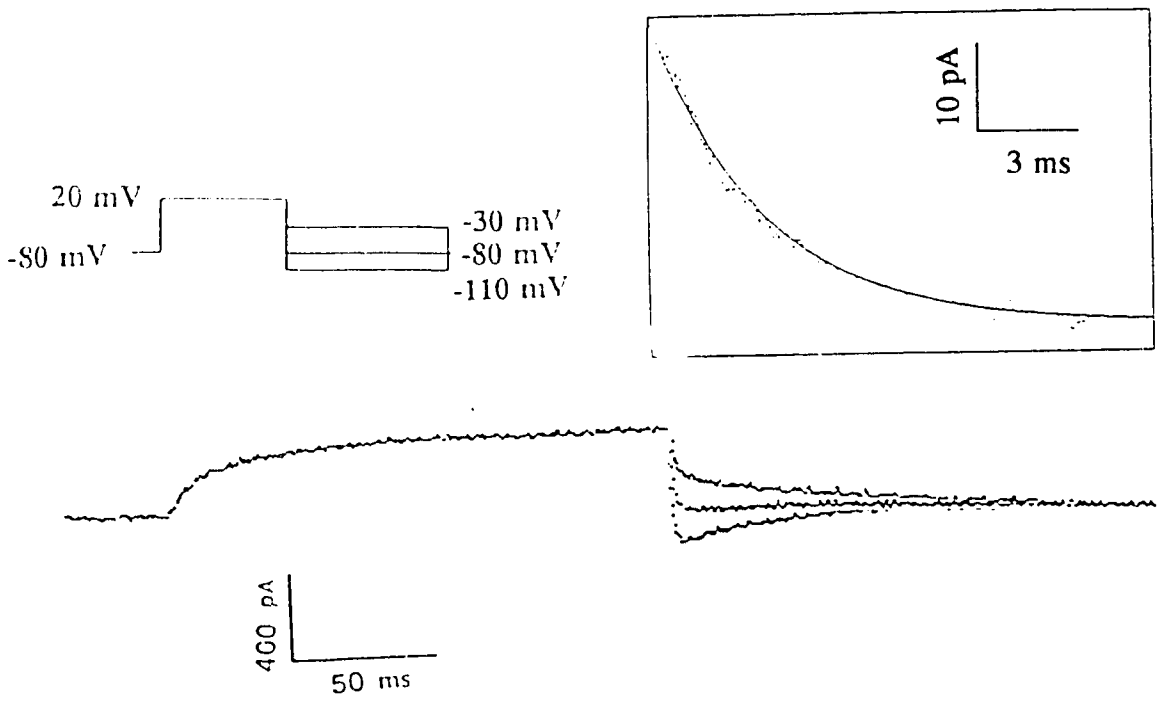

Figure 3-7. Tail current analysis of reversal potential in a primary cultured VSMC. A representive experiment showing instantaneous tail currents at three different repolarizing potentials. The decay of the current (the positive tail at a repolarizing potential of -30 mV) elicited from a holding potential of -80 mV to a test potential of +20 mV could be fitted by a single exponential (τ=2.5 ms, r^2=0.98).

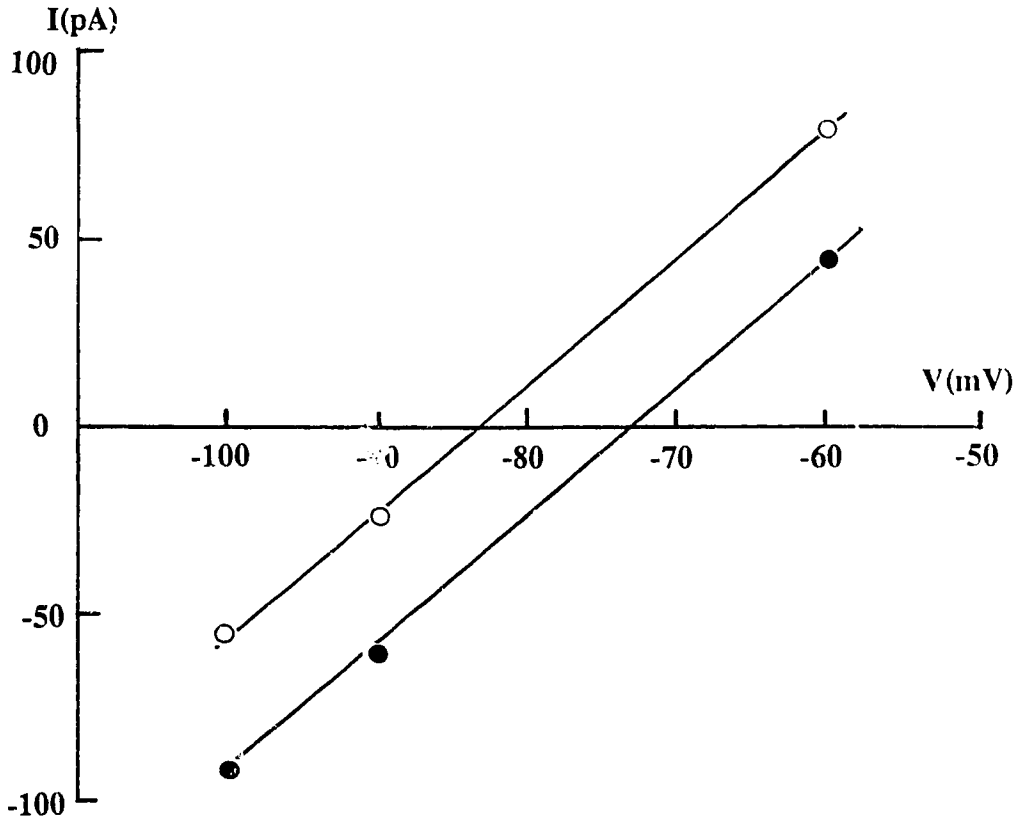

Figure 3-8. Effect of extracellular K on the reversal potential of I_k in VSMCs from rat tail artery. The open circles represent an experiment with $[K^+]_{in}=140$ mM, $[K^+]_{out}=5$ mM, and a reversal potential calculated to be -83.9 mV. The filled circles represent an experiment with $[K^+]_{in}=140$ mM, $[K^+]_{out}=10$ mM and a reversal potential calculated to be -66.5 mV.

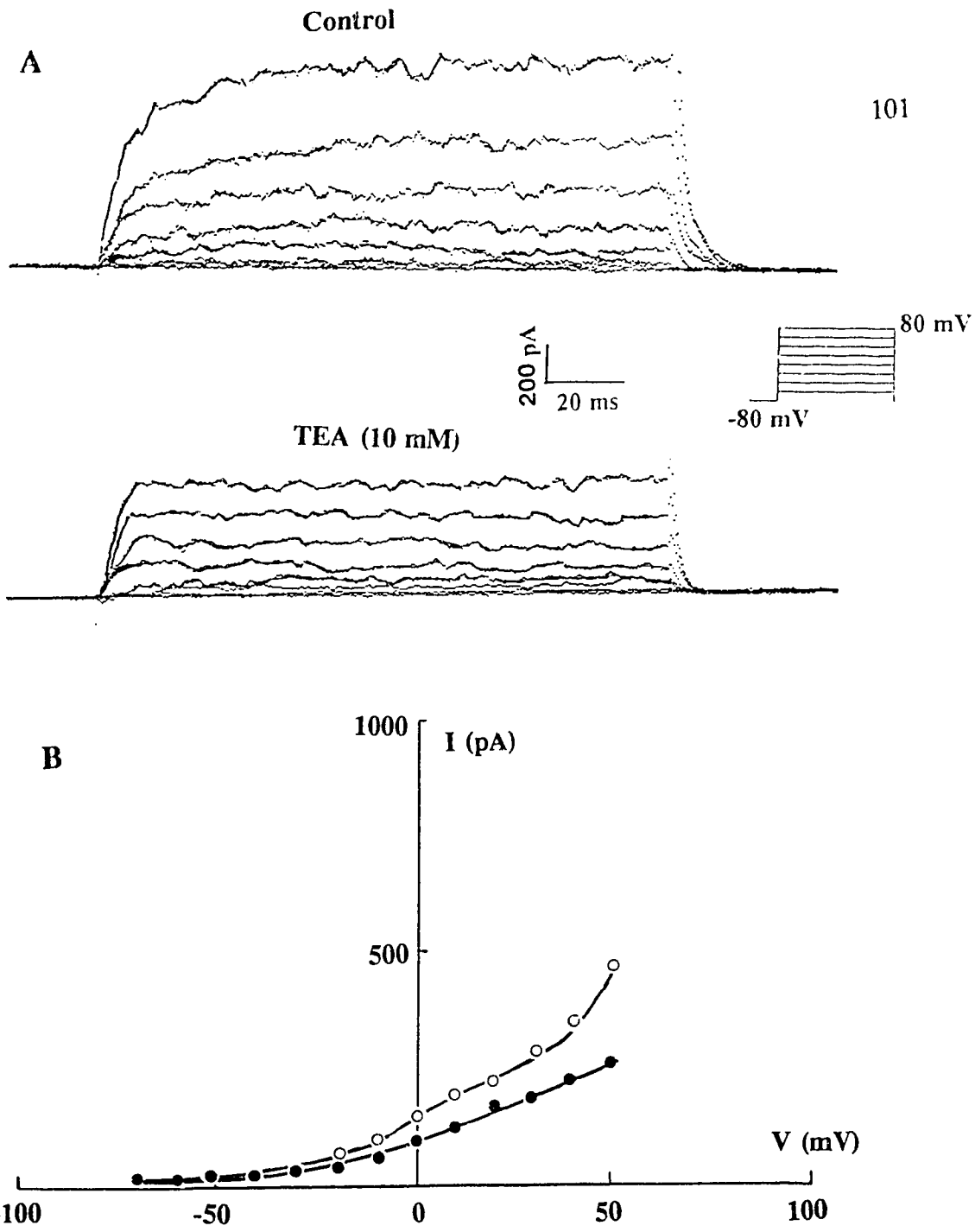

Figure 3-9. The outward K current in a primary cultured VSMC with high intracellular $[Ca^{2+}]_i$ pipette solution (0.2 mM EGTA in the pipette). The current was activated by depolarization from a holding potential of -80 mV. A. The original current (leakage corrected) in the absence or presence of 10 mM TEA. The voltage protocol is also shown. B. The corresponding I-V relationships before (open circles) and after (filled circles) administration 10 mM TEA.

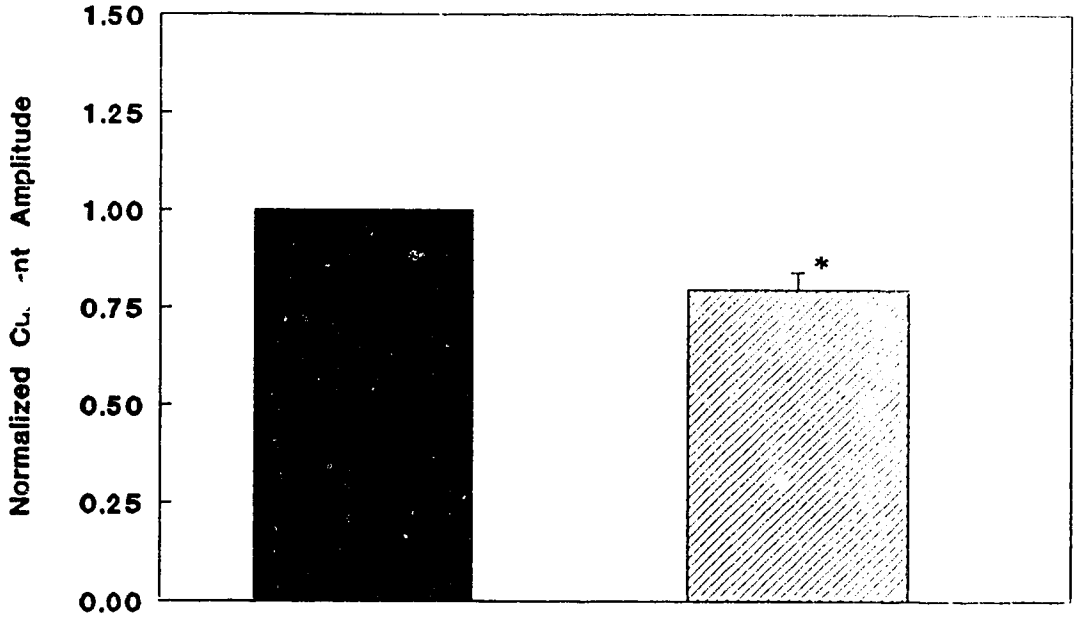

Figure 3-10. Effect of nifedipine on the outward current recorded in VSMCs under high $[Ca^{2+}]_i$ conditions. The VSMCs were treated with nifedipine (1 μM) for 10 min (cross-hatched bar). Current amplitudes were measured at a test pulse of +30 mV and were normalized to the corresponding control. *$p<0.05$ compared with the control values (solid bar). N=4.

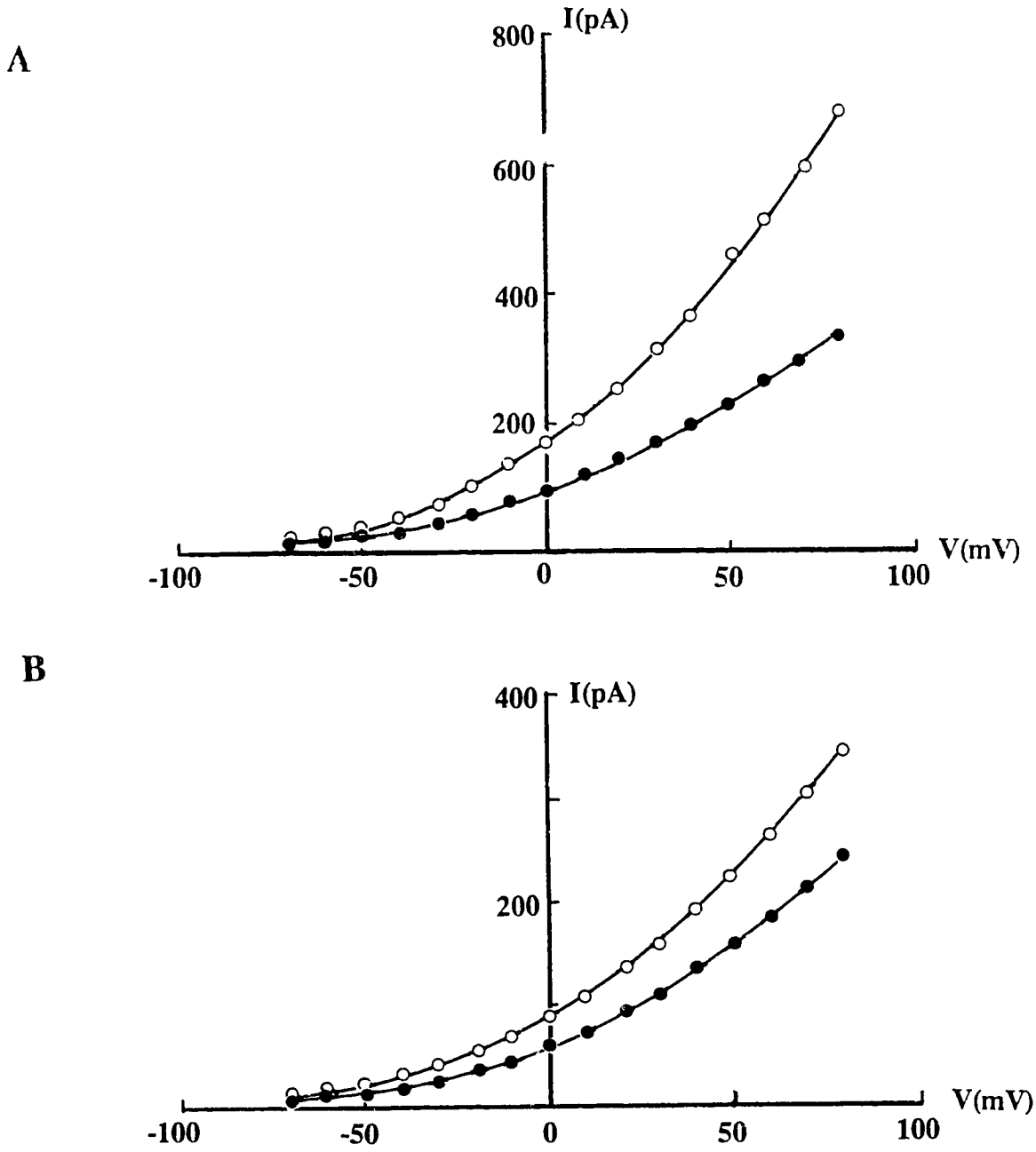

Figure 3-11. Effects of β-DTX on I_k in primary (A) and subcultured (B) VSMCs. β-DTX (200 nM, filled circles) was added to the bath solution and the current was recorded for various potentials after 10 min, and compared with corresponding control records (open circles). I-V relationships are shown for both control experiments (open circles) and for VSMCs exposed to β-DTX (filled circles).

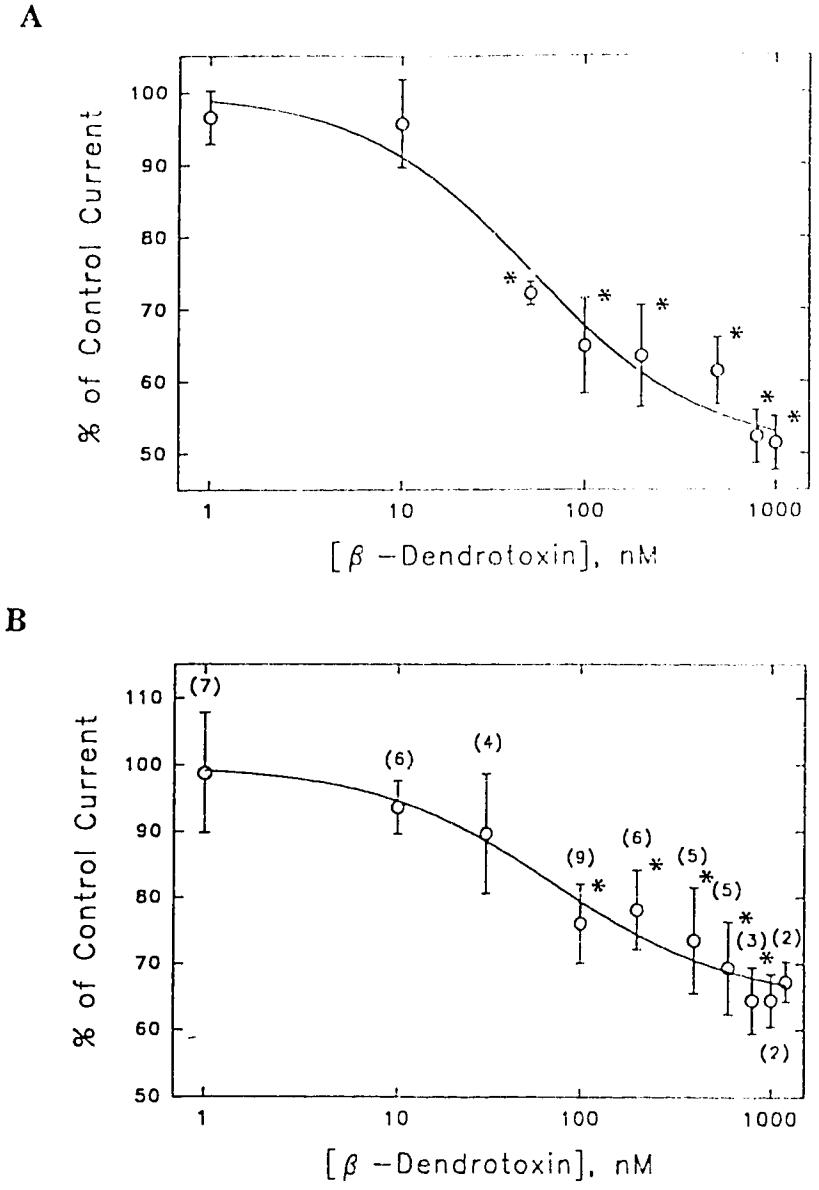

Figure 3-12. The effect of β-DTX on I_k in primary and subcultured VSMCs as a function of concentration. A. Effect of β-DTX concentration (1-1000 nM) on I_k in VSMCs (primary culture). Data points were obtained as described and fitted to a Michaelis-Menton equation (smooth line). The concentration at which half-maximal inhibition (EC_{50}) occurred was calculated from the fitted curve to be 5.1 x 10^{-8}M (N=3). B. Effect of β-DTX concentration (1-1000 nM) on the inhibition of I_k in VSMCs (subcultured). Data were analyzed as in A. The EC_{50} for inhibition was 7.1 x 10^{-8}M (n given for each point in parentheses). In A and B, statistical significance was determined using analysis of variance in conjunction with the Newmann-Keuls Test (*$p<0.05$ *vs.* control).

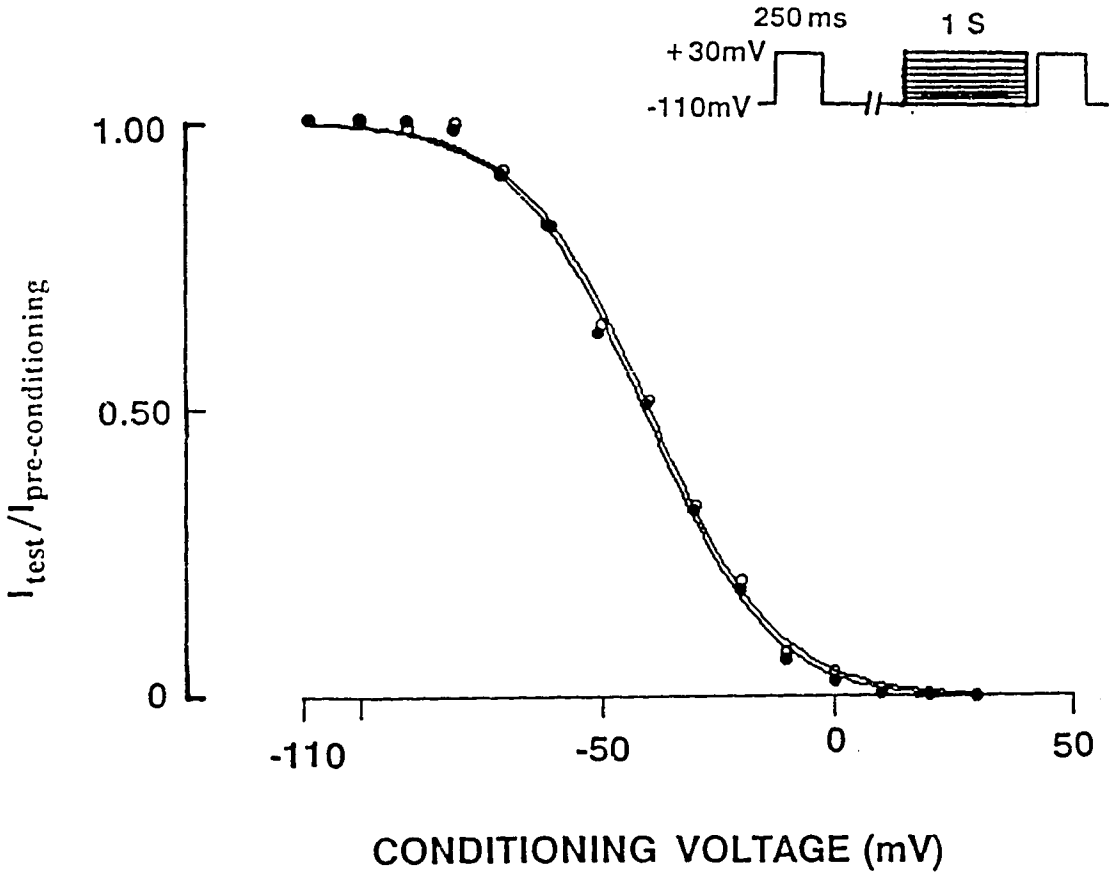

Figure 3-13. Effect of β-DTX on steady-state inactivation of I_k in a primary cultured VSMC. Test pulses before and after administration of 200 nM β-DTX were preceded by a 1 sec condition-ing pulse to various membrane potentials. The amplitude of the current was measured and normalized to the value obtained with a conditioning pulse of -110 mV and plotted as a function of the conditioning potential. The application of 200 nM β-DTX (filled circles) did not shift the steady-state inactivation curve from the control (open circles). The curves were fitted by the Boltzmann equation. The V_h for a primary cultured cell was -40 mV, and the slope factor was 12.9. The results obtained from this single primary cultured cell are representative of those seen in six other primary and subcultured cells.

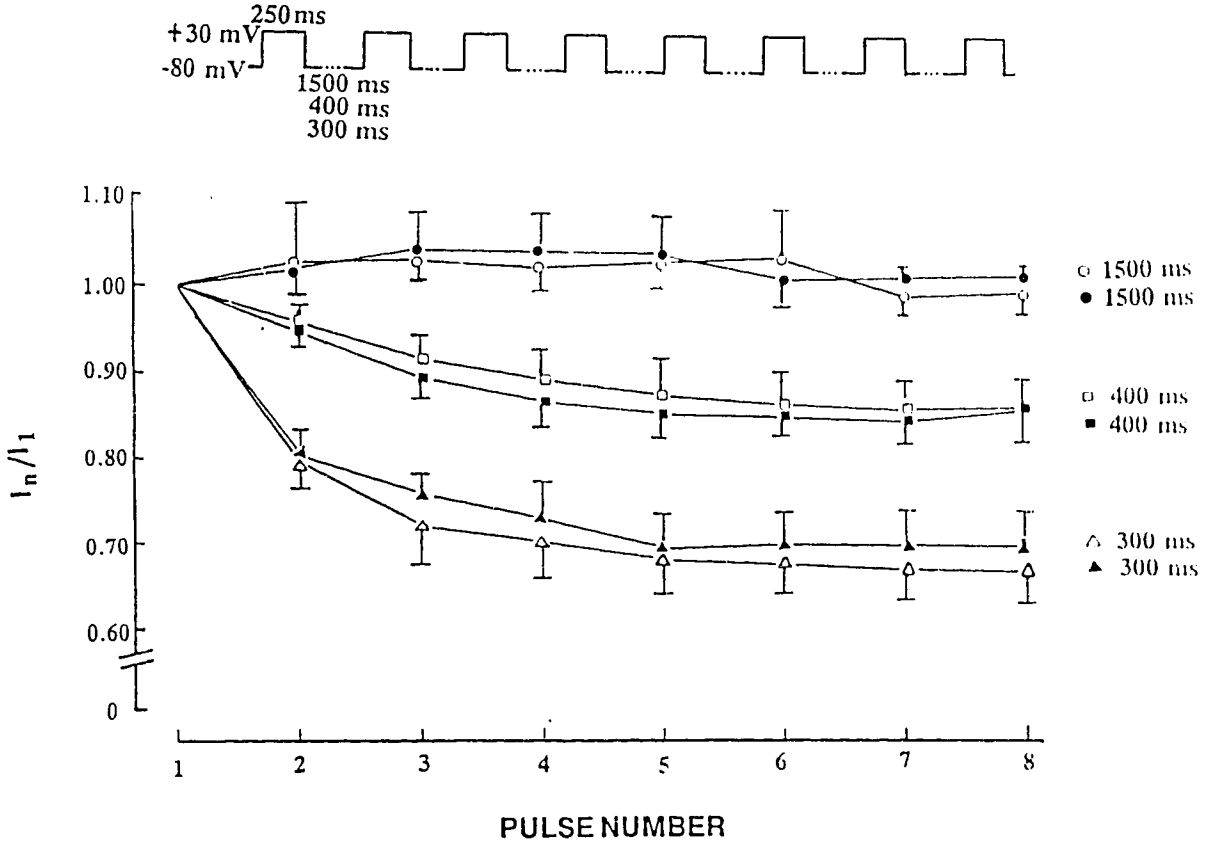

Figure 3-14. Effect of β-DTX on use-dependence block of I_k in primary cultured VSMCs. Trains of eight test pulses (250 msec, +30 mV) were delivered at various intervals (1500, 400 and 300 msec). Peak amplitudes were normalized to the first current in each series and plotted as a function of the pulse number. A progressive attenuation was seen as the interpulse interval was decreased. After exposure to 200 nM β-DTX (filled symbols), the proportional reduction in peak amplitude of I_k was not significantly different from that of the control (open symbols). N=4.

