

MECHANISM OF ACTION OF
VASOACTIVE INTESTINAL PEPTIDE
ON ION CHANNELS IN ENDOTHELIAL CELLS

By

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A Thesis

Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

September, 1993

ACTION OF VASOACTIVE INTESTINAL PEPTIDE
IN ENDOTHELIAL CELLS

DOCTOR OF PHILOSOPHY (1993)
(Biomedical Sciences)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: **MECHANISM OF ACTION OF VASOACTIVE
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ENDOTHELIAL CELLS**

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NUMBER OF PAGES: **x, 144**

ABSTRACT

Endothelial cells contain a high density of vasoactive intestinal peptide (VIP) receptors. Since VIP is a potent vasodilator, activation of these receptors may lead to the release of endothelium-dependent relaxing factor (EDRF). Using patch-clamp techniques it was demonstrated that VIP modulates plasma membrane K^+ channels in bovine pulmonary artery endothelial cells. VIP inhibited activity of inwardly rectifying K^+ channels ($I_{K_{in}}$) and activated opening of the Ca^{2+} -dependent K^+ channels (K_{Ca}). Activation of K_{Ca} channels tends to hyperpolarize the cell membrane. This transient hyperpolarization increases the driving force for influx of extracellular Ca^{2+} through nonselective cation channels. This way VIP may contribute to Ca^{2+} influx necessary for the potential EDRF production.

Although VIP activates adenylate cyclase and production of cyclic AMP (cAMP) in many cell types, this study provides evidence allowing us to exclude cAMP as a second messenger for VIP action in endothelial cells. Direct evidence comes from the measurements of cAMP level in bovine pulmonary artery endothelial cells stimulated with VIP and isoproterenol. Isoproterenol increased cAMP level, whereas VIP did not, or even decreased cAMP production in some cases. Indirect evidence comes from the results of patch-clamp experiments. VIP strongly suppressed $I_{K_{in}}$ channel activity, whereas the inhibitory effect of isoproterenol on this channel was very weak (about 20% of the VIP effect). cAMP elevation may reduce the activity of $I_{K_{in}}$ channel, but is not a mediator for the inhibitory effect of VIP on these channels. In

addition, VIP was still able to inhibit $I_{K_{in}}$ channel activity in outside-out patches, where the second messenger system is not operating. This effect was mediated by a G protein that most likely couples directly to the channel. This unidentified G protein revealed GTP- γ -S and cholera toxin sensitivity and resistance to pertussis toxin.

Since elevated intracellular Ca^{2+} is a trigger for EDRF production, it has been demonstrated that modulation of the calcium fluxes at the level of internal stores did influence an influx of extracellular Ca^{2+} . Cyclopiazonic acid (CPA), an inhibitor of SR/ER Ca^{2+} pump, (i) induced K_{Ca} currents, presumably as a consequence of the spontaneous leakage of Ca^{2+} from internal stores, (ii) reduced the $I_{K_{in}}$ currents, and (iii) enhanced influx of extracellular Ca^{2+} through nonselective cation channels. Moreover, CPA activated K_{Ca} currents in endothelial cells by a mechanism independent of 1,4,5-triphosphate (IP_3). An expected consequence of this action of CPA may be an influx of extracellular Ca^{2+} . In parallel studies, it has been demonstrated that CPA relaxed rat aorta in endothelium-dependent manner, suggesting CPA-induced release of EDRF. Ca^{2+} enters the endothelial cell through nonselective cation channels, which can be activated by an agonist or a mechanical stimulus (e.g. stretch). It is still unclear whether these nonselective cation channels in bovine pulmonary artery endothelial cells are controlled by free intracellular Ca^{2+} , empty Ca^{2+} ER stores or an agonist.

ACKNOWLEDGEMENTS

I would like to express my appreciation to all people who contributed to the completion of this thesis. Most of all, I would like to thank Dr. E.E. Daniel for being my supervisor, teacher and mentor. His broad knowledge, as well as, open-minded and interdisciplinary approach to research proved vital to the course of this project.

I am grateful to the remaining members of my supervisory committee, Dr. J. Huizinga, Dr. C. Nurse and Dr. S. Sims for their criticism and guidance during the course of my study. I am thankful to Dr. A. Molleman for his generosity in sharing his time and knowledge with me. I express my appreciation to all the members of the Smooth Muscle Research Group for their friendship during my years in the program.

Finally, I would like to thank my husband, Stan, for his immense patience and silent contribution to my 'success'. His sense of humour and optimism have helped me to keep things in perspective. I am thankful to my children, Peter, Stan, Jr. and Michael, for making everything worthwhile.

To my children

Contents

CHAPTER I

INTRODUCTION

1. The Endothelium	2
2. Vasoactive Intestinal Peptide	4
3. Ion Channels In Endothelial Cells	5
3.1. Inwardly Rectifying K ⁺ channels	5
3.1.1. Basic properties	5
3.1.2. Rectification	7
3.1.3. Physiological significance	8
3.1.4. Stability	8
3.1.5. The role of intracellular calcium	9
3.1.6. Molecular structure	10
3.2. Ca ²⁺ -activated K ⁺ channels	12
3.3. Nonselective cation channels	13
3.3.1. Agonist-activated channels	13
3.3.2. Mechanosensitive channels	15
4. Calcium Fluxes In Endothelial Cells	16
5. The Cyclic AMP Cascade	18
5.1. G proteins	18
5.2. Adenylate cyclase	20
5.3. Phosphodiesterase	20
6. Interaction Between cAMP And Intracellular Calcium	21
6.1. Cyclic AMP-dependent protein kinase	21
6.2. The role of cAMP in endothelial cells	21

CHAPTER II

OBJECTIVES AND HYPOTHESES

1. The Effects of VIP on Ion Channels in Endothelial Cells 24
2. The Role of a G Protein in VIP-stimulated Endothelial Cells 24
3. A VIP Transduction Pathway in Endothelial Cells 25
4. Calcium Signalling in Endothelial Cells 25

CHAPTER III

METHODS AND RESULTS

1. Paper No. 1 27
An endothelial cell-line contains functional vasoactive intestinal polypeptide receptors: they control inwardly rectifying K^+ channels
2. Paper No. 2 34
Cyclic AMP is not a second messenger for transduction of VIP effects on inwardly rectifying K^+ currents in endothelial cells
3. Paper No. 3 60
A G protein mediates effects of vasoactive intestinal polypeptide on K^+ channels in endothelial cells
4. Paper No. 4 86
Cyclopiazonic acid, an endoplasmic reticulum Ca^{2+} -pump inhibitor, enhances Ca^{2+} entry in endothelial cells in an IP_3 independent manner

CHAPTER IV

DISCUSSION

1. The Effects of VIP on K⁺ Channels in Endothelial Cells 120
2. The Role of a G Protein 123
3. Cyclic AMP Is *Not* a Second Messenger for VIP Transduction Pathway 124
4. Calcium Signalling 127
5. Further Directions 128

REFERENCES 131

APPENDIX I 142

APPENDIX II 143

APPENDIX III 144

Abbreviations:

4-AP	- 4-aminopyridine
8-Br-cAMP	- 8-bromo-adenosine 3':5'-cyclic monophosphate
BrHMA	- bromohexamethyleneamiloride
BHQ	- 2',5'-di-(tert-butyl)-1,4-benzohydroquinone
BSA	- bovine serum albumin
[Ca ²⁺] _i	- intracellular free calcium
cAMP	- cyclic adenosine monophosphate
cGMP	- cyclic guanosine monophosphate
ChTx	- charybdotoxin
CPA	- cyclopiazonic acid
db-cAMP	- N ⁶ ,2'-O-dibutyryl-adenosine 3':5'- cyclic monophosphate
DMSO	- dimethyl sulphoxide
EDRF	- endothelium-derived relaxing factor
EDTA	- ethylenediamine tetraacetic acid
EGTA	- ethyleneglycol-bis-(β aminoethyl ether) N,N'-tetraacetic acid
E _K	- equilibrium potential for potassium
ER	- endoplasmic reticulum
FBS	- fetal bovine serum
FORS	- forskolin
GMP-PNP	- 5'-guanylyl imidodiphosphate
G protein	- guanosine triphosphate binding protein
GTP-γ-S	- guanosine 5'-(3'-O-thiotriphosphate), tetralithium salt
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H-8	- N-[2-(methylamino)ethyl]-5-isoquinoline-sulphonamide dihydrochloride
IBMX	- 3-isobutyl-1-methylxanthine
I _{K(Ach)}	- acetylcholine-activated inwardly rectifying K ⁺ channel
I _{Kin}	- inwardly rectifying K ⁺ channel
IP ₃	- 1,4,5-trisphosphate
IP ₄	- 1,3,4,5-tetrakisphosphate
ISO	- isoproterenol
K _{Ca}	- Ca ²⁺ -dependent K ⁺ channel
MEM	- minimum essential medium
NO	- nitric oxide
PACAP	- pituitary adenylate cyclase activating polypeptide
PBS	- phosphate buffered saline
SK&F 96365	- 1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride
SR	- sarcoplasmic reticulum
TCA	- trichloroacetic acid
TEA	- tetraethylammonium
VIP	- vasoactive intestinal peptide

CHAPTER I

INTRODUCTION

1. The Endothelium

Endothelial cells form a monolayer of cells lining the lumen of blood vessels. Among many physiological functions endothelial cells control the contractile state of underlying smooth muscle. The endothelium synthesizes and secretes vasodilatory substances, prostacyclin, endothelium-derived relaxing factor (Moncada et al., 1977; Furchgott and Zawadzki, 1980) and a vasoconstrictor substance, endothelin (Yanagisawa et al., 1988). Endothelium-derived relaxing factor (EDRF) is believed to play a key role in the regulation of vascular tone.

The chemical nature of EDRF has been identified as nitric oxide (Palmer et al., 1987; Ignarro, 1990). Nitric oxide (NO) is synthesized in endothelial cells from the precursor L-arginine by Ca-dependent NO synthase (Palmer et al., 1988) (fig. 1). Thus, an increase in the intracellular Ca concentration in endothelial cells is the trigger for EDRF synthesis and release. Formed NO activates soluble guanylate cyclase in the target cell, smooth muscle. The resulting rise in cyclic GMP level in the smooth muscle cells is responsible for their relaxation (Ignarro et al., 1981; Moncada et al., 1991).

Many physiological functions of endothelium are mediated through specific receptors located on the cell membrane. The manner in which these receptors activate EDRF release differ. Some agonists (e.g. bradykinin, acetylcholine) stimulate the metabolism of phosphoinositol and increase the intracellular levels of inositol trisphosphate (IP_3), which then leads to Ca mobilization (Colden-Stanfield et al.,

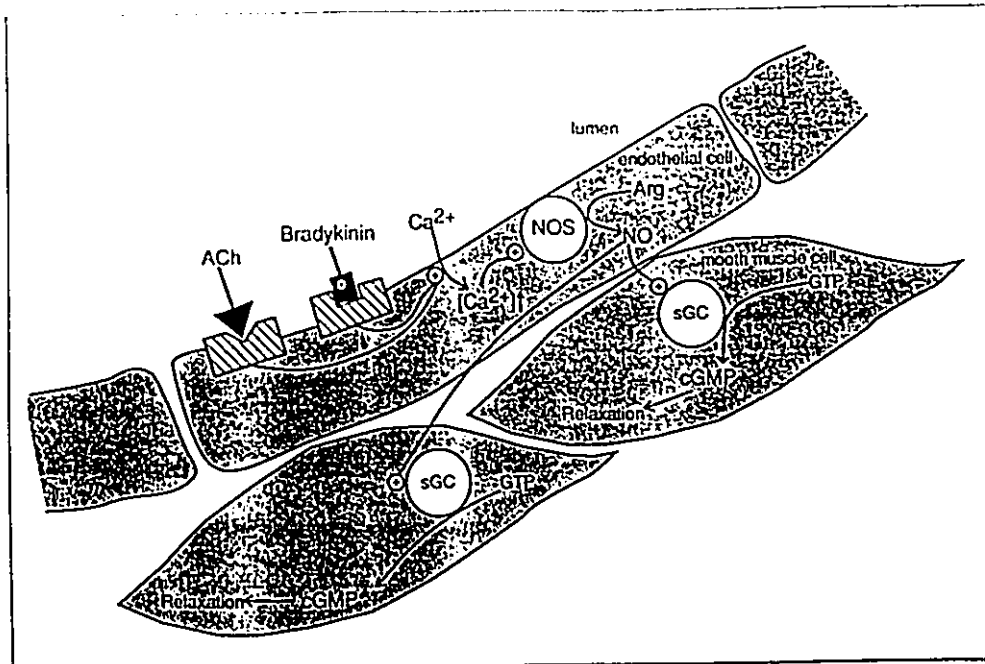


Figure 1
 The signal transduction pathway of endothelium-dependent vascular relaxation. Ach, acetylcholine; NOS, nitric oxide synthase; cGMP, cyclic GMP; Arg, arginine (from Knowles and Moncada, 1992).

1987; Freay et al., 1989; Laskey et al., 1990; Himmel et al., 1993). Other agonists stimulate EDRF release by activation of a cyclic AMP cascade (e.g. isoproterenol) (McEwan et al., 1990; Gray and Marshall, 1992; Zheng et al., 1993b).

This study showed for the first time that endothelial cells contain a high density of functional VIP receptors. Since VIP is known to be a powerful vasodilator, it is possible that the relaxant action of VIP may be mediated by the endothelium. The objective of this work was to study the effect of VIP on ion channels in endothelial cells and a potential second messenger system activated by VIP.

2. Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is 28-amino-acid residue polypeptide with potent vasodilator properties. VIP acts primarily as a neurotransmitter and is widely distributed in both the central and peripheral nervous system. VIP is present in the terminals of perivascular nerves, where is colocalized with another neurotransmitter, acetylcholine (Lincoln and Burnstock, 1990). VIP is also present in the blood where its plasma concentration is normally low (20 pmol/l) (Dockray, 1987).

The mode of action of VIP on blood vessels is so far controversial. Some authors reported that responses to this peptide are dependent on the presence of endothelium in rat aorta, rabbit mesenteric artery, human celiac artery and cultured vascular smooth muscle cells (Davis and Williams, 1984; Sata et al., 1988; Itoh et al., 1985; Thom et al., 1987; Ganz et al., 1986). Others reported endothelium-independent action of this peptide, sometimes in the same blood vessel that has the endothelium-dependent action, in rat aorta (Schoeffer and Stoclet, 1985), cerebral arteries (Duckles and Said, 1982; Lee et al., 1984). However, the VIP-induced relaxation obtained in rat aorta by Schoeffer and Stoclet was markedly small, both in the absence and presence of endothelium, although isoproterenol relaxed this vessel to a great extent. VIP receptors have been shown to be in several tissues, intestinal epithelium (Laburthe et al., 1984), pancreas (Bissonnette et al., 1984), liver (Couvineau and Laburthe, 1985). In most of the tissues stimulation of VIP receptors led to activation of adenylate cyclase and an increase of the cellular cyclic AMP

(cAMP). Stimulation of VIP receptors found in cultured rat aorta smooth muscle cells caused an increase in cAMP level (Hirata et al., 1985; Nabika et al., 1985). Sata et al. (1988) have reported relaxation of rat aorta in response to VIP and the accumulation of cAMP; both responses were endothelium-dependent.

3. Ion Channels In Endothelial Cells

3.1. Inwardly Rectifying K^+ Channel

3.1.1. Basic properties

An inwardly rectifying K^+ channel ($I_{K_{in}}$) is the predominant channel present in cultured endothelial cells (Takeda and Klepper, 1990; Bregestovski and Ryan, 1989). This channel is activated by hyperpolarization. Hyperpolarization evokes large inward currents, while depolarization gives rise only to small outward currents. These currents reveal high K^+ selectivity and therefore reverse very near equilibrium potential for K^+ (E_K). The endothelial cell membrane behaves as an 'inward rectifier' because it conducts large inward K^+ currents at potentials negative to E_K , but only small outward currents at potentials positive to E_K (fig. 2 A, B).

Potassium as a current carrier through the inward rectifier was identified by observing the reversal potential as a function of bath K^+ concentration. The reversal potential was shifted with changes in external K^+ as predicted by the Nernst

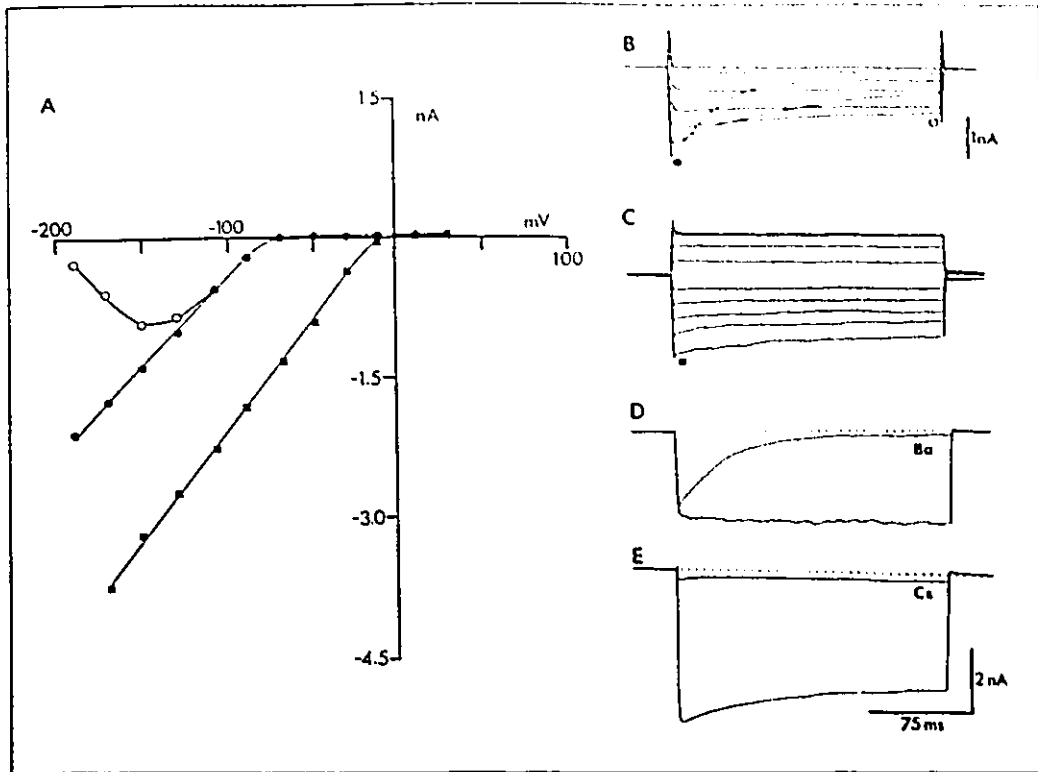


Figure 2
 Whole-cell inward rectifier K^+ current. A. I-V relations for peak (\bullet) and steady state (\circ) current as illustrated in B for physiological solutions (Na^+ external/ K^+ internal) and for symmetrical K^+ solutions (\blacksquare). B. Inward currents activated by hyperpolarizing steps from -190 mV to 30 mV every 20 mV from a holding potential of -70 mV. C. Currents after replacement of the bath Na^+ by K^+ (pulses from -170 to 30 mV). D. Blocking effect of external Ba^{2+} (0.2 mM) in symmetrical K^+ solution. E. Blocking effect of Cs^+ (1 mM). Dotted lines indicate zero current level (from Takeda & Klepper, 1990).

equation. Another piece of evidence establishing this K^+ selectivity is the fact that the conductance of the inward current increased with increasing external K^+ concentration (fig. 2 A, C).

Pharmacology is another tool for identifying a channel type. The inward rectifier is blocked by cesium and barium (10^{-4} - 10^{-3} M) (fig. 2 D, E). The standard K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) are less

effective blockers causing 60 - 80% reduction in the magnitude of the inward rectifier current (Cooper et al., 1991).

One of the characteristic properties of inward rectifiers is that the current inactivates significantly in response to more negative potentials (fig. 2 A, B). This voltage-dependent inactivation is caused by external Na^+ . In Na^+ free bath solution, this steady-state inactivation is abolished. Therefore, external Na^+ acts as a blocking agent of inward rectifier. Here, it is worthwhile to mention that Na^+ does not have its own voltage-operated channel in endothelial cells (Johns et al., 1987; Takeda and Klepper, 1990). The slope conductance for single I_{Kin} channel currents (cell-attached, inside-out, outside-out patches) is 25 pS, on the average, when measured in symmetrical conditions (140 mM K^+ on both sides of the membrane patch) (Bregestowski and Ryan 1989; Takeda and Klepper, 1990).

3.1.2. Rectification

It has been suggested that inward rectification is due to an intracellular ion which blocks the channels on depolarization (Hagiwara and Takahashi, 1974). Recently, it has been reported that physiological levels of internal Mg^{2+} serve as a potent blocker of the outward current of the inward rectifier in cardiac muscle (Matsuda et al., 1987; Matsuda, 1988). However, in endothelial cells internal Mg^{2+} seems to play a minor role in the inward rectification, suggesting that inward rectification is most likely caused by an intrinsic gating mechanism (Silver and DeCoursey, 1990). Internal Mg^{2+}

was also not required for inward rectification in the lens epithelial cells (Cooper et al., 1991).

3.1.3. Physiological Significance

Although the inward rectifier has been identified more than 40 years ago (Katz, 1949), its physiological significance remains obscure. It has been suggested that in endothelial cells inward rectifier helps to maintain the resting membrane potential of the cell. Small efflux of K^+ through the $I_{K_{in}}$ channel at membrane potentials above E_K helps to keep the resting membrane potential close to E_K . If some hyperpolarizing influences in a cell bring the membrane potential to values more negative than the resting potential (e.g. increased activity of Na^+-K^+ pump), a large influx of K^+ through $I_{K_{in}}$ below E_K will prevent the excessive hyperpolarization and bring the membrane potential back to its resting value (Kubo et al., 1993).

3.1.4. Stability

It has been demonstrated in a few cell types that activity of $I_{K_{in}}$ channels decays with time. In most cells it has been shown that activity of the $I_{K_{in}}$ channel depends on the presence of ATP in the intracellular solution (McCloskey and Cahalan, 1990). The $I_{K_{in}}$ conductance decayed after 10-20 min of whole-cell recording, while no decay was observed with 0.5 mM ATP in the pipette solution after 20 min of recordings.

Therefore, it is likely that the $I_{K_{in}}$ channel activity is maintained by cytoplasmic ATP, which is lost during dialysis of the cell contents (McCloskey and Cahalan, 1990). A similar dependence of the $I_{K_{in}}$ channels on ATP was demonstrated in cardiac myocytes (Trube and Hescheler, 1984). In bovine pulmonary artery endothelial cells we demonstrated that the whole-cell current recordings were stable for at least 20 min. when 0.5 mM ATP was present in the pipette solution (APPENDIX II). Single channel currents recorded from the cell-attached or excised patches were also stable for at least 10 to 20 min when 4 mM ATP was present in the 'intracellular' solution (data not shown).

3.1.5. The Role of Intracellular Calcium

Intracellular free Ca^{2+} $[Ca^{2+}]_i$ regulates many plasma membrane ionic currents in a wide variety of cells: outward K^+ (Pallota et al., 1981), Cl^- (Barish, 1983), nonselective cation currents (Partridge and Swandulla, 1988). It is not clear whether $I_{K_{in}}$ channel activity depends on $[Ca^{2+}]_i$. Some evidence suggests that $I_{K_{in}}$ channel may also be modulated by $[Ca^{2+}]_i$. An increase in the $[Ca^{2+}]_i$ has been reported to inactivate the $I_{K_{in}}$ currents in cardiac cells (Vandenberg, 1987), *Aplysia* neurons (Kramer and Levitan, 1988) and mast cells (Mukai et al., 1990).

In cardiac ventricular cells, single channel activity in inside-out patches was maintained by lowering the $[Ca^{2+}]_i$ ($<1 \mu M$), while it disappeared rapidly with higher $[Ca^{2+}]_i$ levels (10 μM -1 mM) in the bath solution (Vandenberg, 1987). In *Aplysia*

neurons, the influx and intracellular accumulation of Ca^{2+} leads to the prolonged inactivation of the I_{Kin} channel. In rat tumor mast cells, the increased Ca^{2+} decreased open probability of the channel in inside-out patches and had no apparent effect on the single channel conductance (Mukai et al., 1990). The rundown of the channel activity at an excision was excluded, since Ca^{2+} -dependent inactivation of the channel activity was observed both in the presence and absence of ATP (Mukai et al., 1990).

In contrast, in endothelial cells it has been reported that bradykinin, which is known to activate phosphatidyl inositol turnover resulting in mobilisation of Ca^{2+} from the internal stores, activated rather than inhibited the I_{Kin} channel activity (Colden-Stanfield et al., 1990). Furthermore, acetylcholine which stimulates a rise in $[\text{Ca}^{2+}]_i$ level and production of EDRF activated the I_{Kin} channel opening (Clark et al., 1990). However, in the case of acetylcholine, it has been demonstrated in cardiac cells that an acetylcholine-activated inwardly rectifying channel ($I_{\text{K(Ach)}}$) was distinct from the I_{Kin} channel opened on hyperpolarization *per se* (Clark et al., 1990). Experiments in our laboratory provide some evidence suggesting that reduction of activity of I_{Kin} channel in bovine pulmonary endothelial cells may be caused by increased $[\text{Ca}^{2+}]_i$ level (see Paper No. 4).