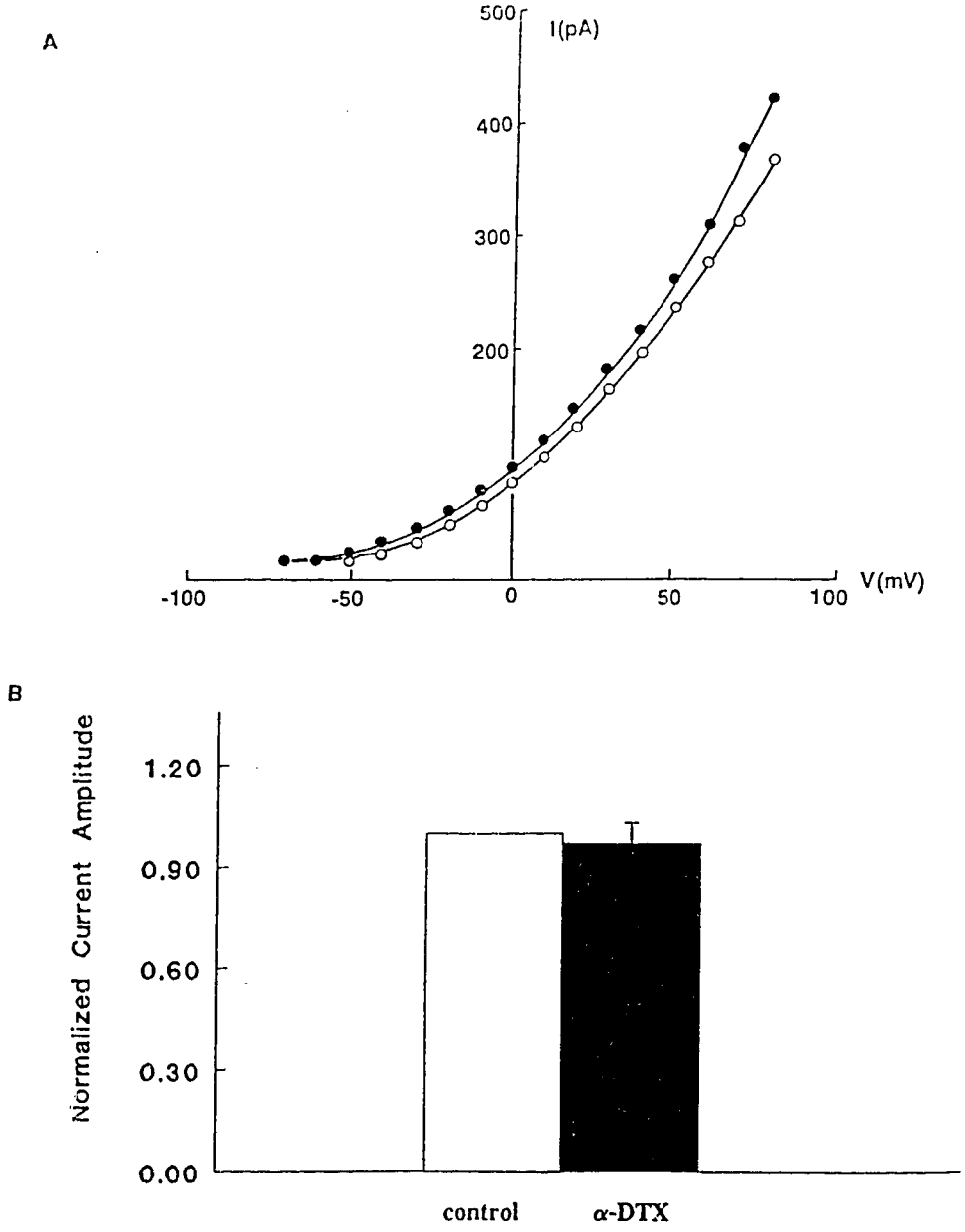

Fig. 3-15. Effect of α-DTX on I_k in primary cultured VSMCs. A. I-V relationship before (open circles) and after (filled circles) the application of 200 nM α-DTX to a primary cultured VSMC. B. Normalized values (test pulse to +30 mV) before (open bar) and after (solid bar) 200 nM α-DTX addition. N=7.

CHAPTER 4

THE EFFECTS OF PGE_2 ON VOLTAGE-DEPENDENT K CHANNELS IN RAT TAIL ARTERY VASCULAR SMOOTH MUSCLE CELLS

I. Introduction

PGE_2 acts through specific surface receptors in an autocrine or paracrine manner (Haluska *et al.*, 1989; Smith, 1989). Effects of PGE_2 have been well established in haemodynamic and cardiovascular disorders. However, the mechanisms underlying these actions are still poorly understood. Since smooth muscle cells that are essential for vascular smooth muscle tone have been found to express various types of K channels (Longmore and Weston, 1990; Nelson *et al.*, 1990), PGE_2 may exert its cardiovascular actions through the modulation of K channels. It has been reported that PGE_2 could modulate K channels in human lymphocytes (Bastin *et al.*, 1990), Madin-Darby canine kidney cells (Steidl *et al.*, 1991), *Xenopus* oocytes (Mori *et al.*, 1989), and rat brain synaptosomes (Ren and Benishin, 1994; Zoltay and Cooper, 1994). However, there have thus far been no reports investigating the action of PGE_2 on K channels in VSMCs.

A recent advance in the field of smooth muscle investigation was the application of the patch-clamp technique to the characterization of ion channels (Small and Foster, 1988). Single VSMCs have recently been successfully isolated from rat tail artery (Wang

et al., 1989; Furspan, 1992; Bolzon *et al.*, 1993; Evans and Kennedy, 1994; Ren *et al.*, 1994), and have been used as a model for peripheral resistance vessels to investigate the ionic basis of various kinds of vasoactive agents such as parathyroid hormone (Pang *et al.*, 1990; Wang *et al.*, 1991) and parathyroid hypertensive factor (Shan *et al.*, 1994). In an effort to gain better insight into the effect of PGE_2 in the control of vascular smooth muscle tone, the present study has investigated the effects of PGE_2 on K channel currents in rat tail artery VSMCs. Since PGE_2 has been considered as a vasodilator in a wide variety of vascular beds, and is though to exert a similar vasodilating action similar to that of PGI_2 (Greeley *et al.*, 1986), the effect of PGI_2 on K channel currents of rat tail artery VSMCs was evaluated and compared to that of PGE_2. These two prostaglandins (PGI_2 and PGE_2) are often listed as the most potent cyclooxygenase metabolites of arachidonic acid, in terms of the actions such as the stimulation of AC activity, dilation of the vascular smooth muscles, increase in cardiac output and blood flow, etc (McGiff and Vane, 1975; McGiff, 1981; Greeley *et al.*, 1986; Cutis-Prior, 1988; Axelord, 1991; Consigny, 1991). They have been considered to be the major vasoactive prostaglandins in cardiac, pulmonary, and uterine vascular beds.

II. Experimental design

These studies were designed to investigate the action PGE_2 on the voltage-dependent K channels (I_k), in both primary cultured and subcultured VSMCs from rat tail arteries (These chanels have been characterized in the previous chapter). To study the

effect of PGE_2 on membrane potential, experiments were carried out using the current-clamp technique. By using the voltage-clamp technique, experiments were designed to investigate the effect of PGE_2 on I_k in rat tail artery VSMCs. Experiments were also performed to determine if the effect of PGE_2 depends on the inactivated state of the channel or on the frequency of electrical stimulation. Because PGE_2 has been considered to exert its action through a specific membrane receptor, attempts were made to verify the involvement of PGE_2 receptors by using meclofenamate, a PGE_2 receptor antagonist (Zelinski-Wooten *et al.*, 1990). To determine if the PGE_2 receptor is coupled to a G-protein(s), the nonhydrolysable GDP analogue, guanosine-5'-O-(2-thiodiphosphate) (GDPβS), a G protein inhibitor, was applied by the pipette. In order to identify the type of G protein(s) involved, two G protein-specific bacterial toxins, cholera toxin (CTX) and pertussis toxin (PTX), were used in the study. Finally, to compare the relative specificity of PGE_2 action, the effect of PGI_2 on K channels was also evaluated in VSMCs from rat tail artery.

III. Results

A. Effect of PGE_2 on resting membrane potential of VSMCs

The membrane potential responses to bath-applied PGE_2 (10 μM) were examined in 25 primary cultured VSMCs: 13 cells (52%) showed a prolonged depolarization by 15.9 $\pm$ 1.3 mV (M $\pm$ S.E.), and 12 cells (48%) showed no change in membrane

potential. Fig. 4-1 shows an example of PGE_2 (10 μM)-induced membrane depolarzation in a primary cultured VSMC. PGE_2 elicited a prolonged depolarization lasting more than 2 minutes with a relative plateau phase. The solvent (ethanol) caused no change in the membrane potential.

B. Effect of PGE_2 on I_k in rat tail artery VSMC

Results presented in Fig. 4-2 show that PGE_2 could significantly inhibit I_k, in both types of VSMCs from rat tail artery. Such inhibition was concentration-dependent (1-200 μM) and was fitted to a Michaelis-Menton equation (Fig. 4-3). The inhibition of I_k at the test potential of +30 mV was normalized to the maximal inhibtion achieved at 10^{-4} M PGE_2 and plotted as a function of the concentration of PGE_2. A 45-50% saturable attenuation of I_k amplitude ($p < 0.05$) was seen with half-maximal inhibition values (EC_{50}) of 3.5 μM and 4.9 μM, in primary and subcultured VSMCs, respectively. The inhibitory effect of PGE_2 on I_k was reversible upon washout. The time course started with steady states of I_k, inhibition of the current amplitudes were seen 5 min after the administration of PGE_2 and reached maximum at about 10 min. The reversal of the inhibition took place 5 to 10 min after wash out of PGE_2, nearly to the control level. Subsequent addition of PGE_2 restored the inhibition of I_k (Fig. 4-4). The activation potential of the I_k was not affected by PGE_2 administration (Fig. 4-2). The effect of PGE_2 (10 μM) was also tested in one VSMC buffered with low EGTA (0.2 mM). A similar degree of inhibition (50%) was seen in the total K current (I_k+Ca^{2+}-activated K current) generated (data not shown).

C. Effect of PGE_2 on the I_k steady-state inactivation curve

In order to determine if the action of PGE_2 on I_k was dependent on membrane potential, complete steady-state inactivation curves of I_k were generated before and 10 min after 50 μM PGE_2 addition (Fig. 4-5). As shown in the voltage-protocol, the cells were voltage-clamped at a conditioning potential from -110 mV to +30 mV for 1 second before application of a 200 msec test pulse to +30 mV. The outward currents (I_k) were normalized to the value of the peak outward current activated from the most negative membrane potential (-110 mV). The steady-state inactivation curves were fitted by the Boltzmann distribution ($I=I_{max}[1+\exp(V-V_h)/k]^{-1}$). The channels were completely inactivated at -110 mV. The I_k currents were usually activated at potentials of about -40 to -30 mV. The voltage at which half of the channels were inactivated (V_h) was calculated to be around -40 mV. V_h and k (slope factor) were -41.85±4.90 mV and -16.93 ±1.37 for control cells (M±S.E., N=4). PGE_2 (50 μM) reduced the channel amplitude, but did not shift the steady-state inactivation curve. The V_h and K values were calculated to be -42.58±4.81 mV and -17.36± 1.55 (M±S.E., N=4), essentially the same as those found under control conditions. These results suggested that the inhibition of I_k by PGE_2 did not depend on the state of the channel (inactivated).

D. The effect of PGE_2 on use-dependent inhibition

To determine the effect of stimulus frequency on the action of PGE_2 on I_k, trains of test pulses at different frequencies were applied in the presence or absence of PGE_2.

The degree of inhibition could be influenced by the frequency and duration of repetitive depolarizations during a series of test pulses. Thus, eight consecutive pulses to +30 mV were applied at different intervals from a holding potential of -80 mV. The duration of each pulse was 250 ms. The peak amplitude of each train of pulses was measured and normalized to the first pulse of each series. The current decreased in amplitude during repetitive stimulation, with interpulse intervals of 1500 to 300 ms, and remained at a constant level from the third pulse onward. In the presence of 50 μM PGE_2, the degree of decrease in amplitude was not significantly different from the control level ($p > 0.05$, Fig. 4-6), suggesting that the inhibition by PGE_2 was not use-dependent nor due to occupation of channel pores.

E. Effects of the PGE_2 receptor antagonist meclofenamate on PGE_2-induced suppression

To determine how PGE_2 might exert its action on I_k, meclofenamate, a PGE_2 receptor antagonist, was added to the cell bath 10 min prior to the addition of PGE_2. Fig. 4-7 shows the effect of sodium meclofenamate (meclo) on PGE_2-induced suppression of I_k. When cells had been pretreated with 1 mM sodium meclofenamate (Zelinski-Wooten *et al.*, 1990) for 10 min, the addition of PGE_2 could not produce the expected suppression of I_k. Meclofenamate itself did not have any effect of on I_k. These results clearly indicate that the action of PGE_2 is through the PGE_2 membrane surface receptor(s).

F. Effects of the G-protein inhibitor GDPβS and toxin pretreatment effects on PGE_2-induced I_k suppression

Further experiments were carried out to determine whether any G proteins are involved in the action of PGE_2 on I_k. GDPβS, a nonhydrolysable GDP analogue employed as a G protein inhibitor, was administered by pipette perfusion 10 min prior to the addition of PGE_2. Fig. 4-8 shows that pretreatment of GDPβS (1 mM) prevented the suppression of I_k induced by PGE_2. Longer pretreatment of GDPβS (up to 30 min) did not produce additional effect. This result suggests that a G protein is very likely to be involved in the action of PGE_2. In order to identify the type of regulatory G protein(s), two bacterial toxins, cholera toxin and pertussis toxin, were used in this study. Each of the two toxins were preincubated with the VSMCs for 8-10 hrs before the experiments. CTX (1 μg/ml) preincubation completely abolished PGE_2-induced I_k attenuation, while PTX (200 ng/ml) preincubation did not affect the action of PGE_2 (Fig. 4-9). These data suggest that the action of PGE_2 on I_k was mediated through the activation of a CTX-sensitive G protein rather than a PTX-sensitive one. Neither CTX, nor PTX, by themselves, significantly affected the current amplitudes (control: 283±27 pA, N=15; CTX: 246±16 pA, N=11; PTX: 262±17 pA, N=7), or the activation potential (data not shown). All test pulses were at +30 mV, from a holding potential of -80 mV.

G. Effect of PGI_2 on I_k in rat tail artery VSMCs

The effect of PGI_2, another major cyclooxygenase product, on I_k was examined and compared with that of PGE_2. The two prostaglandins, PGE_2 and PGI_2, have been reported to possess very similar biological actions in diverse vascular beds. These actions involve vasodilation and the accumulation of cAMP. However, in the present study the effect of PGI_2 on I_k appeared to be different from that of PGE_2. Application of PGI_2 ($5x10^{-6}$ M and 10^{-5} M) to the bath had little effect on I_k (Fig. 4-10). Receptor specificity might be a potential factor in the differing responses to these two prostaglandins. It has, however, been previously reported that these two prostaglandins might exhibit receptor cross-reactivity (Dorn *et al.*, 1992).

IV. Discussion

The results reported in this chapter constituted an attempt to characterize the actions of PGE_2 on K channels in rat tail artery VSMCs. The present study showed that PGE_2, a putative local hormone, inhibited I_k in VSMCs from rat tail artery in a concentration-dependent fashion with half-maximal inhibition values (EC_{50}) of 3.5 μM and 4.9 μM in primary and subcultured VSMCs, respectively. These concentrations of PGE_2 (EC_{50}s) were close to the previously reported lower micromolar concentration range, which was found to be high enough to affect cellular processes. For example, 5 μM PGE_2 caused the activation of a cAMP-mediated K current and a membrane hyperpolarization in *Xenopus* oocytes (Mori *et al.*, 1989), 1.8 μM PGE_2 elicited

intracellular Ca^{2+} release in osteosarcoma cells (Yamaguchi *et al.*, 1988), 5 μM PGE_2 generated IP_3 in rat myometrium (Goureau *et al.*, 1992) and 2.5-3.5 μM PGE_2 stimulated intracellular Ca^{2+} release, IP_3 generation and protein synthesis as well as vascular smooth muscle contraction in rat aorta (Dorn *et al.*, 1992). The inhibition of I_k might contribute to the observed membrane depolarization by PGE_2. At a holding potential of -40 mV (close to the resting membrane potential), about 50% of the I_k channels are activated. Therefore it is possible that the effect of PGE_2 on K channels may account for the membrane depolarization. But because of the time discrepancy (depolarization occurred in seconds compared to minutes for the inhibition of I_k), the membrane depolarization might also be an effect of PGE_2 which was irrelevant, or additional, to its action on the K channel activities. PGE_2 has previously been reported to induce a dramatic membrane depolarization, as seen in the NG-108-15 hybrid cells (Higashida *et al.*, 1984), the neurones of the nucleus tractus solitarius (Matsumura *et al.*, 1993) and the crypt-base of rat distal colon (Siemer and Gogelein, 1993). Short or prolonged membrane hyperpolarization following bath application of PGE_2 has also been recorded in *Xenopus* oocytes (Mori *et al.*, 1989), and canine renal epitheloid cells (Steidl *et al.*, 1991). Steidl *et al.* found that the transient hyperpolarization caused by PGE_2 in these cells was parallelled by an increase in K conductance. The time course of PGE_2-induced IP_3 generation and the subsequent release of intracellular Ca^{2+} was similar to the time course of the initial transient hyperpolarization (Aboolian *et al.*, 1989; Steidl *et al.*, 1991). Such initial hyperpolarization was credited to the activation of Ca^{2+}-activated K channels, due

to the PGE_2-induced release of intracellular Ca^{2+}. The mechanism of PGE_2-induced depolarization is still not clear. It was demonstrated that activation of inward cation channels was responsible for the PGE_2-induced membrane depolarization in neurones of the nucleus tractus solitarius (Matsumura *et al.*, 1993). However, this is not believed to be the sole mechanism of action for the PGE_2-induced membrane depolarization. PGE_2-induced depolarization or hyperpolarization can in part explain its biological actions. PGE_2-induced membrane hyperpolarization has been found to participate in the cellular transmission of bradykinin-induced activation of K channels in MDCK cells (Aboolian *et al.*, 1989). Although the membrane depolarization produced by PGE_2 in VSMCs was relatively small in the present investigation, it might be great enough to elicit some voltage-dependent responses, such as the activation of voltage-dependent Ca^{2+} channels. The membrane depolarization produced by PGE_2 may be of physiological significance concerning the smooth muscle contraction induced by PGE_2.

There are some discrepancies in the results obtained thus far concerning the effect of PGE_2 on K channels in various tissues and species. PGE_2 was found to inhibit Ca^{2+}-activated K channels in rat brain synaptosomes (Ren and Benishin, 1994). However, PGE_2 was also found to promote the opening of receptor-operated (e.g., purinergic, α_2-adrenergic, muscarinic and opioid) K channels that were sensitive to dendrotoxin in rat cortical synaptosomes (Zoltay and Cooper, 1994). Bastin and colleagues (1990) observed that PGE_2 inhibited K currents in human lymphocytes (Jurkat T cell line). This suppression was attributed to an increase in cytosolic cAMP levels, and a subsequent

phosphorylation of channel proteins by the cAMP-dependent protein kinase, i.e., PKA. In *Xenopus* oocytes, it was reported that low micromolar concentrations of PGE_2 activated a cAMP-mediated K current (Mori *et al.*, 1989).

The previous chapter described that the VSMCs from rat tail artery could exhibit at least two classes of K channels, one voltage-dependent, the other Ca^{2+}-activated. However, under the current experimental conditions, it is difficult to separate the Ca^{2+}-activated K current from the total K currents (when the cells were buffered with low EGTA). Thus, it seems to be inconvenient to test the effect of PGE_2 on the Ca^{2+}-activated K current in the present study. Although PGE_2 appeared to display a similar degree of inhibition of the total K current in a single cell with high $[Ca^{2+}]_i$, to that of I_k (about 50%), no conclusion could be drawn thus far whether PGE_2 is able to affect the Ca^{2+}-activated K channels or not.

The present investigation suggested that PGE_2 caused inhibition of I_k without shifting the steady-state inactivation curve, indicating the inhibition of I_k by PGE_2 did not depend on the inactivation state of the channel. The use-dependence experiment suggested that the effect exerted by PGE_2 on the K channel was not likely due to the occupancy of the channel pore, which would reduce the K conductance. Subsequent studies using the receptor blocker, GDPβS, and CTX and PTX provided further evidence that the actions of PGE_2 are mediated through a specific receptor(s) and G protein(s), not a direct interaction with the K channel itself.

Almost all of the actions of PGE_2 are believed to occur via binding to its specific

surface receptors, such as EP_1, EP_2, EP_3. There has, however, been evidence indicating that PGE_2 was also able to cross-react with some other prostanoid receptors such as the TXA_2/TXB_2 or the PGI_2 receptors (Dorn *et al.*, 1992; Coleman *et al.*, 1994). In most tissues analyzed thus far, PGE_2 has been consistently described as the most potent PG receptor agonist (Melien *et al.*, 1988; Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990). Meclofenamate, a potent PGE_2 membrane receptor antagonist, completely blocked the action of PGE_2 on I_k. Such a finding is in agreement with the already well-established receptor-mediated mode of action of PGE_2.

The usual protocol for the electrophysiological investigation of possible G protein involvement is to administer analogues of GTP or GDP to the cells through either microelectrode injection or pipette infusion. This method is based on the work done by pioneers such as Benson and Levitan (1983) in *Aplysia* neurons, Pfaffinger *et al.* (1985) and Breitweiser and Szabo (1985). GDPβS is able to compete with endogeneous GTP, and, thus, antagonize G protein function. CTX and PTX discriminate between two types of signal transducing G proteins (G_s and G_i). CTX catalyses ADP-ribosylation of G_s, leading to a permanent activation of the catalytic unit, whereas PTX causes ADP-ribosylation of G_i, reducing its inhibitory effect on the catalytic unit (Abood *et al.*, 1982; Gilman, 1984; Ui, 1984). The results presented in Fig. 4-8 and Fig. 4-9 strongly indicated that the effect of PGE_2 on I_k, is very likely mediated by G protein(s). It seemed that a cholera toxin-sensitive G protein is coupled to the PGE_2 receptor. However, whether the G protein exerts a local direct action, or whether a subsequent enzymatic

step(s) leading to the generation of any soluble second messenger cascades is involved, needs further investigation.

There is considerable evidence to support the hypothesis that PGI_2 modulates vascular smooth muscle tone and blood pressure in both physiological and pathological conditions. PGI_2 is one of the most potent platelet aggregation inhibitor, adenylate cyclase activator and vasodilator. PGE_2 has been considered to have a similar action to that of PGI_2 in the regulation of vascular smooth muscle tone, blood pressure, cardiac output, blood flow, and other cardiovascular functions. These two prostaglandins, which are both produced in large amount as the principal arachidonic acid metabolites in vasculature (Curtis-Prior, 1988), may have additive effects. However, in some blood vessels PGE_2 is produced in greater amounts than PGI_2 (Curtis-Prior, 1988). Unlike PGE_2, PGI_2 may act as a circulating hormone because it is not removed from the circulation on passage through the lung (Curtis-Prior, 1988). PGE_2 and PGI_2 have shown striking similarities in the regulation of many cardiovascular functions such as vascular smooth muscle tone, blood pressure, etc, in a variety of vascular beds (Greeley *et al.*, 1986; Curtis-Prior, 1988). It is believed that the participation of these prostaglandins in the regulation of vascular responsiveness in the organ functions is part of a protective mechanism against the excessive activities of pressor hormones. These prostaglandins thus control the regional blood flow by regulating the local vascular smooth muscle tone.

Although PGE_2 and PGI_2 exhibit similar actions in many tissues, they still exert different, and sometimes opposite, effects. PGE_2 produced vasoconstriction in various

tissues such as the coronary artery and renal vasculature. These effects were rarely observed with PGI_2 (Baer and McGiff, 1979). When renal blood flow and systemic blood pressure were measured as PGE_2 or PGI_2 was perfused into the aorta, both prostaglandins were found to decrease the blood pressure significantly. However, they exhibit different actions on renal vascular resistance. At present, very little is known concerning the effect of PGI_2 on K channels in VSMCs.

The results from the present study indicated that although there are many similarities between these two prostaglandins, PGE_2 exerted specificity with respect to the regulation of K channel conductance in VSMCs, while at comparable concentrations PGI_2 had no effect on the K channel conductance in these cells.

Conclusion

The results presented in this chapter demonstrated that PGE_2 inhibited a voltage-dependent K channel in cultured VSMCs from rat tail artery through a receptor-mediated process involving the activation of G protein(s). The action of PGE_2 showed some specificity since PGI_2 did not exert a similar action on I_k. These observations have provided strong evidence that PGE_2 might contribute to the regulation of vascular smooth muscle tone. The possibility that PGE_2 might act through one or several signal pathway(s) is intriguing and will be discussed in the following chapter.

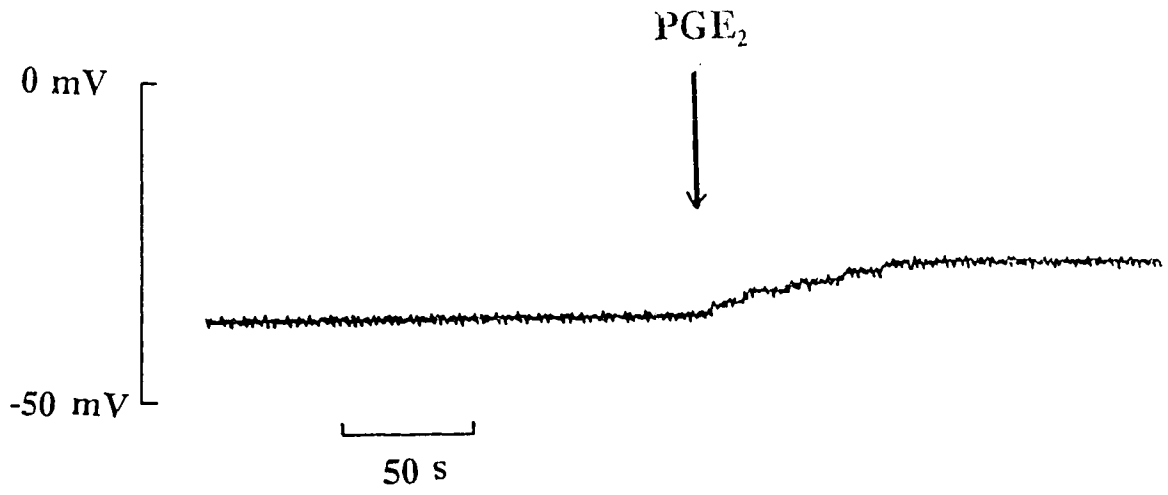

Figure 4-1. Effect of PGE_2 on membrane potential in a primary cultured VSMC. The membrane potential was recorded before and after the application of PGE_2 (50 μM), indicated by an arrowhead. The solvent (ethanol) caused no change in the membrane potential.

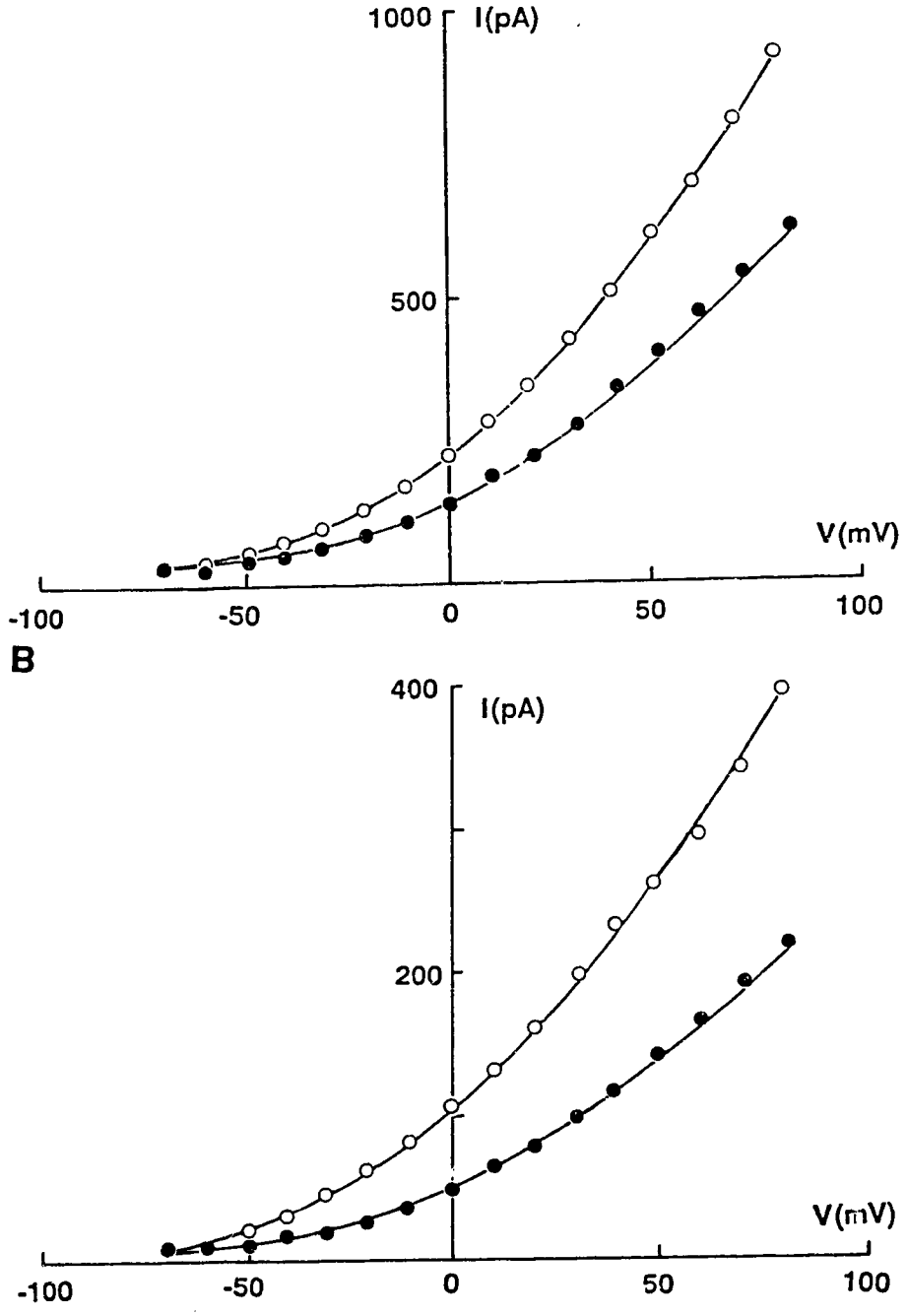

Figure 4-2. Effect of PGE_2 on I_k in primary cultured (A) and subcultured (B) VSMCs from rat tail artery. The amplitudes of the current were recorded and the I-V relationships of I_k were shown before (open circles) and 10 min after (closed circles) exposure to PGE_2.