3.1.6. Molecular structure

Recently an inward rectifier K^+ channel of mouse macrophage cell line has been

cloned and functionally expressed (Kubo et al., 1993). The proposed structural model of $I_{K_{in}}$ represents a major departure from the basic structure of voltage-gated K^+ channels. Voltage-gated K^+ channels are characterized by six membrane-spanning segments (S1-S6), a putative voltage sensor (S4 segment) and an S5-S6 linker (H5 region) responsible for ion conduction (fig. 3). Compared with other voltage-gated K^+ channels, the inward rectifier K^+ channel represents a more reduced structure. The $I_{K_{in}}$ channel polypeptide contains two membrane-spanning segments (M1 and M2), which may correspond to the S5 and S6 segments of voltage-gated K^+ channels. A sequence in between the two hydrophobic segments reveals great similarity with the H5 sequence. This suggests that this region has most or all of the structure that constitutes a K^+ channel pore, through which ions flow across the membrane. The $I_{K_{in}}$ channel does not contain any other hydrophobic segments that would correspond to S1, S2 and S3 of the voltage-gated channels. It also lacks the highly charged S4 sequence that is likely to be a membrane-spanning voltage sensor. A similar topology has been proposed for an inwardly rectifying ATP-regulated K^+ channel (Ho et al., 1993).

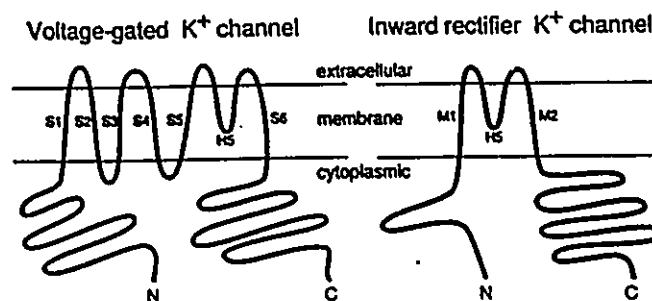


Figure 3
Proposed structural model of K^+ channels (from Kubo et al., 1993).

3.2. Ca^{2+} -activated K^+ channel

By definition, activity of the Ca^{2+} -activated K^+ channel (K_{Ca}) is modulated by changes in $[\text{Ca}^{2+}]_i$. Increased Ca^{2+} concentration at the intracellular membrane surface increases Ca^{2+} -activated K^+ channel activity (Pallota et al., 1981). Membrane depolarization in excitable cells leads to influx of Ca^{2+} through voltage-activated Ca^{2+} channels and can increase the activity of these K^+ channels. K^+ efflux results in quick repolarization, which terminates the influx of Ca^{2+} into the cell and also decreases the activity of the Ca^{2+} -activated K^+ channels.

Membrane depolarization, however, is not a prerequisite for the K_{Ca} channel activity. Agonists that raise intracellular Ca^{2+} level at the resting membrane potential by, for example, stimulation of phosphatidyl inositol turnover, can also stimulate Ca^{2+} -activated K^+ channel activity (Berridge, 1986). The resulting increase in K^+ efflux may then hyperpolarize the cell membrane (Lewis et al., 1990). In endothelial cells, many vasoactive agonists cause increase in $[\text{Ca}^{2+}]_i$ level, both due to release from internal stores and influx of external Ca^{2+} (Laskey et al., 1991). It has been demonstrated that the Ca^{2+} -activated K^+ channel was activated following application of bradykinin, acetylcholine, histamine and thrombin (Colden-Stanfield et al., 1990; Sakai, 1990; Bregestovski et al., 1988; Takeda and Klepper, 1990). A corresponding bradykinin- and acetylcholine-dependent hyperpolarization (Daut et al., 1989; Busse et al., 1988) has also been reported. In fact, endothelial cells do not have voltage-operated Ca^{2+} channels, therefore depolarization does not cause Ca^{2+} influx (Adams

et al., 1989; Nilius, 1991; Takeda et al., 1987). Instead, transient hyperpolarization produced by some agonists creates a driving force for Ca^{2+} influx. Ca^{2+} enters endothelial cells through nonselective cation channels (see below).

In addition to modulation of the Ca^{2+} -activated K^+ channel by depolarization and Ca^{2+} concentration, the activity of this channel can be increased by the process of phosphorylation (Ewald et al., 1985). Elevated cAMP in a cell can lead to activation of protein kinase A, which phosphorylates the channel. This cAMP-dependent phosphorylation of protein activates K^+ efflux presumably by increasing the sensitivity of the Ca^{2+} -activated K^+ channel to Ca^{2+} (Lewis et al., 1990). In cells other than endothelial cells, agonists that increase the intracellular cAMP level, e.g. antidiuretic hormone in kidney cells (Guggino et al., 1985) and VIP in lacrimal cells (Lechleiter et al., 1988), stimulate Ca^{2+} -activated K^+ channel and secretion of fluid.

3.3. Nonselective Cation Channels

3.3.1. Agonist-activated channels

Voltage-operated Ca^{2+} channels are not present in most of endothelial cell types (Adams et al., 1989; Johns et al. 1987; Takeda et al. 1987). Their presence was reported so far only in capillary endothelial cells (Bossu et al., 1989). Therefore, the question arises how Ca^{2+} , an ion that plays a central role in controlling the membrane ionic currents and secretion, enters the interior of endothelial cell?

Evidence suggest that Ca^{2+} influx can occur through nonselective cation channels activated by an agonist or mechanical stimuli (see below).

Some agonists induce an inward current at the resting potential associated with an increase in membrane conductance, as was demonstrated with bradykinin, thrombin and histamine (Johns et al., 1987; Lodge et al., 1988; Schilling et al., 1988; Bregestovski et al., 1988; Jacob et al., 1988; Hallman et al., 1988). These inward currents reveal poor selectivity among cations. In bovine pulmonary endothelial cells, inward whole-cell currents activated by bradykinin and thrombin were cation nonselective, were sensitive to Na^+ , Ca^{2+} , Ba^{2+} and reversed around 0 mV (Johns et al., 1987; Lodge et al., 1988). In human umbilical vein, histamine also induced similar whole-cell current which was decreased in the absence of external Ca^{2+} (Bregestovski et al., 1988). In inside-out patches this channel was activated directly by raising the internal Ca^{2+} concentration and had a slope conductance of 20 pS, and reversed at 0 mV. This evidence suggests that Ca^{2+} entry under physiological conditions could occur through these nonselective cation channels when the endothelial cell is stimulated by some agonists.

In a broad range of cells, including cardiac cells, mast cells, and neurons, nonselective cation channels are activated by intracellular Ca^{2+} (Partridge and Swandulla, 1988). Control mechanisms of this class of channels in endothelial cells are still poorly understood. A recent study has implicated both intracellular Ca^{2+} and inositol 1,3,4,5-tetrakisphosphate (IP_4) in controlling the influx of extracellular Ca^{2+} (Luckhoff and Clapham, 1992). Other studies, however, indicate that the Ca^{2+}

content of the ER controls the Ca^{2+} permeability of the plasmalemma (Dolor et al., 1992; Schilling et al., 1992).

3.3.2. Mechanosensitive channels

There are two components of the force applied to the endothelial surface during blood flow. One is a perpendicular pressure (stretch) and the other is a tangential (frictional) component called shear stress. It has been demonstrated that stretch can activate nonselective cation single-channel currents in endothelial cells in cell-attached patches (Lansman et al., 1987). This channel is permeable for Na^+ , K^+ and Ca^{2+} and single-channel conductances for these ions were 36, 56 and 19 pS, respectively. It has been suggested that influx of Ca^{2+} through this channel can occur under physiological conditions.

It has been demonstrated in many endothelial cells preparations that shear stress increases $[\text{Ca}^{2+}]_i$ level (Shen et al., 1992; Mo et al., 1991) or leads to the calcium-dependent production of vasoactive substances, EDRF (Rubanyi et al., 1986) or prostacyclin (Frangos et al., 1985). Mechanically activated channels would be the best candidates for this Ca^{2+} influx (Davis and Tripathi, 1993). Furthermore, osmotic stress also activated nonselective cation channel in aortic endothelial cells (Ling and O'Neil, 1992). This hypotonic stress-induced channel conducted Ca^{2+} current (28 pS). Activity of this channel was reduced by removal of extracellular Ca^{2+} and was blocked by gadolinium (Ling and O'Neil, 1992).

Mechanical stimuli generate diverse responses in a variety of endothelial cell types. They cause changes not only in ionic conductance (Olesen et al., 1988), intracellular Ca^{2+} level (Shen et al., 1992; Mo et al., 1991), but also in adenylate cyclase activity (Watson, 1991) and generation of inositol trisphosphate (Nollert et al., 1990). These effects are similar to second-messenger responses resulting from agonist-receptor coupling. Recent evidence suggests that the microfilament network (mainly F-actin) is the principal transmission structure. F-actin is linked to integrins, the plasma membrane proteins, which may transduce mechanical stress to biochemical signals by tyrosine kinase phosphorylation (Davis and Tripathi, 1993).

4. Ca^{2+} Fluxes In Endothelial Cells

Intracellular free Ca^{2+} regulates a range of cellular responses in endothelial cells. An increased $[\text{Ca}^{2+}]_i$ level is necessary for the synthesis and release of EDRF (Palmer et al., 1988). The measurement of Ca^{2+} level in endothelial cells in response to an agonist stimulation (e.g. bradykinin) consists of two components: a transient spike and the plateau. The initial spike is associated with release of Ca^{2+} from intracellular stores of the endoplasmic reticulum (ER), whereas the plateau depends on extracellular Ca^{2+} concentration (Laskey et al., 1991)

In endothelial cells influx of external Ca^{2+} occurs through nonselective cation channels activated by an agonist or mechanical stimulus (see above) (fig. 4). Ca^{2+} is extruded by an ATP-driven plasmalemma Ca^{2+} pump. The release of Ca^{2+} from the

internal stores occurs through IP_3 -sensitive Ca^{2+} channels present in the ER membrane. This Ca^{2+} release is balanced by the ER Ca^{2+} -pump (Laskey et al., 1991).

Although the influx of external Ca^{2+} is relatively voltage independent (controlled by receptor occupation or mechanical stimulus), membrane potential (E_m) nevertheless plays an important role in the regulation of Ca^{2+} entry. Membrane potential affects the electrochemical gradient ($E_m - E_{Ca}$), which modulates the driving

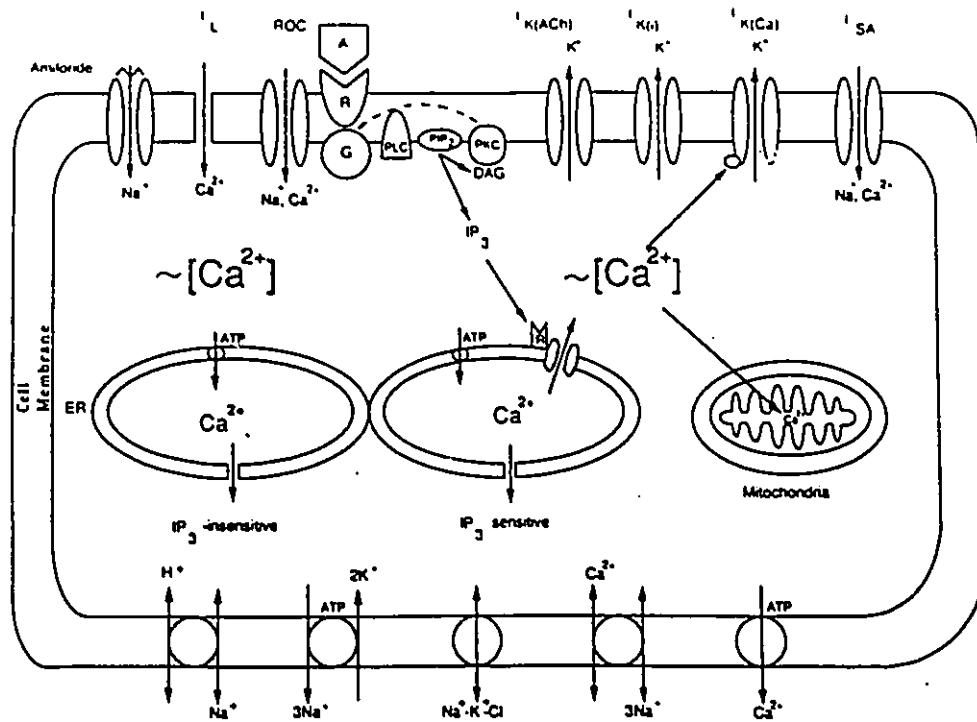


Figure 4

Ion channels and calcium fluxes in endothelial cells. Calcium entry may occur through leak channels (I_L), stretch-activated channels (I_{SA}), or receptor-operated channels (ROC). Binding of an agonist (A) to a receptor (R), which may be coupled by a G protein (G) to phospholipase C (PLC), leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). This leads to the production of IP_3 and DAG. Calcium may be extruded from the cell by $ATP-Ca^{2+}$ pump and by Na^+-Ca^{2+} exchange. Other factors that may affect calcium homeostasis and membrane potential: Ca^{2+} -activated K^+ channel ($I_{K(Ca)}$), inward rectifier ($I_{K(I)}$), and acetylcholine-activated channel ($I_{K(ACh)}$), the $Na^+-K^+-Cl^-$ antiporter, the Na^+-H^+ exchanger, and the Na^+-K^+ pump (from Adams et al., 1989).

force for Ca^{2+} fluxes (Adams et al., 1989; Luckhoff and Busse, 1990). The membrane potential in endothelial cells is regulated mainly by two K^+ channels: inwardly rectifying and outward Ca^{2+} -activated K^+ channels. Inwardly rectifying K^+ channels contribute to the maintenance of the resting membrane potential (see above). Efflux of K^+ through Ca^{2+} -activated K^+ channels produces transient hyperpolarization which shifts the membrane potential towards more negative values and therefore increases the driving force for Ca^{2+} entry (Daut et al., 1989; Busse et al., 1988).

5. The Cyclic AMP Cascade

5.1. G proteins

An intracellular guanyl nucleotide regulatory protein (G protein) is intercalated between the cell surface receptor and the enzyme adenylate cyclase. Adenylate cyclase catalyses the formation of cAMP from ATP in the cell. The G protein enhancing cAMP formation is likely to be stimulatory G_s protein. The G protein associated with the inhibition of cAMP formation is believed to be inhibitory G_i protein. Both G_s and G_i proteins consist of three subunits α , β and γ (Northup, 1985). Only the α subunits of the G_s and G_i proteins are distinct entities. Although the present discussion focuses on the regulation of adenylate cyclase, G proteins control many other cellular processes. G proteins regulate other enzymes (e.g. phospholipase C) and ion channels (e.g. K^+) (Birnbaumer and Brown, 1990).

Guanosine 5'-triphosphate (GTP) is essential for the activity of a G protein. There are two important features of the action of GTP with a G protein. First, receptor stimulation (i.e. occupancy of the receptor by an agonist) triggers the binding of GTP to α subunit of the G protein. This results in the dissociation of the α - β - γ complex into the β - γ subunit and complex of α and GTP. It is the α -GTP complex that regulates the activity of adenylate cyclase. Second, the α subunit possesses GTPase activity that will hydrolyze the bound GTP. The degradation of GTP permits the reassociation of the α - β - γ complex and the termination of the control over adenylate cyclase activity (Kebabian, 1992).

Analogs of GTP resistant to hydrolysis by GTPase, 5'-guanylyl imidodiphosphate (GMP-PNP) and 5'-3'-0-thiotriphosphate (GTP- γ -S) are useful tools for studying a G protein activity. Owing to their insensitivity to GTPase activity, they bind to α subunit and promote permanent dissociation of α - β - γ complex. Consequently, they increase adenylate cyclase activity for longer periods than GTP does.

Cholera and pertussis toxins are useful tools to examine G_s and G_i , respectively. Cholera toxin, the secretory product of *Vibrio cholerae*, catalyses the transfer of the ADP-ribose to α subunit of G_s . This ADP-ribosylation of α and GTP complex inhibits GTPase activity and prolongs activation of adenylate cyclase (Northup, 1985). Pertussis toxin, the secretory product of *Bordetella pertussis*, catalyses the transfer of the ADP-ribose to the carboxyl terminus of α subunit of G_i . This ADP-ribosylation of α subunit and GTP complex sustain the inhibition of adenylate cyclase (Reisine and Law, 1992).

5.2. Adenylate Cyclase

Adenylate cyclase, the plasma membrane enzyme, catalyses the formation of cAMP from ATP. There are at least 3 forms of the enzyme (type I,II,III) (Krupinski, 1991). Activity of the enzyme can be changed by receptor or a G protein stimulation. However, activity of type I can be increased by calcium and calmodulin (Kebabian, 1992). Forskolin, a naturally occurring diterpene from *Coleus forskohlii*, can also increase adenylate cyclase activity. Forskolin seems to directly bind to the catalytic subunit of adenylate cyclase. This binding has low affinity, unless the catalytic subunit of adenylate cyclase is complexed with the α subunit of G_s protein (Seamon, 1985). Forskolin requires both the catalytic subunit of adenylate cyclase and the α subunit of G_s protein for high-affinity binding and maximal stimulation of cAMP production. The 1,9-dideoxyforskolin, an analog that can not stimulate adenylate cyclase, is a useful inactive control in many experiments (Laurenza et al., 1989).

5.3. Phosphodiesterase

Phosphodiesterases are a family of enzymes which catabolize cyclic nucleotides. The most widely-used phosphodiesterase inhibitors are nonselective inhibitors, theophylline, 3-isobutyl-1-methylxanthine (IBMX) and more selective, M&B 22948 and rolipram (Beavo and Reifsnyder, 1990; Kebabian, 1992).

6. Interaction Between cAMP And Intercellular Ca^{2+}

6.1. cAMP-Dependent Protein Kinase

One of the functions of cAMP as a second messenger is activation of protein kinase. The cAMP-dependent protein kinase is an enzyme that catalyses the phosphorylation of the target proteins. In many diverse tissues, activation of protein kinase A by cAMP decreases the level of intracellular Ca^{2+} . This process may involve several mechanisms. First, phosphorylation of the IP_3 receptor may diminish release of Ca^{2+} from endoplasmic reticulum, as was demonstrated in brain (Suppattapone et al., 1988). Second, phosphorylation of phospholipase C may decrease activity of this enzyme and subsequent production of IP_3 which mobilizes Ca^{2+} from internal stores, as suggested in studies with airway smooth muscle (Hall et al., 1989). Third, phosphorylation of a regulatory protein phospholamban in the SR membrane may enhance sequestration of Ca^{2+} into the SR of heart muscle (Exton, 1987). In other cell types, such as smooth muscle, other mechanism also participate; e.g., decreased activity of myosin light chain kinase at any given Ca/calmodulin level (Rasmussen et al., 1991).

6.2. Role of cAMP in Endothelial Cells

The role of endothelial cAMP in modulation of $[\text{Ca}^{2+}]_i$ is still unclear. Luckhoff

et al. (1990) reported that an increase in cAMP reduced ATP-induced Ca^{2+} release in bovine aortic endothelial cells. However, Brock et al (1988) and Buchan and Martin (1991) reported that ATP-, bradykinin- and thrombin-induced plateau phases of $[\text{Ca}^{2+}]_i$ was elevated by cAMP in the same tissue. Forskolin, isoproterenol also induced elevation of intracellular Ca^{2+} when added during the plateau phase of agonist-induced increases in $[\text{Ca}^{2+}]_i$ (Buchan and Martin, 1991).

Kuhn et al. (1990) reported that cAMP does not modulate EDRF release from bovine aortic endothelial cells. In pig aortic endothelial cells, however, it has been demonstrated that forskolin, adenosine and isoproterenol enhanced agonist-induced (bradykinin, ATP) rise in intracellular Ca^{2+} and subsequent formation of EDRF (Graier et al., 1992b; Gray and Marshall, 1992). None of the stimulators of adenylate cyclase alone affect $[\text{Ca}^{2+}]_i$ significantly. The effects of these stimulators were mimicked by the membrane permeable cAMP analogs (dibutyryl-cAMP) and were antagonized by the protein kinase C inhibitor, H-8 (N-[2- (methylamino)ethyl]-5-isoquinoline-sulphonamide dihydrochloride). These data suggest that cAMP-dependent phosphorylation modulates Ca^{2+} signalling in endothelial cells, but this process needs more elucidation.

CHAPTER II

OBJECTIVES AND HYPOTHESES

1. *Objective:* The Effects of VIP on Ion Channels in Endothelial Cells

Hypothesis: VIP modulates an outward Ca^{2+} -dependent K^+ channel and inwardly rectifying K^+ channel activity. The significance of this modulation is the fact that it may increase driving force for calcium entry, which is necessary for the hypothetical, VIP-stimulated production of EDRF.

Results: VIP activates an outward Ca^{2+} -dependent K^+ current and inhibits inwardly rectifying K^+ channel activity. Activation of K_{Ca} tends to hyperpolarize the cell membrane. Direct measurement of the potential EDRF release was not performed.

2. *Objective:* The Role of a G Protein in VIP-stimulated Endothelial Cells

Hypothesis: A G protein is involved in the transduction pathway between VIP receptor and K^+ channel.

Results: The VIP receptor couples to a G protein which *directly* modulates inwardly rectifying K^+ channels. This unidentified G protein is sensitive to a GTP analog (GTP- γ -S) and cholera toxin and resistant to pertussis toxin.

3. *Objective:* A VIP Transduction Pathway in Endothelial Cells

Hypothesis: Since VIP activates adenylate cyclase and production of cAMP in many cell types, it was expected that cAMP cascade is a VIP transduction pathway in endothelial cells.

Results: This study allows me to exclude cAMP as a second messenger for VIP action in endothelial cells. Direct evidence comes from the measurements of VIP-stimulated cAMP level. Electrophysiological experiments (the VIP effect on inwardly rectifying K⁺ channels in isolated patches, where the second messenger is not operating and the effect of exogenous cAMP activators on these channels) provide an indirect evidence allowing us to eliminate the cAMP cascade as the VIP transduction pathway.

4. *Objective:* Calcium Signalling in Endothelial Cells

Hypothesis: Modulation of the calcium fluxes at the level of internal stores influences an influx of extracellular calcium, which is necessary for EDRF production.

Results: A transient increase in intracellular Ca²⁺ due to depletion of

internal Ca^{2+} stores enhances an influx of extracellular calcium.
Calcium enters the endothelial cell through nonselective cation
channels.

CHAPTER III

METHODS AND RESULTS

PAPER No. 1

AN ENDOTHELIAL CELL-LINE CONTAINS FUNCTIONAL VASOACTIVE
INTESTINAL POLYPEPTIDE RECEPTORS: THEY CONTROL INWARDLY
RECTIFYING K⁺ CHANNELS

Published in the *European Journal of Pharmacology* in 1992

Ewa Pasyk's contribution:

- (i) cell culturing
- (ii) performance of all electrophysiological experiments

An endothelial cell-line contains functional vasoactive intestinal polypeptide receptors: they control inwardly rectifying K⁺ channels

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Received 2 May 1991, revised MS received 20 November 1991, accepted 3 December 1991

Bovine endothelial cells cultured from pulmonary artery (ATCC cell line No. 209) were found to contain a high density of [¹²⁵I]-VIP (vasoactive intestinal polypeptide) binding sites. These were found to be saturable and to be fit by a single binding site model (K_d 1.8 nM; B_{max} 534 fmol/mg protein). Studies of association and dissociation of [¹²⁵I]-VIP to this site revealed that binding was fully reversible and yielded a K_d value similar to that from equilibrium binding. However competition studies showed that VIP competed for binding at two sites (K_{i1} 1.2 × 10⁻¹¹ M, K_{i2} 4.7 × 10⁻⁹ M; N₁ = 21%, N₂ = 77%; K_i a dissociation constant for inhibitor; N percentage of occupied receptors). [Phe¹]VIP also competed at two sites, but VIP-(10-28), PHM, [4-Cl-D-Phe⁶,Leu¹⁷]VIP and [D-Ala⁴]VIP displaced all specific VIP binding in a simple competitive manner. These VIP binding sites were shown to be functional. In patch clamp studies VIP 10⁻⁸-10⁻⁷ M inhibited opening of inwardly rectifying K⁺ channels on hyperpolarization. These channels were affected appropriately by alteration in the K⁺-gradient and by Ba²⁺ or Cs⁺. The VIP antagonist [4-Cl-D-Phe⁶,Leu¹⁷]VIP prevented or reversed the effects of VIP. These results show that functional VIP receptors are present in high density in an endothelial cell line and provide a possible model for analysis of the molecular biology of these receptors.

Endothelial cells; Patch-clamp; VIP receptors; VIP analogs; VIP binding sites; [4-Cl-D-Phe⁶,Leu¹⁷]VIP

1. Introduction

VIP (vasoactive intestinal polypeptide) is a neuropeptide widely distributed in both the central and peripheral nervous systems. VIP has been demonstrated to act on a wide array of tissues, including the blood vessels, in various species. The mode of action of VIP on blood vessels is so far controversial for the fact that responses to this peptide have been reported that are either dependent on the presence of endothelium (Davies and Williams, 1984; Ganz et al., 1986; Itoh et al., 1985; Thom et al., 1987), or are independent (Brum et al., 1985; Duckles and Said, 1982; Greenberg et al., 1985; Sata et al., 1986; Schoeffer and Stoclet, 1985). Because of such conflicting reports, the status of VIP receptors on endothelial cells remains uncertain.