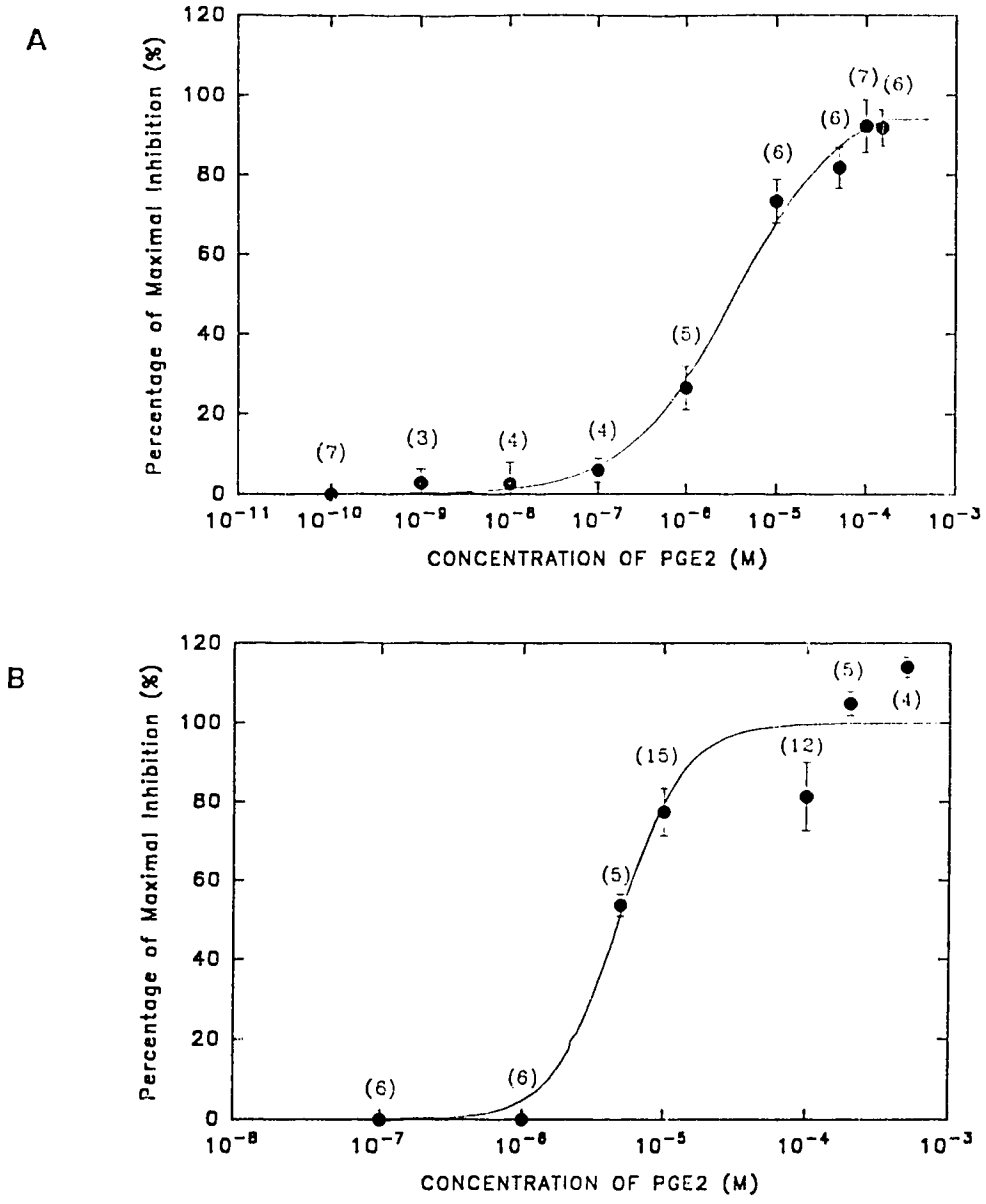

Figure 4-3. The concentration-dependent response of PGE_2 on I_k in primary and subcultured VSMCs. A. Inhibition of I_k by PGE_2 (1-200 μM) in primary cultured cells. The data points were fitted to a Michaelis-Menton equation (smooth line). The concentration at which the half-maximal effect (EC_{50}) occurred was calculated from the fitted curve to be 3.5 μM. B. The concentration-dependent effect of PGE_2 on I_k in subcultured cells. The data were analyzed as in A. The EC_{50} was calculated to be 4.9 μM. N is given for each point in parentheses.

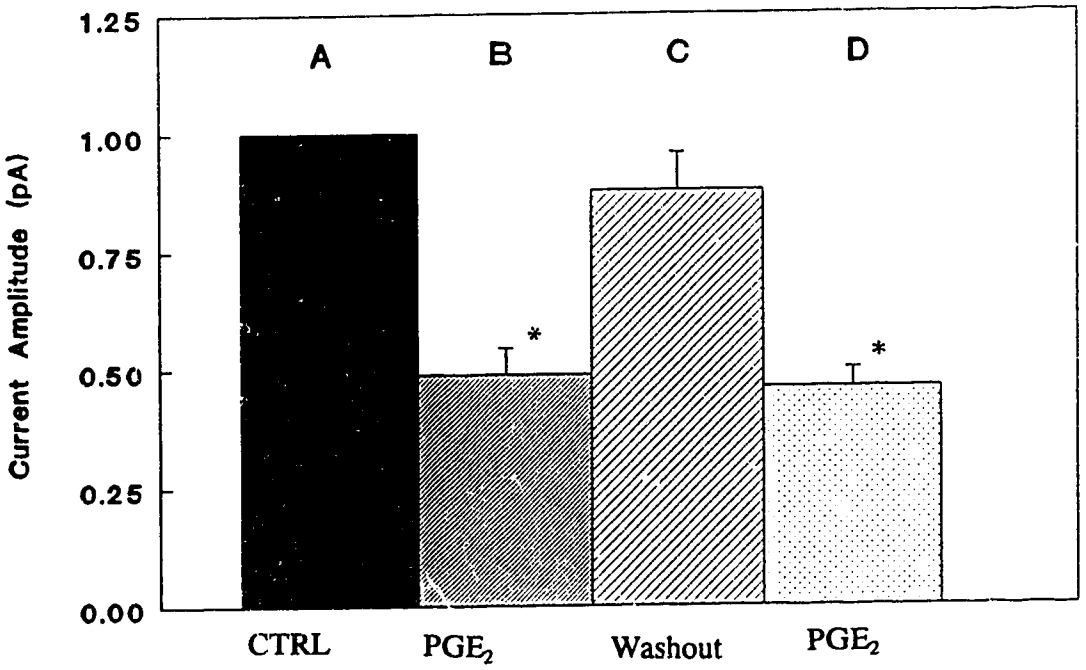

Figure 4-4. Effect of wash out on PGE_2-induced inhibition of I_k. (A). The control period. (B). The inhibition by 10 μM PGE_2. (C). Wash out period. (D). Re-administration of PGE_2. The VSMCs were voltage-clamped at a holding potential of -80 mV and depoiarized to +30 mV. Values are M $\pm$ S.E., N=3 (*$p<0.05$ *vs.* control). N=3.

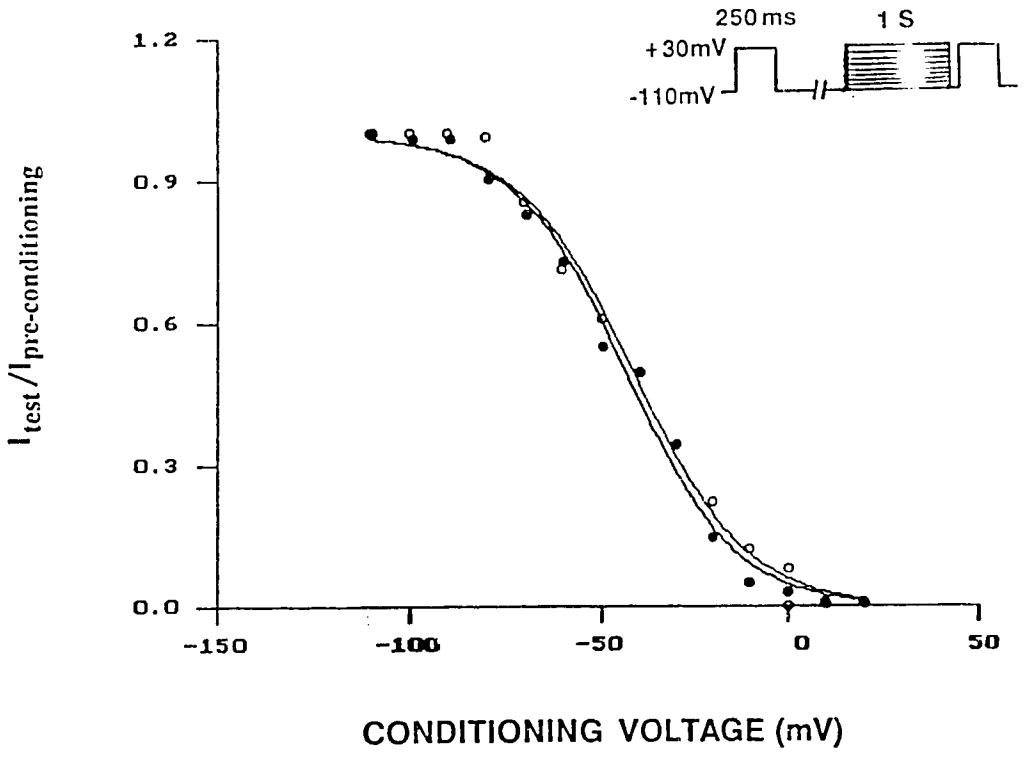

Figure 4-5. Effect of PGE_2 on steady-state inactivation of I_k. Test pulses before and after 50 μM PGE_2 were preceded by a 1-sec conditioning pulse to various membrane potentials. The amplitude of the current was measured and normalized to the value obtained with a conditioning pulse of -110 mV and plotted as a function of the conditioning potentials. The curves were fitted to the data points using a least-squares fit by Boltzmann's equation: $I=I_{max}[1+\exp(V-V_h)/K]^{-1}$, where V_h and K for control were -41.4 mV and -15.3, -43.8 mV and -14.9 after PGE_2, respectively. The results obtained from this primary cultured VSMC were respresentative of those seen other VSMCs. V_h= -41.8 $\pm$ 4.9 mV; K= -16.9 $\pm$ 1.4 for control; V_h= -42.6 $\pm$ 4.8 mV, K= -17.4 $\pm$ 1.6 after PGE_2 (50 μM) treatment. N=4.

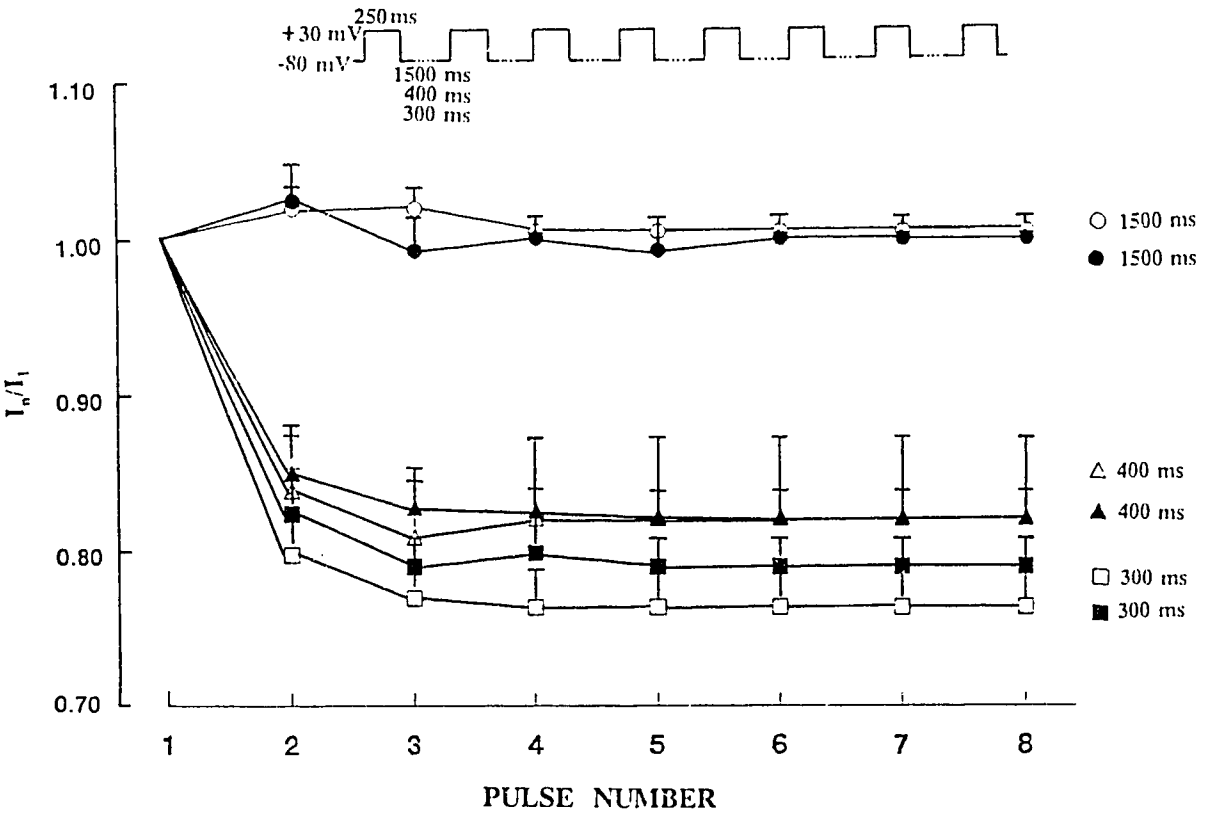

Figure 4-6. Effect of PGE_2 on use-dependent inhibition of I_k in primary cultured VSMCs. Trains of eight test pulses (250 msec, +30 mV) were applied at various interpulse intervals (1500, 400, 300 msec). The peak amplitudes were normalized to the first current in each series and plotted as a function of the pulse number. After expose to 50 μM PGE_2 (filled symbols), the proportional reduction in peak amplitude of I_k was not significantly different from the control cells (open symbols). N=4.

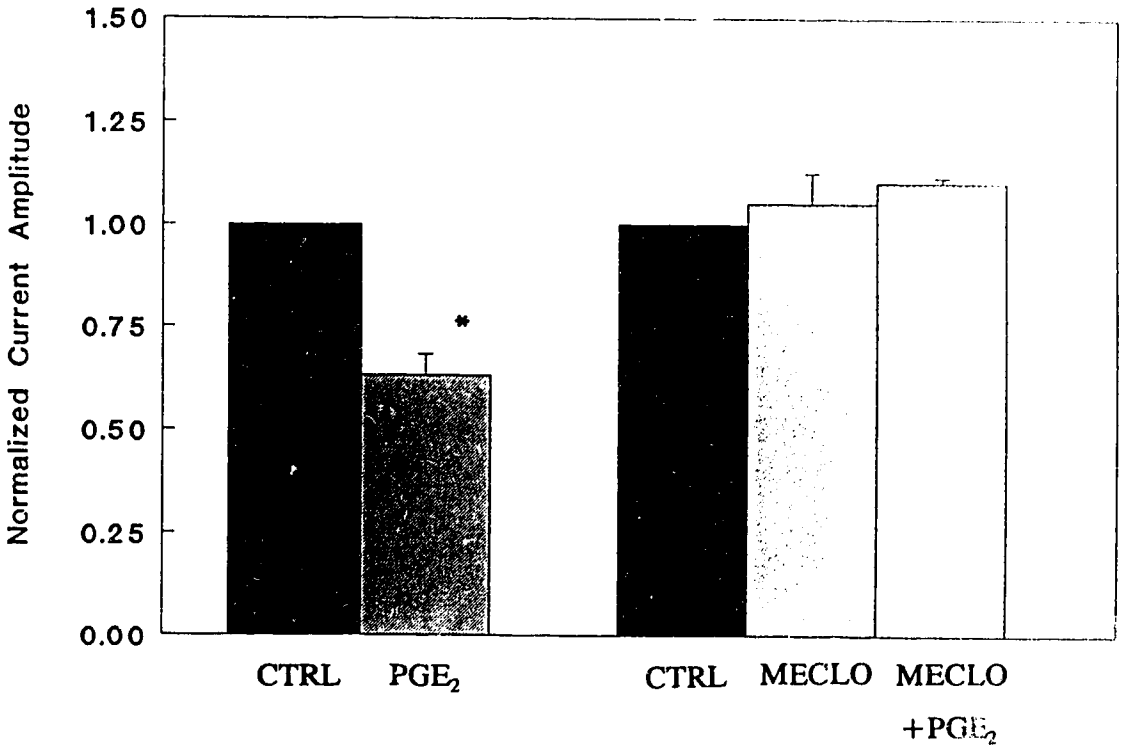

Figure 4-7. Effect of sodium meclofenamate (meclo) on PGE_2-induced inhibition of I_k in primary cultured VSMCs. The cells were pretreated with 1 mM meclofenamate for 10 min, then exposed to PGE_2 (10 μM). *$p < 0.05$ *vs.* control. N=6 for both meclofenamate treated and untreated cells.

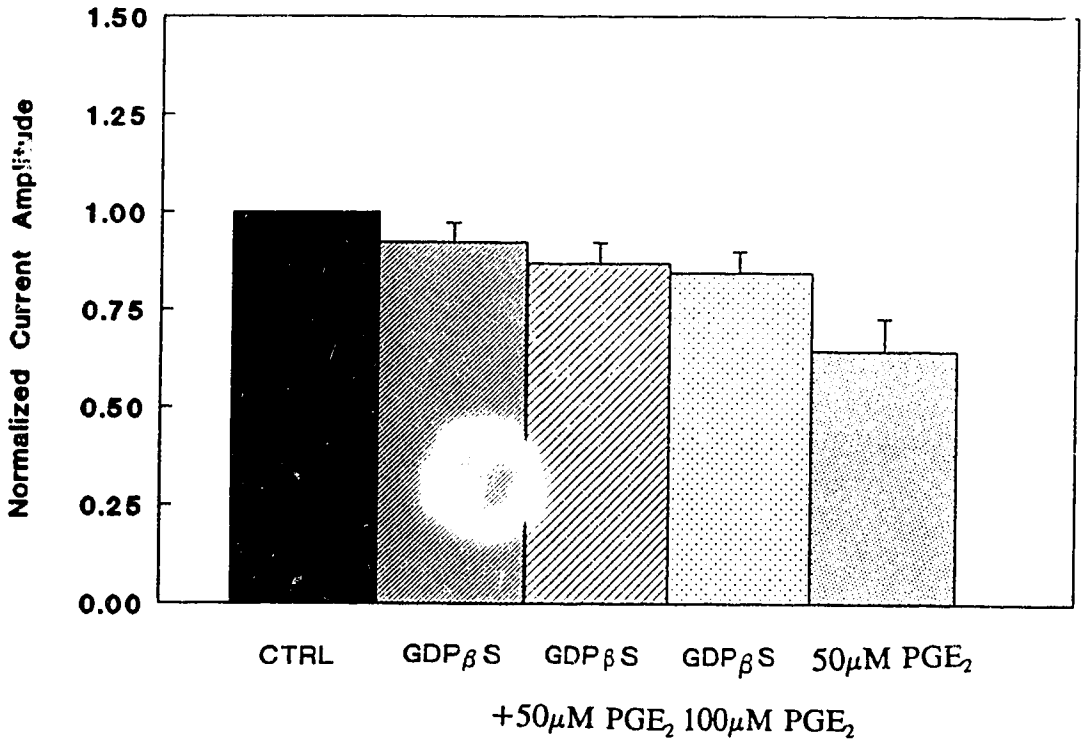

Figure 4-8. Effect of GDPβS on PGE_2-induced inhibition of I_k in primary cultured VSMCs. The cells were pretreated by the intracellular application of GDPβS (1 mM) for 10 min, then exposed to PGE_2 (50 μM or 100 μM) for 10 min. Current recordings were normalized to the respective controls (the current recordings immediately after membrane penetration). Values are M±SE, N=7.

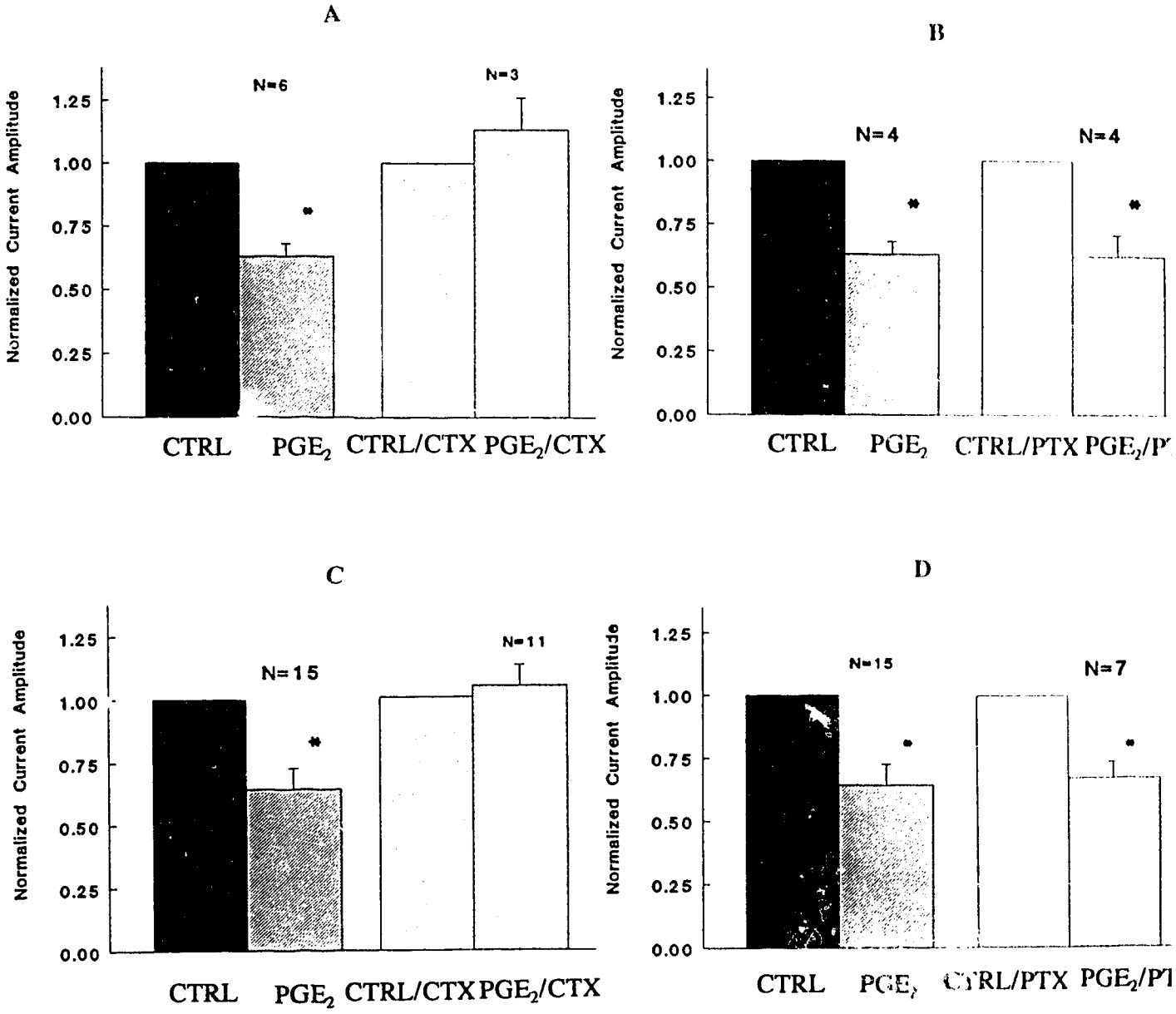

Figure 4-9. Effect of CTX and PTX pretreatment on PGE_2-induced inhibition of I_k. Primary cultured VSMCs were preincubated with and without CTX (A. a&b subunits, 1 μg/ml) or PTX (B. 200 ng/ml) for 8-10 hrs. The cells were then treated with or without PGE_2 for 10 min. The currents from PGE_2 treated cells were normalized to the respective controls. Subcultured cells were preincubated with or without CTX (C) or pertussis toxin (D). Values are M$\pm$SE (*$p<0.05$ *vs.* control).

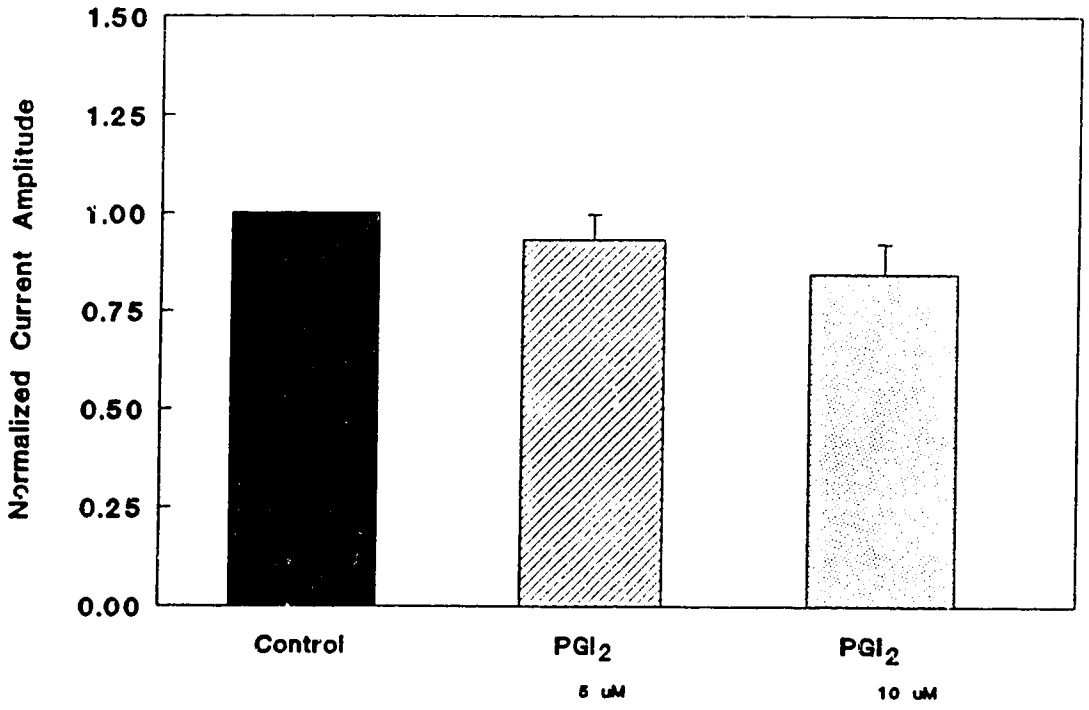

Figure 4-10. Effect of PGI_2 on I_k in primary cultured VSMCs. Cells were treated with various concentrations [$5x10^{-6}$ M (N=7), 10^{-5} M (N=5)] of PGI_2 for 10 min (cross-hatched bars). Current amplitudes were measured at a test potential of +30 mV and were normalized to the corresponding controls (solid bar).

CHAPTER 5

SECOND MESSENGERS INVOLVED IN THE INHIBITION OF K CHANNELS BY PGE_2 IN VASCULAR SMOOTH MUSCLE CELLS

I. Introduction

PGE_2 exerts its biological actions by binding to specific membrane receptors (Haluska *et al.*, 1989; Smith, 1989). An interaction of the receptors with G proteins has already been demonstrated by radioligand binding and biochemical methods (Negishi *et al.*, 1989; Sonnenburg *et al.*, 1990). In some cases, G proteins have been confirmed to be directly coupled to an ion channel, but in most cases it is believed that a second messenger system(s) may be involved. As demonstrated earlier, the diverse physiological actions of PGE_2 have been attributed to the activation of two major signal transduction systems, namely, the cAMP-PKA and the PKC pathways (Goureau *et al.*, 1992). PGE_2 has been shown to stimulate AC activity in various types of cells (Molnar *et al.*, 1987; Sonnenburg and Smith, 1988; Kozawa *et al.*, 1992; Coleman *et al.*, 1994). More recently, PGE_2 has also been reported to activate phospholipase C in fibroblasts (Yamashita and Takai, 1987), osteoblasts (Farndale *et al.*, 1988), adrenal chromaffin cells (Yokohama *et al.*, 1988), astrocytes (Kitanaka *et al.*, 1991) and human platelets

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(cAMP assay was courtesy of Dr. Anthony K. Ho and Miss Heather A. Boyd)

(Vezza *et al.*, 1993). Other than these two major signal transduction pathways, PGE_2 has also been reported to activate phospholipase D (PLD) in human erythroleukemia cells through an increase of $[Ca^{2+}]_i$ (Wu *et al.*, 1992). Activation of PLD, however, is able to generate DG, and thus, can also activate PKC (Nishizuka, 1994).

In the last chapter, PGE_2 was found to inhibit I_k in both primary and subcultured VSMCs from rat tail artery. The effect was believed to be due to a receptor-mediated process involving a cholera toxin-sensitive and/or pertussis toxin-insensitive G proteins. However, whether or not any second messenger systems participate the PGE_2-induced effects still remains unclear. It has been previously shown that cAMP and cAMP-dependent protein kinase (PKA) phosphorylation of the channel protein mediated the modulation of K channels by PGE_2 in human lymphocytes and *Xenopus* oocytes (Mori *et al.*, 1989; Bastin *et al.*, 1990). PGE_2 has been reported to activate AC (AC) and thus cause the accumulation of cAMP in diverse tissues, strongly supporting the role of cAMP as a putative second messenger for PGE_2 action. AC may also be regulated by the binding of other hormones or neurotransmitters to their membrane receptors. These membrane receptors are coupled to AC by at least two known regulatory G proteins, G_s or G_i (Gilman, 1984). cAMP has been implicated in the control of ion channel activity either directly or indirectly via the cAMP-dependent protein phosphorylation. In vascular smooth muscles from rat tail artery, the elevation of intracellular cAMP has been demonstrated to be directly related to the closure of the voltage-dependent Ca^{2+} channel in VSMCs (Wang *et al.*, 1991) and the relaxation of helical smooth muscle strips (Pang

et al., 1985; 1986).

Although coupling of the PGE_2 receptors to the AC complex, through either G_s or G_i, is one possible explanation for [illegible] K channel actions of PGE_2, other mechanisms cannot be ignored. Studies have demonstrated that PGE_2 can stimulate PLC activity and thus induce PIP_2 hydrolysis, resulting in the formation of DG and IP_3 which leads to the activation of PKC and elevation of $[Ca^{2+}]_i$ (Sonnenburg and 1988; Yamaguchi *et al.*, 1988, Yokohama *et al.*, 1988; Aboolian *et al.*, 1989). There has also been some evidence suggesting that PGE_2 could modulate K channels through the activation of PKC. The Ca^{2+}-activated K channel in rat brain synaptosomes was inhibited by PGE_2 via PKC activation (Ren and Benishin, 1994). Similar to the cAMP-PKA system, PKC is another signalling system in which G protein(s) may be involved to couple the PGE_2 receptor to PLC (Gilman, 1987). Alternatively, PGE_2 could also activate PKC at sites distal to IP_3 and DG generation (Piau *et al.*, 1989). Interaction between the PLC-PKC and cAMP-PKA cascades has been well described in many systems, including smooth muscle cells (Nishizuka, 1984a; 1984b; Berridge, 1987; Yamaguchi *et al.*, 1988; Di Marzo *et al.*, 1991), and was recently reported to be the mechanism for PGE_2 action in osteoblast-like cells (Kawase *et al.*, 1991; Kozawa *et al.*, 1992), neuroblastoma cells (Kanba *et al.*, 1991), and gastric parietal cells (Choquet *et al.*, 1990). Therefore, it is possible that the effect of PGE_2 is mediated through both cAMP production and PKC activation. However, the precise mechanism of the postreceptor events induced by PGE_2, has not yet been elucidated in the VSMCs.

Two types of voltage-dependent Ca^{2+} channels (L- & T-) have been characterized on VSMCs from rat tail artery (Wang *et al.*, 1989). The long-lasting, L-type Ca^{2+} channel is the dominant channel conducting the influx of extracellular Ca^{2+}. Although PGE_2 has been reported to regulate, or perhaps activate Ca^{2+} in a number of cell types (Yokohama *et al.*, 1988; Aboolian *et al.*, 1989; Negishi *et al.*, 1989; Kandasamy and Hunt, 1990; Mochizuki-Oda *et al.*, 1991), there has been no evidence regarding the role of PGE_2 on the voltage-dependent Ca^{2+} channel in VSMCs. As mentioned earlier, PGE_2 was capable of stimulating PI hydrolysis, and thus, promoting intracellular Ca^{2+} mobilization in certain cell types such as bovine adrenal chromaffin cells (Ito *et al.*, 1991a; Mochizuki-Oda *et al.*, 1991). However,it is not known whether a similar action occurs in VSMCs. Clarification of the role of PGE_2 on Ca^{2+} channels and intracellular Ca^{2+} in the vasculature would definitely provide more direct evidence for the mechanism of action of PGE_2 in the control of vascular smooth muscle tone.

The purpose of this chapter is to characterize the signal transduction mechanisms underlying PGE_2-induced inhibition of I_k, and the role of PGE_2 on Ca^{2+} channels as well as $[Ca^{2+}]_i$. The whole-cell variant of the patch-clamp technique, radioimmunoassay (RIA) and Fura-2 method were used to investigate enzymically dispersed VSMCs from rat tail artery.

II. Experimental design

The studies were designed to investigate the intracellular messenger systems involved in the action of PGE_2 on I_k in rat tail artery VSMCs. Several pharmacological studies using the cAMP agonists forskolin, Db-cAMP, Sp-cAMPs, and the cAMP antagonist Rp-cAMPs were carried out to determine the role of cAMP in PGE_2 action. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used to determine the possible site of action of PGE_2. PKC activators such as TPA, 4β-phorbol 12, 13-dibutyrate (PDBu) as well as the protein kinase inhibitor staurosporine were studied to determine the possible involvement of the PKC system. Direct assay of PGE_2 on cAMP production in VSMCs was undertaken with RIA method. To investigate if there is any cross-talk between the two second messenger systems, the pharmacological effects of activation of one messenger on I_k in the presence of the inhibitors for the other second messenger cascade were investigated. Furthermore, the effect of the PKC activator TPA on cAMP production was also carried out in VSMCs. The effect of PGE_2 on L-type Ca^{2+} channels was examined. To explore the role of PGE_2 on $[Ca^{2+}]_i$, intracellular Ca^{2+} determination was conducted using the Fura-2 method.

III. Results

A. Effect of Rp-cAMPs on PGE_2-induced inhibition of I_k

Fig. 5-1 shows that Rp-cAMPs (300 μM), an antagonist of PKA, was able to eliminate the suppressive action of PGE_2 on I_k, in both primary and subcultured VSMCs.

The extent of PGE_2-induced inhibition was reduced significantly ($p > 0.05$ compared with control). Rp-cAMPs did not have any effect on I_k in these cells. All the test pulses were at +30 mV.

B. The effect of the cAMP agonists forskolin, Db-cAMP and Sp-cAMPs on I_k

Further support for the concept that the activation of AC was involved in the mechanism of PGE_2-induced inhibition of I_k came from the studies on the effects of direct activation of AC. In these experiments, AC was directly activated by adding the cAMP analogues Db-cAMP (1 mM), Sp-cAMPs (100 μM) or the AC activator forskolin (5 μM) into the extracellular solution (Fig. 5-2). All three agents mimicked the PGE_2 action by attenuating the I_k amplitude, with maximal inhibition of 35%, 50% and 38% in primary VSMCs, 30%, 40% and 36% in subcultured VSMCs, respectively, at the test pulse of +30 mV.

C. The effect of the phosphodiesterase inhibitor IBMX on PGE_2-induced inhibition of I_k

To determine if PGE_2 could cause cAMP accumulation through affecting its hydrolyzing enzyme phosphodiesterase (PDE), VSMCs were preincubated with IBMX, a PDE inhibitor, in the bath before PGE_2 was administered. The result showed that pretreatment of the cells with 100 μM IBMX, a concentration that produced a maximium response in VSMCs (data not shown), suppressed the I_k amplitude by 24.7%. However,

addition of PGE_2 (10 μM), in the presence of IBMX, produced an additional 27.0% inhibition of the I_k amplitude ($p < 0.05$ compared with IBMX alone), indicating that PGE_2 did not likely induce cAMP accumulation by inhibiting the degradation activity of PDE (Fig. 5-3). This result, combined with those studies using the cAMP antagonist and agonists, strongly suggested that the accumulation of cAMP through the activation of AC is involved in the modulation of I_k by PGE_2 in these VSMCs.

D. Direct action of PGE_2 on cAMP production

Radioimmunoassay was performed to directly evaluate the effect of PGE_2 on cAMP production in primary cultured VSMCs from rat tail artery. Fig. 5-4 illustrates that incubation of the VSMCs with PGE_2 increased the cAMP content, in a concentration-dependent manner. This observation was in line with the stimulatory effect of PGE_2 on cAMP accumulation seen in many other tissues, as mentioned earlier.

E. The effect of the kinase inhibitor staurosporine on PGE_2-induced inhibition of I_k

As stated above, PGE_2 might inhibit the I_k in VSMCs from rat tail artery through cAMP accumulation. However, there were some indications that PKC activation could be another important mechanism of PGE_2 action. This possibility was tested by using staurosporine, a potent kinase inhibitor that interacts with the ATP binding sites (Tamaoki *et al.*, 1986). Pretreatment of the cells with staurosporine (10^{-8} M) for 10 min

slightly but significantly deminished the PGE_2-induced inhibition of I_k. However, at a higher concentration (10^{-7} M), staurosporine almost completely abolished the PGE_2-induced inhibition of I_k (Fig. 5-5), indicating a possible involvement of PKC activation in the action of PGE_2.

F. Short- and long-term incubation of the PKC activator TPA on I_k

To further verify the involvement of PKC, the primary and subcultured VSMCs were pretreated with TPA (1 μM), a phorbol ester that activates PKC, either for 10 min or for 24 hrs, which is known to lead to PKC down-regulation due to PKC depletion after prolonged exposure. The control current values for short and long (24 hrs) term incubation of TPA were 160 $\pm$ 30 pA (N=7) and 148 $\pm$ 20 (N=12), respectively ($p>0.05$). Activation of PKC for a short incubation with TPA produced an inhibition of I_k. However, prolonged incubation prevented the PGE_2 (50 μM)-induced inhibition of I_k (Fig. 5-6).

G. The inhibition of PKC on cAMP-induced action on I_k

Fig. 5-7 shows that the inhibitory effects of I_k by direct PKA activation with cAMP analogues were not affected by the blockade of PKC activation, via TPA down-regulation. The VSMCs were pretreated with TPA (1 μM) for 24 hrs before forskolin (1 μM), Db-cAMP (1 mM) or Sp-cAMPs (100 μM) were administered.

H. The effect of PKC activator TPA on cAMP production

To further investigate the interaction between the cAMP system and the PKC cascade, the effects of PKC activation on cAMP production were quantitated using radioimmunoassay. Fig. 5-8 shows that the PKC activator TPA was able to promote cAMP accumulation in primary cultured VSMCs. This effect was concentration-dependent, however, smaller than the effect induced by PGE_2 itself.

I. The inhibition of cAMP-PKA on TPA-induced action on I_k

It has been demonstrated (Fig. 5-8) that the activation of PKC could lead to cAMP accumulation and the subsequent activation of the cAMP-PKA system, revealing an interplay of the two second messenger systems. To determine whether PKA activation could interact with the PKC system, the VSMCs were therefore pretreated with Rp-cAMPs (300 μM) before TPA (1 μM) was administered. Fig. 5-9 shows that the pretreatment with Rp-cAMPs slightly reduced the TPA-induced inhibition of I_k ($p > 0.05$ *vs.* TPA alone). This observation suggested that the effect of TPA on I_k was, at least in a large portion, not affected by the cAMP-PKA signal transduction pathway.

J. The effect of PGE_2 on the voltage-dependent Ca^{2+} channel in VSMCs from rat tail artery

Two types of voltage-dependent Ca^{2+} channels (L- and T-) have been described and studied previously in similiar cell preparations (Wang *et al.*, 1989; Wang , 1991).

The effect of PGE_2 on the L-type Ca^{2+} channel, the major Ca^{2+} current in VSMCs of rat tail artery, was investigated in order to gain further information on the mechanisms of action of PGE_2 on rat tail artery vascular smooth muscles. From a holding potential of -40 mV, a voltage-dependent, long-lasting inward-rectifying current was generated (Fig. 5-10A). PGE_2 (10 μM) inhibited the amplitude of the Ca^{2+} current by 30.0% (Fig. 5-10B). This inhibition of the Ca^{2+} channel was considered to be related to the ability of PGE_2 to stimulate cAMP accumulation, as cAMP has been firmly demonstrated to cause an inhibition of the L-type Ca^{2+} channel current in VSMCs from rat tail artery (Wang *et al.*, 1991). The final concentration of the solvent (ethanol) was less than 0.5%, and did not have any effect on the Ca^{2+} channel currents.

K. The effect of PGE_2 on $[Ca^{2+}]_i$ in VSMCs from rat tail artery

To avoid any alterations to the action of PGE_2 on rat tail artery vascular smooth muscles that might be caused by the non-physiological factors such as the disturbed ion gradients and the use of charge carrier (Ba^{2+}) in the patch-clamp studies, the effect of PGE_2 on intracellular Ca^{2+} concentration was determined using the Fura-2 measurement in primary cultured VSMCs. Intracellular Ca^{2+} determinations revealed that PGE_2 (up to 50 μM) did not affect the basal $[Ca^{2+}]_i$ (Control: 90.6 $\pm$ 15.6 nM; PGE_2: 98.0 $\pm$ 14.1 nM, N=9, $p>0.05$), indicating that PGE_2 had no effect on either intracellular Ca^{2+} mobilization or Ca^{2+} channel opening in VSMCs from rat tail artery. This result (no intracellular Ca^{2+} mobilization by PGE_2) is in agreement with the data from the study of

PGE_2 on extracellular Ca^{2+}-omitted tension development, which will be discussed in the next chapter. However, PGE_2 (10 μM) was found to significantly potentiate the increase of $[Ca^{2+}]_i$ induced by 30 mM KCl (Fig. 5-11).

IV. Discussion

The results reported in this chapter characterized the specificity of the PGE_2-induced postreceptor interaction with possibly two different second messenger systems on the inhibition of I_k, in VSMCs from rat tail artery. It is believed that PGE_2, like most neurotransmitters and hormones, exerts its actions through cytosolic second messengers, which include such diverse molecules as cAMP, Ca^{2+}, IP_3 and DG. Second messengers such as cAMP and DG activate specific protein kinases, i.e., cAMP-dependent protein kinase (PKA) and protein kinase C, respectively (Scott and Soderling, 1992). These are known to be the essential second messenger pathways for various physiological processes (Ho *et al.*, 1988a; Di Marzo *et al.*, 1991), such as modulation of the activity of a large number of enzymes, membrane ion channels as well as structural proteins. K channels are widely distributed on the VSMC plasma membranes, where they play a key role in controlling many cellular functions, and most importantly, the vascular smooth muscle tone (Longmore and Weston, 1990). It is believed that many vasoactive substances could exert their action on K channels through intracellular second messengers.

A. The cAMP-PKA cascade in mediating the action of PGE_2 on I_k

Intracellular cAMP concentration is dependent upon the activity of AC, which catalyses the conversion of ATP to cAMP, and by PDE, which degrades cAMP to 5'-AMP. The AC activity can be regulated by the binding of agonists to the membrane receptors, which are coupled to AC by at least two regulatory G proteins, G_s and G_i. In the present study, the inhibition of I_k by PGE_2 was found to be mediated by cAMP accumulation and the subsequent activation of PKA. This was indicated by the ability of forskolin, Db-cAMP and Sp-cAMPs to mimick, and the ability of Rp-cAMPs to abolish the inhibition of I_k by PGE_2, in both primary and subcultured VSMCs. Radioimmunoassay data also suggested that PGE_2 directly induced cAMP accumulation in VSMCs from rat tail artery. The finding that maximal inhibition of the PDE activity by 100 μM IBMX did not prevent the inhibitory effect of PGE_2 indicated that the accumulation of cAMP was likely due to the activation of AC, instead of the inhibition of PDE. These results, combined with those from chapter 4 suggested that the inhibition of I_k by PGE_2 was due to the activation of AC via a G protein-mediated process.

Modulation of K channel activity by cAMP has been reported in a number of cases. In *Xenopus* oocytes and node of Ranvier, addition of cAMP amplified the K current (Seelig and Kendig, 1982; Van Renterghem *et al.*, 1985). In cultured chick sensory neurones, forskolin suppressed a voltage-dependent K current (Dunlap, 1985). It was also reported that intracellular perfusion of cAMP or forskolin rapidly decreased the K current amplitude in murine B cells (Choquet *et al.*, 1987) and in oligodendrocytes

(Soliven *et al.*, 1988). Grega and Macdonald (1987) applied compounds that stimulate AC (forskolin, cholera toxin, and prostaglandin E_1) to mouse sensory neurones in culture and found the blockade of a voltage-dependent K channel.Such inhibition of the voltage-dependent K channel was responsible for prolonging the action potential duration. It has been hypothesized that cAMP exerts its action by a subsequent phosphorylation of the K channels via a cAMP-dependent protein kinase, i.e., PKA (Levitan, 1985), while a possible direct effect of cAMP on K channels should not be excluded.

B. The PKC cascade in mediating the action of PGE_2 on I_k

In smooth muscle cells and neurones, another protein kinase, PKC, activated by DG (or synthetic phorbol ester compounds) and intracellular Ca^{2+}, has also been reported to regulate a number of K currents (Baraban *et al.*, 1985; Colby and Blaustein,1988; Walsh and Kass, 1988; Lester *et al.*, 1991; Moran *et al.*, Ren and Benishin, 1994). In the present study, it was found that the PGE_2-induced inhibition of I_k was blocked by PKC inhibition with staurosporine or PKC down-regulation by prolonged exposure to TPA. TPA, a potent tumor-promoting phorbol ester that can activate PKC, also produced an inhibition of I_k. These results suggested that the activation of PKC may be involved in the mechanism of action of the PGE_2-induced inhibition of I_k. The fact that TPA could produce a larger (65%) inhibition of I_k than PGE_2 (45%) might be due to the possibility that PGE_2 caused less than complete activation of PKC, e.g., PGE_2 activated a narrower spectrum of PKC isozymes than TPA. Activation of PKC has been shown to directly

inhibit different types of K channels. Lester *et al.* (1991) found that when DG and arachidonic acid, another PKC activator, were applied to the *Hermissenda* photoreceptor, they acted synergisticly to reduce the voltage-dependent K current. PGE_2 was also found to inhibit a Ca^{2+}-activated K channel in rat brain synaptosomes through a PKC-dependent mechanism (Ren and Benishin, 1994). Further studies described in this chapter revealed that the TPA-induced inhibition was not, at least in large portion, influenced by the cAMP-PKA pathway, indicating the trivial contribution of the cAMP-PKA cascade to TPA-induced inhibition of I_k. However, the fact that Rp-cAMPs blocked the PGE_2-induced inhibition of I_k but only managed to reverse 20% of the TPA-induced inhibition indicated a possible discrepancy in PKC activation. It might be possible that TPA activated certain PKC isozymes which could not be activated by PGE_2. This was also in line with the finding that PGE_2 produced less inhibition of I_k compared with TPA.

C. The interaction between the two signal transduction pathways

There has been considerable evidence that there is interaction between these two major signal transduction pathways. The results presented here suggested that the PGE_2-induced inhibitory effect of I_k might involve interactions between the two major signal transduction systems, namely cAMP-PKA and PKC. The activation of PKC seemed to modify the cAMP-PKA pathway, however, such was not likely the case *vise versa*.

The modulation of the cAMP-PKA signal transduction pathway by PKC has been found to be complex. Treatment of cells with phorbol esters, structural analogues of DG,

were considered to exert various actions, i.e. either an augmentation or attenuation of agonist-induced cAMP accumulation, depending on the type and length of phorbol ester treatment as well as the cell type. The diversity in cellular regulation may also be related to the number of PKC isozymes present in the cells which may be differentially activated by agonists and phorbol esters. At least eleven family members of PKC isozymes have been characterized so far (Nishizuka, 1994).

In the present study, it was shown that pretreatment of the VSMCs with the protein kinase inhibitor staurosporine, a potent inhibitor via interaction with the kinase ATP binding sites, or the prolonged exposure to TPA to down-regulate PKC, abolished the inhibitory action of PGE_2 on I_k amplitude. The difference in the degree of action of staurosporine might be due to the fact that a lower concentration of staurosporine caused a less than complete inhibition of protein kinase C, or a less extent of inhibition of PKA. Staurosporine, although usually referred as a PKC inhibitor, is not very selective between PKC and PKA in some tissues and cells. It is able to affect PKA activity at a relatively higher concentration compared to that of PKC inhibition. However, the pretreatment of staurosporine did not affect the inhibition of I_k induced directly by the cAMP analogues Db-cAMP, Sp-cAMPs as well as forskolin. Direct activation of PKC by TPA caused cAMP accumulation. These results indicated that the activation of PKC might participate the PGE_2-induced inhibition of I_k through potentiation of the cAMP accumulation. The inability of PKC inhibition to abolish the inhibition of I_k by the cAMP analogues and forskolin has, however, called into question the possible site of action of PKC in the

PGE_2-induced action. In addition to its regulatory effects on the cAMP-PKA system, PGE_2 was also found to stimulate the phospholipase C pathway and to activate PKC in many tissues and cells (Sennenburg and Smith, 1988; Yamaguchi *et al.*, 1988; Yokohama *et al.*, 1988; Aboolian *et al.*, 1989; Hayaishi *et al.*, 1989). The PKC activation has been documented to induce cAMP accumulation in various tissues (Nagshineh *et al.*, 1986; Yoshimasa *et al.*, 1987; Gusovsky and Gutkind, 1991; Hagel-Bradway *et al.*, 1991; Eikvar *et al.*, 1993), although the site(s) of interaction in the cAMP-PKA pathway is still not defined. This has been demonstrated again in VSMCs with the cAMP assay in the present study. The fact that the PGE_2-induced inhibition of I_k was not affected by pertussis toxin (see chapter 4) suggested that PGE_2 could probably activate PKC through a pertussis toxin-insensitive G protein(s) (G_q, the G protein specific for PLC) or at site(s) distal to the PI hydrolysis, which have been shown previously in other tissues (Negishi *et al.*, 1989; Piau *et al.*, 1989; Kitanaka *et al.*, 1991). Such a hypothesis on the interactions of the second messenger signal transduction mechanisms has gained more support from the studies in other tissues. The results presented here were consistent with the view that PKC activation could either directly stimulate AC activity or potentiate agonist-induced cAMP accumulation. Bos *et al.* (1991) reported that TPA, at the concentration greater than 1 μM, could potentiate the PGE_2-induced cAMP accumulation in fetal rat osteoblasts. The action was probably due to the activation of AC. Pretreatment of the cells with pertussis toxin did not prevent this action. Tachado *et al.* (1993) reported that PKC activation was involved in the cAMP formation due to $PGF_{2\alpha}$

desensitization. The biological effects of PGE_2 on regulation of progesterone production in monkey luteal cells has also been reported to involve both PKC activation and cAMP accumulation (Michael *et al.*, 1993). Although no suggestions for the mechanism of these effects were advanced by these investigators, the following considerations on the sites of action by PKC activation may shed some light on it.

The data obtained suggested a possible role of PKC in mediating the AC activity in VSMCs. Similar findings have been widely documented in different tissues, such as adipocytes and osteoblasts (Katada *et al.*, 1985; Nagshineh *et al.*, 1986; Yoshimasa *et al.*, 1987; Gusovsky and Gutkind, 1991; Hagel-Bradway *et al.*, 1991). PKC activation by phorbol esters has been shown to have a dual action, providing positive feed-forward as well as negative feedback control on cAMP accumulation, depending on the cell types and the experimental conditions. PKC activation by phorbol ester has been reported to reduce agonist-stimulated cAMP production (Mukhopadhyay and Schumader, 1985; Rebois and Patel, 1985; Kawase *et al.*, 1991). Kawase *et al.* (1991) found that pretreatment of the osteoblast-like cells with TPA increased the basal cAMP level, but markly reduced the PGE_2-stimulated cAMP production in the presence of IBMX. However, the same study also found that TPA exerted no effect on forskolin- or cholera toxin-stimulated cAMP production. It was thus considered that PKC activation acted directly (e.g., by phosphorylation) on the PGE_2 receptors to decrease their affinity and thereby reduced the PGE_2-stimulated cAMP accumulation.

D. The possible sites of action of PKC

The target sites of PKC activation on the cAMP-PKA system are so far not clear. It is possible that each individual component of the receptor-G protein-AC-PDE complex could become a potential site of action for PKC. This is supported by several lines of evidence. Firstly, PKC activation may act to regulate the surface membrane receptor, thus producing biological effects involving up- or down-regulation of the receptor (Watson *et al.*, 1993). Alternatively, it is also possible that the protein phosphorylation with prolonged exposure of the cells to TPA might desensitize the membrane receptors, other than depleting the cytosolic PKC. Secondly, PKC activation has been shown to interact with the regulatory G proteins and the effects involve protein phosphorylation (Katada *et al.*, 1985; Yoshimasa *et al.*, 1987). Phosphorylation of the regulatory G protein (presumed to be G_i) removes the tonic inhibition of the AC complex. There has been no evidence, so far, that G_s could be modified by PKC phosphorylation. Thirdly, by examining the receptor-mediated cAMP metabolism in the presence of the phosphodiesterase inhibitor, PKC activation could modify the AC activity, by interacting with the catalytic unit of the enzyme (Katada *et al.*, 1985). Finally, the cAMP accumulation induced by TPA has been found to involve a inhibitory action of the phosphodiesterase isozymes (Watson *et al.*, 1993). The data presented in this chapter more or less suggested that the most likely target site(s) of PKC action might be beyond AC activation.

Substantial progress has been made recently towards the characterization of the

"cross-talk" mechanism between these two messenger systems in a variety of tissues (Yoshimasa *et al.*, 1987; Abdel-Latif, 1991; Houslay, 1991). The data presented here are in accordance with the previous result that PGE_2 or other PGs stimulated AC through activation of PKC in osteoblast-like cells and bovine iris sphincter (Kozawa *et al.*, 1992; Tachado *et al.*, 1993). It is possible that the binding of PGE_2 to its receptor could activate PKC either directly, or indirectly through a pertussis toxin-insensitive G protein. The activation of PKC may in turn lead to the modification in the receptor-stimulated cAMP-PKA signal transduction pathway (Johnson and Toews, 1990). A schematic diagram is presented showing the possible mechanism of action of PGE_2 in modulating the voltage-dependent K channels in VSMCs (Fig. 5-12).

E. The role of PGE_2 in the modulation of Ca^{2+} channels and $[Ca^{2+}]_i$

In VSMCs of the rat tail artery, an increase in cAMP was found to inhibit the L-type voltage-dependent Ca^{2+} channel (Wang, 1991; Wang *et al.*, 1991). This finding was in accordance with the result from tension studies in rat tail artery helical strips that an increase of intracellular cAMP caused relaxation of the muscle strips (Pang *et al.*, 1985; 1986). Many investigations appeared in the last decade concerning the role of cAMP in the vascular smooth muscle tone (Khalil *et al.*, 1987; Consigny, 1991; Rembold, 1992). It is now generally accepted that an increase in the intracellular cAMP level will lead to the smooth muscle relaxation and vasodilation, although with exceptions (Brown *et al.*, 1989). The production of cAMP has been considered to be an important mechanism for

the vascular functions of many vasodilators. These vasodilators, including parathyroid hormone, epinephrine, dopamine, PGI_2 and PGE_2, are able to activate the AC when their membrane receptors are occupied (Consigny, 1991). The accumulation of cAMP then leads to the activation of cAMP-dependent protein kinase, i.e., PKA. PKA is believed to be responsible for the vasodilating or muscle relaxing effect of cAMP through one of the following mechanisms: (1) a decrease in $[Ca^{2+}]_i$, either through reducing Ca^{2+} influx through Ca^{2+} channel, or via enhancing Ca^{2+} extrusion by Ca^{2+} pump. (2) a decrease of the $[Ca^{2+}]_i$ sensitivity to phosphorylation by phosphorylating the myosin light chain kinase at a site that reduces its affinity to Ca^{2+}-calmodulin complex. (3) the uncoupling of the force from myosin phosphorylation. (4) the opening of K channels which repolarize or hyperpolarize the VSMCs (Consigny, 1991; Rembold, 1992). The present study showed that PGE_2 could promote cAMP accumulation in VSMCs from rat tail artery. This seemed to fit in with the observation that PGE_2 inhibited the L-type voltage-dependent Ca^{2+} channel in the same cells. It could thus be speculated that the inhibitory effect of PGE_2 on Ca^{2+} channel might be related to its ability to induce cAMP accumulation, although direct evidence is still lacking.

The Fura-2 determination of the intracellular Ca^{2+} concentration, however, yielded results which contradicted the patch-clamp study. PGE_2 was found to potentiate the KCl-stimulated increase of $[Ca^{2+}]_i$, suggesting an enhancing effect on Ca^{2+} entry. The results that will be presented in the next chapter also indicate that PGE_2 increases tension development in rat tail artery helical strips, indicating that the overall effect of PGE_2 on

$[Ca^{2+}]_i$ is likely to be an increase. The lack of response of PGE_2 on basal $[Ca^{2+}]_i$ suggested that PGE_2 did not affect the intracellular Ca^{2+} mobilization from the intracellular stores, such as SR or promote influx of extracellular Ca^{2+}. However, PGE_2 by itself did cause contraction of the helical strips, which has traditionally been considered to be proportional to $[Ca^{2+}]_i$. PGE_2 has been reported previously to increase $[Ca^{2+}]_i$ in other types of cells such as osteoblasts (Yokohama *et al.*, 1988; Ito *et al.*, 1991a). These discrepancies in the results from the patch-clamp study (Ca^{2+} channel) and the Fura-2 measurement might possibly be explained as follows: the effect of PGE_2 on voltage-dependent Ca^{2+} channel might be relatively minor and is likely to be compensated by other PGE_2-induced unknown mechanisms which may increase the Ca^{2+} entry or Ca^{2+} sensitivity. The difference in the age of VSMCs might also be a factor, as 1-2 days old VSMCs were used in patch-clamp studies compared to those of 4-5 days in Fura-2 studies because of the time to reach confluence.