VIP receptors have been demonstrated on various

tissues by radioligand binding studies. Thus VIP receptors on bovine retina (Swedlund and Rosenzweig, 1990), guinea pig pancreas (Bissonnette et al., 1984), rat liver (Couvineau and Laburthe, 1985), rat intestinal epithelium (Laburthe et al., 1984), cultured colonic adenocarcinoma cells (Muller et al., 1985) and human lymphoblasts (O'Dorisio et al., 1981) have been characterized by radioligand binding studies.

Sata et al. (1988) have observed relaxation of rat aorta in response to added VIP and the accumulation of c-AMP; both of these responses were dependent on the presence of endothelium. Functional VIP receptors on cultured smooth muscle cells from rat aorta have also been demonstrated, which accumulate c-AMP in response to VIP (Hirata et al., 1985; Nabika et al., 1985).

To our knowledge, no such studies have been carried out on endothelial cells in order to discern the mode of action of VIP on blood vessels and to characterize these receptors. In the present studies, we have carried out radioligand binding studies, and have examined the electrophysiological response of cultured bovine pulmonary artery endothelial cells to VIP.

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2. Materials and methods

2.1. Cell culture

Cultured bovine pulmonary artery endothelial cells (ATCC cell line No. 209) have been used. This cell line was initiated from artery lumen scrapings dispersed by enzyme treatment. All of the electrophysiological experiments have been performed on individual confluent cells. These cells were slightly attached to the bottom of the Petri dish and appeared bright under phase contrast microscope.

2.2. Harvesting the cells for binding

The cells were obtained by scraping the confluent cultures with teflon cell scraper in phosphate buffered saline (PBS). The cells were then centrifuged at $300 \times g$ for 10 min and resuspended in PBS and frozen at -70°C .

2.3. Electrophysiology

Standard patch clamp techniques for whole cell recording have been employed (Hamill et al., 1981). Patch electrodes, which were heat polished, had resistances of 2–5 M Ω and were filled with intracellular solution containing (in mM): 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA and 20 HEPES. The pH of the intracellular solution was adjusted to 7.3 with KOH, and the solution was filtered before use (pore size 0.2 μm). The normal external bath solution contained (mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose, 10 HEPES and pH was adjusted to 7.3 with NaOH. High K⁺ external bath solution was made by replacing NaCl with KCl (pH 7.3 with KOH).

Test solutions were applied by perfusion (2–3 ml/min) or added to the bath to get final concentration as indicated. All experiments were conducted at room temperature. Currents were measured with an Axopatch-1C patch-clamp amplifier and recorded online by the computer (IBM-AT). Data were filtered with a lowpass Bessel filter (–3 dB at 1 KHz) and digitized at a sampling frequency of 5 KHz using pCLAMP software (Axon Instruments, Inc.).

2.4. ¹²⁵I-VIP Binding

¹²⁵I-VIP were purchased from Amersham (Arlington Heights, IL). VIP, VIP-(10–28), PHM, [4-Cl-D-Phe⁶,Leu¹⁷]VIP were from Peninsula Lab Inc. [D-Ala⁴]VIP and [Phe¹]VIP were supplied by Dr. D.H. Coy. The binding was performed in the reaction buffer consisting of 25 mM Tris-HCl, 2 mM MgCl₂, 1% BSA buffer pH 7.4. Twenty microliters of appropriately diluted ¹²⁵I-VIP were incubated with 150 μl cells (20–30

μg) diluted in the reaction buffer, 10 μl (200 KIU) aprotinin and 20 μl buffer (for total binding) or 10^{–8} M VIP (for nonspecific binding), each in triplicate, at 21°C for 30 min (the effect of temperature on binding in these cells was not examined). The reaction was then stopped by the addition of 3 ml ice cold buffer and immediate filtration over Whatman glass fibre GF/F filter discs (0.2% polyethyleneimine presoaked overnight). The tubes and filters were washed with additional 3 \times 3 ml buffer. The radioactivity retained on the filters was measured in a Beckman 5500 gamma scintillation counter. Non-specific binding of ¹²⁵I-VIP was about 25% of the total binding. The competition experiments were performed in triplicate with 100 pM ¹²⁵I-VIP. These procedures are similar to those described by Rorstad et al. (1990).

3. Results

Bovine pulmonary artery endothelial cells from the ATCC cell line express an inwardly rectifying K⁺ current ($n = 70$ cells) reported in other endothelial cell types (Takeda et al., 1987; Takeda and Klepper, 1990; Bregestovski and Ryan, 1989; Colden-Stanfield et al., 1990). This current (figs. 1A, 2A) resembles the classical inward rectifier of skeletal and cardiac muscle and osteoclasts (Standen and Stanfield, 1979; Sakmann and Trube, 1984; Sims and Dixon, 1989). Strong inward rectification of currents was observed in I–V curves (figs. 1C, 2C).

To demonstrate the K⁺-selective nature of the inward current, experiments were conducted with external bath solution made by replacing NaCl with KCl. When 140 mM [K⁺]_{out} was applied, the I–V curve of inward currents was shifted to the right along the voltage axis and crossed the axis at 0 mV, the value close to the expected K⁺ equilibrium potential predicted by the Nernst equation for symmetrical concentrations of K⁺ inside and outside the cell.

In another series of experiments effects of barium and cesium as blocking agents of inward rectifying K⁺ currents were examined. BaCl₂ (0.5 mM) suppressed the amplitude of the inward current (fig. 1). Similarly, CsCl (0.1 mM) caused reversible block of the inward current.

Finally, the effect of VIP on the inward rectifier was examined. When endothelial cells were exposed to exogenous VIP (10^{–8} to 10^{–7} M; $n = 15$ cells), the amplitude of the control current was reduced in all examined cells (fig. 2). The extent to which this current was reduced was different in different cells. In some cells VIP blocks the inward rectifier completely (fig. 3B). The recovery from the VIP effect was not observed until VIP antagonist was applied. When the VIP receptor antagonist ([4-Cl-D-Phe⁶,Leu¹⁷]VIP) was

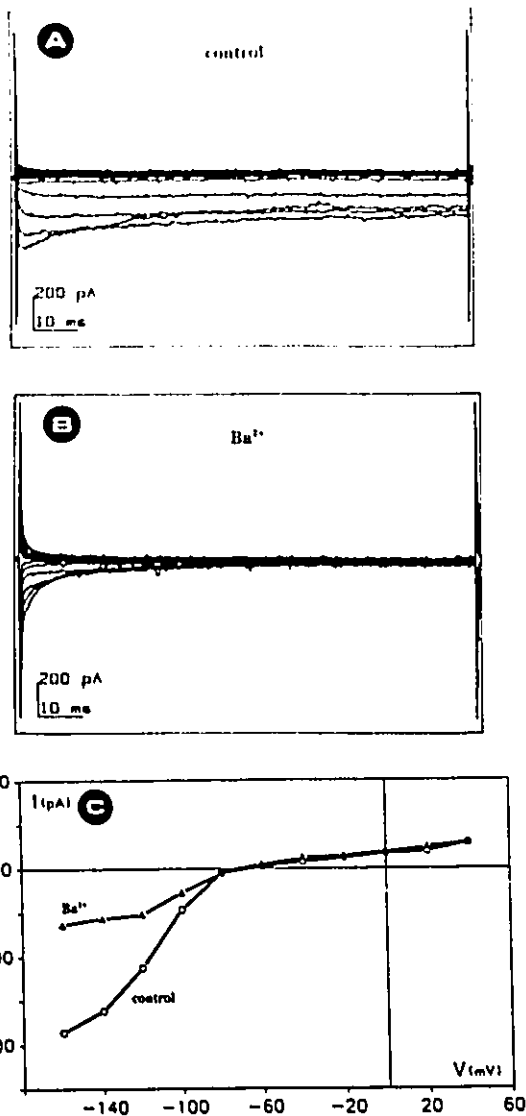


Fig. 1. Effect of barium on inward rectifier current in a single endothelial cell. (A) Control currents obtained with normal external solution for voltage steps given every 3 s in 20 mV steps over the range -160-40 mV from the holding potential of -50 mV. (B) Current traces after addition of BaCl₂ (0.5 mM) to the external solution. (C) The instantaneous current-voltage relationships for the families of currents shown in (A) and (B). Ba²⁺ blocked current amplitude at negative potentials. This figure is representative of three experiments.

applied exogenously, the VIP effect was fully reversible ($n = 4$ cells). The significant blockade of the effect of VIP on inward rectifier was obtained with this antagonist at a concentration of 10^{-6} M. Figure 3 shows one of the experiments where recovery from the VIP effect was obtained using this antagonist.

The binding of VIP increased linearly with increasing concentrations of the radioactive ligand. Binding was saturable and Scatchard transformation of the data revealed the apparent homogeneity of the binding sites (fig. 4) as inferred from the linearity of the Scatchard

plot and the value of the Hill coefficient close to unity ($nH = 0.98 \pm 0.02$). The affinity constant, K_d was 1.8 ± 0.65 nM and maximum number of binding sites (B_{max}) was 534 ± 114 fmol/mg protein. Association and dissociation rate constants measured by three determinations using 100-200 pM ¹²⁵I-VIP binding to endothelial cells at 21°C were $0.09 \text{ min}^{-1} \text{ nM}^{-1}$ and 0.107 min^{-1} , respectively (fig. 5), the K_d was 1.19 nM, calculated from the association and dissociation rate constants. This is consistent with the K_d value from saturation. VIP and its analogues competed for binding to the endothelial cell VIP receptors with differing potencies. Native VIP competed most effectively (fig. 6). The

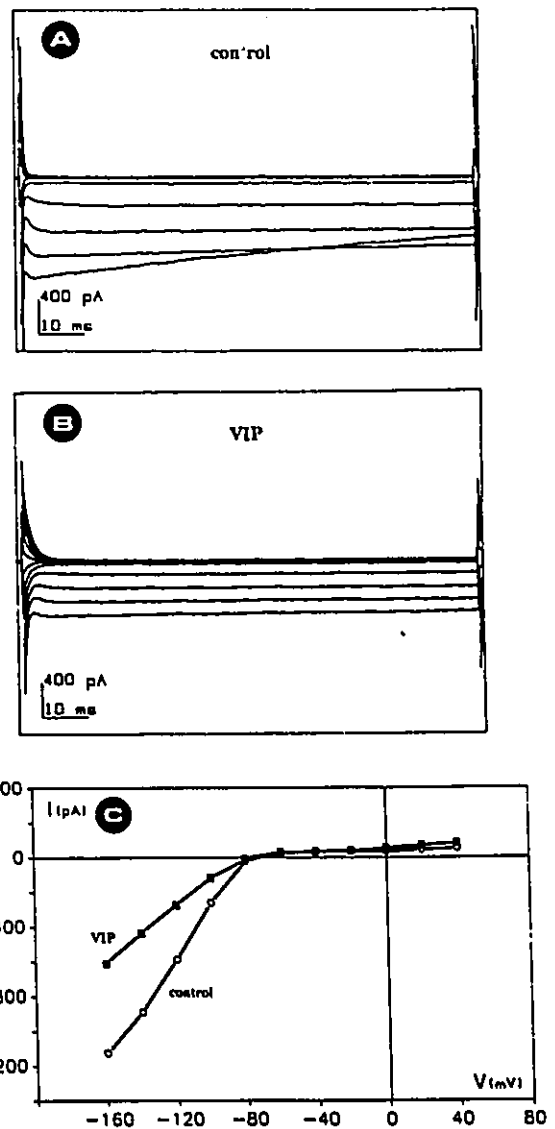


Fig. 2. Effect of VIP on inward rectifying K⁺ current in an endothelial cell. (A) Current evoked by command pulses given every 3 s in 20 mV steps over the range -160-40 mV from the holding potential of -50 mV. (B) VIP (10^{-8} M) reduces the amplitude of the inward current. (C) The instantaneous voltage relationships for the currents in normal external solution (control) and after addition of VIP. This figure is representative of 15 experiments.

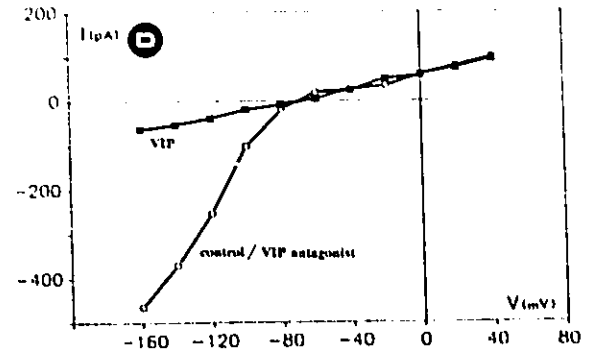
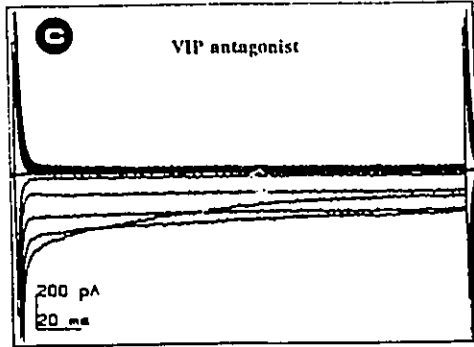
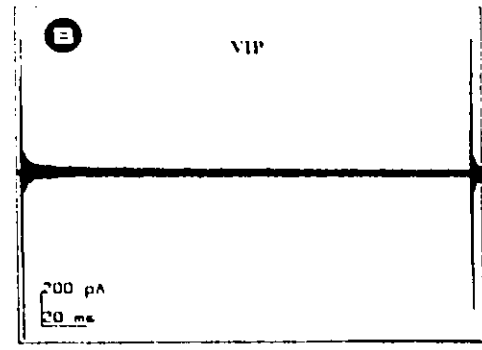
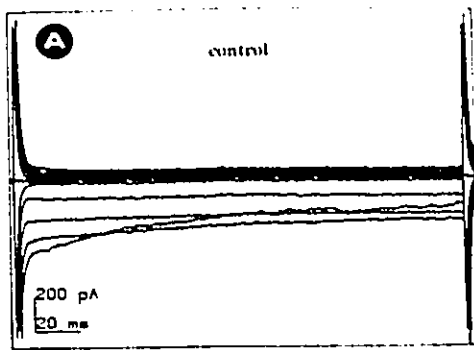


Fig. 3. Effect of VIP and VIP antagonist on inward rectifying K^+ current in an endothelial cell. (A) Control currents evoked by command voltages given every 3 s in 20 mV steps over the range -160 – -40 mV from the holding potential -60 mV. (B) VIP (10^{-7} M) blocks the inward current. (C) VIP antagonist (10^{-6} M) causes full recovery from the VIP effect. (D) The instantaneous I–V curves for the currents in normal solution ('control'), after addition of VIP and after applying VIP antagonist (superimposed with 'control'). This figure is representative of four experiments.

competition curve of the highly homologous peptides PHM, VIP-(10–28), $[Phe^1]VIP$ were approximately parallel to and shifted to the right of the competition curve of VIP. The potency order of competition was $VIP > [Phe^1]VIP > [D-Ala^4]VIP > [4-Cl-D-Phe^6,Leu^{17}]VIP > PHM > VIP-(10-28)$. The IC_{50} values of these peptides are shown in table 1. However, as is apparent from fig. 6A,B and table 1, the slopes of

displacement for VIP and $[Phe^1]VIP$ were significantly less than unity, an observation indicative of the presence of multiple binding sites. Computer analysis of the non-linear best fit by the CDATA program 87 (EMF software, Knoxville, TN) consistently gave significantly better fit for a two site model as compared to a one site model. However, many peptides including PHM, VIP-(10–28), $[4-Cl-D-Phe^6,Leu^{17}]VIP$ and $[D-Ala^4]VIP$ failed to interact with high affinity at any site. $[Phe^1]VIP$ did interact with both high and low affinity sites such as was reported for vascular smooth muscle VIP receptors (Rorstad et al., 1990).

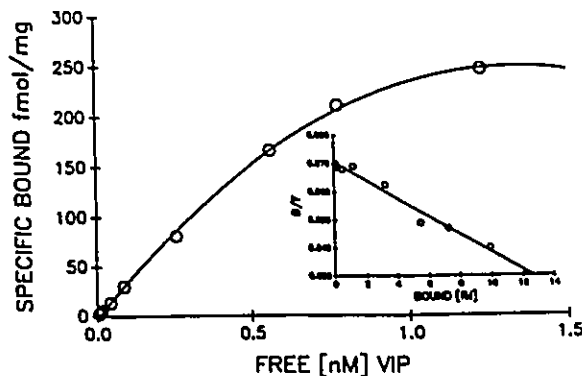


Fig. 4. Binding of ^{125}I -VIP as a function of increasing concentration of the radioligand. For binding conditions refer to the Methods section. Inset depicts the computer-generated Scatchard plot of the same data. The plot, a representative of three such experiments performed in triplicate for total and non-specific binding.

4. Discussion

This study shows that a bovine endothelial cell line contains a high density of functional VIP receptors. Activation of these receptors by VIP raises c-AMP levels (Ahmad, unpublished) and inhibits opening of inwardly rectifying K^+ -channels on hyperpolarization. It remains to be demonstrated whether c-AMP elevation is the basis of the K^+ -channel effect. It also remains to be demonstrated that the G-protein involved in VIP receptor interaction which acts to raise

TABLE I

Two site analysis of competition data (n = 3). K_{d1} is dissociation constant for competitors at high affinity binding sites. When only one binding site was found, the K_d value was placed under K_{d1} . K_{d2} is dissociation constant for competitors at low affinity binding sites. N_1 is percentage of high affinity receptors, N_2 is percentage of low affinity receptors. Slopes are from pseudo-Hill plots.

Compound	IC_{50} (nM)	K_{d1} (nM)	N_1 (%)	K_{d2} (nM)	N_2 (%)	Slope
VIP	3.17 ± 0.75	0.012 ± 0.01	21.30 ± 4.48	4.66 ± 0.97	77 ± 5.20	0.43 ± 0.15 ^a
VIP (10-28)	730 ± 462	505 ± 321				0.85 ± 0.14
PHM	469 ± 140	439 ± 140				0.85 ± 0.11
[4Cl-D-Phe ⁶ ,Leu ¹⁷]VIP	16.9 ± 10.9	11.4 ± 7.4				0.74 ± 0.11
[Phe ¹]VIP	6.30 ± 3.55	1.69 ± 0.17	60.99 ± 5.90	208 ± 39	33.10 ± 6.56	0.52 ± 0.14
[D-Ala ⁴]VIP	13.9 ± 2.8	15.8 ± 2.3				0.96 ± 0.17

^a Significantly less than 1 ($P < 0.05$).

e-AMP is G_i and that this interacts directly or indirectly with inwardly rectifying K^+ -channels.

VIP does have actions on endothelial cells *in vivo* as documented in the introduction. However as noted there seems to be variation in whether VIP-induced vasodilation is endothelial-dependent (suggesting that VIP acts to release EDRF (endothelium-derived relaxing factor) or another endothelial relaxing factor) or independent (suggesting an action on smooth muscle). It is unclear how inhibition of opening of inwardly

rectifying K^+ -channels might contribute to release of EDRF. More detail of the underlying electrophysiological mechanisms of EDRF release is needed to evaluate how VIP actions would affect release of relaxing factors.

The physiological significance of VIP action to inhibit opening of K^+ -channels on hyperpolarization of these cells is unclear. If other hyperpolarizing influences in a cell bring the membrane potential to values more negative than the resting potential, closing of inwardly rectifying K^+ -channels by VIP may contribute to repolarization. These channels may also contribute to the determination of the resting potential and inhi-

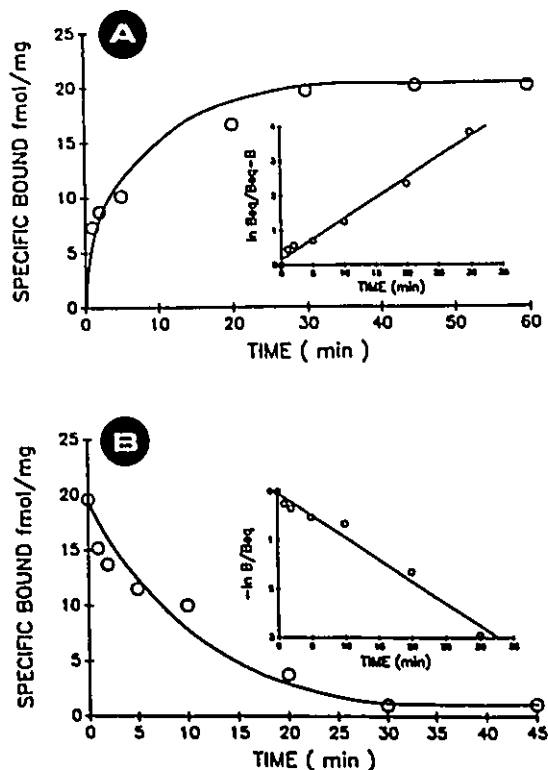


Fig. 5. Kinetics of association (A) and dissociation (B) of ^{125}I -VIP binding to endothelial cells. Reaction was started by addition of endothelial cells (20 μ g) to incubation tubes 100–200 pM radioligand. Dissociation was initiated after 30 min by addition of excess (1 μ M) unlabelled VIP. This figure is representative of three such experiments done in triplicate ($k_{+1} = 0.09 \text{ min}^{-1} \text{ nM}^{-1}$, $k_{-1} = 0.107 \text{ min}^{-1}$, $K_d = 1.19 \text{ nM}$).

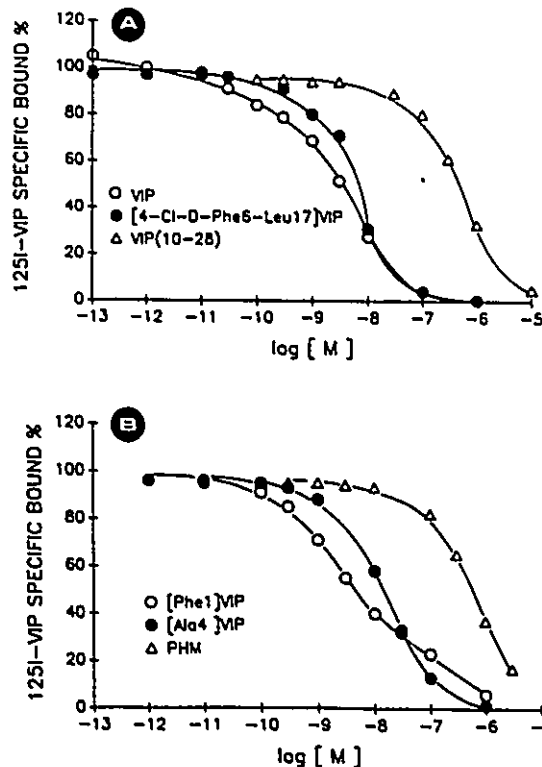


Fig. 6. (A,B) Competition of ^{125}I -VIP binding. This figure is representative of three such experiments done in triplicate. Graphs show computer generated displacement curves and results are plotted as percentage of specific binding in absence of any competition.

bition of their opening by VIP would prevent this and enhance recovery of excitability.

Rorstad et al. (1990) reported that vascular and neural VIP binding sites could be distinguished by several VIP analogs. Among those ligands we examined, [D-Ala⁴]VIP was 7–8 times more potent than [Phe¹]VIP in displacing VIP from binding on nerves and 2–4 times less potent in displacing VIP from binding on blood vessels. In our studies [D-Ala⁴]VIP was less potent than [D-Phe¹]VIP, more like vascular binding sites. Moreover, [¹²⁵I]-VIP binding was displaced by [D-Phe¹]VIP with a shallow slope suggesting two binding sites, one high affinity and one low affinity. [D-Ala⁴]VIP displacement revealed only one binding site like the antagonist [4-Cl-D-Phe⁶,Leu¹⁷]VIP. Thus, these VIP receptors may resemble receptors studied by Rorstad et al. (1990) on blood vessels and these receptors appear to be functional.

We have shown previously (Ahmad et al., 1990) that the same cell line possess β -adrenoceptors. It is possible that both β -adrenoceptors and VIP receptors control inwardly rectifying K⁺-channels; this is currently under study. In conclusion, this study demonstrates that an endothelial cell line stably expresses functional VIP receptors. This cell provides a readily available model for further study of the molecular pharmacology of VIP receptors and their function.

Acknowledgements

Supported by the Medical Research Council and the National Science and Engineering Council of Canada.