Conclusion

There is accumulating experimental evidence indicating that PGE_2 plays a critical physiological role in the control of vascular smooth muscle tone. This effect could be largely attributed to the modulation of PGE_2 on the vascular smooth muscle K channel activity. The data presented in this chapter and the previous chapter have shown that PGE_2 inhibited a voltage-dependent K current. Evidence is presented that the inhibition

is mediated via the activation of the cAMP-PKA and possibly the PKC signal transduction pathways. Pharmacological studies have shown that cAMP-PKA and PKC activation may be individually involved in the PGE_2-induced inhibition. Staurosporine and PKC down-regulation reduced or blocked the PGE_2-induced inhibition of I_k, but did not exert any effect on cAMP-induced inhibition of I_k. This brought about the notion of second messenger "cross-talk" in the action of PGE_2. Radioimmunoassay demonstrated that TPA stimulated the cAMP accumulation. The cAMP-PKA pathway did not seem to have a novel interaction with the PKC cascade. A possible subcellular mechanism of action is shown in Fig. 5-12: PGE_2 acts on the membrane surface receptor(s), the binding of the receptor(s) induces the activation a cholera toxin-sensitive, pertussis toxin-insensitive regulatory G protein(s). Two cytosolic messenger systems, the cAMP-PKA pathway and possibly the PKC pathway, are then activated. The activation of each of the two cascades could exert an inhibition on the voltage-dependent K current (I_k). There is evidence suggesting the "cross-talk" between the two pathways. A possible activation of PKC by PGE_2, either through a pertussis toxin-insensitive G protein or at a site distal to PI hydrolysis, stimulates cAMP accumulation probably through the activation of the AC, although direct evidence is still lacking on the target sites of action. The activation of PKC may mediate the effect of PGE_2 on cAMP accumulation and ultimately the inhibition I_k, although PGE_2 may stimulate cAMP accumulation directly. The cAMP-PKA pathway activated directly by PGE_2 may exert the inhibition of I_k by itself, with or without the mediation of PKC activation. Also the cAMP-PKA pathway does not appear

to have a novel action on the PKC pathway. Finally, PGE_2 was found to inhibit the L-type voltage-dependent Ca^{2+} channel. This effect may be related to an elevation of the intracellular cAMP level. However, contradictory data were obtained from intracellular Ca^{2+} determination study using Fura-2. PGE_2 was found to have no effect on basal $[Ca^{2+}]_i$, but potentiated the KCl-stimulated increase in $[Ca^{2+}]_i$. These observations indicated that the effect of PGE_2 on Ca^{2+} channel in VSMCs might be relatively minor in contributing to the level of $[Ca^{2+}]_i$.

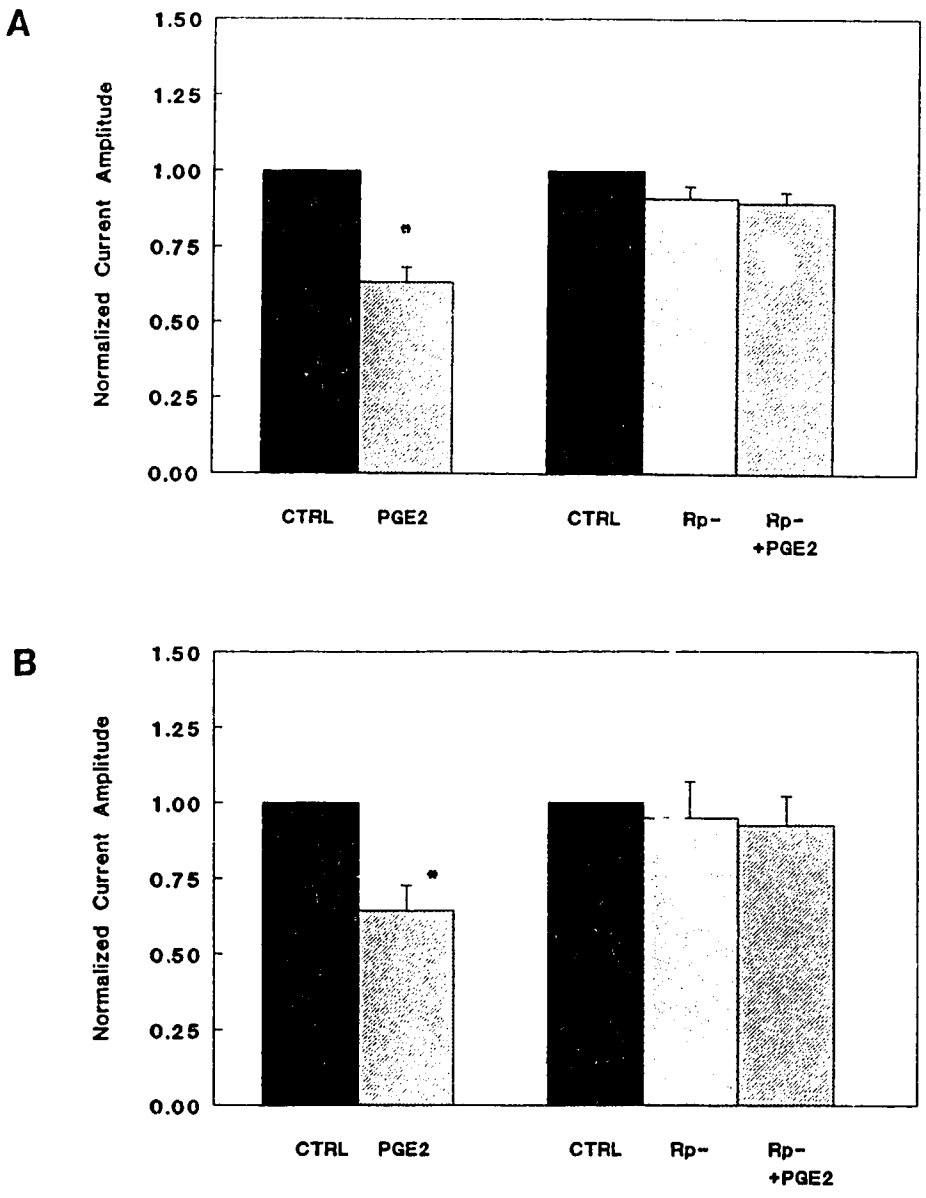


Figure 5-1. Effect of Rp-cAMPs on PGE_2-induced inhibition of I_k in primary cultured (A) and subcultured (B) VSMCs. Rp-cAMPs (Rp-, 300 μM) was applied to the bath 10 min before the addition of PGE_2 (50 μM). *$p<0.05$ compared with control. Values were normalized to the respective control. N=3 and 6 for primary cultured and subcultured VSMCs, respectively.

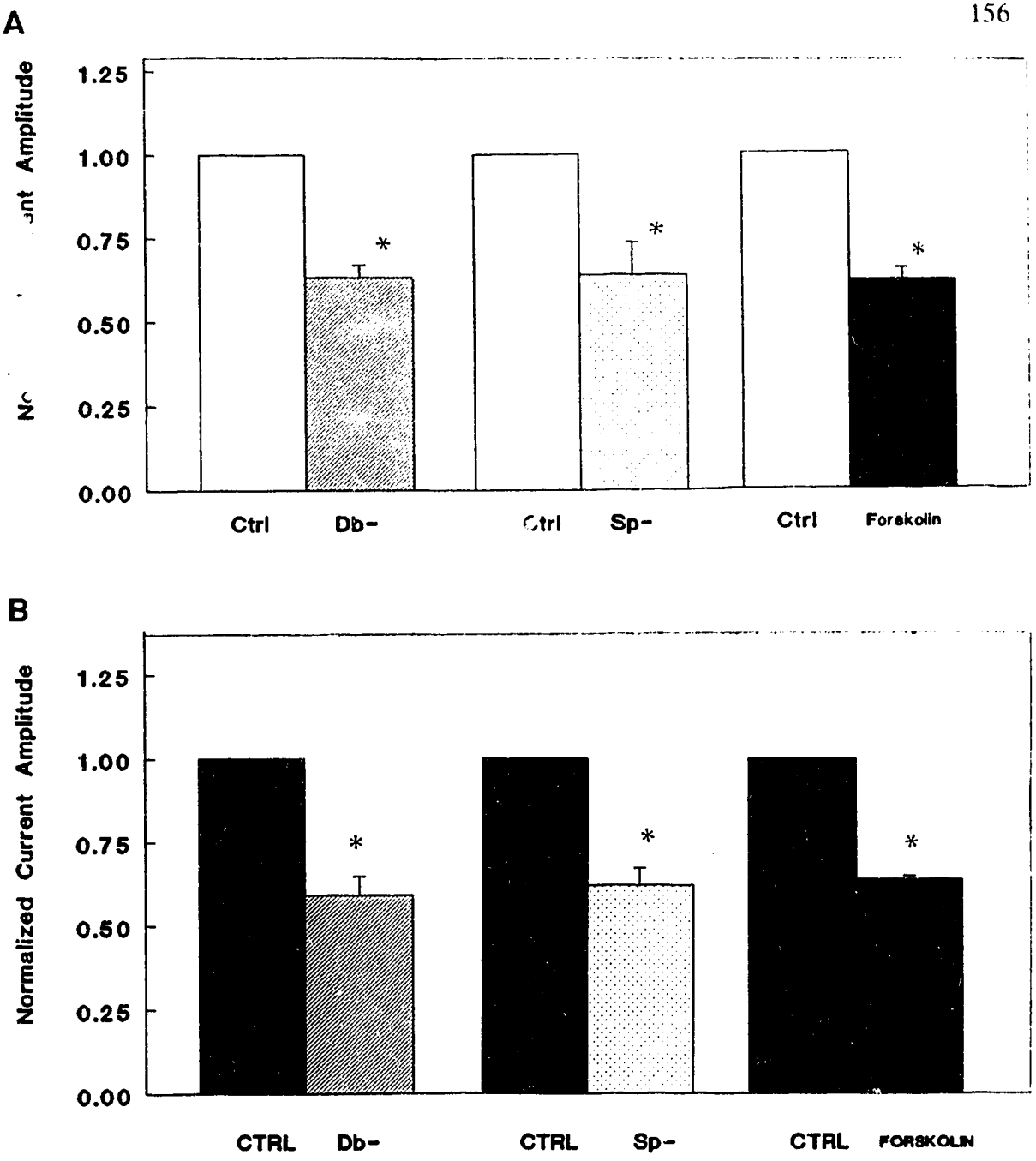


Figure 5-2. Effects of Db-cAMP (1 mM), Sp-cAMPs (100 μM) and forskolin (5 μM) on I_k in primary cultured (A) and subcultured (B) VSMCs. Drugs were added to the bath and the amplitude of the current was recorded after 10 min. Normalized values are M$\pm$SE, *$p<0.05$ *vs.* controls. N=4 and 5, for primary and subcultured VSMCs, respectively.

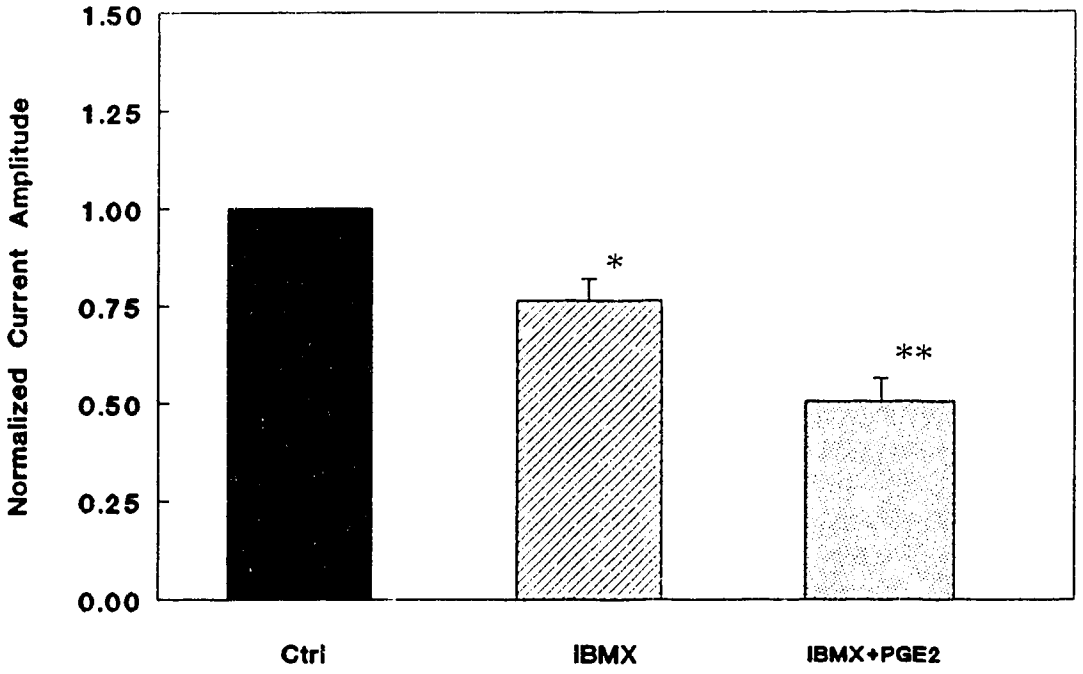


Figure 5-3. Effect of IBMX on PGE_2-induced inhibition of I_k in primary cultured VSMCs. The cells were treated with IBMX (100 μM) for 10 min before being exposed to PGE_2 (10 μM). Current amplitudes were normalized to the corresponding control and are shown as M$\pm$S.E. *$p<0.05$ *vs.* control, **$p<0.05$ *vs.* IBMX alone. N=5.

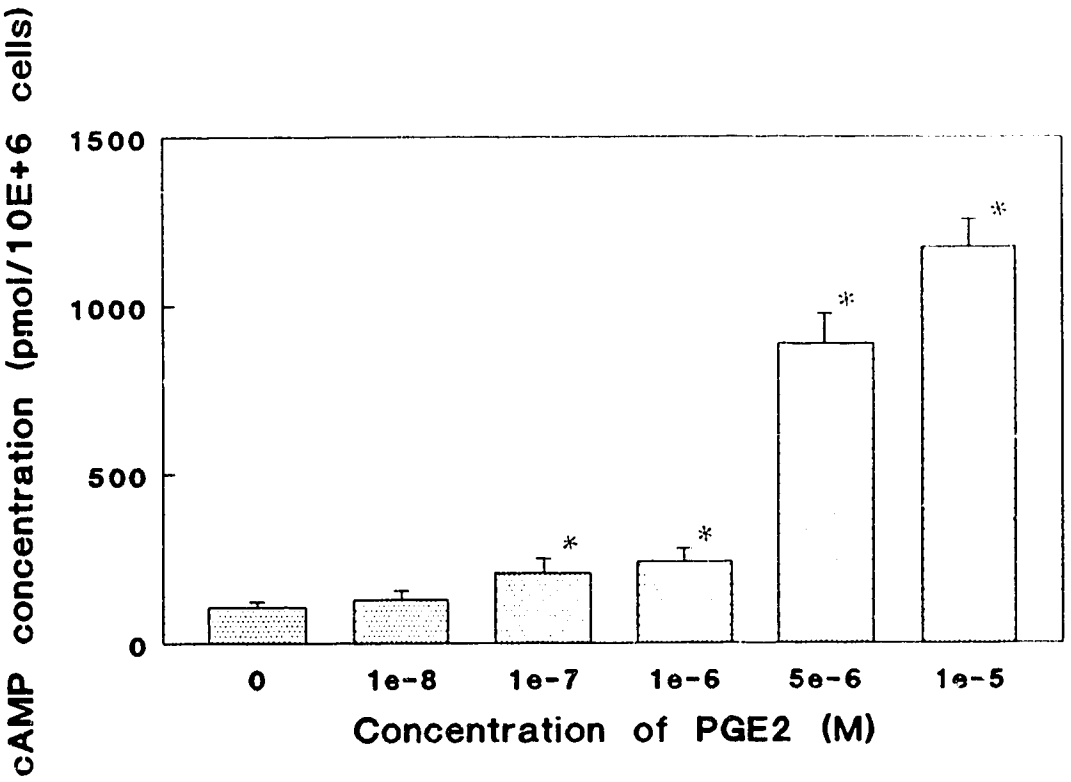


Figure 5-4. Effect of PGE_2 on cAMP accumulation in primary cultured VSMCs. The VSMCs were preincubated with various concentrations of PGE_2 for 20 min, before the cAMP content was determined with RIA. The data shown are representative of four experiments performed in duplicate. Values are M $\pm$ S.E., *$p<0.05$ *vs.* control. Test of significance was performed using analysis of variance in conjunction with the Newman-Keuls multiple range test.

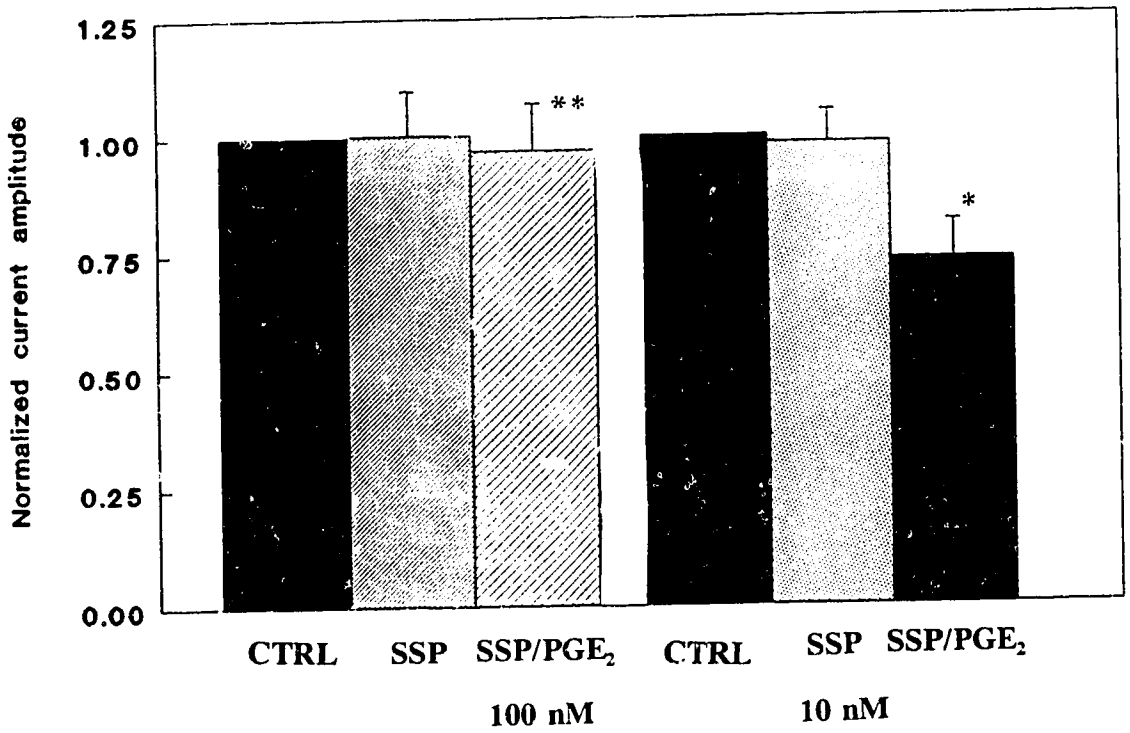


Figure 5-5. Effect of staurosporine on PGE_2-induced inhibition of I_k in primary cultured VSMCs. The cells were pretreated with staurosporine (10 nM or 100 nM) for 10 min before exposure to PGE_2 (50 μM). Currents were normalized to the control and are shown as M$\pm$S.E., N=4 and 9 for low and high concentrations of staurosporine, respectively. * $p<0.05$ *vs.* staurosporine alone. ** N.S. not significantly different from the staurosporine alone.

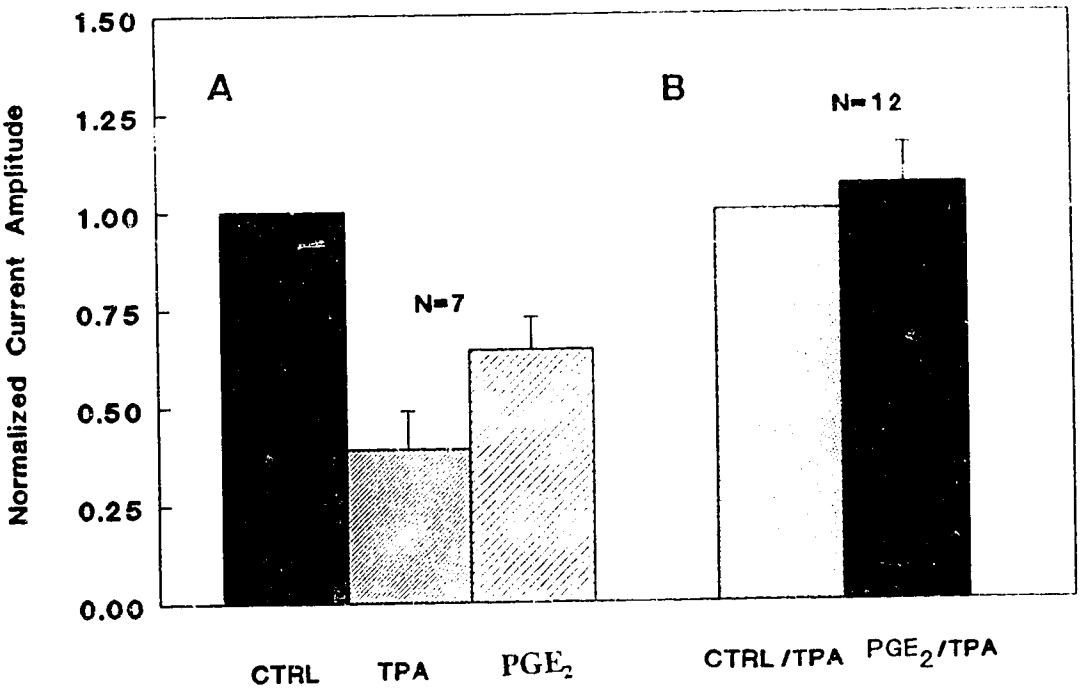


Figure 5-6. Effects of short and prolonged incubation of primary cultured VSMCs with TPA on I_k. A. TPA (100 nM) was present for 10 min prior to the measurement (N=7). B. Cells were preincubated with TPA (1 μM) for 24 hrs to cause down-regulation of PKC. The currents were then recorded before and 10 min after PGE_2 (50 μM) was added (N=12). Bars represent M$\pm$SE *$p<0.05$ *vs.* control.

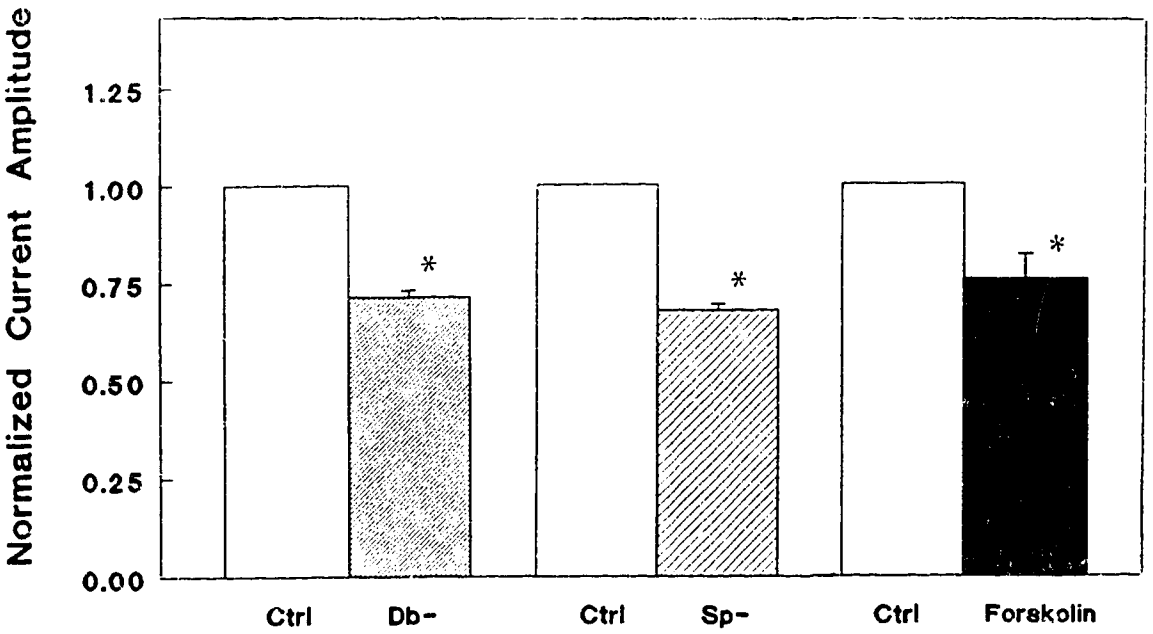


Figure 5-7. Effect of PKC inhibition on cAMP-induced inhibition of I_k. The VSMCs were pretreated with TPA (1 μM) for 24 hrs to cause down-regulation of PKC, the currents were then recorded before (open bars) and 10 min after (cross-hatched or solid bars) addition of forskolin (1 μM), Db-cAMP (1 mM) and Sp-cAMPs (100 μM). Bars represent M$\pm$S.E. *$p<0.05$ *vs.* control. N=4 for each drug.

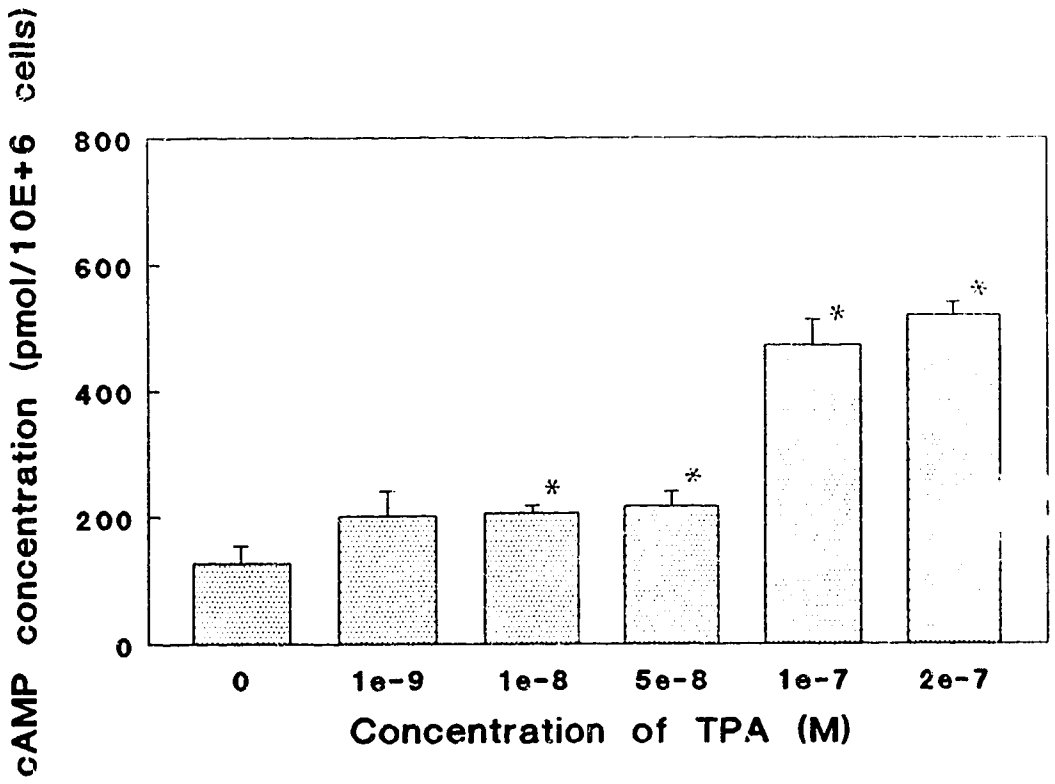


Figure 5-8. Effect of the PKC activator TPA on cAMP production in primary cultured VSMCs. The cells were preincubated with various concentrations of TPA for 20 min, before the cAMP assay. The data shown are representative of four experiments performed in duplicate. Values are M$\pm$S.E., *$p < 0.05$ *vs.* control. Test of significance was performed using analysis of variance in conjunction with the Newman-Keuls multiple range test.

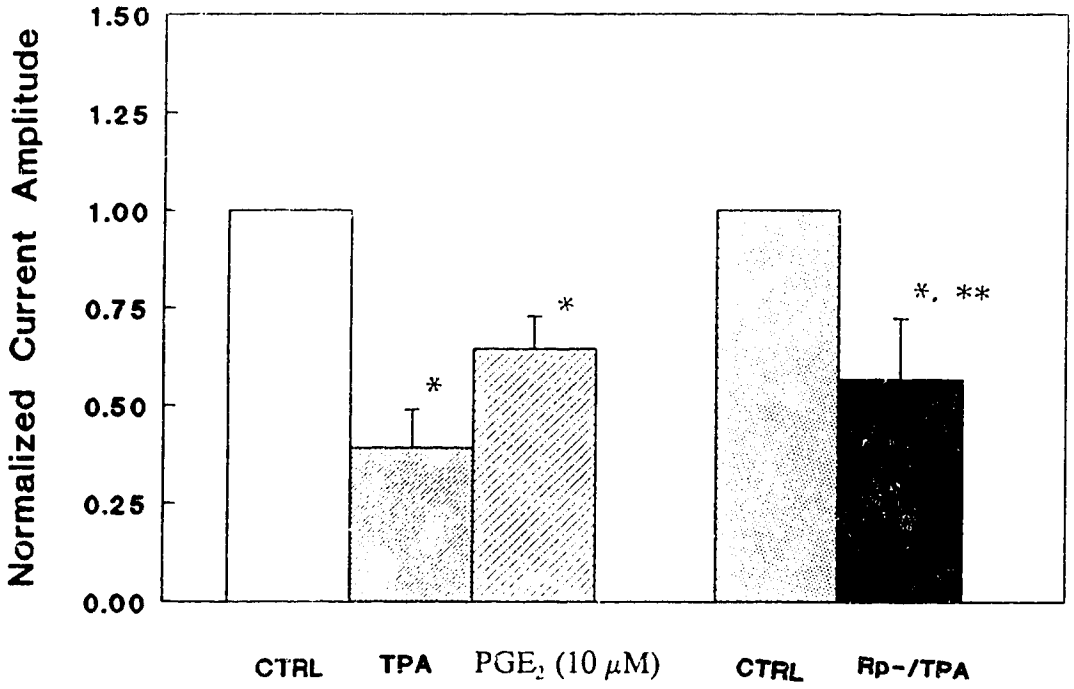


Figure 5-9. Effect of Rp cAMPs (300 μM) on TPA (1 μM)-induced inhibition of I_K. The VSMCs were pretreated with Rp-cAMPs (Rp-) for 10 min. The currents were then recorded before (solid bars) and 10 min after (cross-hatched bars) the addition of TPA. Bars represent M $\pm$ S.E., *$p<0.05$ *vs.* control, **N.S. not significantly different from TPA alone. N=7 for Rp- untreated cells, and N=5 for Rp- treated cells.