References

- Ahmad, S., P. Chretien, E.E. Daniel and S.H. Shen, 1990, Characterization of beta adrenoceptors on cultured endothelial cells by radioligand binding, *Life Sci.* 47, 2365.
- Bissonnette, B.M., M.J. Collen, H. Adachi, R.T. Jensen and J.D. Gardner, 1984, Receptors for vasoactive intestinal peptide and secretin on rat pancreatic acini, *Am. J. Physiol.* 246, G710.
- Bregestovski, P. and U.S. Ryan, 1989, Voltage-gated and receptor-mediated ionic currents in the membrane of endothelial cells, *J. Mol. Cell Cardiol.* 21 (Supplement 1), 103.
- Brum, J.M., V.L.W. Go, P.M. Vanhoutte and A.A. Bove, 1985, Evidence for VIP-ergic control of coronary vasoregulation, *Reg. Pept.* 3, S37 (Abstract).
- Colden-Stanfield, M., W.P. Shilling, L.D. Possani and D.L. Kunze, 1990, Bradykinin-induced potassium current in cultured bovine aortic endothelial cells, *J. Membr. Biol.* 116, 227.
- Couvineau, A. and M. Laburthe, 1985, The rat liver vasoactive intestinal peptide binding site. Molecular characterization by covalent cross-linking and evidence for differences from the intestinal receptor, *Biochem. J.* 225, 473.
- Davies, J.M. and K.I. Williams, 1984, Endothelial-dependent relaxant effects of vaso-active intestinal polypeptide and arachidonic acid in rat aortic strips, *Prostaglandins* 27, 195.
- Duckles, S.P. and S.I. Said, 1982, Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation, *Eur. J. Pharmacol.* 78, 371.
- Gane, P., A.W. Sandrock, S.C. Landis, J. Leopold, M.A. Gimborne Jr. and R.W. Alexander, 1986, Vasoactive intestinal peptide, vasodilation and cyclic AMP generation, *Am. J. Physiol.* 251, H755.
- Greenberg, B., K. Rhoden and P. Barnes, 1985, Endothelial relaxant factor is present in human pulmonary artery, *Circulation* 72, Suppl. III, 265.
- Hamill, O.P., A. Marty, E. Neher, B. Sakmann and E.J. Sigworth, 1981, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflügers Arch.* 391, 85.
- Hirata, Y., M. Tomita, S. Takata and T. Fujita, 1988, Functional receptors for vasoactive intestinal peptide in cultured vascular smooth muscle cells from rat aorta, *Biochem. Biophys. Res. Commun.* 132, 1079.
- Itoh, T., T. Sasaguri, Y. Makita, Y. Kanmura and H. Kuriyama, 1985, Mechanisms of vasodilation induced by vasoactive intestinal polypeptide in rabbit mesenteric artery, *Am. J. Physiol.* 249, H231.
- Laburthe, M., B. Breant and C. Rouyer-Fessard, 1984, Molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium by covalent cross-linking. Evidence for two classes of binding sites with different structural and functional properties, *Eur. J. Biochem.* 139, 181.
- Muller, J.M., J. Louis, J. Fantini, B. Abadie, F. Giannellini, J. Marvaldi and J. Pichon, 1985, Covalent cross-linking of vasoactive intestinal peptide (VIP) to its receptor in intact colonic adenocarcinoma cells in culture (HT 29), *Eur. J. Biochem.* 151, 411.
- Nabika, T., Y. Nara, Y. Yamori, W. Lovenberg and J. Endo, 1985, Angiotensin II and phorbol ester enhance isoproterenol and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells, *Biochem. Biophys. Res. Commun.* 131, 30.
- O'Dorisio, M.S., N.S. Hermina, T.M. O'Dorisio and S.P. Balcerzak, 1981, Vasoactive intestinal polypeptide modulation of lymphocyte adenylate cyclase, *J. Immunol.* 127, 271.
- Rorstad, O.P., I. Wanke, D.H. Coy, A. Fournier and M. Huang, 1990, Selectivity for binding of peptide analogs to vascular receptors for vasoactive intestinal peptide, *Mol. Pharmacol.* 37, 971.
- Sakmann, B. and G. Trube, 1984, Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart, *J. Physiol. (London)* 347, 641.
- Sata, T., H.P. Misra, E. Kubota and S.I. Said, 1986, Vasoactive intestinal polypeptide relaxes pulmonary artery by an endothelium-independent mechanism, *Peptides* 7, Suppl. 1, 225.
- Sata, T., T. Linden, L.W. Liu, E. Kubota and S.I. Said, 1988, Vasoactive intestinal peptide evokes endothelium-dependent relaxation and cyclic AMP accumulation in rat aorta, *Peptides* 9, 853.
- Schoeffer, P. and J.C. Stoclet, 1985, Effect of vasoactive intestinal polypeptide (VIP) on cyclic AMP level and relaxation in rat isolated aorta, *Eur. J. Pharmacol.* 109, 275.
- Sims, S.M. and S.J. Dixon, 1989, Inwardly rectifying K⁺ current in osteoclasts, *Am. J. Physiol.* 256 (Cell. Physiol. 25) C1277.
- Standen, N.B. and P.R. Stanfield, 1979, Potassium depletion and sodium block of potassium currents under hyperpolarization in frog sartorius muscle, *J. Physiol. (London)* 294, 497.
- Swedlund, A.P. and S.A. Rosenzweig, 1990, Characterization of vasoactive intestinal peptide receptors in retina, *Exp. Eye Res.* 51, 317.
- Takeda, K., V. Schini, H. Stoeckel, 1987, Voltage-activated potassium, but not calcium currents in cultured bovine aortic endothelial cells, *Pflügers Arch.* 410, 385.
- Takeda, K. and M. Klepper, 1990, Voltage-dependent and agonist-activated ionic currents in vascular endothelial cells: A review, *Blood Vessels*, 27, 169.
- Thom, S., A. Hughes, G. Martin and P.S. Sever, 1987, Endothelium-dependent relaxation in isolated human arteries and veins, *Clin. Sci.* 73, 547.

PAPER No. 2

CYCLIC AMP IS NOT A SECOND MESSENGER FOR TRANSDUCTION OF
VIP EFFECTS ON INWARDLY RECTIFYING K⁺ CURRENTS IN
ENDOTHELIAL CELLS

In revision for the *European Journal of Pharmacology*

Ewa Pasyk's contribution:

- (i) cell culturing
- (ii) preparation of cells for cAMP measurements
- (iii) performance of all electrophysiological experiments

CYCLIC AMP IS NOT A SECOND MESSENGER FOR TRANSDUCTION OF
VASOACTIVE INTESTINAL PEPTIDE EFFECTS ON INWARDLY
RECTIFYING K⁺ CURRENTS IN ENDOTHELIAL CELLS

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Abstract

Our studies provide both direct and indirect evidence suggesting that activation of vasoactive intestinal peptide (VIP) receptors does not lead to an elevation of cyclic AMP (cAMP) level in bovine pulmonary artery endothelial cells. Direct evidence comes from the measurements of cAMP level after stimulation with VIP and isoproterenol. Isoproterenol increases cAMP in endothelial cells about 120-130% over the basal. VIP does not elevate cAMP in endothelial cells and even decreases it in some cases. In addition, in the patch-clamp experiments VIP exerted inhibitory effect on the inwardly rectifying K^+ current ($I_{K_{in}}$) (about 62% decrease). The effect of isoproterenol on this current was very weak (about 14% decrease). The magnitudes of effects evoked by other activators of the cAMP cascade (forskolin, cAMP analogs) on this channel were intermediate between those of VIP and isoproterenol. Although cAMP elevation can reduce the activity of $I_{K_{in}}$ channel in endothelial cells, it is not responsible for the inhibitory effect of VIP on this channel.

VIP receptor may be directly coupled to these channels or may act through a second messenger other than cAMP.

cAMP, VIP, isoproterenol, inward rectifier, patch-clamp

Introduction

Endothelium consists of a single layer of cells, which line the inner wall of the blood vessels and play an important role in the control of vascular reactivity. Several physiological functions of endothelium are mediated through the receptors located on the cell membrane. Activation of these receptors leads to an increase in the intracellular Ca^{2+} concentration (Johns et al., 1987). This increase in the Ca^{2+} activity is the trigger for synthesis and release of a number of relaxing or contracting factors, such as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) and endothelin (Yanagisawa et al., 1988). EDRF, which recently has been identified as nitric oxide or a closely related compound (Palmer et al., 1987), seems to play a key role in the regulation of the vascular tone.

Recently it was demonstrated that cultured bovine pulmonary artery endothelial cells contain a high density of vasoactive intestinal peptide (VIP) receptors (Pasyk et al., 1992). It has been known for a long time that VIP is capable of relaxing blood vessels. In endothelial-dependent relaxation by VIP (Davies and Williams, 1984; Thom et al., 1987), activation of the endothelial VIP receptors may lead to release of nitric oxide or another relaxant factor. Studies in the previous paper characterized binding properties of VIP to its endothelial receptors and demonstrated that these receptors are functional. Activation of VIP receptors inhibits inwardly rectifying K^+ current, the predominant current found in these cells observed in the whole-cell configuration.

VIP receptors have been shown to be in several tissues, intestinal epithelium (Laburthe et al., 1984), pancreas (Bissonnette et al., 1984), liver (Couvineau and Laburthe, 1985). In most of the tissues stimulation of VIP receptors led to activation of adenylate cyclase and an increase of the intracellular cyclic AMP (cAMP). Stimulation of VIP receptors in cultured rat aorta smooth muscle caused an increase in cAMP level (Hirata et al., 1985; Nabika et al., 1985). Sata et al. (1988) have reported a VIP stimulated elevation of cAMP and relaxation of rat aorta in an endothelium-dependent manner.

Although VIP activates adenylate cyclase and production of cAMP in many cell types, this study presents evidence allowing us to exclude cAMP as a second messenger for VIP action in endothelial cells. cAMP elevation can be associated with reduced inwardly rectifying K^+ channel activity in bovine pulmonary artery endothelial cells, but is not a mediator for the inhibitory effect of VIP on these channels.

Materials and Methods

Cell culture

Cultured bovine pulmonary artery endothelial cells (American Type Culture Collection, ATCC cell line #209) have been used. This cell line was initiated from artery lumen scrapings dispersed by enzyme treatment. All of the experiments have been performed on individual confluent cells.

2.2. Cyclic AMP measurements

Cells were propagated into 25 cm² tissue culture flasks (FALCON, Franklin Lakes, NJ.). When the estimated number of cells in each flask reached 0.3 - 0.5 x 10⁶ (after 12 - 24 hours), the medium was aspirated and cells were rinsed 3 times with Hank's Basic Salt Solution (GIBCO BRL, Burlington, Ont.). After the rinsing, small amount of Hank's solution (1 ml) was added to each flask to cover the cell layer. Cells were incubated in CO₂ incubator for 30 - 45 min. at 37⁰C. Then cells were exposed to isoproterenol (10⁻⁵ M, 10⁻⁶ M) for 4 minutes or VIP (10⁻⁶ M, 10⁻⁷ M) for 5 min. Some cells were preincubated in 3-isobutyl-1-methylxantine (IBMX) for 10 min. IBMX (5x10⁻⁴ M) was added to 1 ml of Hank's solution at the end of 45-min. incubation. After the incubation the cells were lysed by the addition of 10% trichloroacetic acid (TCA). All liquid from each flask was put into a test tube and was then dried in a Speed Vac.

For cAMP levels determination, cAMP Scintillation Proximity assay kit (Amersham, Oakville, Ont.) was used. The buffer from the kit was used to dissolve the product in each test tube; this solution was then assayed. The experiment was done on 4 different occasions. Each time one flask was set aside for protein determination, and two for basal level output determination. For basal level the cells were not exposed to VIP or isoproterenol, and lysed with TCA. For protein determination the cells could be preincubated with IBMX but then the Hank's solution was removed and the cells were covered with 1 ml distilled H₂O. Then cells were dried down, redissolved, and assayed by protein assay kit (PIERCE, Rockford, IL).

Electrophysiology

Standard patch clamp techniques for whole cell recording have been employed (Hamill et al., 1981). Patch electrodes were heat polished and had resistances of 3 - 6 M Ω . They were filled with intracellular solution containing (in mM): 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES and 0.5 ATP. The pH of the intracellular solution was adjusted to 7.3 with KOH, and the solution was filtered before use (pore size 0.2 μ m). The normal external bath solution contained (mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose, 10 HEPES and the pH was adjusted to 7.3 with NaOH. High K external bath solution was made by replacing NaCl with KCl (pH = 7.3 with KOH). Na⁺ free solution was obtained by replacing NaCl with N-methylglucamine (pH = 7.3 with HCl). All experiments were conducted at room temperature.

Currents were measured with an Axopatch-1C patch-clamp amplifier and recorded on line by a computer (IBM-AT). Data were filtered with a low pass Bessel filter (-3 dB at 1 KHz) and digitized at a sampling frequency of 5 KHz using pCLAMP software (Axon Instruments, Inc.). The whole-cell currents were not corrected for leakage. Series resistance and capacitance transients were not compensated. The transient currents were subtracted using pCLAMP software only in some cells (figs. 3, 5). Instantaneous currents for I-V curve were measured after settling of capacitive currents, usually between 10th to 20th ms after the beginning of the voltage pulses. Test solutions were applied by perfusion (2 - 3 ml/min) or added to the bath to get a final concentration as indicated.

Chemicals

The following drugs were used: forskolin (Sigma, St Louis, MO, USA) and 1,9-dideoxyforskolin (Calbiochem, La Jolla, CA, USA), both dissolved in dimethyl sulphoxide (DMSO); 8-bromoadenosine 3':5'-cyclic monophosphate, N⁶,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate, isoproterenol, arterenol, timolol, 3-isobutyl-1-methylxanthine (IBMX), trichloroacetic acid (TCA) (Sigma, St Louis, MO, USA); vasoactive intestinal peptide (VIP) (Calbiochem, La Jolla, CA).

Results

Cyclic AMP level after stimulation with VIP and isoproterenol.

Conditions (agonist concentrations) in cAMP assay were kept similar to the patch-clamp techniques for comparison. Although patch-clamp experiments were done in the absence of IBMX, an inhibitor of phosphodiesterase (Kebabian, 1992), measurements of cAMP level were performed both in the presence and absence of IBMX. In the presence of IBMX, the absolute basal level of cAMP was significantly increased (fig. 1 B).

In both cases, in the presence or absence of IBMX, isoproterenol (10^{-6} M and 10^{-5} M) increased cAMP level over basal (fig. 1 A, B). A higher increase was observed with IBMX (about 130%; fig. 1 B). VIP (10^{-7} M and 10^{-6} M) did not affect cAMP levels in assay with IBMX and even appeared to decrease cAMP level in the absence of IBMX, especially at concentration of 10^{-7} M (fig. 1 A).

Statistical difference among these four group means (VIP 10^{-7} , VIP 10^{-6} , ISO 10^{-6} , ISO 10^{-5}) was compared by one way analysis of variance (ANOVA). The difference among the group means in the absence of IBMX (VIP 10^{-7} vs. ISO 10^{-6} and VIP 10^{-7} vs. ISO 10^{-5}) was significant ($p < 0.05$). In the presence of IBMX, the difference among the group means (VIP 10^{-6} vs. ISO 10^{-6} and VIP 10^{-6} vs. ISO 10^{-5}) was marginally significant ($p \geq 0.05$). Data are expressed as mean \pm SEM.

Inward rectifier K⁺ current.

The inward rectifier was the predominant current observed in the whole-cell configuration in bovine pulmonary artery endothelial cells. It was activated by hyperpolarization to -160 mV from a holding potential of -70 or -60 mV. Depolarization (up to 40 mV) elicited only a small outward current. Similar to other inward rectifiers, increased extracellular K⁺ (140 mM KCl) in bovine pulmonary artery endothelial cells shifted the reversal potential close to 0 mV ($n = 3$, data shown in APPENDIX I) (Bregestovski and Ryan, 1989). The current was suppressed by inward rectifier K⁺ - channel blocking agents such as Ba²⁺, Cs⁺ and Na⁺ ($n = 7$; data not shown) (Takeda and Klepper, 1990).

Effects of isoproterenol and norepinephrine on the inward rectifier.

When isoproterenol or norepinephrine, the β -adrenergic agonists, were applied to the endothelial cells, they exerted a relatively weak effect; i.e., they slightly reduced the amplitude of the inward rectifier current (fig. 2). These effects were reversible.

The number of cells stimulated with isoproterenol was $n = 23$, with norepinephrine $n = 5$ (data not shown). The magnitude of this reduction was about 14% of the control (table 1). Isoproterenol and norepinephrine concentration ranges were between 10^{-6} to 10^{-4} M. Timolol (5×10^{-5} M), a non-selective β adrenergic antagonist, blocked the effect of norepinephrine (10^{-5} M) ($n = 3$ cells; data not shown).

Effects of forskolin and cAMP analogs on the inward rectifier.

Forskolin (10^{-6} to 10^{-5} M), an activator of adenylate cyclase, reduced the amplitude of the inward rectifier current (figs. 3 and 4; $n = 10$ cells). In some cells the effects were fully or partially reversible. Forskolin not only reduced the $I_{K_{in}}$ channel activity, but also slowed down the inactivation kinetics of these channels at the potential of -160 mV (fig. 3). 1,9-Dideoxyforskolin (10^{-6} to 10^{-5} M), a forskolin analog which does not activate adenylate cyclase (Laurenza et al., 1989), did not affect the inward current when applied extracellularly in the whole cell configuration ($n = 4$ cells; data not shown). The membrane-permeant cAMP analogs, 8-Br-cAMP and dibutyryl-cAMP (2 - 3 mM) were used to mimic the production of intracellular cAMP. These analogs reversibly reduced the inward rectifier K^{+} currents (fig. 5; for 8-Br-cAMP $n = 4$ cells; for db-cAMP $n = 3$, data not shown). The time course of changes in $I_{K_{in}}$ current after application of forskolin and 8-Br-cAMP are shown in fig. 6 A. For comparison time course of VIP and isoproterenol action is given in fig. 6 B.

Comparison of the magnitude of the effects on the inward rectifier.

Table 1 shows the average percentage decrease in the amplitude of the inwardly rectifying K^+ current after application of VIP (10^{-7} M), isoproterenol (10^{-6} M), forskolin (10^{-6} M) and 8-Br-cAMP (10^{-3} M). VIP data were taken from the previous paper (Pasyk et al., 1992). All cells tested with a drug at the indicated concentrations were taken into consideration in calculations.

Statistical difference between two measurements, control and drug-affected currents was compared by paired t test and was significant ($p < 0.009$). Statistical difference among all four group means shown in table 1 (average decrease with VIP, isoproterenol, forskolin and 8-Br-cAMP) was compared by one way analysis of variance (ANOVA). This difference was also significant ($p < 0.0001$).

Discussion

This study presents evidence that stimulation of recently described endothelial VIP receptors (Pasyk et al., 1992) leading to a decrease in $I_{K_{in}}$ currents does not depend upon an increase in intracellular cAMP level. Since VIP activates adenylate cyclase and production of cAMP in many cell types (Hirata et al., 1985; Nabika et al., 1985; Sata et al., 1988), it was expected that cAMP may also serve as a second messenger for VIP transduction pathway in endothelial cells. This expectation was initially reinforced by the observation that other agents like forskolin or 8-Br-cAMP, which are known to elevate cAMP level also inhibited $I_{K_{in}}$ currents. However, our

studies provide both direct and indirect evidence allowing us to rule out cAMP as a second messenger for VIP action in endothelial cells. Direct evidence comes from the measurement of cAMP levels in bovine pulmonary artery endothelial cells after stimulation with VIP and isoproterenol. Isoproterenol is known to raise cAMP in many endothelial cell types (McEwan et al., 1990). Our cAMP level measurements confirmed this mode of isoproterenol action in bovine pulmonary artery endothelial cells (fig. 1). Stimulation with VIP did not elevate basal cAMP level in our cells. In some cases, VIP seemed even to decrease the basal cAMP production (fig. 1 A) (discussed below). In the presence of IBMX, an inhibitor of phosphodiesterase, the enzymes which catabolize cyclic nucleotides, both basal and agonist-stimulated levels of cAMP were increased, as expected. VIP did not elevate cAMP production, however, above the basal level (fig. 1 B).

Indirect evidence suggesting that cAMP is not a second messenger for VIP transduction signalling in endothelial cells comes from patch-clamp experiments. The effects of isoproterenol and VIP on $I_{K_{in}}$ currents, the predominant currents in endothelial cells, were significantly different (table 1). VIP strongly reduced or blocked $I_{K_{in}}$ channel activity (Pasyk et al., 1992), whereas the inhibitory effect of isoproterenol on this channel was very weak (about 20% of the VIP effect) (fig. 2). Recent competition binding studies in this cell line suggested that about 25% of total population of receptors are β_1 type and the remaining 75% is the mixture of β_2 type and atypical β receptors (Ahmad et al., 1990). Although the inhibitory effect of β adrenergic agonists on $I_{K_{in}}$ was very weak (about 14% of the control), the effect of

norepinephrine (10^{-5} M) was reversed by timolol (5×10^{-5} M), an unselective β adrenergic antagonist (Barnes, 1988). This timolol mode of action confirmed the involvement of β adrenoceptors.

To elucidate further how cAMP affects $I_{K_{in}}$ channels in bovine pulmonary artery endothelial cells, we applied activators of the cAMP cascade, forskolin and cAMP analogs. Forskolin, a potent agonist-independent activator of adenylate cyclase, and the membrane-permeant cAMP analogs (8-Br-cAMP and db-cAMP) reduced the $I_{K_{in}}$ current (figs. 3, 4, 5). The magnitudes of the effects evoked by these compounds were intermediate to those evoked by VIP and isoproterenol (table 1). It is likely that very weak inhibitory effect of isoproterenol and relatively weak inhibitory effect of forskolin and cAMP analogs on the $I_{K_{in}}$ channel activity can be explained by cAMP-dependent phosphorylation of the $I_{K_{in}}$ channel. It has been demonstrated in many cell types that cAMP-dependent protein kinase phosphorylates the K^{+} channel and this process changes activity of the channel (Kebabian, 1992).

Since forskolin was reported to exert also some other effects than stimulation of adenylate cyclase in some cells, as a control we applied 1,9-dideoforskolin ($n = 4$ cells; data not shown), a forskolin analog which does not activate adenylate cyclase (Laurenza et al., 1989). Forskolin (10^{-6} M) did have a moderate inhibitory effect on the $I_{K_{in}}$ current in these cells (table 1 and fig. 3). An additional aspect of forskolin effect on the $I_{K_{in}}$ current was an observation that forskolin slowed down the inactivation kinetics of this current at the potential of -160 mV (fig. 4). Inactivation kinetics was not affected by cAMP analogs or isoproterenol, whereas VIP changed

it in only about 10% of examined cells (Pasyk et al., 1992). The effect of forskolin on inactivation kinetics has been demonstrated already in other cell types (Hoshi et al., 1988; Krause et al., 1988). This action of forskolin in endothelial cells requires further elucidation. Since it was not produced by the inactive analog, it appears to be a consequence of an effect on protein kinase A.

The difference in mechanism of action of forskolin and cAMP analogs was reflected in the time course of their effects, as well as in inactivation kinetics. 8-Br-cAMP acted within 30 s, while a forskolin effect was observed after 6 to 15 min. (fig. 6 A). Forskolin activates adenylate cyclase which catalyses a reaction leading to an elevation in cAMP, whereas 8-Br-cAMP or db-cAMP are stable cAMP analogs which readily cross the membrane. The time delay in forskolin action may be caused not only by the time required for adenylate cyclase activation, but also by the distribution, the concentration or other properties of this enzyme in the endothelial cell membrane. In comparison, the effect of VIP and isoproterenol on the $I_{K_{in}}$ current was observed after 2 to 4 minutes (Fig. 6 B). Despite variations in the quantitative extent of activation, studies with forskolin and cAMP analogs strongly suggest that cAMP elevation can inhibit $I_{K_{in}}$ currents.

Additional evidence allowing us to rule out cAMP as a second messenger for VIP action in endothelial cells is the effect of VIP on the $I_{K_{in}}$ activity in the isolated patches. Although in isolated patches a second messenger system is considered not to operate (see also Chapter IV), VIP was still able to inhibit $I_{K_{in}}$ channel activity in outside-out patches. This effect was mediated by a G protein that most likely

couples directly to the channel (Pasyk and Daniel, submitted).

Our evidence, however, does not exclude existence of a putative VIP-activated second messenger other than cAMP. Recent studies suggest that VIP and peptides homologous with VIP, such as pituitary adenylate cyclase activating polypeptide (PACAP) do not only activate adenylate cyclase and increase intracellular cAMP levels in various tissues, but also stimulate the phosphatidylinositol cascade and mobilizes intracellular Ca^{2+} (Canny et al., 1992; Deutch and Sun, 1992; Tatsuno et al., 1992; Watanabe et al., 1992). Further studies are necessary to evaluate whether VIP affects IP_3 production in endothelial cells. If Ca^{2+} is a putative second messenger for VIP transduction pathway, then the possible increase in Ca^{2+} may contribute to the decrease of cAMP level after stimulation with VIP (fig. 1 A). Such an effect of elevated Ca^{2+} on cAMP production has been reported in many cell types (Exton, 1987). In addition, it has been demonstrated in our laboratory that increased $[\text{Ca}^{2+}]_i$ may be an inhibitor of I_{Kin} channel activity in bovine pulmonary artery endothelial cells (Pasyk et al., submitted).

In conclusion, this study excludes the cAMP cascade as an expected VIP transduction pathway in bovine pulmonary artery endothelial cells. The results suggest that cAMP does reduce I_{Kin} channel activity, the main channel present in endothelial cells, but is not a mediator for the inhibitory effect of VIP on this channel.

Acknowledgements

Supported by MRC of Canada and PMAC - Health Research Foundation. The authors thank Dr. J. Huizinga and Dr. A. Molleman for useful discussion and suggestions during the course of this study.