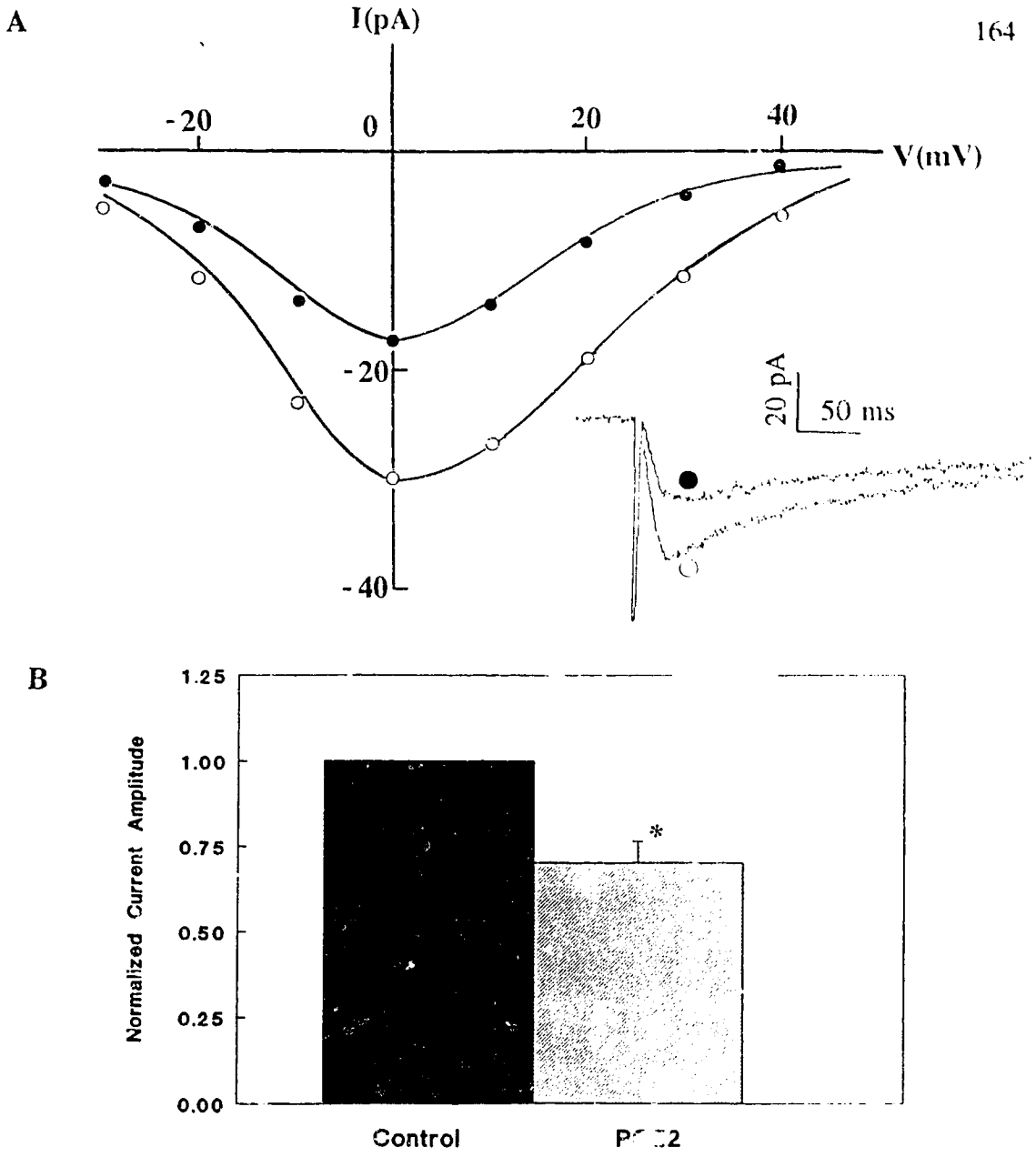


Figure 5-10. Effect of PGE_2 on Ca^{2+} channel currents in primary cultured VSMCs. A. I-V relationships of Ca^{2+} currents before (open circles) and 10 min after the addition of PGE_2 (10 μM, filled circles) in a primary cultured VSMC. Inset: original current records measured at +10 mV before (open circles) and after (filled circles) PGE_2. B. Effect of PGE_2 on peak Ca^{2+} channel currents (N=6). The VSMCs were voltage-clamped at a holding potential of -40 mV. Values are normalized to the respective control and expressed as M±S.E. *$p<0.05$ *vs.* control.

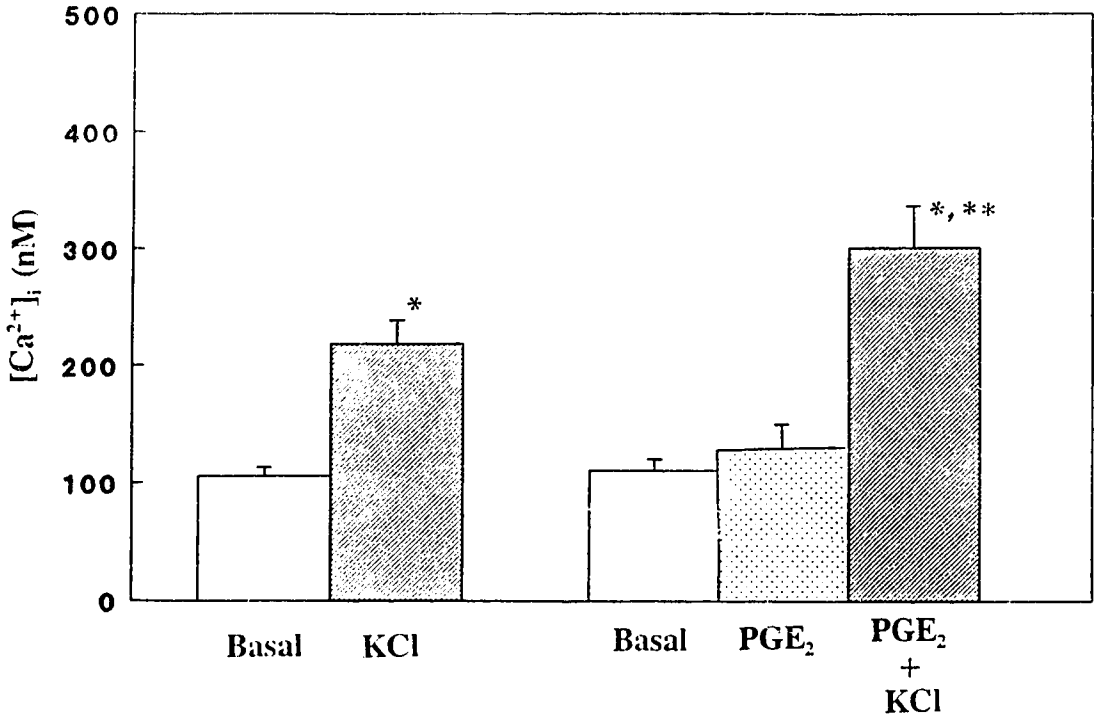


Figure 5-11. Effect of PGE_2 on $[Ca^{2+}]_i$ in primary cultured VSMCs. Histograms summarizing the effects of PGE_2 (10 μM) on basal (empty bars) and KCl-stimulated $[Ca^{2+}]_i$. In potentiation experiments, the cells were first stimulated with KCl (solid bar), then washout, followed by the addition of PGE_2 before KCl was readministered (cross-hatched bar). Values are M$\pm$S.E.. *$p<0.05$ *vs.* basal level, ** $p<0.05$ *vs.* KCl-stimulated level. N=7.

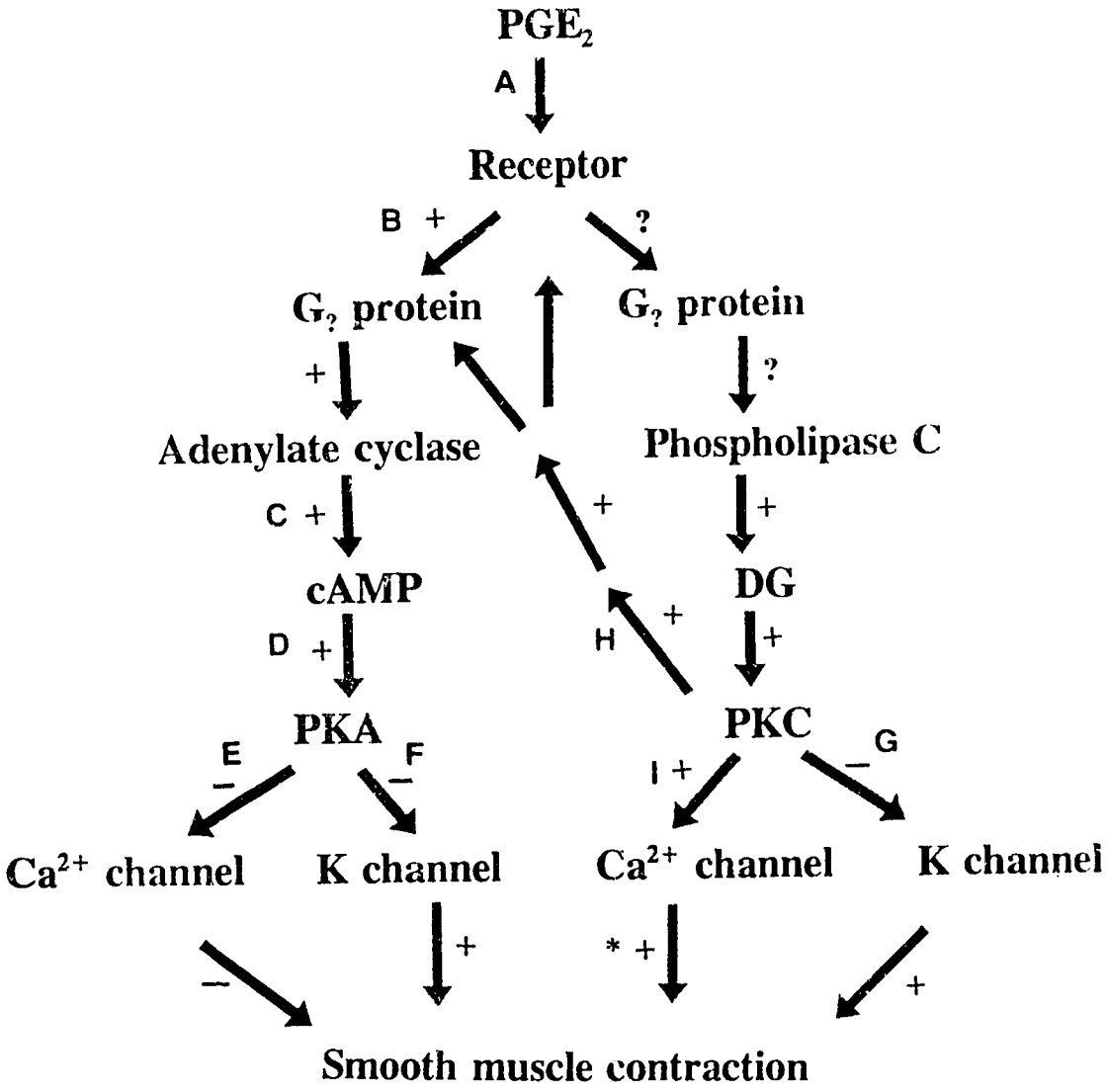


Figure 5-12. Scheme showing the possible mode of action of the two second messenger systems cAMP-PKA and PLC-PKC, mediating the action of PGE_2 in VSMCs from rat tail artery. + and -, indicate stimulation and inhibition, respectively. ? implies an uncertain mechanism. Supporting data are: A. Fig. 4-7; B. Fig. 4-8, Fig. 4-9; C. Fig. 5-2, Fig. 5-4; D. Fig. 5-2; E. Wang *et al.*, 1991; F. Fig. 5-1; G. Fig. 5-5, Fig. 5-6; H. Fig. 5-6, Fig. 5-7, Fig. 5-8; I. Li and Karpinski, unpublished data.

CHAPTER 6

THE EFFECT OF PGE_2 ON TENSION IN ISOLATED RAT TAIL ARTERY HELICAL STRIPS

I. Introduction

The involvement of PGE_2 in the control of vascular smooth muscle tone is well known. Smooth muscle cells exhibit considerable heterogeneity in electrical excitability and responsiveness to specific hormones and neurotransmitters, as might be expected due to the wide variety of tissues in which they are localized. Such heterogeneity may contribute to the diverse and sometimes opposite effects of PGE_2. Both vasorelaxation (Greeley *et al.*, 1986; Axelrod, 1991; Klemm *et al.*, 1991) and vasoconstriction (Camu *et al.*, 1992; Dorn *et al.*, 1992; Jaiswal *et al.*, 1993) have been attributed to PGE_2, revealing a potential tissue specificity. Being largely produced in the circulation from VSMCs and vascular endothelial cells, PGE_2 is believed to possess the capacity of affecting local vascular tone in many tissues either directly, or indirectly through the modulation of vascular responses to hormones, neurotransmitters, or even changes in oxygen tension (Smith and McGrath 1993). The actions of PGE_2 as well as other E-series prostaglandins have been reported to be directly related to their ability to stimulate AC activity, thus causing cAMP accumulation in various types of cells including vascular smooth muscle (Consigny, 1991). This is similar to the activity of prostacyclin, another novel vasodilator. The circulating level of PGE_2 has been reported to contribute to the development of hypertension in rat and human (McGiff *et al.*, 1981; Klemm et al., 1991), and it is also implicated in the

pathogenesis of a great variety of cardiovascular disorders. The vasoactive properties of PGE_2 appear to be complex, and can depend on a wide variety of factors including the particular tissue preparation, the presence or absence of intact endothelium, the level of basal vascular tone, and especially the interaction of multiple signal pathways at the subcellular level. It has been postulated that these diverse or even opposite actions of PGE_2 on cell function may be attributed to the interaction of PGE_2 with at least two major signal transduction systems, one already mentioned is the cAMP-PKA pathway, and the other has recently been found to be as the PLC-PKC pathway (Goureau *et al.*, 1992; Kozawa *et al.*, 1992). Therefore, the discrepancy in vascular effects of PGE_2 may be reasonable, given that different signalling pathways may be activated. These second messenger systems may alter smooth muscle contraction by increasing or decreasing intracellular Ca^{2+} concentration, and, thus, indirectly affect the extent of myosin light chain phosphorylation, which is key for muscle contraction.

PGE_2 has long been known to be a vasorelaxant like PGI_2 (Greeley *et al.*, 1986). However, it has also been reported to have contractile effects in variety of vascular and non-vascular tissues from a number of species, including rat (Malik and McGiff, 1975; Dorn *et al.*, 1992), guinea-pig (Bennett *et al.*, 1975a&b; Ishizawa, 1991), cow (Camu *et al.*, 1992; Lograno *et al.*, 1992), rabbit (Pairet *et al.*, 1986; Burakoff and Percy, 1992) and human (Bennett *et al.*, 1981; Secher *et al.*, 1982; Camu *et al.*, 1992). Although subcellular signalling mechanisms have been postulated for the contractile responses to PGE_2 (Hanasaki and Arita, 1989; Hanasaki *et al.*, 1990; Goureau *et al.*, 1992), concisely defined mechanisms are, however, still unclear. In this chapter, rat tail artery helical strips (endothelium denured due to surgical process) are used as a model of peripheral resistence vessels to study the role of PGE_2 on vascular tone, as well as

the subcellular signalling pathway(s) involved.

II. Experimental design

The experiments described here were designed to examine the role of PGE_2 in the local control of vascular smooth muscle tone in rat tail arteries. The response of PGE_2 on isometric tension development was investigated using helical strips from rat tail artery precontracted with KCl and norepinephrine (NE), in the presence or absence of extracellular Ca^{2+}. Pharmacological studies were performed using the PKC activators TPA and PDBu, as well as the kinase inhibitors staurosporine, 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine hydrochloride (H-7, Hidaka *et al.*, 1984) and the PKA inhibitor N-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004, also used as a PKC negative control for H-7, Kawamoto and Hidaka, 1984), to determine possible subcellular mechanisms of PGE_2 action. An extracellular Ca^{2+} dependence study of the effect of PGE_2 on tension development was also undertaken, using either the Ca^{2+} channel blocker nifedipine or omission of the extracellular Ca^{2+}.

III. Results

A. The effect of PGE_2 on tension in isolated rat tail artery helical strips

Fig. 6-1 illustrates that PGE_2 evoked a biphasic response on isometric tension development in rat tail artery helical strips. The response was characterized by a initial rising contractile phase followed by a sustained plateau. When successive concentrations of PGE_2 varying between 10^{-9} and 10^{-4} M were applied to the muscle strips, with fixed time in between each addition, a concentration-dependent contraction was seen. The half-maximal response (EC_{50})

was calculated to be 9.6 μM. The data points were fitted to a Michaelis-Menton equation (smooth line) (Fig. 6-2). The helical strips have been precontracted with 60 mM KCl or 1 μM NE to verify the responsiveness. The maximal concentration of the ethanol, the vehicle of PGE_2, was 0.1%, which had no discernible effect on tension of the helical strips. Extracellular Ca^{2+} (2 mM) was provided in the organ bath.

B. The effect of the kinase inhibitors staurosporine and H-7 on PGE_2 induced tension development

In order to study the possible subcellular mechanisms involved in the tension response of PGE_2, the kinase inhibitors staurosporine (100 nM) or H-7 (20 μM) were added to the organ bath with 2 mM extracellular Ca^{2+} once the PGE_2-induced contraction of rat tail artery helical strips had reached a plateau (see Fig. 6-1 for example). The results indicated that both agents dramatically inhibited the contraction of strips induced by PGE_2 (20 μM), as shown in Fig. 6-3. The abolition of PGE_2-induced contraction by staurosporine (100 nM) was completely irreversible even after prolonged washing of the tissue, whereas it was partially reversible for H-7 upon washout. These results suggested that PKC activation was involved in the PGE_2-induced increase in tension.

C. The effect of PKC activator on tension in rat tail artery helical strips

To further verify the involvement of PKC in the PGE_2-induced contractile responses, two PKC activators, TPA (100 nM) and PDBu (100 nM) were applied to the bath which contained 2 mM extracellular Ca^{2+}. Fig. 6-4 shows that both agents could mimick the contractile effect

of PGE_2. The contractile profile of the two PKC activators were qualitatively the same. These data strongly supported the notion that PKC activation is involved in the contraction of the rat tail artery helical strips.

D. The effect of PKA inhibitors Rp-cAMPs and HA1004 on PGE_2 induced tension development

To test whether the contractile response of PGE_2 was due to the activation of cAMP-PKA which could cause an increase in influx of extracellular Ca^{2+}, as suggested by Brown *et al.* (1989) and Ishikawa *et al.* (1993), tissues were exposed to 10 μM PGE_2 in the presence of the PKA inhibitors Rp-cAMPs (100 nM) or HA1004 (10 μM). However, neither Rp-cAMPs nor HA1004 affected the PGE_2-induced contraction in rat tail artery strips (Fig. 6-5).

E. The effect of extracellular Ca^{2+} on the contractile response of rat tail artery helical strips to PGE_2 and PKC activators

After a stable tension increment had been induced by PGE_2 or the PKC activators, TPA or PDBu, the Ca^{2+} entry blocker nifedipine (100 nM) was added to the organ bath (2 mM Ca^{2+}), and completely blocked the contractile responses of helical strips, returning the tension back to the baseline level (Fig. 6-6). Alternatively, omission of extracellular Ca^{2+} from the bath solution, along with addition of 1 mM EGTA in the organ bath, eliminated the contractile responses of rat tail artery helical strips to PGE_2 (20 μM) or the PKC activators TPA (100 nM), or PDBu (100 nM) (Fig. 6-7). These results supported the notion that the PGE_2-induced as well as the PKC activators-induced contraction in rat tail artery helical strips are dependent on the

entry of extracellular Ca^{2+}.

IV. Discussion

The present findings demonstrated, for the first time, that PGE_2 caused a concentration-dependent contractile response in rat tail artery helical strips. Such a response could be blocked by the kinase inhibitors staurosporine and H-7. In contrast, the PKC activators TPA and PDBu mimicked the contractile response of PGE_2. PKA inhibition with either Rp-cAMPs or HA1004 did not affect the PGE_2-induced tension development. Since the Ca^{2+} entry blocker nifedipine also eliminated the contractile responses of rat tail artery muscle strips to PGE_2 as well as PKC activators, it could thus be inferred that PGE_2 might exert its contractile response through activation of PKC, or might share the same mechanism of [illegible] activation of PKC. The activation of PKC might then cause the influx of ex[illegible] owever, the patch-clamp studies described in Chapter 5 demonstrated th[illegible] ype Ca^{2+} channel, and thus, reduced the Ca^{2+} influx. These data (fro[illegible] -clamp studies) have raised the possibility that PGE_2 may enhance [illegible] cause the contractile response in vascular smooth muscles of the rat tai[illegible]. The intracellular Ca^{2+} pool did not seem to be affected by PGE_2, since the contractile response of PGE_2 appeared to depend entirely on extracellular Ca^{2+}.

It is usually proposed that vasoactive agents stimulate smooth muscle contraction by releasing Ca^{2+} from the intracellular stores such as sacroplasmic reticulum, or by opening membrane Ca^{2+} channels (Khalil *et al.*, 1987; Consigny, 1991; Morgan *et al.*, 1991). Two kinds

of Ca^{2+} channels may be involved, one voltage-dependent, the other ligand-gated. These kinds of Ca^{2+} channels have been reported to be sensitive to different Ca^{2+} entry blockers, so that the channels could be differentiated pharmacologically. The use of a specific Ca^{2+} blocker can provide further information regarding which Ca^{2+} pool is involved in smooth muscle contraction (Godfraind, 1986; 1988). In vascular smooth muscle, extracellular Ca^{2+} has been considered to be the major source of Ca^{2+} for tension development. The dependence of action potential generation on Ca^{2+} is the key factor determining the active state of VSMCs (Van Breeman, 1977). This especially holds true for small arteries, like tail arteries which possess a relatively small volume of sacroplasmic reticulum. Thus, the capacity of intracellular Ca^{2+} stores is much restricted. This notion was confirmed in the present study by the data from the nifedipine, extracellular Ca^{2+} omission and Fura-2 experiments. It suggested that a dihydropyridine (DHP)-sensitive Ca^{2+} channel is involved in the transmembrane influx of Ca^{2+} ions.

In vascular smooth muscle, most contractile stimuli cause tension development, either in length shortening or in force, through increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Ca^{2+} triggers a series of reactions leading to the contractile response. Ca^{2+} first binds to the Ca^{2+} binding protein calmodulin to form a Ca^{2+}-calmodulin complex. This complex then removes the autoinhibition of the myosin light chain kinase (MLCK) (Means *et al.*, 1991). The activated Ca^{2+}/calmodulin-dependent MLCK then phosphorylates the 20 kdalton myosin light chains on serine 19 and activates the Mg^{2+}-ATPase on the myosin (Ikebe *et al.*, 1988). The phosphorylated (activated) myosin cyclically interacts with the actin filaments to form a "latch bridge", generating force or shortening. So the force of contraction or shortening of the muscle is basically dependent on the intracellular Ca^{2+} concentration. However, some studies in smooth

muscle have found that phosphorylation by Ca^{2+}-dependent MLCK cannot always account for the tension development. Some Ca^{2+}-dependent pathways other than those resulting in the activation of MLCK may also transduce the signal-contraction coupling, the most important of these are the second messenger systems such as cAMP-PKA, PLC-PKC. These cytosolic second messengers can alter smooth muscle contractility by controlling the $[Ca^{2+}]_i$ level or the $[Ca^{2+}]_i$ sensitivity of phosphorylation (through MLCK) (Rembold, 1989; McDaniel *et al.*, 1991; Rembold, 1992), and thus, indirectly affect the MLC phosphorylation and muscle contractility. There has been evidence indicating that second messenger systems may mediate signal-contraction coupling of various vasoactive agents, such as substance P and vasopressin, in smooth muscle contraction (Bolton, 1979; Holzer and Lippe, 1985; Grillone *et al.*, 1988).

PGE_2 has been widely documented to be an activator of AC, although, in rare cases, it could inhibit cAMP accumulation. The most prominant vasodilating action of PGE_2 could be attributed to its action on cAMP accumulation (Consigny, 1991). It has been commonly accepted that an increase in cytosolic cAMP level would lead to a decrease in $[Ca^{2+}]_i$ or $[Ca^{2+}]_i$ sensitivity of phosphorylation, and thus smooth muscle relaxation (Mueller and Van Breemen, 1979; Pang *et al.*, 1985; Khalil *et al.*, 1987; Consigny, 1991; Rembold, 1992). Alternatively, the activation of cAMP-dependent protein kinase, PKA, will lead to the phosphorylation of MLCK which causes a decrease in the extent of MLCK activity by reducing its affinity to Ca^{2+}/calmodulin (Rembold, 1992). Numerous studies have been carried out to directly prove this inverse relationship between cAMP levels and $[Ca^{2+}]_i$ or $[Ca^{2+}]_i$ sensitivity to phosphorylation (Karaki, 1989; Murray, 1990). Both decreases in $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ sensitivity to phosphorylation, due to the accumulation of cAMP would directly correlate with smooth muscle relaxation. Activation

of AC has been implicated in the actions of a number of vasodilatory agents, including β-adrenergic receptor agonists such as epinephrine and isoproterenol (Bulbring and Tomita, 1987), calcitonin gene-related peptide (Shoji *et al.*, 1987), dopamine and prostaglandins E_2 and I_2 (Consigny, 1991). The action of cAMP on rat tail artery force development does not seem to be an exception, as cAMP has been shown to block the voltage-dependent Ca^{2+} channel in VSMCs (Wang, 1991), and decrease the tension in helical strips (Pang *et al.*, 1985), from rat tail artery. The experiments described herein were founded on the increasing realization, in the light of more recent information, that cAMP accumulation will lead to vasorelaxation. In the previous chapter, it was demonstrated that PGE_2 stimulated activation of AC, leading to a cAMP accumulation in single VSMCs. Hence, a vasodilation was expected in the rat tail artery helical strips. However, the study described in the present chapter revealed a contractile response of PGE_2 on the helical strips of rat tail artery. Further investigation found that PKA inhibition with either Rp-cAMPs or HA1004, also a weak PKC inhibitor, did not affect the PGE_2-induced development tension. These results indicated that the cAMP-PKA pathway was not likely to be the predominant signal transduction pathway mediating the action of PGE_2 on vascular smooth muscle tone in rat tail artery.

Although cAMP has been commonly postulated as a vasodilator, the lack of vasodilation in response to cAMP accumlation has been sparsely reported and found to be not surprising, given that some controversy appeared recently regarding the role of cAMP in force generation. In a recent study, Ishikawa *et al.* (1993) found that under physiological conditions, the increase in intracellular cAMP level displayed a biphasic action on $[Ca^{2+}]_i$. A moderate rise in intracellular cAMP level led to an amplification of the inward Ca^{2+} current, whereas a higher

level of cAMP led to an inhibition of the Ca^{2+} current. cAMP dependent protein kinase A has also been shown to increase the influx of extracellular Ca^{2+} through phosphorylation the L-type voltage-dependent Ca^{2+} channel, leading to an increase of $[Ca^{2+}]_i$ (Brown *et al.*, 1990). However, due to the inability of Rp-cAMPs and HA1004 to abolish the PGE_2-induced tension development, cAMP and cAMP-dependent protein kinase phosphorylation were not believed to responsible for the contractile response of PGE_2 on helical strips of rat tail artery.

Other than its action on the cAMP-PKA system, PGE_2 has also been reported in a number of cases to stimulate the PLC-PKC pathway (Yamashita and Takai, 1987; Farndale *et al.*, 1988; Yokohoma *et al.*, 1988; Kitanaka *et al.*, 1991). One metabolite of PLC enzymatic reaction, IP_3, has been proposed to act as a second messenger for intracellular Ca^{2+} mobilization in various tissues, including smooth muscle (Berridge and Irvine, 1984, Abdel-Latif, 1991). The other major product of PLC enzymatic reaction is DG, an endogenous activator of PKC, which, in turn, controls a diversity of cellular processes (Nishizuka, 1984a; 1984b; 1986). Recent studies have claimed a key role for PKC in enhancing the vascular smooth muscle tone in both normotensive and hypertensive subjects (Bilder *et al.*, 1990; Makita and Yasuda, 1990; Neusser *et al.*, 1993). It was suggested that the phosphorylation changes induced directly or indirectly by the activation of PKC might be an important mechanism in smooth muscle contraction (Park and Rasmussen, 1986; Khalil *et al.*, 1987; Rasmussen *et al.*, 1987). Soloviev and Bershtein (1992) found that the increase in vascular smooth muscle tone in the aorta of hypertensive rats could be abolished by the inhibition of PKC. However, it was suggested that not all the PKC inhibitors have vasorelaxant or antihypertensive effects at the concentrations which inhibit PKC activity (Buchholz *et al.*, 1991).

There are many studies suggesting that PKC is activated by contractile agonists such as endothelin and angiotension II in smooth muscle and intact tissues (Griendling *et al.*, 1986; 1989; Barrett *et al.*, 1986; Haller *et al.*, 1990; Rembold, 1992). It is believed that the activation of PKC causes an increase in the vascular smooth muscle tone through one of the following mechanisms: (1) protein phosphorylation of myosin light chains. (2) increase in the transmembrane Ca^{2+} influx from the extracellular pools. (3) mobilization of Ca^{2+} from the intracellular pools. (4) elevation of the $[Ca^{2+}]_i$ sensitivity of phosphorylation (Chattergee and Tejada, 1986; Khalil *et al.*, 1987; Rembold, 1992). The similarities in the actions of PGE_2 and the PKC activators TPA and PDBu in causing the tension development, supported the notion that

y act through PKC activation or at least share some similar mechanism of action with PKC. This hypothesis was further supported by evidence from the pharmacological studies with the kinase inhibitors staurosoprine and H-7. The PKC activator PDBu produced a smaller contractile response compared to that of TPA. This was probably due to the weaker PKC activation by PDBu compared with that of TPA, or alternatively, the deficiency of specific PDBu binding sites on the tissue. Staurosporine is a potent kinase inhibitor, which exerts its inhibitory effect by competely occupying the kinase ATP-binding sites and preventing the protein phosphorylation. This kinase inhibitor is able to bind to the cytoskeletal ATP-binding sites tightly, which could account for the irreversibility of its effects even after wash out. H-7 was only able to partially block (by about 50%) the effects of PGE_2 and this partial blockade was not completely reversed by wash out. The lack of complete blockade of PGE_2-induced action is most likely due to the fact that H-7 blocks only 40% of PKC activity *in vitro* (Ho *et al.*, 1988a). No effect was seen with the weak PKC inhibitor HA1004, implying that these effects are related to

the PKC-inhibiting action of H-7 rather than any effect on cAMP-dependent kinase. With the demonstration of an extracellular Ca^{2+}-dependence for the contractile response of PGE_2, intracellular Ca^{2+} mobilization can be ruled out. However, the exact mechanisms for the contractile response of PGE_2 via PKC activation are still not clear. Both regulation of smooth muscle contraction via MLCK phosphorylation by PKC and the increase in the transmembrane Ca^{2+} influx through membrane Ca^{2+} channels, either voltage- or receptor-gated, may be playing roles, since they are both dependent on Ca^{2+}. The modulation of K channels by PKC activation may be another possible mechanism, as opening or closure of K channels may regulate the $[Ca^{2+}]_i$.

There have been other reports describing the mode of action of PKC activation on vascular smooth muscle tone. Nakajima *et al.* (1991) have reported that 12-deoxyphorbol 13-isobutyrate (DPB), a PKC activator, induced a sustained contraction in vascular smooth muscle without causing a detectable change in $[Ca^{2+}]_i$ in Ca^{2+}-free media in rat thoracic arterial strips. In a different study, Nakajima *et al.* (1993) found that both DPB and PDBu increased $[Ca^{2+}]_i$. Thus, the conclusion can be made that PKC activation could induce contraction through two distinct mechanisms, one dependent on and the other independent of the $[Ca^{2+}]_i$. It has also been suggested that phorbol esters such as as PDBu, and TPA could induce smooth muscle contraction by causing direct translocation of the different isoforms of cytosolic PKC to the membrane (Bazan *et al.*, 1993).

The role of PKC activation in vascular smooth muscle tone is still not clear. There is evidence suggesting that the DG-PKC pathway could function as a negative control system for a variety of cellular systems (Berridge, 1987; Abdel-Latif, 1991). PKC activation by DG could

act on membrane receptors as well as receptor-coupled subcellular messenger systems, and thus produce a negative feedback control on signal-contraction coupling in smooth muscle leading to an inhibition of vasoconstriction.

Conclusion

These studies have demonstrated that on one hand PGE_2 activates a PKC signalling system, whereas, on the other hand, it possesses the ability to stimulate AC, as described in the previous chapter. The prevalence of one of the two mechanisms of or the coexistence of both appears to be a feature of PGE_2 depending on the vascular beds investigated. Nonetheless, PGE_2 exhibited a concentration-dependent vasoconstricting effect in rat tail artery, which was used as a model resistance vessel. The contractile action is most likely due to activation of the PKC system. The activation of PKC, which is Ca^{2+}-dependent, probably causes a transmembrane Ca^{2+} influx from the extracellular Ca^{2+} pool through a dihydropyridine sensitive Ca^{2+} channel. Intracellular Ca^{2+} mobilization is not thought to be involved in the contractile action of PGE_2 on rat tail artery helical strips.

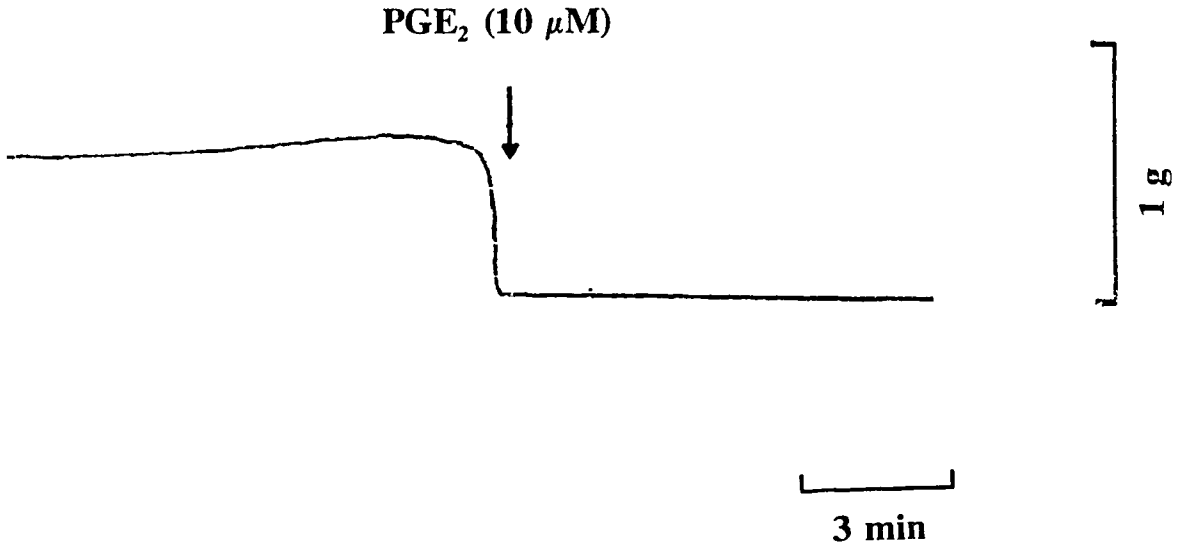


Figure 6-1. Effect of PGE_2 on tension in isolated rat tail artery helical strips. A typical response (contraction) of the rat tail artery helical strips to PGE_2, in normal KHS with 2 mM Ca^{2+}. The addition of PGE_2 (10 μM) is indicated by the arrow. The trace reads from right to left.

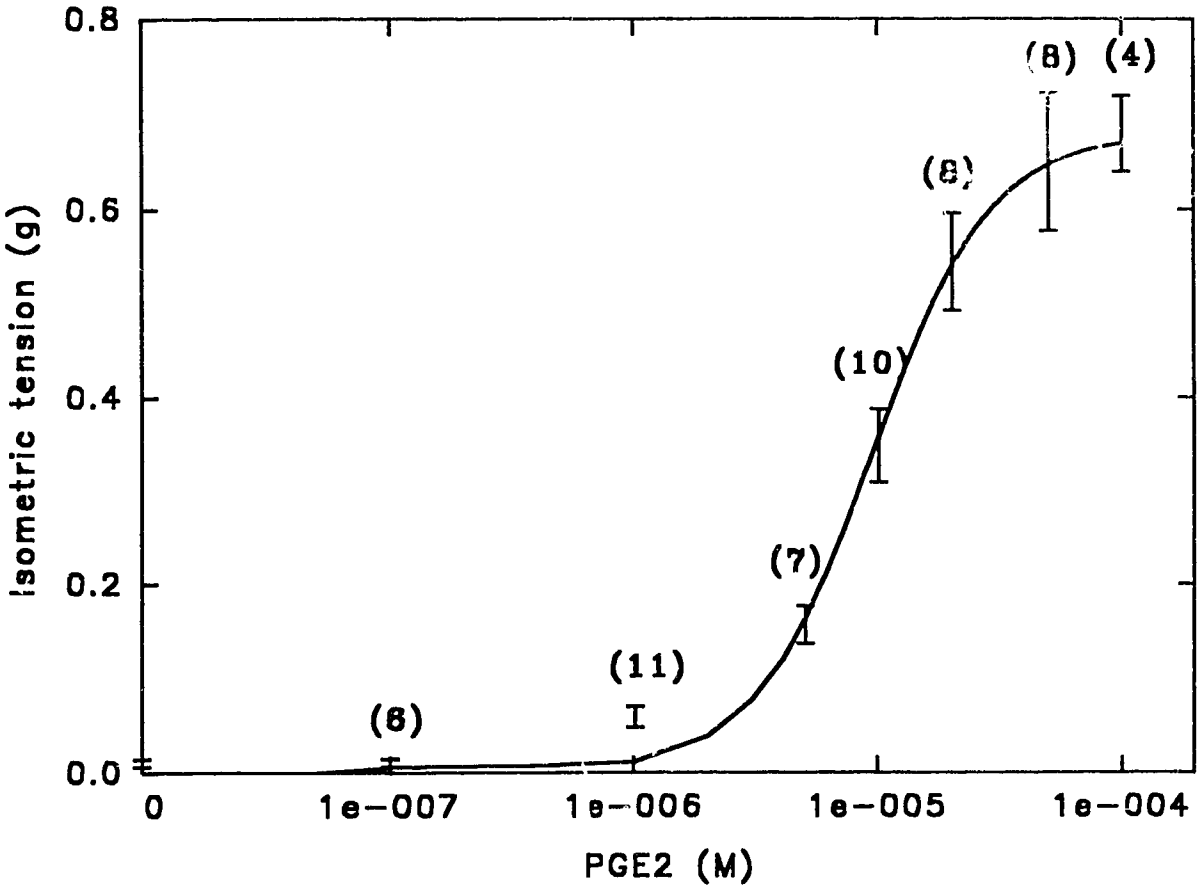


Figure 6-2. The concentration-dependent response of PGE_2 (10^{-9} to 10^{-4} M) on isometric tension development in rat tail artery helical strips. The data points were fitted to a Michaelis-Menton equation (smooth line). The concentration at which the half-maximal effect (EC_{50}) occurred was calculated from the fitted curve to be 9.6 μM. The slope factor was calculated to be 1.14. Values are shown as M $\pm$ S.E. (N given for each point in the parentheses).

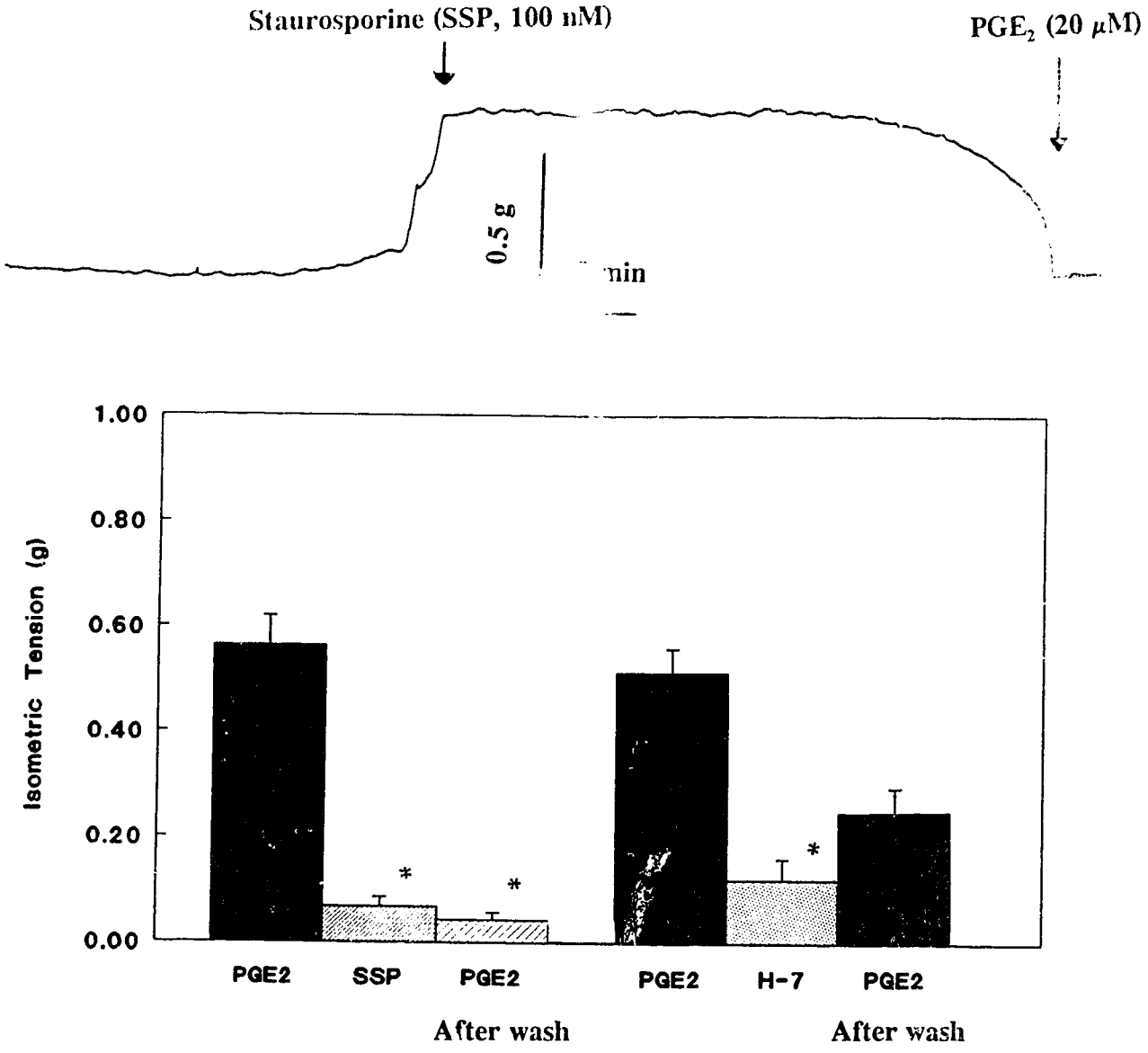


Figure 6-3. Effects of staurosporine and H-7 on PGE_2-induced increase in tension in rat tail artery helical strips. A. A representative trace of the preparation that was precontracted to a stable level with 20 μM PGE_2 before addition of staurosporine (100 nM) or H-7 (20 μM). All administrations are indicated by the arrowheads. The trace reads from right to left. B, Histogram summarizing the effects of staurosporine (100 nM) and H-7 (20 μM). Values are M $\pm$ S.E. (N=6 for staurosporine, N=4 for H-7).

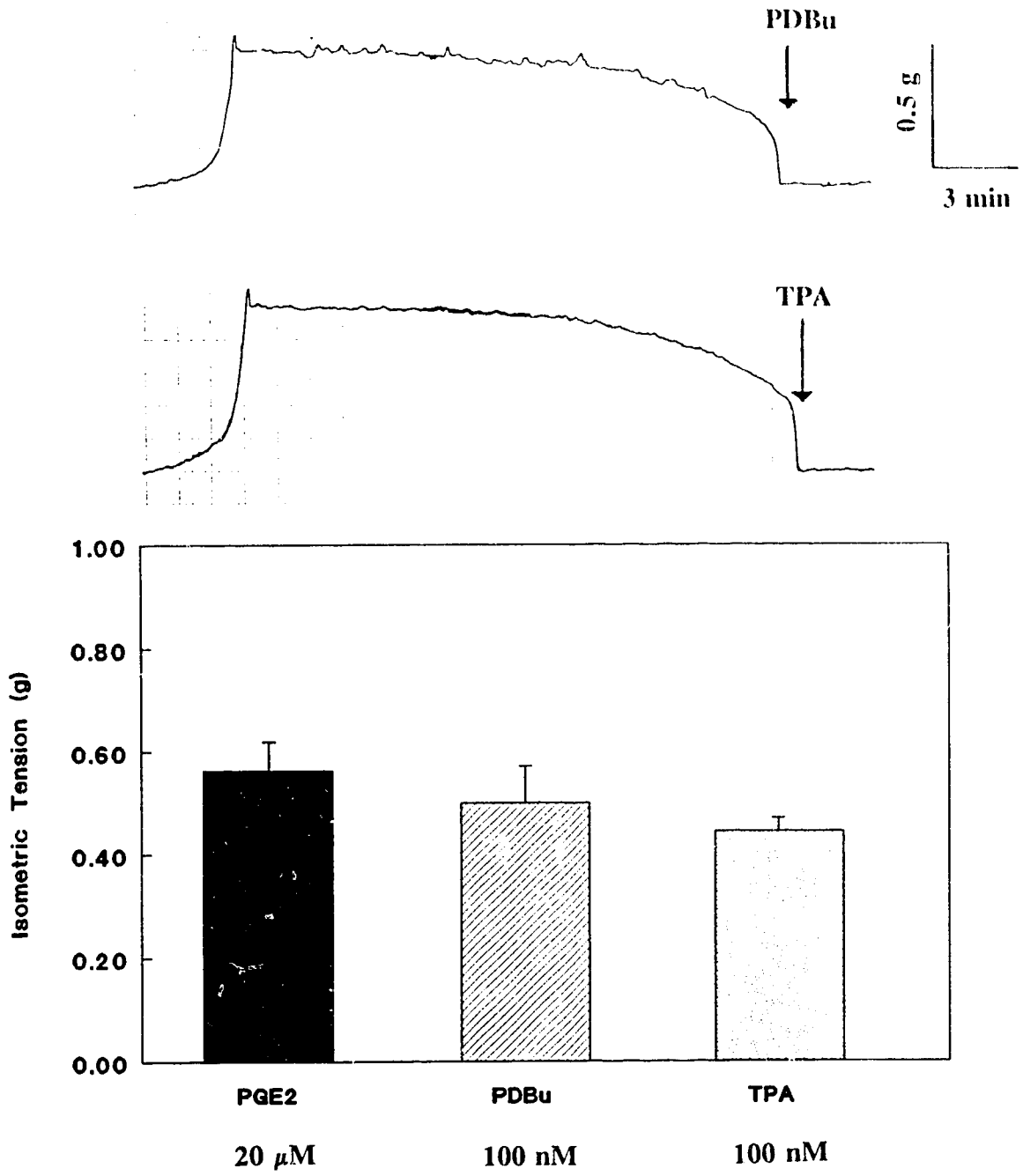
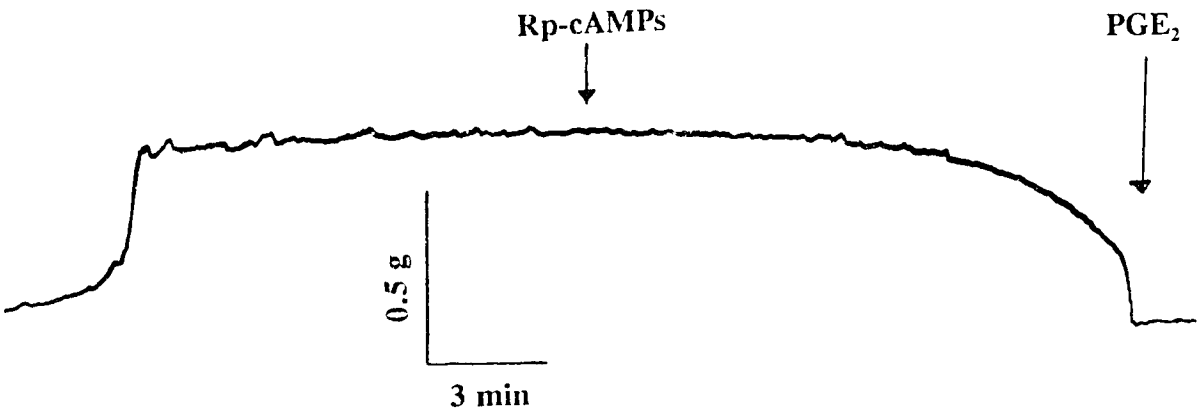


Figure 6-4. Effects of PKC activators TPA (100 nM) and PDBu (100 nM) on tension in rat tail artery helical strips, with 2 mM Ca^{2+} in the bath. Values are M±S.E., N=4. The traces read from right to left.



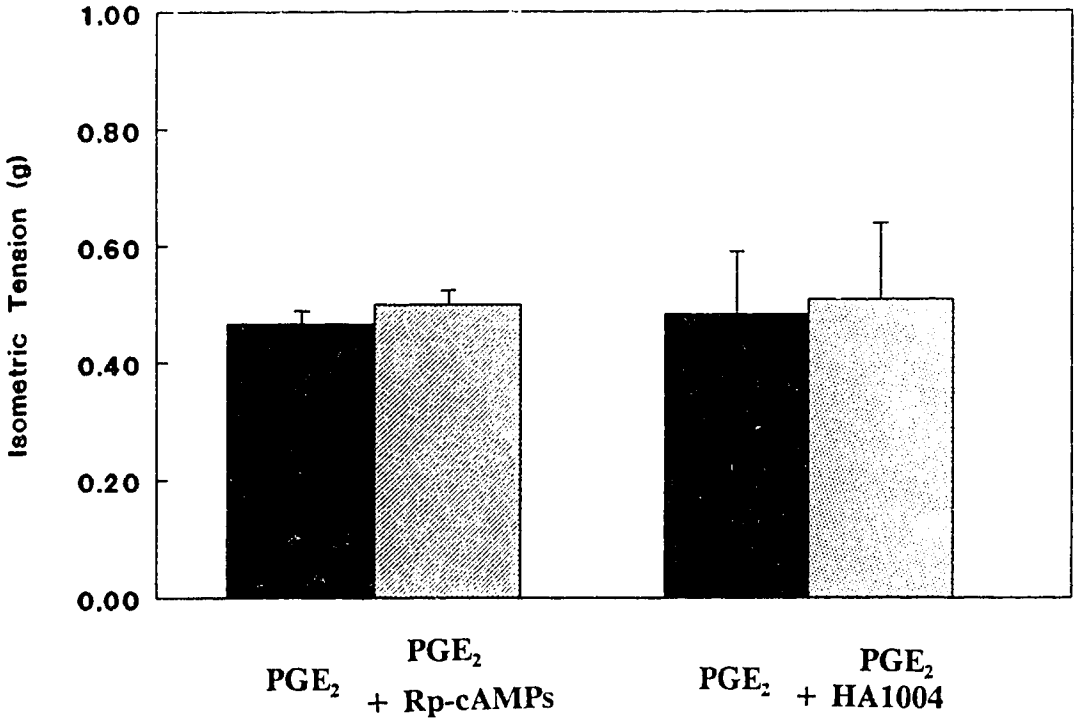


Figure 6-5. Effects of the PKA inhibitors Rp-cAMPs (300 μM) and HA1004 (10 μM) on the PGE_2-induced contractile response in rat tail artery helical strips with 2 mM Ca^{2+} in the bath. The tissues were precontracted to a stable level with 20 μM PGE_2 before PKA blockers were added. Values are M$\pm$S.E., N=3. $p>0.05$ compared with control. The trace reads from right to left.

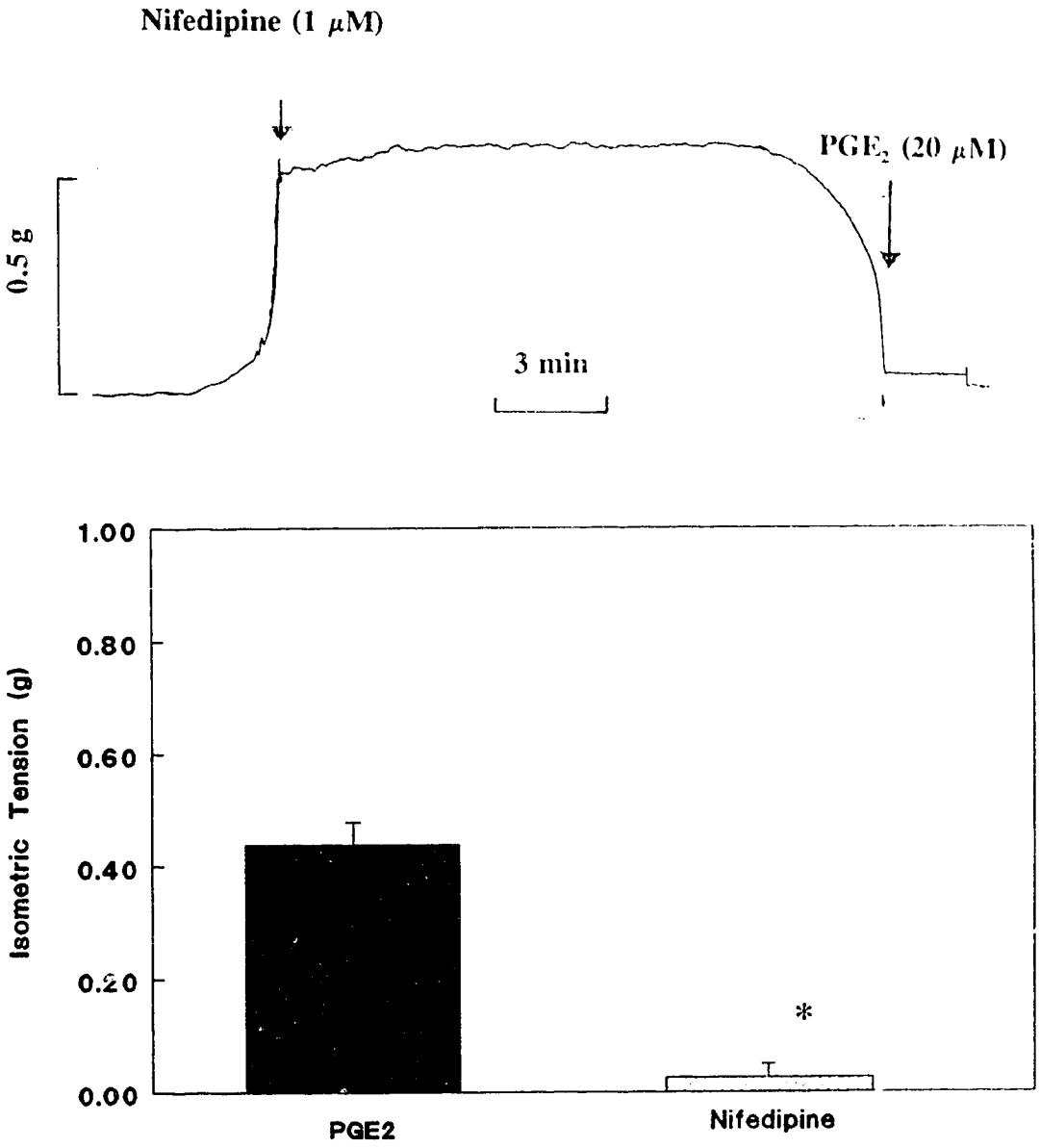


Figure 6-6. Effect of the Ca^{2+} entry blocker nifedipine (10^{-6} M) on the contractile response of rat tail artery helical strips to 20 μM PGE_2 (with 2 mM Ca^{2+} in the bath). The preparations were precontracted with PGE_2 before nifedipine was added. Values are M ± S.E. N=4. The trace reads from right to left.