References

- Ahmad, S., P. Chretien, E.E. Daniel and S.H. Shen, 1990, Characterization of beta adreno-ceptors on cultured endothelial cells by radioligand binding, *Life Sci.* 47, 2365.
- Barnes, P.J., 1988, The Airways: Neural Control in Health and Disease, In: *Lung Biology in Health and Disease*, (Dekker, New York City) p.57.
- Bissonnette, B.M., M.J. Collen, H. Adachi, R.T. Jensen and J.D. Gardner, 1984, Receptors for vasoactive intestinal peptide and secretin on rat pancreatic acini, *Am. J. Physiol.* 246, G710.
- Bregestovski, P.D. and U.S. Ryan, 1989, Voltage-gated and receptor-mediated ionic currents in the membrane of endothelial cells, *J. Mol. Cell Cardiol.* 21 (Suppl. 1), 103.
- Canny, B.J., S.R. Rawlings and D.A. Leong, 1992, Pituitary adenylate cyclase activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes, *Endocrinology* 130, 211.
- Couvineau, A. and M. Laburthe, 1985, The rat liver vasoactive intestinal peptide binding site. Molecular characterization by covalent cross-linking and evidence for differences from the intestinal receptor, *Biochem. J.* 225, 473.
- Davies, J. M. and K. I. Williams, 1984, Endothelial-dependent relaxant effects of vaso-active intestinal polypeptide and arachidonic acid in rat aortic strips, *Prostaglandins* 27, 195.
- Deutch, P.J. and Y. Sun, 1992, The 38 amino acid form of pituitary adenylate cyclase activating polypeptide stimulates dual signalling cascades in PC12 cells and promotes neurite outgrowth, *J. Biol. Chem.* 267, 5108.
- Exton, J.H., 1987, Calcium signalling in cells, molecular mechanisms, *Kidney Int.* 32 (Suppl. 23), S68.
- Furchgott, D. F. and J. V. Zawadzki, 1980, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle to acetylcholine, *Nature (London)* 288, 373.
- Hamill, O.P., A. Marty , E. Neher, B. Sakman and F.J. Sigworth, 1981, Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches, *Pflug. Arch.* 391, 85.
- Hirata, Y., M. Tomita, S. Takata and T. Fujita, 1985, Functional receptors for vasoactive intestinal peptide in cultured vascular smooth muscle cells from rat aorta,

Biochem. Biophys. Res. Commun. 132, 1079.

Hoshi, T., S.S. Garber and R.W. Aldrich, 1988, Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation, *Science* 240, 1652.

Johns, A., T.W. Lategan, N.J. Lodge, U.S. Ryan, C. Van Breemen and D.J. Adams, 1987, Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells, *Tissue and Cell* 19, 733.

Kebabian, J., 1992, The cyclic AMP cascade: A signal transduction system, *Neurotransmissions* 2, 1.

Krause, D., S.C. Lee and C. Deutsch, 1988, Forskolin effects on the voltage-gated K^+ conductance of human T cells, *Pflug. Arch.* 412, 133.

Laburthe, M., B. Breant and C. Rouyer-Fessard, 1984, Molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium by covalent cross-linking. Evidence for two classes of binding sites with different structural and functional properties, *Eur. J. Biochem.* 139, 181.

Laurenza, A., E.M. Sutkowski and K.B. Seamon, 1989, Forskolin: a specific stimulator of adenyl cyclase or a diterpene with multiple sites of action? *TIPS* 10, 442.

McEwan, J.R., H. Parsaee, D.C. Lefroy and J. MacDermot, 1990, Receptors linked to adenylate cyclase on endothelial cells, in: *The Endothelium: An Introduction to Current Research*, ed. J.B. Warren (Wiley-Liss, New York) p.45.

Nabika, T., Y. Nara, Y. Yamori, W. Lovenberg and J. Endo, 1985, Angiotensin II and phorbol ester enhance isoproterenol and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells, *Biochem. Biophys. Res. Commun.* 131, 30.

Palmer, R.M., A.G. Ferige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, *Nature (London)* 327, 524.

Pasyk, E., M. Inazu and E.E. Daniel, 1993, Cyclopiazonic acid, an endoplasmic reticulum Ca^{2+} -pump inhibitor, enhances Ca^{2+} entry in endothelial cells in an IP_3 independent manner, *Am. J. Physiol.* (submitted).

Pasyk, E., Y.K. Mao, S. Ahmad, S.H. Shen and E.E. Daniel, 1992, An endothelial cell-line contains functional VIP receptors: They control inwardly-rectifying K^+ channels, *Eur. J. Pharmacol.* 212, 209.

Sata, T., J. Linden, L.W. Liu, E. Kubota and S.I. Said, 1988, Vasoactive intestinal peptide evokes endothelium-dependent relaxation and cyclic AMP accumulation in rat aorta, *Peptides* 9, 853.

Takeda, K. and M. Klepper, 1990, Voltage-dependent and agonist-activated ionic currents in vascular endothelial cells: A review, *Blood Vessels* 27, 169.

Tatsuno, I., T. Yada, S. Vigh, H. Hidaka and A. Arimura, 1992, Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) increase cytosolic free calcium concentration in cultured rat hippocampal neurons, *Endocrinology* 131, 73.

Thom, S., A. Hughes, G. Martin and P.S. Sever, 1987, Endothelium-dependent relaxation in isolated human arteries and veins, *Clin. Sci.* 73, 547.

Watanabe, T., Y. Masuo, H. Matsumoto, et al., 1992, Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline, *Biochem. Biophys. Res. Commun.* 173, 252.

Yanagisawa M., H. Kurihara, S. Kimura, G. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, *Nature (London)* 332, 411.

Figure Legends

Figure 1

Measurements of cyclic AMP level in endothelial cells stimulated with VIP (10^{-7} M and 10^{-6} M) and isoproterenol (10^{-6} M and 10^{-5} M).

A. cAMP levels over basal (100%) in the absence of IBMX. The mean absolute value of basal cAMP levels in all experiments without IBMX is 114.7 ± 3.1 pmol/mg (n = 4).

B. cAMP levels over basal (100%) in the presence of IBMX (5×10^{-4} M). The mean absolute value of basal cAMP levels in all experiments with IBMX is 2.6 ± 0.4 nmol/mg (n = 4).

Figure 2

Effect of isoproterenol on inward rectifying K^+ currents in an endothelial cell.

A. Current traces evoked by voltage step of -140 mV given from the holding potential of -70 mV in normal solution and after addition of isoproterenol (10^{-5} M).

B. The instantaneous I-V curves for the currents in normal solution and after application of isoproterenol evoked by voltage pulses given every 5 s in 20 mV steps over the range -160 to -20 mV from the holding potential of -70 mV. This figure is representative of 23 experiments.

Figure 3

Effect of forskolin on inwardly rectifying K⁺ currents in an endothelial cell.

A. Control currents evoked by command voltages given every 5 s in 20 mV steps over the range -160 to -40 mV from the holding potential -70 mV.

B. Forskolin (10⁻⁶ M) reduced the inward current and slowed inactivation kinetics at the potential of -160 mV.

C. The instantaneous I-V curves for the currents shown in A and B. This figure is representative of 8 experiments.

Figure 4

Effect of forskolin on inwardly rectifying K⁺ currents in an endothelial cell.

A. Control currents evoked by command voltages given every 3 s in 20 mV steps in the range -160 to 40 mV from the holding potential -70 mV.

B. Forskolin (10⁻⁶ M) blocks the inward current completely.

C. Recovery from forskolin action at 5th min. after the beginning of wash-out.

D. The instantaneous I-V curves for the currents shown in A and B. This figure is representative of 2 experiments.

Figure 5

Effect of 8-Bromo-cAMP on inwardly rectifying K⁺ currents in an endothelial cell.

A. Control currents evoked by command voltages given every 5 s in 30 mV steps over the range -160 to 20 mV from the holding potential of -70 mV.

B. 8-Br-cAMP (2 mM) reduced the inward current.

C. Recovery from 8-Br-cAMP effect.

D. The instantaneous I-V curves for currents shown in A and B. This figure is representative of 4 experiments.

Figure 6

Time course of changes evoked by forskolin, 8-Br-cAMP (A) and VIP, isoproterenol (B). The changes were recorded at the voltage step -140 mV from a holding potential of -70 mV from four different endothelial cells. I/I_{con} means the value of the current amplitude in relation to the control current at 10-th ms of the applied pulse. (↓)

Drugs were applied directly to the bath to get a final concentration as indicated. (↑)

In A, the beginning of wash out of 8-Br-cAMP and forskolin; in B, the beginning of wash out of isoproterenol and application of VIP antagonist ([4-Cl-D-Phe⁶,Leu¹⁷]VIP) (10⁻⁶ M). Perfusion rate 4 ml/min.

TABLE 1

The decrease of I_{Kin} current amplitude was measured at the same potential, -140 mV. 100% was an amplitude of the control current at this potential at 10th ms. The mean absolute values of control currents are given in parentheses. Data are expressed as mean±SEM.

Drug		Average decrease (%)	
VIP	10^{-7} M	61.7 (-275 pA)±7.8	(n = 9 cells)
Isoproterenol	10^{-6} M	13.9 (-727 pA)±1.1	(n = 16 cells)
Forskolin	10^{-6} M	46.5 (-259 pA)±7.0	(n = 10 cells)
8-Br-cAMP	10^{-3} M	33.3 (-309 pA)±7.6	(n = 4 cells)

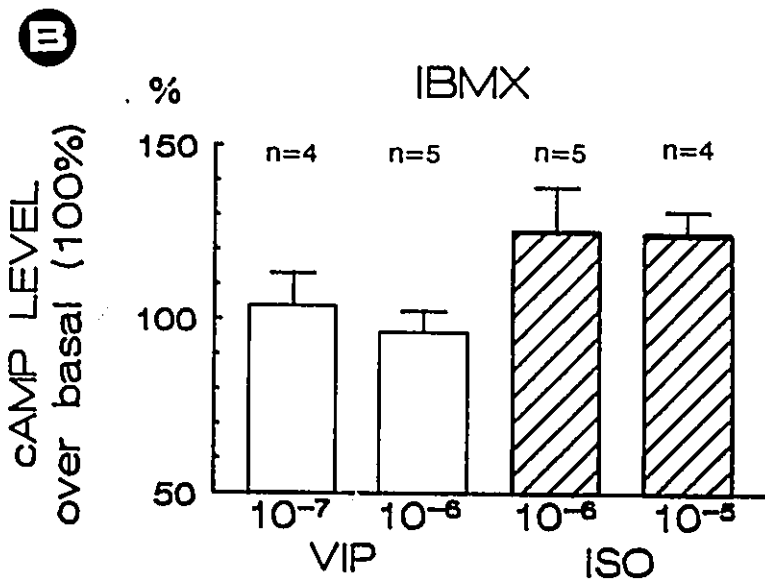
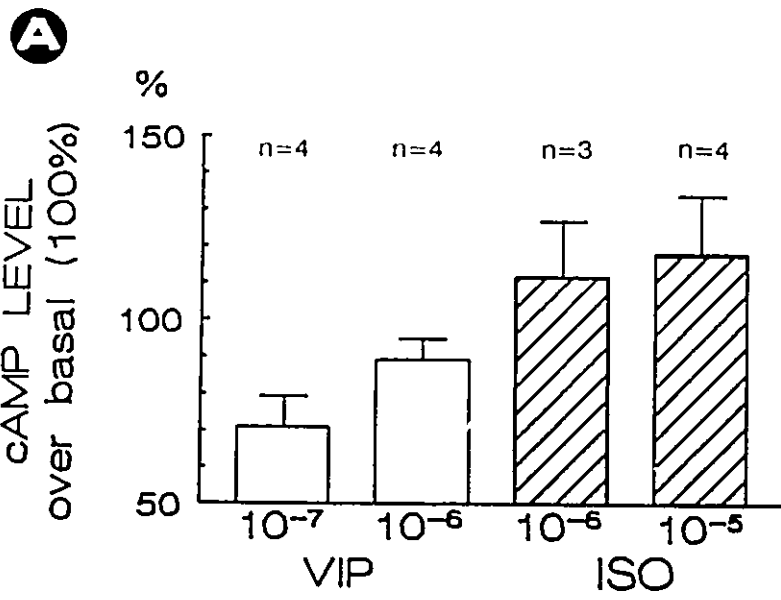


Figure 1

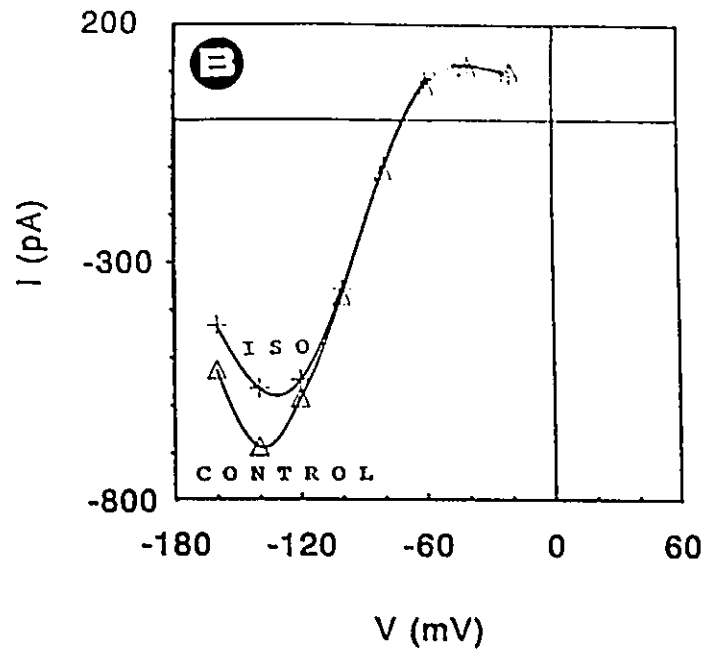
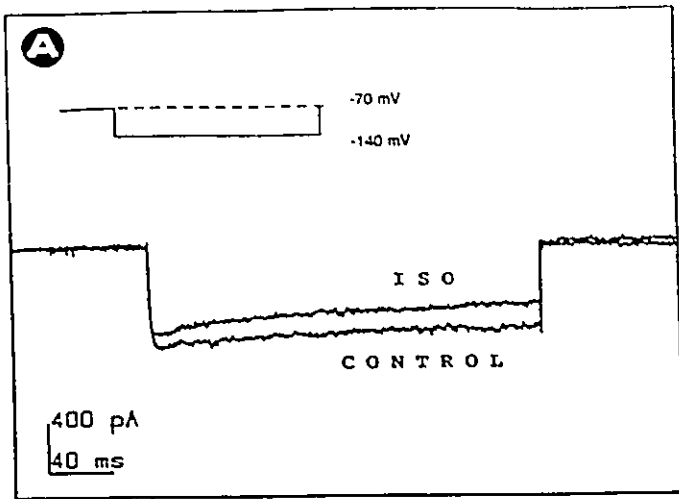


Figure 2

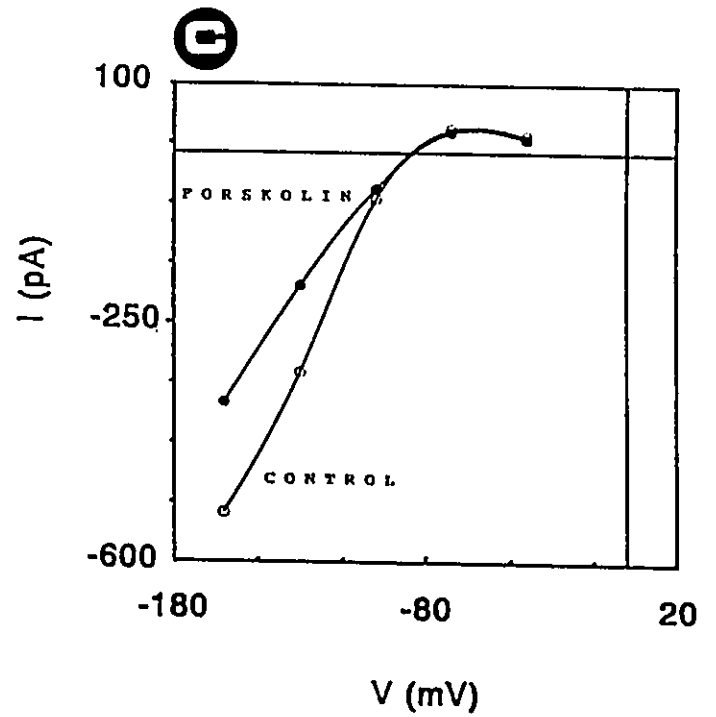
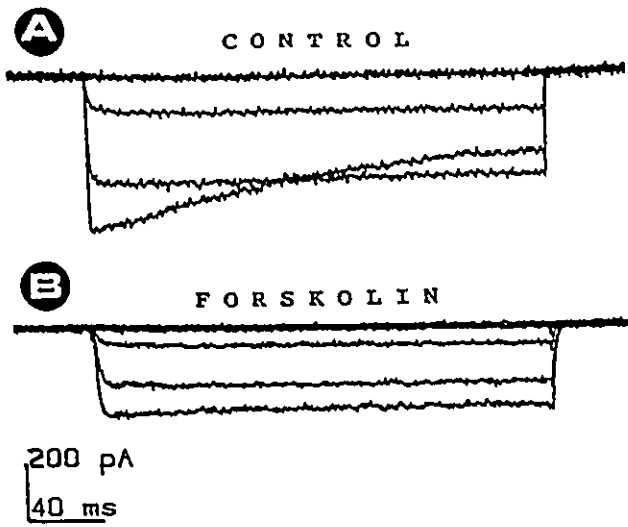


Figure 3

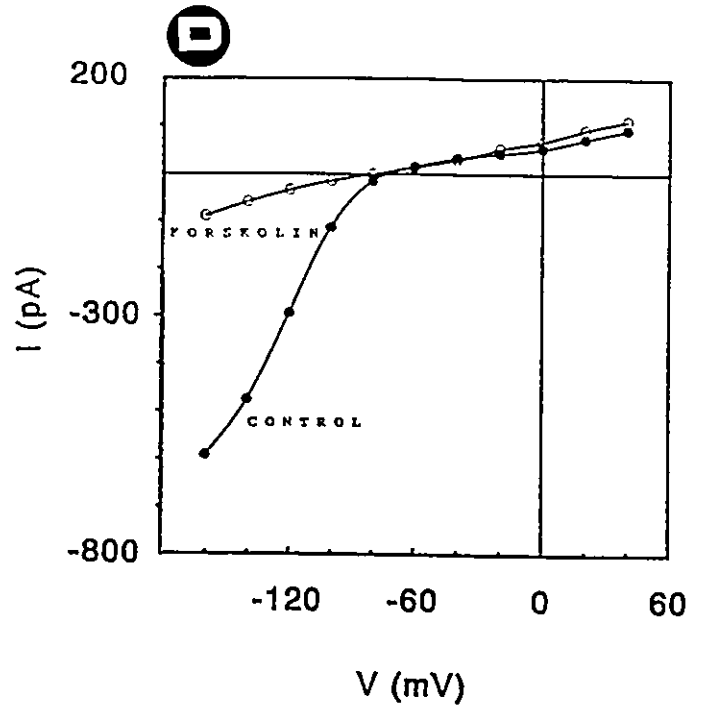
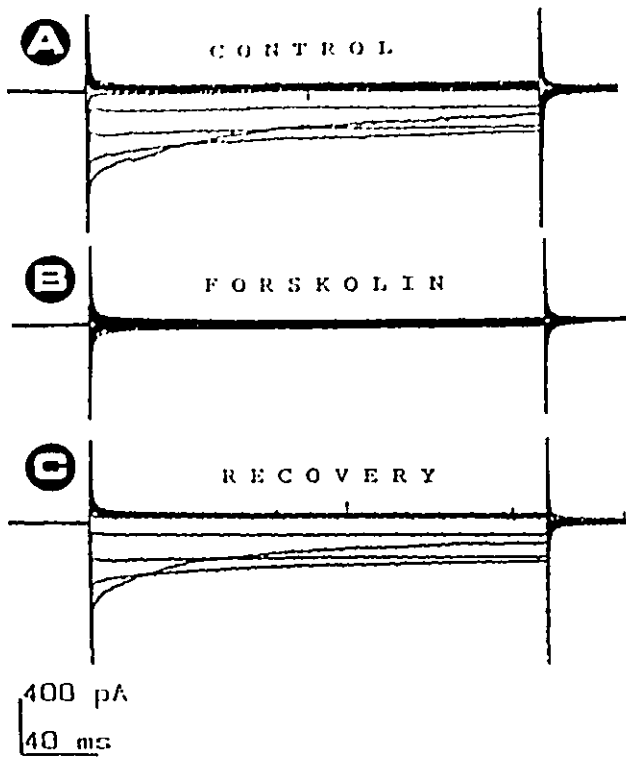


Figure 4

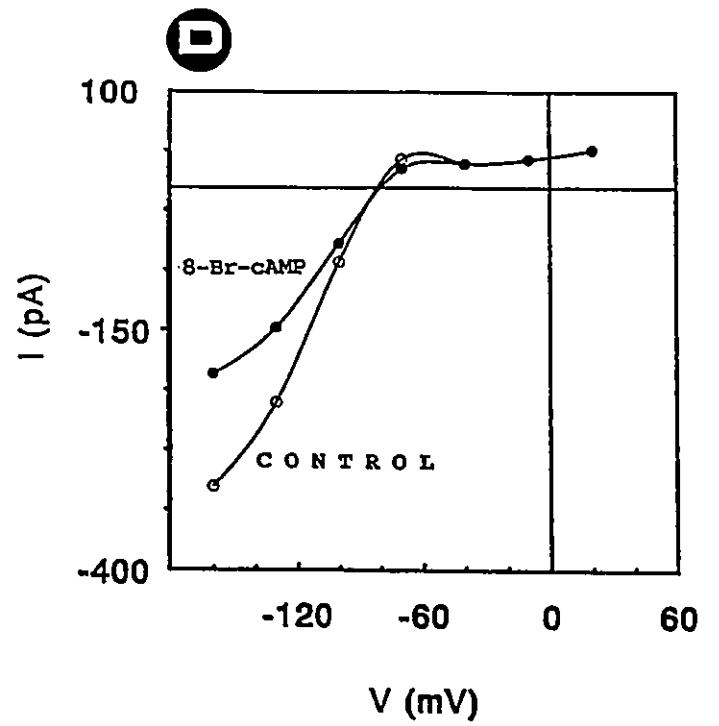
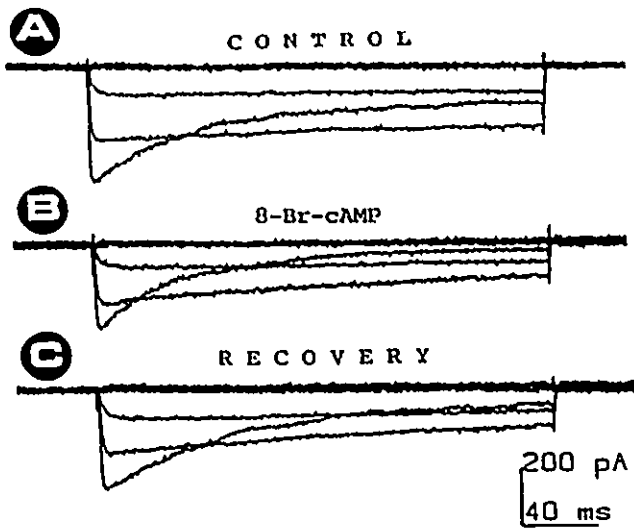