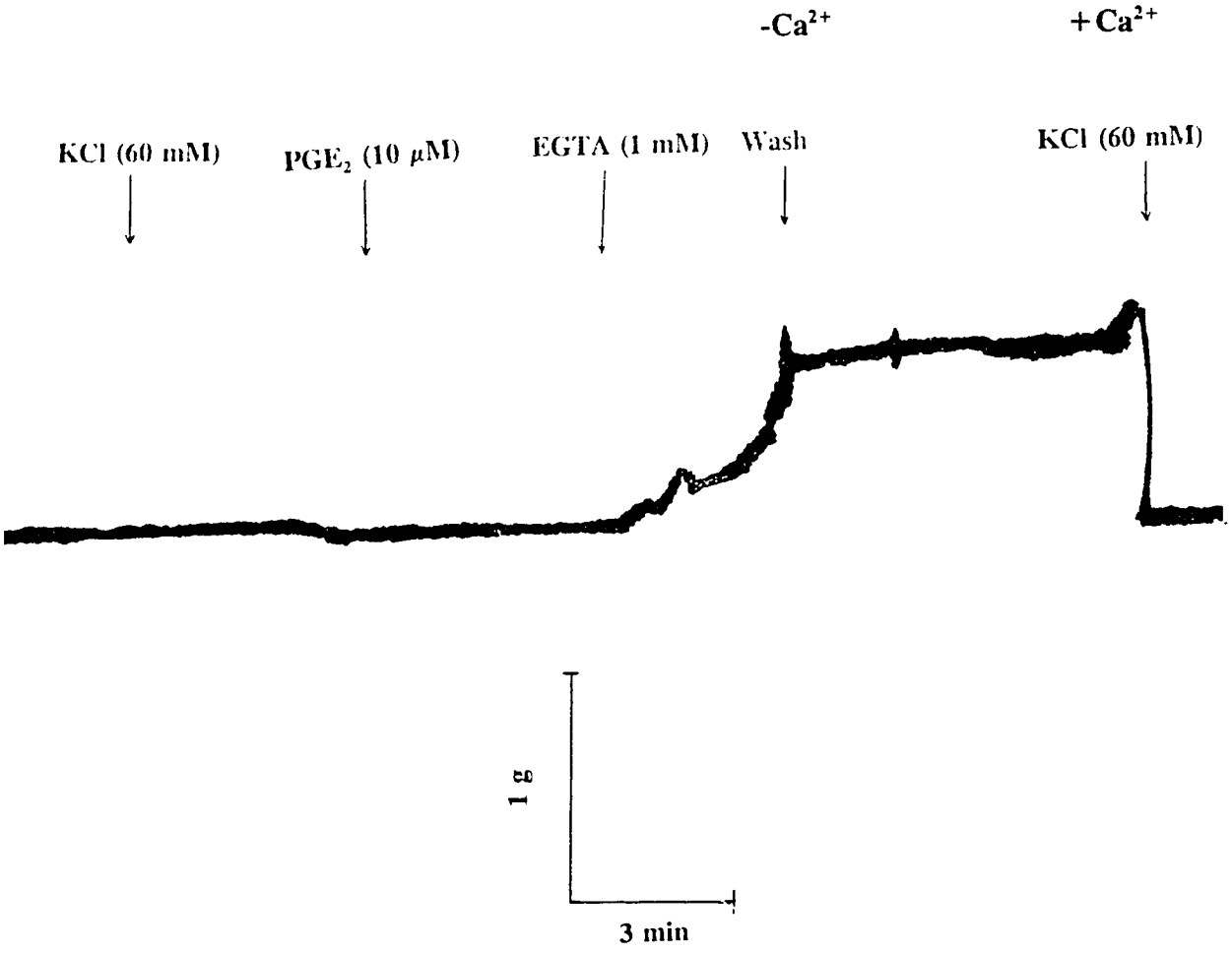


Figure 6-7. A representative trace showing the effect of omitting the external Ca^{2+} (along with addition of 1 mM EGTA in the organ bath) on the contractile response of PGE_2 (20 μM) in a rat tail artery helical strip. The trace reads from right to left. It was seen in four other similar experiments.

CHAPTER 7

GENERAL DISCUSSION AND SUMMARY

I. General Discussion

A. Characterization of the voltage-dependent, delayed outward rectifier K channel in rat tail artery VSMCs.

It has been widely accepted that K channels play a critical role in the regulation of vascular smooth muscle tone. Many vasoactive substances have been suggested to exert their actions on vascular smooth muscle via modulation of the K channel conductance, which leads to either physiological or pathological effects in the cardiovascular system. This is of particular clinical significance in understanding the pathogenesis and therapeutic strategy of the cardiovascular disorders such as hypertension.

Vascular smooth muscle tone in small vessels is the major contributing factor to the total peripheral resistance of the blood flow, compared with that of the large capacitance or conduit vessels. The small resistance vessels differ in a number of ways from the large arteries and veins. Vasoconstriction that contributes to the regulation of

blood pressure and blood flow is drawing more attention in the regulation of the cardiovascular responses, since the cardiovascular system has been considered to be in a state of constant vasodilation (Moncada, 1994). Conductances of ion channels such as Ca^{2+} and K channels, are the major factors determining the cellular electrical excitability, $[Ca^{2+}]_i$ and eventually, the local vascular smooth muscle tone (Longmore and Weston, 1990). However, most experimental approaches which have been applied to the ion channels of VSMCs have focused on the large capacitance or conduit veins and arteries such as the portal vein and aorta. Thus, with the importance of the small resistance vessels to vascular smooth muscle tone in mind, investigation of the ion channels in smooth muscle cells of the small resistance vessels is critical.

One major focus of this thesis was to characterize the voltage-dependent, delayed rectifier K channel in VSMCs from rat tail artery. Different methods for the isolation of these VSMCs, cultured or non-cultured, have been well described and modified over the past few years (Wang *et al.*, 1989; Furspan, 1992; Bolzon *et al.*, 1993; Evans and Kennedy, 1994; Ren *et al.*, 1994). Two types of voltage-dependent Ca^{2+} channels, L- and T-, have been characterized in cultured VSMCs (Wang *et al.*, 1989) and various work has been carried out, on cells prepared using this protocol, to study the mechanism of action of different vasoactive substances (Pang *et al.*, 1990a; Wang, 1991; Wang *et al.*, 1991; Shan *et al.*, 1993; Shan *et al.*, 1994). It has extended our knowledge of the electrical activity of the peripheral resistance vessels, especially with the indication that ion channels may play different roles in the small resistance vessels than in the large

vessels (Quayle *et al.*, 1993). Results presented in this thesis, for the first time, demonstrated the presence of a voltage-dependent, delayed outward rectifier K current (I_k) in both primary cultured and subcultured VSMCs from rat tail artery. From a holding potential of -80 mV, stepwise depolarization generated a voltage-dependent, delayed outward rectifier K current. The currents were activated at membrane potentials between -45 and -40 mV, and showed almost no inactivation. With very similar kinetic and pharmacological profiles, the outward currents from the subcultured VSMCs appeared to retain the characteristics of the ancestor primary cultured VSMCs, although there have been indications that the culture procedure could cause changes in channel protein expression (Chamley-Campbell *et al.*, 1979; Owens *et al.*, 1987; Yuan *et al.*, 1993a).

During the course of this study, very similar voltage-dependent K channel currents were also obtained in acutely isolated, non-cultured VSMCs by two other laboratories (Bolzon *et al.*, 1993; Evans and Kennedy, 1994). However, there are some pharmacological discrepancies, such as the sensitivity to aminopyridines, between the K currents from cultured and non-cultured VSMCs (Evans and Kennedy, 1994), supporting the notion that the culture procedure may affect certain aspects of the K channel properties (Chamley-Campbell *et al.*, 1979; Owens *et al.*, 1987; Yuan *et al.*, 1993a).

The close agreement between the reversal potential of the outward currents and the K equilibrium potentials under different experimental conditions provided good evidence that K^+ was the primary conducting ion responsible for the sustained outward current. The resistance of the outward currents to the Ca^{2+} channel blockers nifedipine,

La^{3+} and Cd^{2+}, Cl^- channel blockers DIDS and SITS, strongly suggested that neither a Ca^{2+}-sensitive component, nor outward rectification through a Cl^- channel contributed to the outward current.

The inhibition of the outward current by TEA, coupled with the non-responsiveness to 4-AP, charybdotoxin and glibenclamide, have offered further information on the pharmacological profile of the outward K current as the delayed rectifier K channel (I_k). However, the results also showed that the outward currents were not completely blocked by TEA. Tail current analysis only exhibited a single exponential function for the deactivation of the I_k current. It cannot exclude the possibility that two kinetically similar delayed rectifier K channels subtypes might co-exist in VSMCs, with only one being sensitive to TEA.

Most VSMCs exhibit at least two types of K currents, one voltage-dependent, the other Ca^{2+}-activated (Longmore and Weston, 1990). The outward currents displayed distinct voltage-dependence, as measured by stepwise voltage-clamp depolarization, in both low and high intracellular $[Ca^{2+}]_i$ VSMCs. However, a Ca^{2+}-activated K component was believed to contribute to the outward rectification in VSMCs with high $[Ca^{2+}]_i$ that could be achieved by reducing the pipette EGTA level (to 0.2 mM). This Ca^{2+}-activated K component was sensitive to application of a DHP blocker of Ca^{2+} channels, nifedipine, suggesting its activation was secondary to the entry of extracellular Ca^{2+}.

B. Characterization of the delayed rectifier K current with β-dendrotoxin.

This section of the thesis constituted an attempt to characterize a specific, high-affinity probe that blocks the voltage-dependent K channels. The development of the structure-function relationship of the voltage-dependent K channels has been hindered largely due to the lack of specific, high-affinity pharmacological probes. Several dendrotoxins from snake venom (such as α-dendrotoxin, dendrotoxin-I) have shown selective inhibitory actions on different types of the voltage-dependent K channels such as I_k and I_A. α-Dendrotoxin acceptors have been solubilized and purified (Black and Dolly, 1986; Sorensen and Blaustein, 1989; Scott *et al.*, 1994a; 1994b). It has been proposed that the acceptors for the dendrotoxins are a family of voltage-dependent K channels, and they might be one of the four subunits forming the tetramer of the K channel protein. Up-to-date, most work has been confined to the nervous system. It is thus of great practical value for any dendrotoxin that can be used as a potential probe of the voltage-dependent K channels in VSMCs. This thesis has characterized, for the first time, the inhibitory properties of β-dendrotoxin from the Eastern Green Mamba *Dendroaspis angusticeps* on the voltage-dependent, delayed rectifier K channel current (I_k) in VSMCs from rat tail artery. β-Dendrotoxin elicited a concentration-dependent inhibition of I_k in both primary and subcultured VSMCs. The primary cultured cells were slightly more sensitive to β-dendrotoxin (with a maximal inhibition of 45% compared to that of 35% in subcultured VSMCs).

The inability to completely block the I_k by β-dendrotoxin indicated the possible existence of a β-dendrotoxin-insensitive component of the outward K current. This might be in accordance with the TEA sensitivity data, i.e. the β-dendrotoxin-sensitive component was also TEA-sensitive, or *vice verse*. The EC_{50}s of inhibition on I_k (51 nM and 71 nM for primary cultured and subcultured VSMCs, respectively) were in a range (low nanomolar) similar to the K_ds for binding (Sorensen and Blaustein, 1989). These were considered to be high-affinity which was suitable for an ideal pharmacological probe in the identification, purification and characterization of certain K channel proteins. The inhibition of I_k by β-dendrotoxin did not depend on membrane potential (state of how many channels are activated/inactivated). The use-dependency experiment suggested that β-dendrotoxin did not cause the inhibition of I_k through physical occupancy of the channel pore. It is possible that β-dendrotoxin may exert its action in a similar fashion as ACh acts on nicotinic ACh receptor (Karlin, 1980; Noda *et al.*, 1983), i.e. the binding of β-dendrotoxin to certain portion (acceptor) of the K channel protein causes a conformational change of the channel protein structure, and thus leads to the change of the channel conducting capability. The non-responsiveness of I_k to α-dendrotoxin exhibited the specificity of dendrotoxins on the voltage-dependent K channels from VSMCs, since α-dendrotoxin has been reported to block transient outward "I_A" K currents in neurones and the "I_k" currents in some cells (Stansfeld *et al.*, 1987).

C. The effects of PGE_2 on voltage-dependent, delayed rectifier K current in rat tail artery VSMC.

Single VSMCs have recently been successfully isolated and cultured from rat tail artery (Wang *et al.*, 1989; Ren *et al.*, 1994), and have been used to investigate the ionic basis of various kinds of vasoactive agents such as parathyroid hormone and parathyroid hypertensive factor (Pang *et al.*, 1990; Wang *et al.*, 1991; Shan *et al.*, 1994). These cells have been considered to be a suitable model for the VSMCs from peripheral resistance vessels. The effect of the vasoactive eicosanoid metabolite PGE_2 on the voltage-dependent, delayed rectifier K channel currents from VSMCs has been thoroughly investigated, in order to gain more information on the ionic basis of its cardiovascular actions.

PGE_2 has been reported to elicit both vasodilation and vasoconstriction, depending on the vascular beds. Considerable heterogeneity of VSMCs, in term of electrical excitability or responsiveness to specific hormones and neurotransmitters, has been demonstrated and may contribute to the diverse and sometimes opposite effects of PGE_2. In chapter 6 of this thesis, the tension study on rat tail artery helical strips suggested that PGE_2 exerted vasoconstriction as the dominant effect on the vascular smooth muscles from rat tail artery. This observation has gained support from the investigation on the membrane electrical properties, which provided ionic explanations, at least partially, for such a vasoconstricting effect. The current-clamp study indicated that PGE_2 depolarized

the membrane. Under the whole-cell voltage-clamp conditions, PGE_2 was found to inhibit the voltage-dependent, delayed rectifier K channel currents (I_k). The inhibition exhibited a concentration-dependence with EC_{50}s of 3.5 μM and 4.9 μM for primary and subcultured VSMCs. These concentrations were consistent with the previous reported concentrations of PGE_2 (low micromolar range) to promote cAMP accumulation, elicit intracellular Ca^{2+} release and vascular smooth muscle contraction, *etc.* (Yamaguchi *et al.*, 1988; Mori *et al.*, 1989; Dorn *et al.*, 1992). This indicated the feasibility of extrapolating the present findings to other biological effects, *in vivo* or *in vitro*, elicied by PGE_2. The steady-state inactivation experiment indicated that the effect of PGE_2 did not depend on the membrane potential or the (receptor) site of PGE_2 action did not fall into the membrane electrical field. The PGE_2-induced inhibition was not use-dependent either, indicating that the inhibition was not achieved by the physical blockade of the I_k channel pore.

Further studies on the cellular mechanism of action demonstrated that the PGE_2 receptor antagonist meclofenamate and regulatory G protein inhibitor GDPβS blocked the PGE_2-induced inhibition of I_k. Identification of the G proteins involved in PGE_2-induced I_k inhibition by the use of two bacterial toxins, cholera toxin and pertussis toxin provided strong evidence of the involvement of a cholera toxin-sensitive and/or pertussis toxin-insensitive regulatory G protein. Taken together, these findings were in agreement of the well-described classical mode of action for PGE_2; i.e. as a local hormone, PGE_2 exerts its biological action through binding of the specific surface membrane receptors, and

activation of certain membrane G proteins. The data in chapter 5 has provided additional evidence of the involvement of cytosolic second messenger cascades.

D. Second messengers involved in the inhibition of K channels by PGE_2 in rat tail artery VSMCs.

Cytosolic second messenger systems are often involved in the action of various hormones and neurotransmitters. The effect of PGE_2 on I_k was believed to contribute to the regulation of vascular smooth muscle tone of PGE_2. In this thesis, intensive pharmacological studies have been performed towards elucidating the subcellular mechanism of action of PGE_2 on the inhibition of I_k currents. It is believed that the cAMP-PKA signal transduction pathway is involved in the PGE_2-induced modulation of I_k. Studies using the cAMP analogues Db-cAMP, Sp-cAMPs, Rp-cAMPs as well as the AC activator forskolin provided direct evidence for the involvement of the cAMP-PKA pathway in the inhibitory effect of PGE_2. This was in line with the early finding of the involvement of a cholera toxin-sensitive G protein (i.e. G_s) in the inhibition of I_k by PGE_2, as uncoupling of G_s subunits directly leads to the activation of AC. cAMP and cAMP-dependent PKA activation have been reported to inhibit certain voltage-dependent K channels in a number of tissues (Choquet *et al.*, 1987; Choquet and Korn, 1988; Soliven *et al.*, 1988). Radioimmunoassay directly showed that PGE_2 could stimulate cAMP accumulation in isolated VSMCs. However, further studies with the PKC activator

TPA and the kinase inhibitor staurosporine as well as down-regulation of PKC with prolonged exposure to TPA have also raised the possibility of the involvement of PKC in the PGE_2-induced inhibition of I_k. Although pharmacological profiles have shown that activation of either of the two pathways might inhibit I_k, they were more likely to interact with each other, or function synergistically. Interaction between these two messenger systems ("cross-talk") has been documented for PGE_2 in a number of tissues. In VSMCs, the possible mechanism of action of the PGE_2-induced inhibition of I_k may be as follows: Firstly, PGE_2 binds to its specific surface membrane receptor(s); secondly, the binding of PGE_2 to its membrane receptor activates a cholera toxin-sensitive and/or pertussis toxin-insensitive G protein; thirdly, two second messenger pathways may be activated. One is the cAMP-PKA pathway. Activation of AC, which is likely to be mediated by a cholera toxin-sensitive G protein, converts ATP to cAMP. The other second messenger pathway may be involved is the PLC-PKC pathway. The activation of PKC may be mediated either via a pertussis toxin-insensitive G protein, or via a direct activation mechanism at sites distal to PLC. However, the enzymatic action of PKC may potentiate the activation of the cAMP-PKA pathway, although the site of action is still not clear. Finally, accumulation of cAMP leads to the activation of PKA, which then phosphorylates K channel proteins to cause the inhibition. However, activation of PKC may itself also directly inhibit I_k, probably via protein phosphorylation. This latter process may not involve the cAMP-PKA pathway.

These studies in VSMCs from rat tail artery have demonstrated that on one hand

PGE_2 might activate the PKC signalling system, whereas on the other hand, it possessed the ability to stimulate cAMP-PKA pathway. The interplay between the cAMP-PKA and PKC signal transduction systems or the prevalence of one of the two messenger cascades appeared to be a feature of PGE_2, dependent on the vascular beds investigated.

E. The effects of PGE_2 on voltage-dependent Ca^{2+} channels and intracellular $[Ca^{2+}]_i$.

There has been accumulating experimental and clinical evidence indicating that PGE_2 plays an important physiological as well as pathological role in the regulation of vascular smooth muscle tone. In vascular smooth muscle, most contractile stimuli cause force development through the increase of intracellular Ca^{2+} concentration. Ca^{2+}, which triggers a series of reactions leading to a contractile response, has been commonly accepted as the second messenger coupling the excitation-contraction in smooth muscles. The force or shortening of VSMCs basically depends on the intracellular Ca^{2+} concentration. The second messenger cascades such as cAMP-PKA, PLC-PKC can alter smooth muscle contractility by controlling the level of $[Ca^{2+}]_i$ and thus, indirectly affecting the MLC phosphorylation and smooth muscle contractility. It is known that an increase in cytosolic cAMP level leads to a decrease in $[Ca^{2+}]_i$ (Khalil *et al.*, 1987; Consigny, 1991; Rembold, 1992). Alternatively, the activation of cAMP-dependent protein kinase A, leads to the phosphorylation of MLCK which causes a decrease in the

extent of MLCK activity by reducing its affinity to Ca^{2+}/calmodulin complex. Numerous studies have been carried out to demonstrate this inverse relationship between cAMP levels and $[Ca^{2+}]_i$, or myosin sensitivity to Ca^{2+} (Karaki, 1989; Murray, 1990). The experiments described in this part of the thesis were founded on the increasing realization, in the light of more recent information, that cAMP accumulation will lead to vasodilation (Pang *et al.*, 1985; 1986; Consigny, 1991). The action of cAMP on rat tail artery force development does seem to be an exception, as cAMP has shown to cause decrease in tension in rat tail artery helical strips (Pang *et al.*, 1985). In this thesis, PGE_2 has been found to inhibit a voltage-dependent, long-lasting (L-type) Ca^{2+} channel from the VSMCs from rat tail artery. This data fit with the notion that elevation of intracellular cAMP level would lead to the closure of voltage-dependent Ca^{2+} channels, since PGE_2 had been shown to cause cAMP accumulation. Earlier studies also provided direct evidence that cAMP decreased the L-type voltage-dependent Ca^{2+} channel in VSMCs from rat tail artery (Wang *et al.*, 1991). Thus, the inhibitory effect of PGE_2 on Ca^{2+} channels seemed to be directly related to its stimulation of cAMP accumulation. However, this inhibitory effect of PGE_2 on voltage-dependent Ca^{2+} channel might only play a minor role in overall smooth muscle function, since PGE_2 potentiated the KCl-induced increase of $[Ca^{2+}]_i$ in the Fura-2 study. Since the overall effect of PGE_2 on the rat tail artery vascular smooth muscle was a contractile response, the intracellular Ca^{2+} concentration was likely to be elevated by PGE_2. PGE_2 did not affect the resting $[Ca^{2+}]_i$, indicating that it did not affect the intracellular Ca^{2+} mobilization from the intracellular

stores or stimulate influx of extracellular Ca^{2+}. The conflicting results between the electrophysiological study of Ca^{2+} channels and the Fura-2 study suggested that the effect of PGE_2 on Ca^{2+} channels might be compensated or shadowed by the other mechanisms that may enhance the Ca^{2+} influx.

F. The effect of PGE_2 on tension in rat tail artery helical strips.

The vasoactive properties of PGE_2 appear to be complex, exhibiting both vasodilation and vasoconstriction, depending on a variety of factors such as the types of vascular beds, the condition of the endothelium, the level of basal vascular smooth muscle tone, and especially, the interaction of different cytosolic messenger pathways. Studies have demonstrated an increased vascular smooth muscle tone to PGE_2 (Camu *et al.*, 1992; Dorn *et al.*, 1992; Jaiswal *et al.*, 1993). It has been reported that PGE_2 elicited the contractile response on many vascular and non-vascular tissues from a number of animal species, including rat (Milik and McGiff, 1975; Dorn *et al.*, 1992), guinea-pig (Bennett *et al.*, 1975a; 1975b; Ishizawa, 1991), cow (Camu *et al.*, 1992; Lograno *et al.*, 1992), rabbit (Pairet *et al.*, 1986; Burakoff and Percy, 1992) and human (Bennett *et al.*, 1981; Secher *et al.*, 1982; Camu *et al.*, 1992). Although mechanisms have been postulated for the contractile responses to PGE_2, such as cross-reactivity of receptors (Dorn *et al.*, 1992) and elevation of the intracellular Ca^{2+} (Yokohama *et al.*, 1988; Goureau *et al.*, 1992), the concise mechanisms are, however, still poorly

understood.

PGE_2 has been widely documented to stimulate, although, in rare cases, inhibit cAMP accumulation (Nagshineh *et al.*, 1986; Molnar *et al.*, 1987; Hagel-Bradway *et al.*, 1991; Coleman *et al.*, 1994). The prominent vasodilating effect of PGE_2 can thus be attributed to its action on cAMP accumulation, as cAMP accumulation was found to decrease $[Ca^{2+}]_i$ and myosin sensitivity to Ca^{2+}. In this thesis, rat tail artery helical strips were used to explore the mechanism of action of PGE_2 on vascular smooth muscle tone. It has been demonstrated that PGE_2 stimulated cAMP accumulation in isolated VSMCs. Hence, a vasodilation was expected for PGE_2 in rat tail artery smooth muscle strips. However, the present study found that PGE_2 contracted, instead of relaxed the muscle strips. Although the effect of PGE_2 on K channels absolutely required cAMP-PKA activation, the effect of PGE_2 on tension did not seem to involve the cAMP-PKA activation. PKA inhibition with either Rp-cAMP or HA1004 did not affect PGE_2-induced contractile response, indicating that the cAMP-PKA pathway was not the predominant signalling pathway mediating the action of PGE_2 on tension development. Although some controversy regarding the role of cAMP in force generation has appeared recently, as cAMP accumulation was found to cause an increase in the Ca^{2+} influx (Ishikawa *et al.*, 1993), cAMP was believed to reduce the Ca^{2+} influx and tension development in VSMCs and helical strips from rat tail artery (Pang *et al.*, 1985; Wang *et al.*, 1991). The PGE_2-induced contractile response was, however, found to be blocked by PKC inhibition with staurosoprine and H-7. The similarities in the actions of PGE_2 and PKC activators TPA

and PDBu also supported the notion that PGE_2 acted through PKC activation. With the demonstration of the extracellular Ca^{2+} dependence for the contractile action of PGE_2, intracellular Ca^{2+} mobilization was ruled out. PGE_2 has been reported to stimulate the PLC-PKC pathway (Yamashita and Takai, 1987; Farndale *et al.*, 1988; Kitanaka *et al.*, 1991; Vezza *et al.*, 1993). Recent studies have claimed a key role for PKC in enhancing the vascular smooth muscle tone in both normotensive and hypertensive subjects (Bilder *et al.*, 1990; Makita and Yasuda, 1990; Henrion and Laher, 1993; Neusser *et al.*, 1993). The increase in vascular smooth muscle tone could be abolished by inhibition of PKC. However, PKC inhibitors by themselves do not usually exert vasorelaxant or antihypertensive actions at the concentrations which inhibit PKC activity (Buchholz *et al.*, 1991). This further supported the notion that the cardiovascular system is normally in a state of vasodilation (Moncada, 1994).

It is believed that PKC causes an increase in the vascular tone through one of the following mechanisms: (1). Protein phosphorylation of myosin light chains; (2). Increase in transmembrane Ca^{2+} influx from extracellular pools; (3). Mobilization of Ca^{2+} from intracellular pools; (4). Ca^{2+}-induced Ca^{2+} sensitization; (5). Direct translocation of the isoforms of cytosolic PKC to the membrane. However, there is still considerable debate on the role of PKC in vascular smooth muscle tone (Khalil *et al.*, 1987; Rembold, 1992). In addition to vasoconstriction, PKC could also function as a negative feedback control for a variety of cellular systems such as the signal-contraction coupling in smooth muscle leading to an inhibition of vasoconstriction.

It is usually proposed that vasoactive agents stimulate smooth muscle contraction by releasing Ca^{2+} from intracellular stores such as sacroplasmic reticulum, or by opening membrane Ca^{2+} channels. In vascular smooth muscle of small vessels such as the tail artery, extracellular Ca^{2+} is considered to be the major source of intracellular Ca^{2+} for tension development. A relatively small volume of sacroplasmic reticulum could be found, thus, the capacity of intracellular Ca^{2+} stores is restricted (Van Breemen, 1977). These findings were in agreement with the results from nifedipine and Fura-2 experiments. It suggested that a dihydropyridine-sensitive Ca^{2+} channel was involved in the transmembrane influx of Ca^{2+}.

Nonetheless, PGE_2 exhibited a concentration-dependent vasoconstriction of rat tail artery vascular smooth muscles. The contractile action was most likely due to activation of the PKC system. The activation of PKC, which is Ca^{2+}-dependent, probably caused a transmembrane Ca^{2+} influx from the extracellular Ca^{2+} pool through a dihydropyridine-sensitive Ca^{2+} channel. Intracellular Ca^{2+} mobilization was not involved in the contractile action of PGE_2 on rat tail artery helical strips.

II. Summary

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

1. The study characterized a voltage-dependent, delayed outward rectifier K channel

current (I_k) in both primary and subcultured VSMCs from rat tail artery. The pharmacological and kinetic studies identified that the outward currents were indeed carried solely by the delayed rectifier K channels, which were inhibited by TEA. Neither a Ca^{2+}-activated component nor outward rectification through a Cl^- channel was believed to contribute to the outward currents. These results suggested that VSMCs from rat tail artery can be used as a suitable model for the study of K channels in small resistance vessels. A Ca^{2+}-sensitive component existed in VSMCs with high intracellular Ca^{2+}.

2. β-dendrotoxin, a potential antagonist for the voltage-dependent K channels, inhibited the delayed rectifier K channel currents in both primary and subcultured VSMCs from rat tail artery, in a concentration-dependent manner. The inhibition of I_k by β-dendrotoxin was not dependent on either the membrane potential or the physical occupancy of the channel pore.

3. Under the current-clamp conditions, PGE_2 caused a membrane depolarization. Under the whole-cell voltage-clamp conditions, PGE_2 suppressed the delayed rectifier K currents in a concentration-dependent manner. This effect was a receptor-mediated process. A cholera toxin-sensitive and/or pertussis toxin-insensitive G protein was responsible for the postreceptor activation of subcellular signal transduction machinery.

4. The cAMP-PKA second messenger pathway was involved in the mediation of the

PGE_2-induced inhibition of I_k. However, pharmacological evidence suggested that the PKC pathway might be also activated by PGE_2, although direct evidence is still lacking. The activation of either pathway individually might induce the inhibition of I_k, the interaction between the two signal transduction pathways "cross-talk" was, however, believed to play an important role in the mechanism of action of PGE_2. It appeared that PGE_2 might activate both cAMP-PKA and PKC pathways in rat tail artery VSMCs. However, the activation of PKC might potentiate the stimulation of the cAMP-PKA pathway. Whereas the cAMP-PKA cascade did not seem to affect the PKC pathway.

5. PGE_2 inhibited the L-type voltage-dependent Ca^{2+} channels in VSMCs from rat tail artery. The inhibitory effect might be directly related to the ability of PGE_2 to stimulate cAMP accumulation. The intracellular Ca^{2+} determination in primary cultured VSMCs with the Fura-2 technique revealed that PGE_2 did not affect the basal $[Ca^{2+}]_i$, but potentiated the KCl-stimulated increase in $[Ca^{2+}]_i$.

6. PGE_2 evoked a concentration-dependent contractile response in rat tail artery helical strips. The contractile response of PGE_2 was likely to be mediated through the activation of PKC and dependent on extracellular Ca^{2+}.

III. Significance

1. This is the first report to characterize a voltage-dependent K channel in VSMCs from the rat tail artery.

2. This thesis described for the first time that β-dendrotoxin inhibited the voltage-dependent K channel in VSMCs, thus providing valuable information on the potential pharmacological tool for the voltage-dependent K channels.

3. The present work explored the possible mechanism of action of PGE_2 in the regulation of the smooth muscle tone. It is possible that PGE_2 might inhibit the voltage-dependent K channel in rat tail artery VSMCs through both the cAMP-PKA and the PKC second messenger pathways. The inhibition of K channel has been documented to be related to the vascular smooth muscle tone. It is further shown that PGE_2 caused a contractile response in the helical strips of rat tail artery, possibly through the activation of PKC. These studies indicate that the subcellular mechanism of PGE_2 on the vascular smooth muscles may be more complicated than the well estabolished activation of the cAMP-PKA pathway.

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