Figure 5

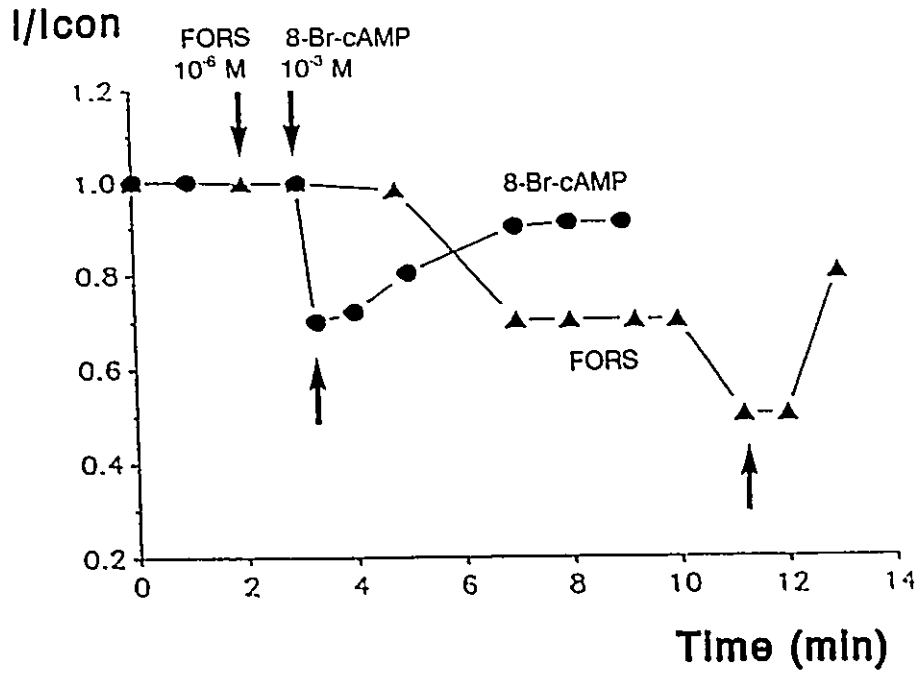
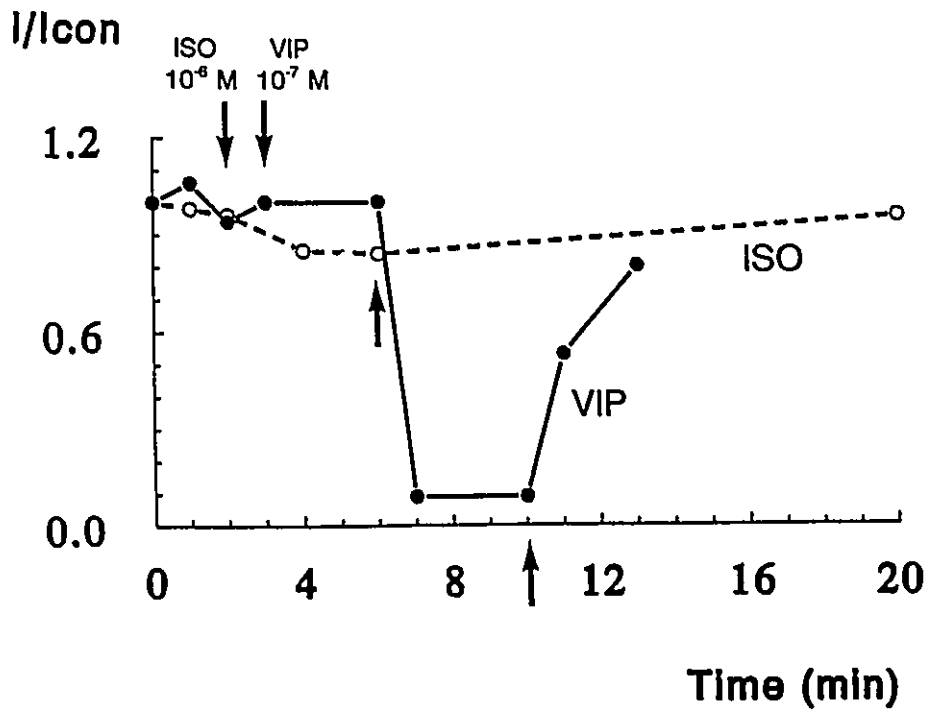
A**B**

Figure 6

PAPER No. 3

A G PROTEIN MEDIATES EFFECTS OF VASOACTIVE INTESTINAL
POLYPEPTIDE ON K⁺ CHANNELS IN ENDOTHELIAL CELLS

Submitted to *Pflügers Archiv* in August 1993

Ewa Pasyk's contribution:

- (i) cell culturing
- (ii) performance of all electrophysiological experiments

A G PROTEIN MEDIATES EFFECTS OF VASOACTIVE INTESTINAL
POLYPEPTIDE ON K⁺ CHANNELS IN ENDOTHELIAL CELLS

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Abstract

We have studied the effects of various G protein activators (a GTP analog, cholera and pertussis toxins) on K^+ channels in cultured bovine pulmonary endothelial cells using patch clamp techniques. We demonstrated that activation of these cells by GTP- γ -S, a non-hydrolysable GTP analog, and cholera toxin led to the inhibition of inwardly rectifying K^+ ($G_{K_{in}}$) conductance. The activity of the $I_{K_{in}}$ channels were pertussis toxin insensitive. GTP- γ -S also contributed to the appearance of an outward K^+ current in some cases. GTP- γ -S and cholera toxin mimicked the effects of vasoactive intestinal polypeptide (VIP) on K^+ channels in endothelial cells. VIP inhibited $I_{K_{in}}$ channel activity in outside-out and cell-attached patches. Our results suggest that VIP receptors interact with $I_{K_{in}}$ channels through a G protein(s). This G protein may couple directly to the $I_{K_{in}}$ channel. In addition, it may couple to a putative VIP-activated second messenger system other than cAMP cascade, which may also contribute to the inhibitory effect on the $I_{K_{in}}$ channel activity. Activation of VIP receptors may lead to the release of endothelium-derived relaxing factor (EDRF) which regulates vascular tone.

G protein, K^+ channels, VIP receptors, endothelial cells, cAMP.

Introduction

GTP-binding proteins (G proteins) have been shown to be involved in regulation of ion channels in several types of excitable (Hescheler et al., 1987; Holz et al., 1986) and nonexcitable cells (McCloskey and Cahalan, 1990). This regulation may occur through a direct coupling of a G protein to an ion channel (Brown and Birnbaumer, 1988; Hescheler et al., 1987) or an indirect control with a second messenger (e.g. Ca^{2+} , cAMP) being a mediator (Brown, 1991; Olate and Allende, 1991; Yatani et al., 1987). Receptor-mediated effects of a G protein on K^+ channels in many cell types appear to involve more than one type of G proteins (Brown, 1991; Fargon et al., 1990; Roerig et al., 1992). A G protein that leads to the stimulation of adenylate cyclase and is sensitive to cholera toxin is likely to be a G_s protein (Brown, 1991; Olate and Allende, 1991; Yatani et al., 1987). A G protein with an inhibitory action on adenylate cyclase and sensitivity to pertussis toxin is likely to be a G_i protein (Brown, 1991; Lerner et al., 1992; Olate and Allende, 1991).

In our experiments, we examined the possible effects of various G proteins activators on the ion channels of endothelial cells. We found that stimulators of a G protein led to the suppression of the inwardly rectifying K^+ (I_{Kin}) channels and appearance of an outward K^+ current in some circumstances. Furthermore, these agents mimicked the effects of vasoactive intestinal polypeptide (VIP) on K^+ channels in endothelial cells (Pasyk et al., 1992). Our results demonstrate that VIP receptors may interact with I_{Kin} channels through a G protein which is sensitive to

the cholera toxin and resistant to pertussis toxin.

Since endothelial cells play an important role in the regulation of vascular tone, pharmacological intervention at the level of a G protein may be of a potential significance, particularly with respect to the production of endothelium-derived relaxing factor (EDRF). Stimulation of endothelial VIP receptors (Pasyk et al., 1992) may also lead to EDRF release. In some blood vessels, VIP-induced vasodilation has been reported to be dependent on the presence of endothelium (Sata et al., 1988; Thom et al., 1987).

Materials and Methods

Chemicals

Tetralithium salt of guanosine 5'-(3'-O-thiotriphosphate) (GTP- γ -S), cholera and pertussis toxins were purchased from SIGMA Chemical Company (St. Louis, MO). Cholera toxin (1 mg/ml) was dissolved in the solution containing: Tris buffer, NaCl, NaN₃ and Na₂EDTA at pH = 7.5. Pertussis toxin (200 μ g/ml) solution contained sodium phosphate and sodium chloride at pH = 7.2. Vasoactive intestinal polypeptide (VIP) and [4-Cl-D-Phe⁶,Leu¹⁷]-VIP, VIP antagonist were obtained from Peninsula Laboratories, Inc. (Belmont, CA). 4-Amino-pyridine (4-AP) and cyclopiazonic acid (CPA) were purchased from SIGMA Chemical Company (St. Louis, MO).

Cell culture

Cultured bovine pulmonary artery endothelial cells were obtained from American Type Culture Collection (ATCC; cell line No. 209). The cells were cultured in Minimum Essential Medium (MEM), supplemented with 20% fetal bovine serum, 0.1% gentamycin and 0.1% fungizone (all materials from GIBCO, Grand Island, NY). The cells were used for experimentation on the second or third day after plating in plastic cell culture dishes while still confluent.

The pretreatment with pertussis toxin was done according to procedures given by Holz et al. (1986). Freshly dissolved pertussis toxin was added to the specially prepared culture medium (devoid of serum and antibiotics) to yield final concentrations of 50-500 ng/ml. The toxin pretreated cells were incubated at 37°C for 12-20 hrs. Control cultured cells were treated exactly the same way as pertussis toxin treated cells, except that toxin was not added.

Electrophysiology

Patch-clamp techniques were used (Hamill et al., 1981): whole-cell, cell-attached and isolated patches. Currents were measured using an Axopatch-1C amplifier (Axon Instruments, Inc.) and recorded on-line by the computer (IBM-AT). Data were filtered with a lowpass Bessel filter (1 or 2 kHz). Records were sampled at 5 kHz using pCLAMP software (Axon Instruments, Inc.).

Patch electrodes which had been heat polished and had resistances between 3 and 6 M Ω were used. For the whole-cell recordings electrodes were filled with (in

mM): 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP. To obtain high EGTA concentrations, the intracellular solution contained (in mM): 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES, 4 Na₂ATP. The pH of these solutions was adjusted to 7.3 with KOH. To provide symmetrical solutions, the patch pipette contained the same solution as in the bath (see below). All the intracellular solutions were filtered before use (pore size 0.2 μm). The external bath solution (high sodium) contained (mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose, 10 HEPES. The pH was adjusted to 7.3 with NaOH.

Tested drugs were applied by perfusion (3-4 ml/min) or added to the bath to get final concentration as indicated. All experiments were conducted at room temperature.

Data analysis

Whole-cell currents. Some whole-cell currents were corrected for leakage, but none of those shown on the figures. Series resistance and capacitance transients were not compensated. The 'instantaneous' currents for the I-V curve were measured after settling of capacitive currents, usually between 10 to 20th ms after the beginning of voltage pulses. The 'steady-state' currents were measured at the end of the 250th ms voltage protocol.

Single channel currents. Unitary current amplitudes and open/closed times were determined from the digitized current values. Brief channel openings and closings of <500 μs in duration were not included in analysis. Open-state probability was

determined for 8-13 s period of recording. The mean open-state probability (P_o) was obtained from the mean open (T_o) and mean closed (T_c) times, according to the relation $P_o = T_o/(T_o+T_c)$.

Results

Effect of VIP on an outward K^+ current

Under the control experimental conditions, when depolarizing voltages from -60 to +80 mV were applied in 20 mV steps from the holding potential of -60 mV, there was no outward current present in most endothelial cells; some cells had a very small outward current as shown in fig. 1. VIP (10^{-7} M) activates an outward current ($n = 4$) and the effect was reversed by VIP antagonist, [4-Cl-D-Phe⁶,Leu¹⁷]-VIP (10^{-6} M). This VIP-activated current depends on the availability of free intracellular Ca^{2+} . When a high concentration of EGTA (11 mM) was used in the pipette, VIP was not able to evoke this outward current (data shown in previous papers (Pasyk et al., 1993; Pasyk et al., 1992). In addition, when cells were pretreated for 8-10 minutes with cyclopiazonic acid (CPA) (20 μ M), an inhibitor of Ca^{2+} -pump of internal Ca^{2+} stores (Seidler et al., 1989; Uyama et al., 1992), VIP did not activate the outward current ($n = 3$; data not shown).

Tenfold increase in $[K^+]_o$ shifted the reversal potential of I-V curve of VIP-activated currents approximately 50-55 mV to the right, as was predicted for K^+ currents by the Nernst equation ($n = 4$ cells; data not shown).

A proportion (20-30%) of endothelial cells reveals the presence of an outward K^+ current on depolarization under our control conditions. This current was increased after VIP (10^{-7} M) application (fig. 2). Figure 2 shows that VIP antagonist (10^{-6} M) reversed the effect of VIP and 4 aminopyridine (4-AP) (1 to 4 mM) then suppressed this outward current ($n = 3$).

Effect of VIP on the $I_{K_{in}}$ currents

It has been demonstrated that VIP inhibits $I_{K_{in}}$ in bovine pulmonary endothelial cells recorded in the whole-cell configuration (Pasyk et al., 1992). This effect was confirmed on the single channel level in the cell-attached ($n = 3$; fig. 3) and outside-out modes ($n = 3$; fig. 4). Single channel currents recorded in these configurations under control conditions were stable for 10-15 min when 4 mM ATP was present in the intracellular solution (for each configuration $n = 3$; data not shown). Slope conductances of the $I_{K_{in}}$ channels in these configurations were between 25 - 26 pS. The probability of channel-opening at the potential of -100 mV under control conditions in the cell-attached and outside-out modes measured when only one channel was present in the patch was $P_o = 0.62 - 0.65$. In the outside-out mode, VIP reduced the probability of opening measured at the same potential to $P_o = 0.15$. In the cell-attached mode, VIP reduced the amplitudes of the unitary currents by 80 to 90%.

Effects of GTP- γ -S on inward and outward K⁺ currents

GTP- γ -S (100 μ M), a non-hydrolysable GTP analog, mimicked the VIP effect when applied to the bath solution in the inside-out patch (fig. 5; n = 4). It reduced the probability of channel opening measured at the potential of -100 mV from 0.65 (control) to $P_o = 0.33$. Under control conditions, single channel currents recorded in this configuration were stable for 9 - 11 min when 4 mM ATP was present in the intracellular solution (n = 3 cells; data not shown). The slope conductance of $I_{K_{in}}$ channels in the inside-out was 28 pS.

In the whole cell configuration, GTP- γ -S (100 μ M) reduced the $I_{K_{in}}$ in a time dependent manner (fig. 6). GTP- γ -S also increased the outward K⁺ current (fig. 7; n = 4).

Effects of cholera and pertussis toxins on the $I_{K_{in}}$ currents

Cholera toxin (500 ng/ml), when applied to the bath solution in the whole cell mode caused the suppression of $I_{K_{in}}$ in 4 out of 6 cells (fig. 8). Cells pretreated with pertussis toxin (50-500 ng/ml) for 12 to 20 hours revealed the same $I_{K_{in}}$ channels activity as cells under the control conditions (n = 8; data not shown). Whole-cell currents recorded under control conditions were stable for at least 20 min. when 0.5 mM ATP was present in the pipette solution (see APPENDIX II).

Discussion

The only readily and regularly observable channel in pulmonary artery endothelial cells under our control conditions is the inwardly rectifying K^+ channel ($I_{K_{in}}$) (Takeda et al., 1987). In our experiments, we demonstrated that the G protein activator (GTP- γ -S) inhibited this $I_{K_{in}}$ conductance on both whole-cell and single channel levels (figs. 5, 6). Since the activity of this channel was cholera toxin sensitive (fig. 8) and pertussis toxin resistant, the involvement of a G_s protein seems likely. Since these G protein activators mimicked the effects of VIP on $I_{K_{in}}$, this suggests that the G protein couples VIP receptors to the $I_{K_{in}}$ channel. The run-down of single channel recordings was excluded by demonstrating the stability of control recordings for 9 - 15 minutes in the presence of ATP in the intracellular solutions (data not shown).

Some evidence suggested that this coupling between a G protein and $I_{K_{in}}$ channel is direct in its nature. Since VIP and GTP- γ -S reduce the $I_{K_{in}}$ in isolated patches, where the second messenger system is assumed not to operate (see also Chapter IV), the G protein activated after VIP receptors stimulation apparently couples directly to the $I_{K_{in}}$ channel. Such a direct coupling between a G protein and $I_{K_{in}}$ has been reported in many other cell types with various agonists (Fargon et al., 1990; Hoyer et al., 1991; McCloskey and Cahalan, 1990; Nakajima et al., 1988).

Results of our previous studies (Pasyk et al., 1993) allow us to rule out cAMP as a second messenger for VIP action in endothelial cells, although VIP activates

adenylate cyclase and production of cyclic AMP (cAMP) in many cell types (Sata et al., 1988; Nabika et al., 1985). Measurements of cAMP level revealed that VIP did not elevate cAMP production in bovine pulmonary artery endothelial cells, whereas isoproterenol did increase its production. In addition, VIP strongly suppressed $I_{K_{in}}$ channel activity, whereas the inhibitory effect of isoproterenol on this channel was very weak (about 20 % of the VIP effect). The magnitude of the effects on $I_{K_{in}}$ channel activity evoked by some activators of the cAMP cascade (forskolin, cAMP analogs) had intermediate values between those of isoproterenol and VIP (Pasyk et al., 1992). Therefore, cAMP elevation may reduce the activity of $I_{K_{in}}$ channel, but is not a mediator for the inhibitory effect of VIP on these channels.

However, we can not exclude existence of any other than cAMP, VIP-activated second messenger in endothelial cells. VIP was also able to inhibit the single $I_{K_{in}}$ channel activity in cell-attached patches when applied to the bath (fig. 3). It is likely that a G protein activated after VIP receptor stimulation couples directly to the $I_{K_{in}}$ channel and it also activates a putative second messenger when interior of the cell remains intact (cell-attached mode). Our results showed that in the outside-out patches, VIP reduced mainly the open probability of the $I_{K_{in}}$ channel (fig. 4). In the cell-attached mode, VIP reduced amplitude of the channel opening (fig. 3). This suggests involvement of two distinct mechanisms of VIP action on the single channel activity in cell-attached and outside-out patches (see also Chapter IV).

An additional aspect of our study was to demonstrate the presence of an outward K^+ current activated by VIP and its dependence on intracellular free Ca^{2+}

(fig. 1). VIP was not able to evoke this current when the pipette contained high concentrations of EGTA which buffered intracellular Ca^{2+} (Pasyk et al., 1992), or when internal Ca^{2+} stores were depleted by CPA (20 μM), a sarcoplasmic reticulum (SR) Ca^{2+} pump inhibitor (Seidler et al., 1989; Uyama et al., 1992). It has been shown that CPA inhibits the Ca^{2+} -transport ATPase in SR vesicles from skeletal muscle (Seidler et al., 1989). CPA also selectively inhibits ATP dependent Ca^{2+} uptake into internal stores in skinned ileal smooth muscle (Uyama et al., 1992) and lymphocytes (Mason et al., 1991). K^{+} selectivity of this VIP-evoked current was demonstrated by an appropriate shift of the I-V curve when K^{+} gradient was changed. Therefore, this VIP-evoked current resembles Ca^{2+} -activated K^{+} current present in several other endothelial cells types (Colden-Stanfield et al., 1987).

When an outward K^{+} current was present under the control conditions (20-30% of cells), VIP seems to increase this current (fig. 2). Since GTP- γ -S, an activator of a G_s protein, activated a whole-cell outward K^{+} current in some cells (fig. 7), it mimicked the VIP effect on the outward K^{+} conductance.

An outward K^{+} current and inwardly rectifying K^{+} current are two classes of K^{+} channels which regulate the membrane potential in endothelial cells (Luckhoff and Busse, 1990). The I_{Kir} channel helps to keep the resting membrane potential close to E_{K} (Kubo et al., 1993). Activation of an outward K^{+} current (as shown with VIP and GTP- γ -S) should tend to hyperpolarize the cell (Luckhoff and Busse, 1990). Any shift of the membrane potential towards negative values creates a driving force for Ca^{2+} entry. Ca^{2+} entry, in turn, is necessary for the synthesis and release of

EDRF (Colden-Stanfield et al., 1987; Luckhoff and Busse, 1990).

In conclusion, our results suggest that VIP receptors interact with K^+ channels through a G protein(s). $I_{K_{in}}$ channels can be controlled directly by an unidentified G protein which is activated by GTP- γ -S or cholera toxin and is resistant to pertussis toxin. In addition, a G protein may couple to a putative VIP-stimulated second messenger other than cAMP cascade, which may also mediate the inhibitory effect on $I_{K_{in}}$ channel activity.

Acknowledgements

Supported by the Medical Research Council and Pharmaceutical Manufacturers Association of Canada.

References

- Brown, A.M., 1991, Ion channels as G protein effectors, *NIPS* 6, 158.
- Brown, A.M. and L. Birnbaumer, 1988, Direct G protein gating of ion channels, *Am. J. Physiol.* 254, H401.
- Colden-Stanfield, M., W.P. Schilling, A.K. Ritchie, S.G. Eskin, L.T. Navarro and D.L. Kunze, 1987, Bradykinin-induced increases in cytosolic calcium and ionic currents in culture bovine aortic endothelial cells, *Circ. Res.* 61, 632.
- Fargon, F., P.A. McNaughton and F.V. Sepuleveda, 1990, Possible involvement of GTP-binding proteins in the deactivation of an inwardly rectifying K^+ current in enterocytes isolated from guinea-pig small intestine, *Pflug. Arch.* 417, 240.
- Hamill, O.P., A. Marty, E. Neher, B. Sakman and F.J. Sigworth, 1981, Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches, *Pflugers Arch.* 391, 85.
- Hescheler, J., W. Rosenthal, W. Trautwein and G. Schultz, 1987, The GTP-binding protein, Go, regulates neuronal calcium channels, *Nature* 325, 445.
- Holz IV, G.G., S.G. Rane and K. Dunlap, 1986, GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels, *Nature* 319, 670.
- Hoyer, J., R. Popp, J. Meyer, H.J. Galla and H. Gogelein, 1991, Angiotensin II, vasopressin and GTP[γ -S] inhibit inward-rectifying K^+ channels in porcine cerebral capillary endothelial cells, *J. Membr. Biol.* 123, 55.
- Kubo, Y., T.J. Baldwin, Y.N. Jan and L.Y. Jan, 1993, Primary structure and functional expression of a mouse inward rectifier potassium channel, *Nature* 362, 127.
- Lerner, R.W., G. D. Lopaschuk and F.M. Olley, 1992, Prostaglandin E2 receptors in the heart are coupled to inhibition of adenylyl cyclase via a pertussis toxin sensitive G protein, *Can. J. Physiol. Pharmacol.* 70, 77.
- Luckhoff, A. and R. Busse, 1990, Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential, *Pflugers Arch.* 416, 305.
- Mason, M.J., C. Garcia-Rodriguez and S. Grinstein, 1991, Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane: comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes, *J. Biol. Chem.* 266, 20856.

McCloskey, M.A. and M.D. Cahalan, 1990, G protein control of potassium channel activity in a mast cell line, *J. Gen. Physiol.* 95, 205.

Nabika, T., Y. Nara, Y. Yamori, W. Lovenberg and J. Endo, 1985, Angiotensin II and phorbol ester enhance isoproterenol and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells, *Biochem. Biophys. Res. Commun.* 131, 30.

Nakajima, Y., S. Nakajima and M. Inoue, 1988, Pertussis toxin-insensitive G protein mediates substance P-induced inhibition of potassium channels in brain neurons, *Proc. Natl. Acad. Sci.* 85, 3643.

Olate, J. and J.E. Allende, 1991, Structure and function of G proteins, *Pharmac. Ther.* Vol. 51, 403.

Pasyk, E., S. Cipris and E.E. Daniel, 1993, cAMP is not a second messenger for transduction of VIP effects on inwardly rectifying K⁺ currents in endothelial cells, *Eur. J. Pharmacol.* (in revision).

Pasyk, E., Y.K. Mao, S. Ahmad, S.H. Shen and E.E. Daniel, 1992, An endothelial cell-line contains functional VIP receptors: They control inwardly rectifying K⁺ channels, *Eur. J. Pharmacol.* 212, 209.

Roerig, S.C., H.H. Loh and P.Y. Law, 1992, Identification of three separate guanine nucleotide-binding proteins that interact with the sigma-opioid receptor in NG108-15 neuroblastoma X glioma hybrid cells, *Moll. Pharmacol.* 41, 822.

Sata, T., J. Linden, L.W. Liu, E. Kubota and S.I. Said, 1988, Vasoactive intestinal peptide evokes endothelium-dependent relaxation and cyclic AMP accumulation in rat aorta, *Peptides* 9, 854.

Seidler, N.W., I. Jona, M. Vegh and A. Martonosi, 1989, Cyclopiazonic acid is a specific inhibitor of the Ca²⁺-ATPase of sarcoplasmic reticulum, *J. Biol. Chem.* 264, 17816.

Takeda, K., V. Shini and H. Stoeckel, 1987, Voltage-activated potassium, but not calcium currents in cultured bovine aortic endothelial cells, *Pflü. Arch.* 410, 385.

Thom, S., A. Hughes, G. Martin and P.S. Sever, 1987, Endothelium-dependent relaxation in isolated human arteries and veins, *Clin. Sci.* 73, 547.

Uyama, Y., Y. Imaizumi and M. Watanabe, 1992, Effects of cyclopiazonic acid, a novel Ca²⁺-ATPase inhibitor, on contractile responses in skinned ileal smooth muscle, *Br. J. Pharmacol.* 106, 208.

Yatani, A., J. Codina, Y. Imoto, J.P. Reeves, L. Birnbaumer and A.M. Brown, 1987, A G protein directly regulates mammalian cardiac calcium channels, *Science* 235, 207.

Figure Legends

Figure 1

A. VIP (10^{-7} M) activates an outward whole-cell current in an endothelial cell. Command voltages were given as illustrated. This figure is representative of 4 experiments.

B. Steady-state I-V curve for the family of currents shown in A.

C. Time course of current changes evoked by VIP. The current amplitudes were measured at +60 mV at 20th ms of the voltage protocol. I/I₀ means the value of the current amplitude in the relation to the control current.

Figure 2

A. VIP (10^{-7} M) increases an outward whole-cell K⁺ current. Command voltages were given as illustrated. This figure is representative of 3 experiments.

B. Steady-state I-V curve for the family of currents shown in A.

Figure 3

A. The effect of VIP (10^{-7} M) on inwardly rectifying K⁺ (I_{Kin}) channel in an endothelial cell. Cell-attached patch with normal Na⁺ solution in the bath and high K⁺ solution in the pipette. Pipette potential was 50 mV. The inward current is shown as deflections downwards. Dashed line represents a baseline. Two channels were present in this patch. This figure is representative of 3 experiments.

B. I-V relationship for the $I_{K_{in}}$ conductance under symmetrical conditions. The applied pipette potentials were scaled by the subsequently measured resting membrane potential (-68 mV) in the whole-cell mode. The slope conductance was 25 pS.

Figure 4

The effect of VIP (10^{-7} M) on the $I_{K_{in}}$ channel in an endothelial cell in the isolated patch (outside-out). Although second messenger system is not present, VIP still inhibits the $I_{K_{in}}$. Applied pipette potential was -90 mV. The inward current is shown as deflections downwards. Slope conductance for the $I_{K_{in}}$ channel in the outside-out patch under symmetrical conditions was 26 pS. This figure is representative of 3 experiments.

Figure 5

A. The effect of GTP- γ -S (100 μ M) on $I_{K_{in}}$ in an endothelial cell. Inside-out configuration with symmetrical solutions in the bath and the pipette. Pipette potential was 30 mV. The inward current is illustrated as deflections downwards. Dashed line represents a baseline. This figure is representative of 4 cells.

B. I-V relationship for the $I_{K_{in}}$ conductance in the inside-out patch under symmetrical conditions. The slope conductance was 28 pS.

Figure 6

A. The effect of GTP- γ -S on the whole-cell $I_{K_{in}}$ in an endothelial cell. Currents were recorded at -160 mV from the holding potential of -70 mV. GTP- γ -S (100 μ M) was added into the pipette solution. The traces were recorded in time as indicated. This figure is representative of 4 experiments.

B. The time course of the inhibition of the $I_{K_{in}}$ current by GTP- γ -S. I/I_0 means an amplitude normalized to the absolute value of the current observed at $t=0$ min.

Figure 7

A. GTP- γ -S activates an outward whole-cell K^+ current in an endothelial cell. GTP- γ -S (100 μ M) was added to the pipette solution. Command voltages were given as illustrated. Time $t = 0$ is the time of the first recording. This figure is representative of 4 experiments.

B. Instantaneous I-V curves for the family of currents shown in A.

Figure 8

A. The effect of cholera toxin (500 ng/ml) on $I_{K_{in}}$ in an endothelial cell. Command voltages were given as illustrated. This figure is representative of 4 experiments.

B. Steady-state I-V curve for the currents shown in A.

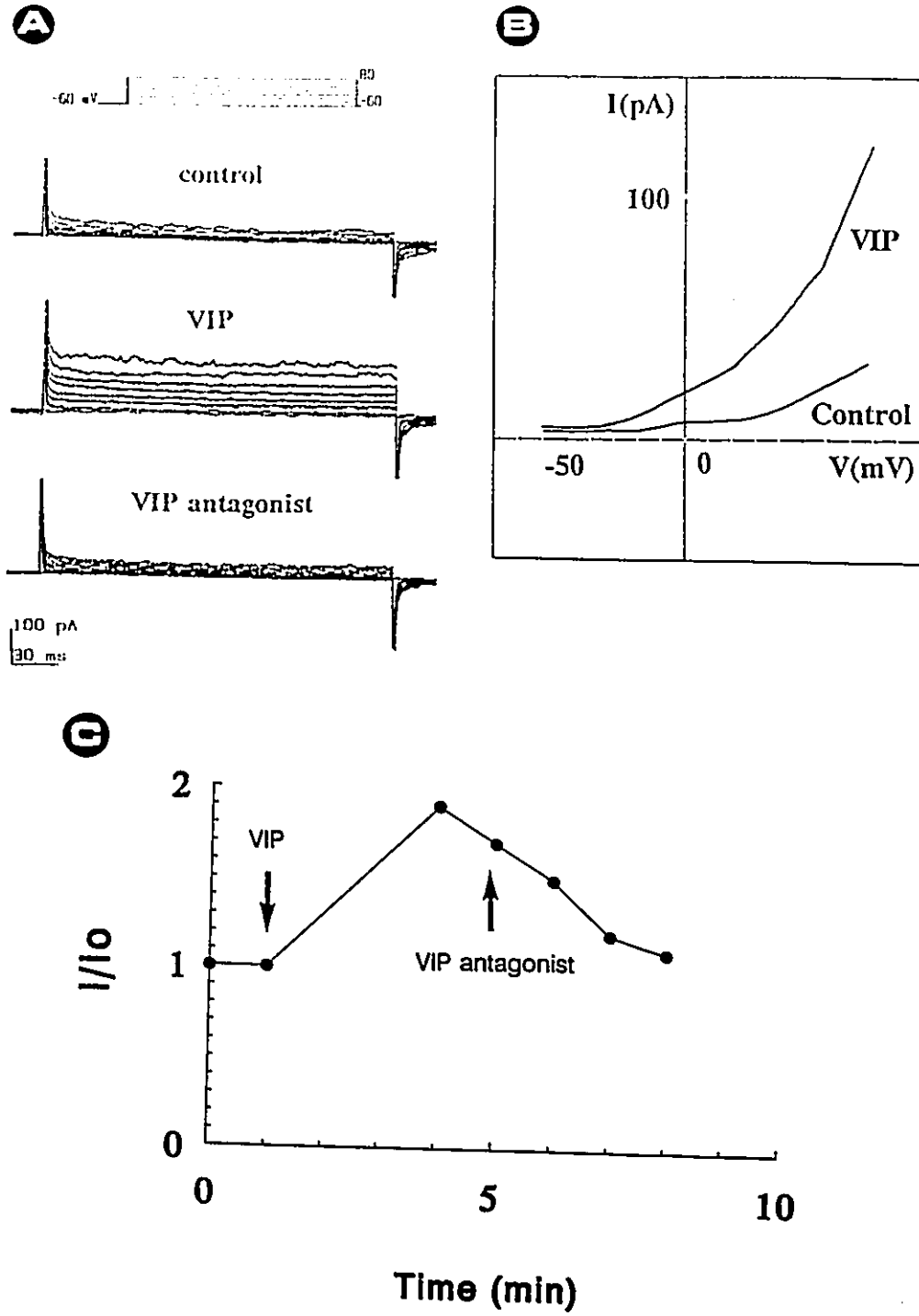


Figure 1

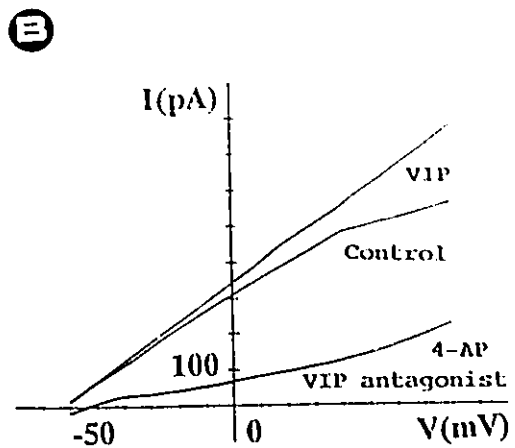
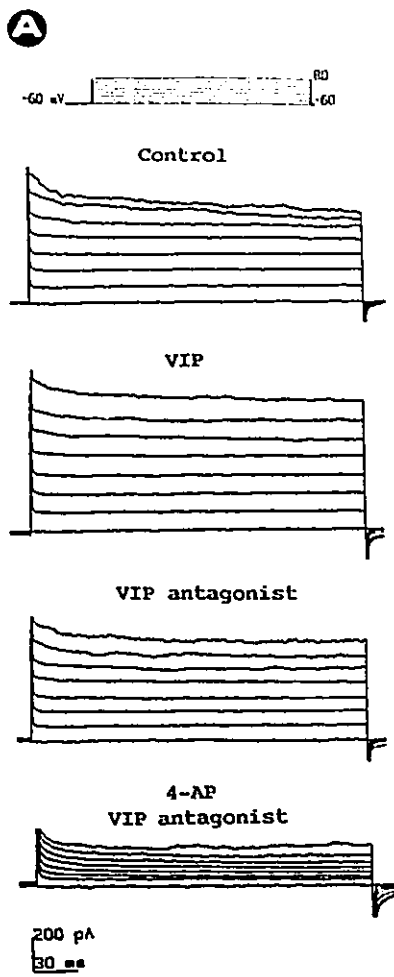


Figure 2

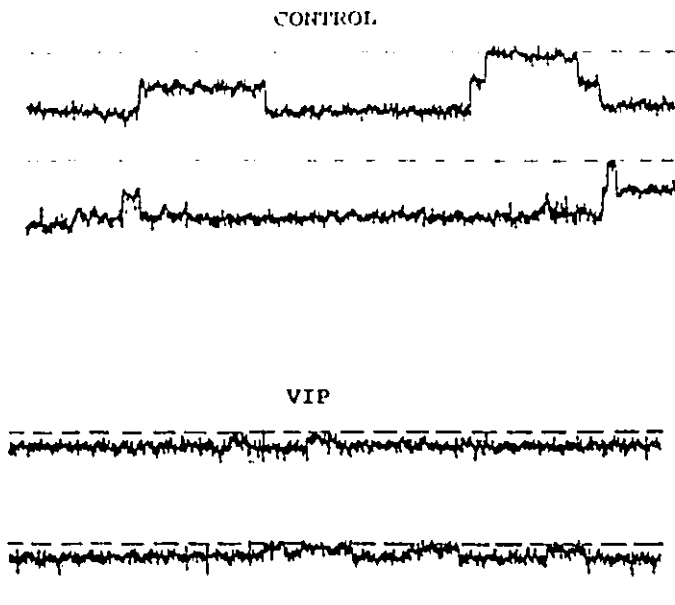
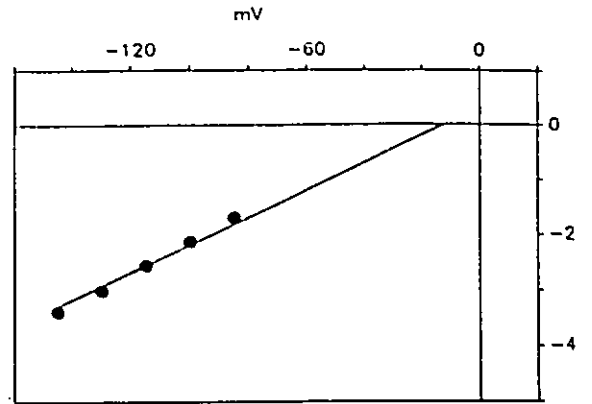
A**B**

Figure 3

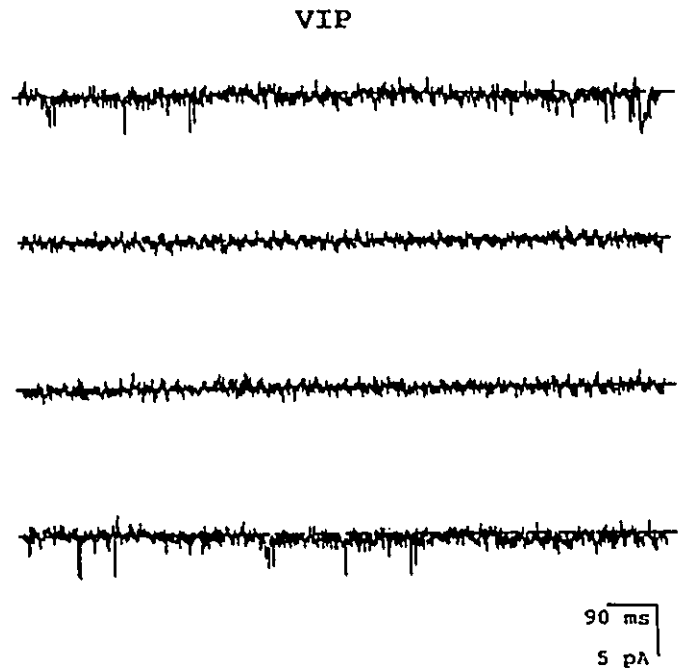
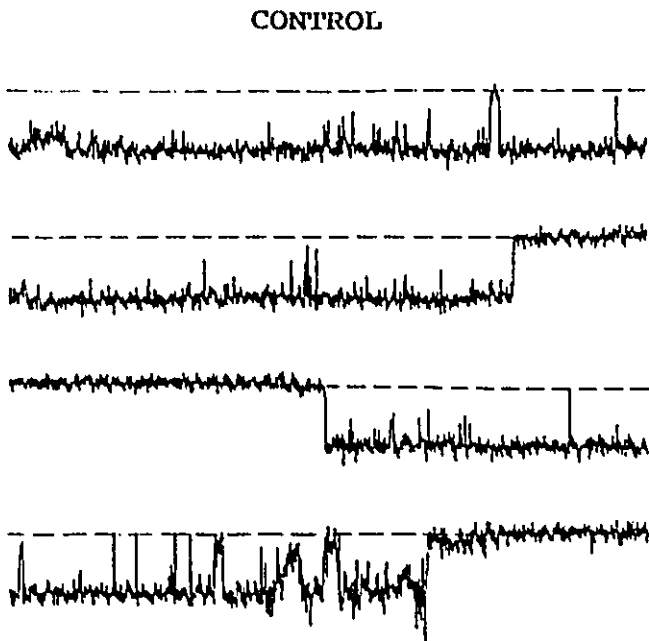
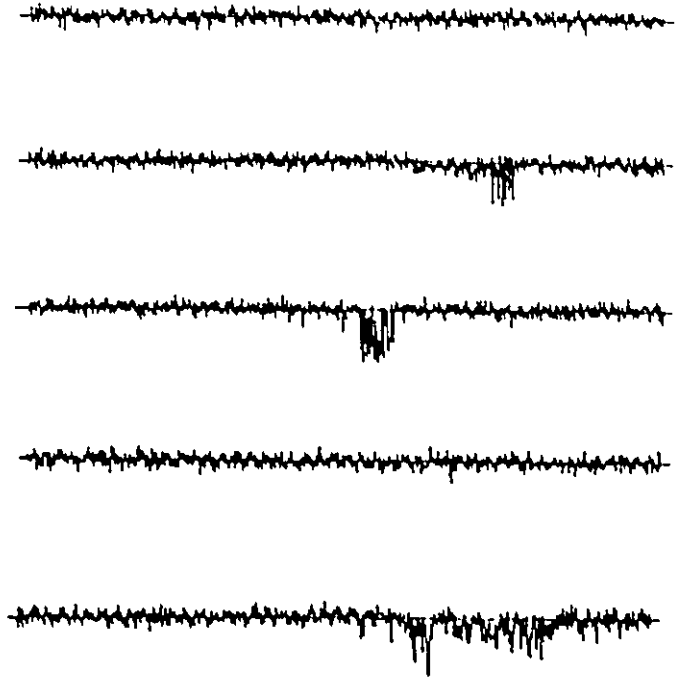
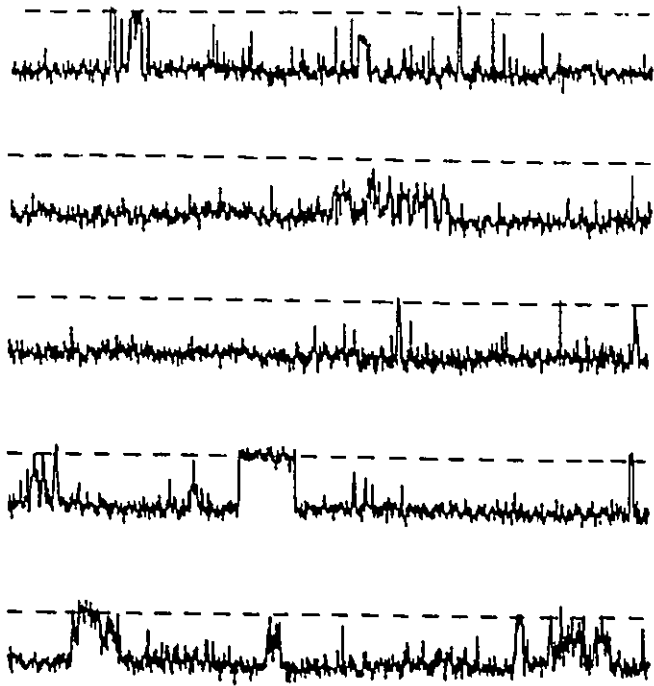


Figure 4

A

Control

GTP- γ S



90 ms
5 pA

B

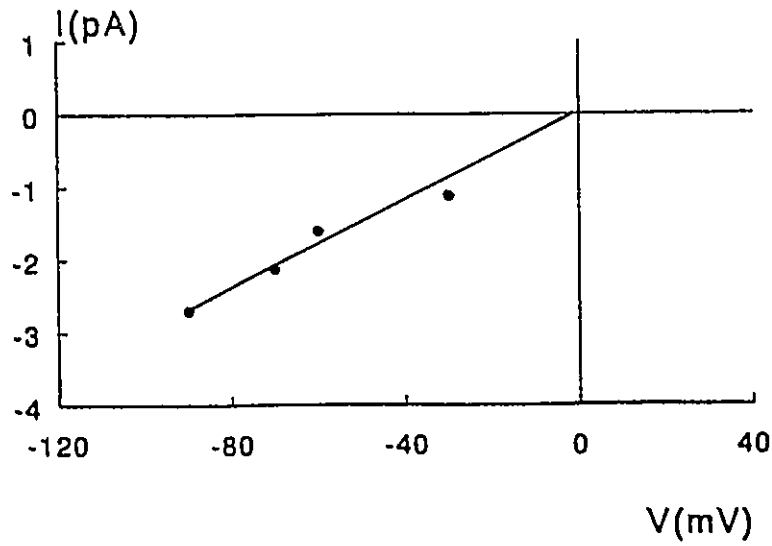


Figure 5

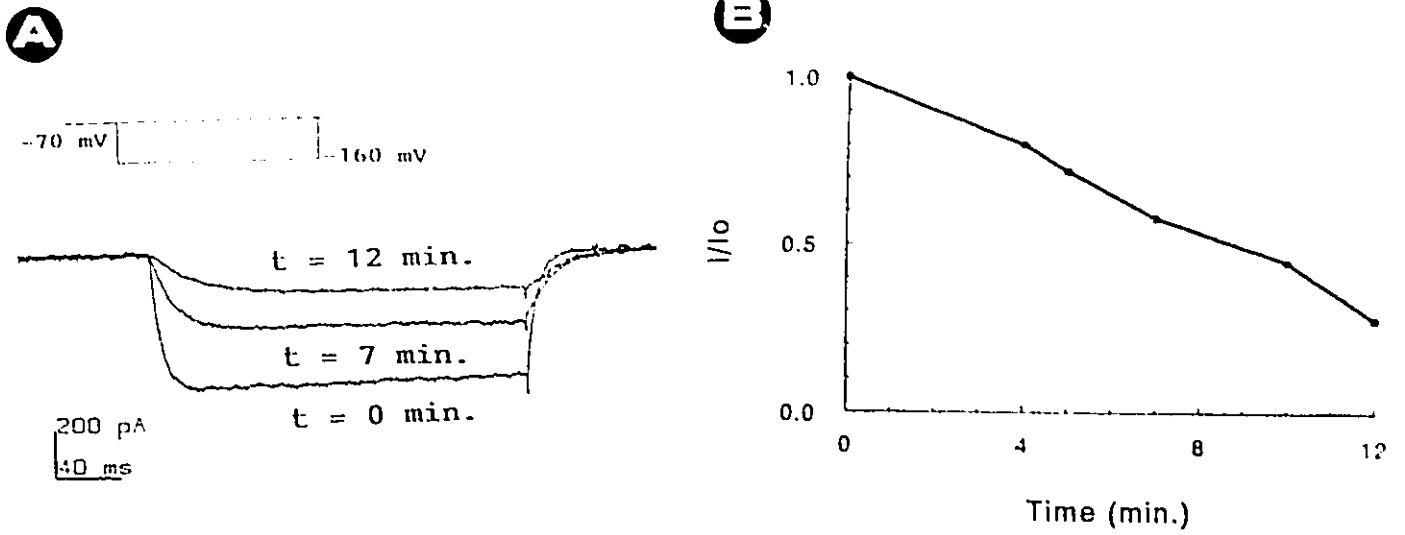


Figure 6

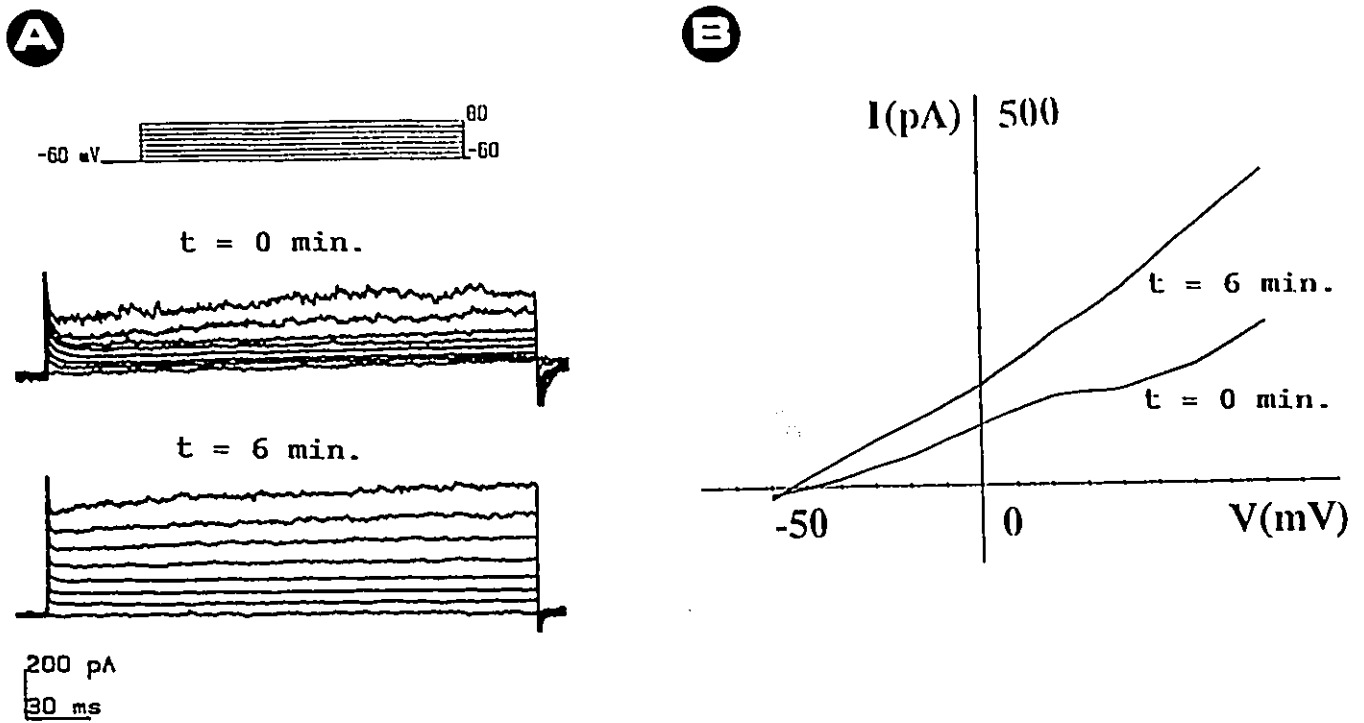


Figure 7

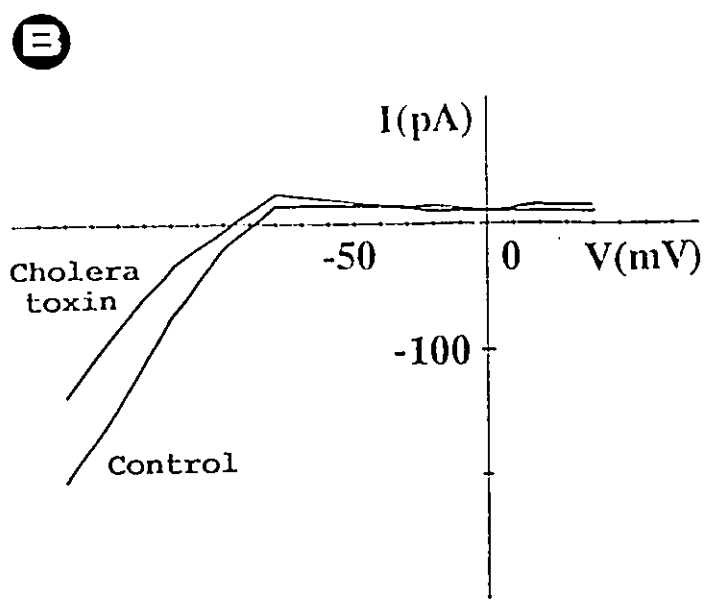
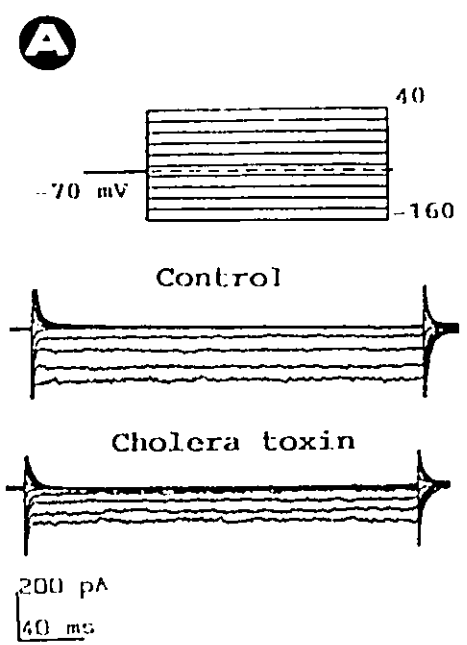


Figure 8

PAPER No. 4

CYCLOPIAZONIC ACID, AN ENDOPLASMIC RETICULUM Ca^{2+} -PUMP
INHIBITOR, ENHANCES Ca^{2+} ENTRY IN ENDOTHELIAL CELLS IN AN IP_3
INDEPENDENT MANNER

In revision for the *American Journal of Physiology*

Ewa Pasyk's contribution:

- (i) cell culturing
- (ii) performance of all electrophysiological experiments except for those demonstrating the effect of CPA on nonselective cation channels (fig. 3)

CYCLOPIAZONIC ACID, AN ER Ca^{2+} -PUMP INHIBITOR, ENHANCES
 Ca^{2+} ENTRY IN ENDOTHELIAL CELLS IN AN IP_3 INDEPENDENT
MANNER

E. Pasyk^{*}, M. Inazu and E.E. Daniel

Running head: CYCLOPIAZONIC ACID ENHANCES Ca^{2+} ENTRY IN
ENDOTHELIAL CELLS

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Abstract

Studies of rat aorta revealed that cyclopiazonic acid (CPA), an inhibitor of the endoplasmic reticulum (ER) Ca^{2+} -pump, released endothelium-derived relaxing factor (EDRF) and relaxed the muscle. We have used CPA to elucidate how this inhibitor of Ca^{2+} uptake into internal stores affects K^+ channels and Ca^{2+} entrance in cultured bovine pulmonary endothelial cells using patch-clamp techniques. CPA increased a Ca^{2+} -dependent outward K^+ current for many minutes, presumably as a consequence of the unbalanced leakage of Ca^{2+} from internal stores and Ca^{2+} entrance across the cell membrane. An expected consequence of this activation of the outward current changes is hyperpolarization of the cell membrane and increased driving force for Ca^{2+} entry. CPA activated the influx of extracellular Ca^{2+} through nonselective cation channels. Ca^{2+} influx through nonselective cation channels could help maintain $[\text{Ca}^{2+}]_i$ elevation and EDRF release. CPA also reduced the inwardly rectifying K^+ current. Inositol 1,4,5-trisphosphate (IP_3) in the patch pipette also produced an increase in outward K^+ currents, which were Ca^{2+} dependent. After depletion of Ca^{2+} internal stores by CPA, the response to IP_3 was abolished. Heparin in the patch pipette reduced the increase in outward currents induced by bradykinin, an agonist known to raise IP_3 and release Ca^{2+} , but did not prevent CPA-induced increases in outward current. Thus, CPA acts to elevate Ca^{2+} -activated currents in endothelial cells by a mechanism independent of IP_3 -induced release and this may lead to EDRF release both directly and as a consequence of Ca^{2+} entry

through nonselective cation channels driven by an increased electrical gradient for Ca^{2+} .

cyclopiazonic acid, cytosolic Ca^{2+} , EDRF, K^+ channels, nonselective cation channel

Introduction

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been shown to selectively inhibit the Ca^{2+} -pump in sarcoplasmic reticulum (SR) of striated (Goeger and Riley, 1989; Kurebayashi and Ogawa, 1991; Seidler et al., 1989), smooth muscle (Uyama et al., 1992) and endoplasmic reticulum (ER) of lymphocytes (Mason et al., 1991). It has been shown that CPA inhibits the Ca^{2+} -transport ATPase in SR vesicles from rat skeletal muscle (Goeger et al., 1988). Kurebayashi and Ogawa (1991) have studied the effects of CPA on Ca^{2+} -ATPase activity of the SR in skinned mammalian skeletal fibres. Recently it has been shown that CPA selectively inhibits ATP dependent Ca^{2+} uptake into internal stores in skinned ileal smooth muscle (Uyama et al., 1992).

Endothelial cells lack voltage-operated Ca^{2+} channels, yet EDRF release requires elevation of intracellular Ca^{2+} (Adams et al., 1989; Nilius, 1991; Takeda et al., 1987). Agonists which release EDRF are presumed to function initially by raising IP_3 levels, thus releasing Ca^{2+} from internal stores and secondarily by promoting entry of extracellular Ca^{2+} (Nilius, 1991). We recently observed that CPA caused sustained relaxation of rat aortic rings contracted by phenylephrine and relaxation was endothelium-dependent and abolished by an inhibitor of NO-synthase and methylene blue (Zheng et al., 1993). These results suggested that the mechanisms of Ca^{2+} elevation in endothelial cells to release EDRF might be independent of IP_3 formation and follow from release of Ca^{2+} from ER.

The objectives of this study were to determine how CPA affected $[Ca^{2+}]_i$ in endothelial cells, the consequences of this for Ca^{2+} entry and the IP_3 -dependence of CPA effects. We evaluated $[Ca^{2+}]_i$ by examining Ca^{2+} -activated K^+ currents. Our studies suggest that CPA inhibits the ER Ca^{2+} -pump of endothelial cells and elevates Ca^{2+} near the cell membrane. The transient cytosolic Ca^{2+} elevation, modulates plasma membrane ion channels and promotes influx of external Ca^{2+} and could promote subsequent production of EDRF by endothelial cells.

Materials and Methods

Materials

Cultured bovine pulmonary artery endothelial cells were obtained from American Type Culture Collection (ATCC cell line No. 209). Minimum Essential Medium (MEM), fetal bovine serum (FBS) and antibiotics were obtained from GIBCO (Grand Island, NY). Tissue culture plastics were from Corning Glass Works (Corning, NY) and Becton Dickinson Labware (Lincoln Park, NJ). Cyclopiazonic acid (CPA), tetraethylammonium (TEA), tetrabutylammonium (TBA), EGTA, HEPES, lanthanum chloride ($LaCl_3$), inositol 1,4,5-trisphosphate (IP_3), low molecular weight heparin (4-6 kDa), dimethyl sulphoxide (DMSO) and 4,4'-diisothio-cyanatostilbene-2,2'-disulfonic acid (DIDS) were purchased from SIGMA (St. Louis, MO). Charybdotoxin (ChTx) was obtained from Accurate Chemicals (Hicksville, NY). CPA was dissolved in DMSO in a concentration 10^{-2} M. A stock solution of 10^{-3} M was

obtained by the addition of distilled water.

Cell culture

This cell line was initiated from artery lumen scrapings dispersed by enzyme treatment. The cells were cultured in Minimum Essential medium, supplemented with 20% fetal bovine serum, 0.1% gentamycin and 0.1% fungizone. The cells were grown in a humidified incubator at 37°C and 5% CO₂. Confluent cell monolayers were passaged using trypsin containing EDTA once every 4 or 5 days. The cells were used for experimentation on the second or third day after plating in plastic cell culture dishes while still confluent.

Patch-clamp technique

The whole-cell patch-clamp technique was employed (Hamill et al., 1981). Currents were measured using an Axopatch-1C amplifier (Axon Instruments, Inc.) and recorded on-line by the computer (IBM-AT). Data were filtered with a lowpass Bessel filter (1 or 2 kHz). Records were sampled at 5 kHz using pCLAMP software (Axon Instruments, Inc.). Series resistance and capacitance transients were not compensated. The 'instantaneous' currents for the I-V curves were measured after settling of capacitance currents, usually between 10 to 30 ms after the beginning of the voltage pulses. The 'steady-state' currents for the I-V curves were measured at the very end of the 250th ms voltage protocol. Only some whole-cell currents were corrected for leakage which is indicated in the figure legend. Transient currents and

time-independent leakage were subtracted using pCLAMP software.

Patch electrodes had resistances between 1.5 to 5 M Ω and were heat polished. For the whole-cell recordings electrodes were filled with different intracellular solutions containing different amount of EGTA and ions (in mM): (a) 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES, 4 Na₂ATP, (b) 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP, (c) 135 KCl, 2 MgCl₂, 1 CaCl₂, 0.3 EGTA, 20 HEPES, 4 Na₂ATP, (d) 132 K-gluconate, 2.2 MgCl₂, 0.3 CaCl₂, 2.2 EGTA, 10 glucose and 5 HEPES, and (e) 135 KCl, 2 MgCl₂, 1 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP. The pH of (a), (b), (c) and (e) solutions was adjusted to 7.3 with KOH; the pH of the (d) solution was adjusted with Tris. The intracellular solutions were filtered before use (pore size 0.2 μ m). In almost all experiments the external bath solution contained (in mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose, 10 HEPES; pH adjusted to 7.3 with NaOH. In the experiment illustrated on fig. 3 different composition of the external bath solution was used (in mM): 140 TEA.Cl, 20 TBA.Cl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 5 HEPES (pH = 7.4 with Tris). Ca²⁺-free external bath solution contained (in mM): 142 NaCl, 4.5 KCl, 1 MgCl₂, 20 HEPES and 5.5 glucose.

Tested drugs were applied by perfusion (3 - 4 ml/min) or added to the bath to get final concentration as indicated. All experiments were conducted at room temperature.

Results

Effects of CPA on outward K⁺ currents in endothelial cells

Figures 1 and 2 show that under the control conditions depolarizing voltage steps (in the range -60 to 80 mV from the holding potential of -60 mV) evoked an outward current in the whole-cell configuration. This current was small or large in its amplitude and was charybdotoxin- and TEA-sensitive (figs. 2 A and 4). When CPA (10 μ M) was applied ($n = 6$), it strongly enhanced the outward current (fig. 1). This current resembled the Ca²⁺-activated K⁺ current reported in endothelial cells and evoked by other agents (Adams et al., 1989; Colden-Stanfield et al., 1987). This CPA-induced activation of the outward current was followed by fluctuations of the current amplitude in time (fig. 1 C). These fluctuations lasted for 10 to 15 min. A wash-out almost fully reversed the CPA effect in some cells. CPA, however, did not evoke the outward current when the pipette contained 11 mM EGTA ($n = 4$ cells; data not shown).

Potassium selectivity of the current was demonstrated by the change in K⁺ gradient and by the reduction of the current by charybdotoxin (for each experiment $n = 3$). When the bath concentration of KCl was increased tenfold (to 50 mM), the reversal potential shifted 50-55 mV to the right as predicted for K⁺ currents by the Nernst equation (data not shown). Charybdotoxin (50 nM) (Miller et al., 1985) prevented the activation of the outward current by CPA (fig. 2 B,C). After wash-out of charybdotoxin, the cell responded to CPA (10 μ M) and this effect was partially

reversible on washing out of CPA.

We also tested the solvent of CPA alone. DMSO at the concentration of 10^{-2} M did not affect either an outward nor the inwardly rectifying K^{+} currents in bovine pulmonary artery endothelial cells (see below) ($n = 3$ cells; data not shown).

Effects of CPA on the inwardly rectifying K^{+} current in endothelial cells

First stability of the whole-cell $I_{K_{in}}$ currents was examined. Control cells were recorded continuously for 19 min. 'Rundown' was not observed even after 19 min in the presence of 0.5 mM ATP in the pipette solution (data shown in APPENDIX II; $n = 4$ cells). The mean absolute value of control currents was 312 ± 48 pA ($n = 5$ cells), measured 250 ms after the beginning of the voltage pulse at -120 mV.

CPA (10 μ M) reversibly reduced inwardly rectifying K^{+} current ($I_{K_{in}}$), the predominant whole-cell current in endothelial cells activated by hyperpolarization ($n = 5$; data not shown). The magnitude of this decrease was $35.4 \pm 7.3\%$ (SE; $n = 5$ cells) of the control current measured as in the control condition. CPA effect was measured after 1-2 minutes of CPA application. Statistical difference between these two measurements was compared by t test and was considered significantly different ($p < 0.005$). In some cells (2 out of 5) this effect was also followed by fluctuations of the inward current amplitude (data shown in APPENDIX III).

Effect of increased $[Ca^{2+}]_i$ on the inwardly rectifying K^{+} currents

When the patch pipette contained a high concentration of EGTA (pipette

contents in mM: 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES and 4 Na₂ATP), the mean amplitude of the whole-cell I_{Kin} current measured at -160 mV potential was 506±54 pA (SE; n = 10; cells were randomly chosen out of 165 examined cells). The measurement of the I_{Kin} current amplitude under the same conditions, except for the presence of 0.3 mM EGTA in the pipette ((e) pipette contents) gave a mean value of 299±57 pA (SE; n = 10; cells were randomly chosen out of 20 examined cells). These two measurements were compared by t test and were considered statistically significant (p<0.05).

Effect of CPA on nonselective cation currents

When an intracellular solution with 132 mM K-gluconate (contents (d) described in Methods) and external solution containing blockers of potassium channels (TEA.Cl and TBA.Cl) were used, a large inward and small outward currents were observed in the whole-cell configuration (fig. 3; n = 8 cells). CPA (10 μM) increased both currents and these currents reversed at -11 mV (fig. 3 B, D). CPA-evoked currents were reversibly blocked by 50 μM lanthanum, an inorganic Ca²⁺ influx blocker (Colden-Stanfield et al., 1987). The reversal potential then returned to -60 mV (fig. 3 C, D).

In another set of experiments, control cells were first recorded in Ca²⁺-free extracellular solution, and then cells were exposed to 3 mM Ca²⁺ in the bath solution (n = 3 cells; data not shown). Addition of extracellular Ca²⁺ evoked a large outward current. This outward current was 20-30 % larger than when cells were treated by

CPA (10 μM) for 4-5 min. before addition of 3 mM Ca^{2+} to the bath ($n = 3$ cells; data not shown).

Effects of IP_3 on outward K^+ currents in endothelial cells.

When IP_3 (10 μM) was present in the pipette, there was a significant transient increase in the outward current measured in the whole-cell configuration (fig. 4; $n = 7$ cells). This effect was maximal at 3 - 5th minutes. The magnitude of this increase was at maximum 59 ± 8 % (SE; $n = 7$ cells) over the control current. The mean absolute value of control currents was 232 ± 47 pA (SE; $n = 7$ cells). The control currents were measured just after the break-through to the whole-cell configuration ($t = 0$), while IP_3 effects were measured after 3 to 4 minutes of the whole-cell recordings. All currents were measured at the same potential (+60 mV) at the end of the 250th-ms voltage pulses protocol. Statistical difference between these two measurements was compared by t test and was considered significantly different ($p < 0.0001$).

Although neither the control currents nor the washout from IP_3 in the cell could be recorded (addition of IP_3 to the pipette solution after break-through was not carried out), transitory increases in the whole-cell currents were observed over time (fig. 4 B). In 'control' experiments significant current changes over time were not observed in the whole-cell mode when intracellular solution had the same composition but without IP_3 (fig. 5; $n = 4$).

IP_3 -evoked outward currents appeared to be Ca^{2+} -activated K^+ currents. When

the pipette solution contained high concentration of EGTA (11 mM), outward currents were not activated (fig. 5; n = 4). When cells were pretreated for 8-10 minutes with CPA (10 μ M), before breakthrough to the cell, IP₃ also did not activate an outward current (fig. 5; n = 4). K⁺ selectivity of the IP₃-induced current was shown by the change in the K⁺ gradient and by the sensitivity to TEA. Under whole-cell symmetrical conditions (both the bath and pipette solutions contained 140 mM KCl), the I-V curve crossed the x axis at 0 mV, as predicted for K⁺ currents by the Nernst equation (n = 3; data not shown). TEA (1 - 5 mM) added to the bath solution reduced both basal current (by 60 - 80 %) and the response to IP₃ (fig. 4; n = 4). The absolute increase in K⁺ current attributed to IP₃ became very small.

Effects of heparin and CPA on an outward current.

When heparin (10 - 20 μ g/ml), an ER IP₃ receptor blocker (6), was present in the pipette solution, the outward whole-cell current was reduced in time (fig. 6; n = 5). In these experiments, cells were prestimulated by bradykinin (50 nM) 1 min before the break-through to the whole-cell configuration. Bradykinin is known to increase IP₃ production in endothelial cells (Adams et al., 1989; Colden-Stanfield et al., 1987; Laskey et al., 1990). Control currents were reduced by 75 \pm 2% (SE; n = 5 cells). The control currents were measured just after break-through to the whole-cell configuration (t = 0), while the effect of heparin was recorded 5 min later. All currents were recorded at the same potential (+60 mV) at the end of 250th-ms voltage pulses protocol. These two measurements was compared by t test and were

significantly different ($p < 0.0001$). The mean absolute value of the control currents was 829 ± 76 pA (SE; $n = 5$ cells).

In another set of experiments, bradykinin (50 - 100 nM) was added to the bath after 4-5 minutes of the whole-cell recordings with heparin (10 μ g/ml) in the patch pipette. In this case, bradykinin was not able to evoke an outward current ($n = 4$; data not shown). However, in other experiments, despite the presence of heparin (10 μ g/ml) in the pipette, CPA (10 μ M) added to the bath solution still was able to evoke significant K_{Ca} current (fig. 7; $n = 7$). The magnitude of this increase was $116 \pm 37\%$ (SE; $n = 7$ cells) over the control current. The control currents were measured 2 to 3 minutes after break through to the whole-cell configuration, at 1 min before the addition of CPA, whereas CPA effect was recorded 3 min after its addition. All currents were recorded at the same potential (+60 mV) at the end of the 250th-ms voltage pulses protocol. These two measurements were compared by t test and were significantly different ($p < 0.005$). The mean absolute value of the control currents was 275 ± 91 pA (SE; $n = 7$ cells).

Finally, bradykinin (50 nM) was added to the bath after 3-4 minutes of the whole-cell recordings with heparin (10 μ g/ml) in the pipette solution. Although heparin prevented the bradykinin-evoked outward K^+ current, CPA (10-20 μ M) applied to *the same cell* was able to evoke an K_{Ca} outward current ($n = 3$; data not shown).

Discussion

Control of intracellular Ca^{2+} in endothelial cells is not fully understood. Ca^{2+} enters an endothelial cell membrane through receptor-operated Ca^{2+} channels, which may be nonselective cation channels (Johns et al., 1987; Nilius, 1991; Schilling et al., 1988). Ca^{2+} entry through these channels is driven by the electrochemical gradient. An important uncertainty is whether this channel is regulated by $[\text{Ca}^{2+}]_i$ levels. In addition, there are stretch-activated non-selective cation channels (Adams et al., 1989; Lansman et al., 1987). Their relationship to the receptor-operated channels is unclear. Ca^{2+} extrusion is very likely to be through a plasmalemma Ca^{2+} pump (Hagiwara et al., 1983) and Na^+ - Ca^{2+} exchange (Adams et al., 1989). However, Na^+ - Ca^{2+} exchange appears to play no significant role in regulating $[\text{Ca}^{2+}]_i$ (Laskey et al., 1990). Uptake and release of Ca^{2+} by ER also plays a role and our study shows that continuous activity of the Ca^{2+} -pump in ER is required to control $[\text{Ca}^{2+}]_i$. Earlier studies established that there was ATP-dependent uptake and IP_3 -mediated release by this ER store in permeabilized endothelial cells (Adams et al., 1989; Freay et al., 1989). We have confirmed the role of IP_3 by the use of IP_3 or heparin in the patch pipette in this study.

Production of EDRF by endothelial cells is mediated by an increase of the intracellular Ca^{2+} activity. This increase of cytosolic Ca^{2+} is caused by Ca^{2+} release from intracellular stores and Ca^{2+} entry through receptor-operated ion channels in the plasma membrane (Adams et al., 1989; Colden-Stanfield et al., 1987; Johns et al.,

1987). The rate of external Ca^{2+} influx can be modulated by the membrane potential, the driving force for Ca^{2+} entry (Luckhoff and Busse, 1990a; Luckhoff and Busse, 1990b). Any shift of the membrane potential towards more negative values would increase the driving force for Ca^{2+} . In our experiments, the membrane potential was regulated by two types of K^+ channels: outward K^+ channels evoked by CPA and inwardly rectifying K^+ channel activated by hyperpolarization and modulated by CPA. Since CPA has been shown to act as a potent and selective inhibitor of the Ca^{2+} -pump of SR in several tissues (Goeger and Riley, 1989; Goeger et al., 1988; Seidler et al., 1989; Uyama et al., 1992) and appeared to release EDRF from endothelial cells (Zheng et al., 1993), we applied it in endothelial cells to demonstrate its effect on these channels and evaluate its potential effects on influx of external Ca^{2+} .

Our results show that CPA (10 - 20 μM) activated an outward K^+ current (fig. 1). This CPA-activated current was present in all cells examined when intracellular solution for the whole-cell recording contained a small amount (0.3 mM) or zero EGTA, and was not observed when cytosolic Ca^{2+} was buffered with a high concentration of EGTA (11 mM) in the pipette solution. This demonstrates: (i) a Ca^{2+} dependence of this current and (ii) that a low level of EGTA in the cell cannot control these K^+ channels, near which Ca^{2+} in high concentration may still occur. Several lines of evidence show that these currents are K^+ currents. Changing the K^+ gradient resulted in an appropriate shift of the I-V curve and established the K^+ selectivity of this current (data not shown). Furthermore, charybdotoxin, a drug that

reduces the 'big' Ca^{2+} -dependent K^+ current (Miller et al., 1985), prevented the CPA effect (fig. 2). These results suggest that the entry of Ca^{2+} into the cell and the unopposed leak from the CPA-sensitive internal Ca^{2+} stores activates the Ca^{2+} -dependent K^+ current.

This Ca^{2+} -dependent K^+ current always underwent significant fluctuations in its amplitude in time (fig. 1 C). These fluctuations may reflect dynamic changes of intracellular Ca^{2+} concentration near K^+ channels caused by the unbalanced Ca^{2+} leakage from internal stores and leakage across the plasmalemma, and by a disequilibrium between Ca^{2+} entry and extrusion at the plasma membrane.

A recent study of $[\text{Ca}^{2+}]_i$ levels in endothelial cells from bovine aorta used thapsigargin and 2',5'-di-(tert-butyl)-1,4-benzohydroquinone (BHQ) as inhibitors of the ER Ca^{2+} -pump (Dolor et al., 1992; Gericke et al., 1993; Thuringer and Sauve, 1992). These studies provided evidence that emptying the internal Ca^{2+} stores led to an IP_3 -independent pathway for Ca^{2+} entry which continued until the store refilled. Thapsigargin caused a sustained or oscillatory activation of K_{Ca} channels in cell-attached patches in bovine aortic endothelial cells (Thuringer and Sauve, 1992).

Another K^+ channel contributing to the changes in the membrane potential of endothelial cells is the inwardly rectifying K^+ current ($I_{\text{K}_{\text{in}}}$) activated upon hyperpolarization. CPA inhibits this $I_{\text{K}_{\text{in}}}$ current. The 'rundown' of $I_{\text{K}_{\text{in}}}$ currents was prevented in our studies by the presence of 4 mM ATP in the pipette solution. Our results also suggest that reduction of $I_{\text{K}_{\text{in}}}$ activity may be caused by increased $[\text{Ca}^{2+}]_i$ level. An increase in the $[\text{Ca}^{2+}]_i$ has been reported to inactivate the $I_{\text{K}_{\text{in}}}$ currents

also in other cell types, cardiac cells, *Aplysia* neurons and mast cells (Kramer and Levitan, 1988; Mukai et al., 1992; Vanderberg, 1987). Therefore, the transient increase in $[Ca^{2+}]_i$ near the plasmalemma may be responsible for the inhibitory effect of CPA on the $I_{K_{in}}$ current. Alternatively, CPA may release EDRF or another factor (Zheng et al., 1993) to act directly on this channel.

Owing to the lack of voltage-operated Ca^{2+} channels in endothelial cells (Adams et al., 1989; Nilius, 1991; Takeda et al., 1987), Ca^{2+} enters the cell through nonselective cation channels activated by an agonist (Johns et al., 1987) or stretch (Lansman et al., 1987). Our results indicate that, since CPA inhibits the refilling of the intracellular stores, the empty stores may be a trigger for the influx of external Ca^{2+} (Putney, 1990) through nonselective cation channels. Increased $[Ca^{2+}]_i$ released from ER stores may also contribute to the activation of these nonselective cation channels, as it has been demonstrated in other tissues (Partridge and Swandulla, 1988; Schlichter, 1992). The negative membrane potential likely created by effects of transient elevation of cytosolic Ca^{2+} on K^+ channels after CPA application also favours transmembrane influx of Ca^{2+} through these channels. Thus CPA may initiate a process to activate nonselective cation channels which provides positive feedback (enhanced Ca^{2+} entry) to allow EDRF release.

Furthermore, our results showed that when K^+ channels were blocked (TEA.Cl and TBA.Cl in the bath solution), CPA activated the influx of extracellular Ca^{2+} through nonselective cation channels (fig. 3). These channels were also permeable for Na^+ and K^+ (Inazu et al., 1993). CPA shifted current-voltage relationship close to

0 mV, the reversal potential for nonselective cation channels under our conditions. In the presence of lanthanum, an inorganic Ca^{2+} entry blocker, currents-voltage curves returned to the reversal potential of -60 mV. Chloride was excluded as a carrier for this channel since Cl^- was replaced with gluconate. In addition, the CPA-evoked current was insensitive to DIDS, the Cl^- channel blocker (Inazu et al., 1993).

We also have studied whether CPA actions resemble or involve a role of IP_3 in bovine pulmonary artery endothelial cells. The release of Ca^{2+} from internal stores due to the presence of IP_3 in the pipette was estimated by the appearance of Ca^{2+} -activated K^+ currents (K_{Ca}). IP_3 evoked a significant transient increase in outward K_{Ca} current (fig. 5). The secondary decrease in the K_{Ca} current may be due to emptying of internal Ca^{2+} stores and pumping out of released Ca^{2+} after prolonged exposure to IP_3 .

CPA pretreatment prevented the appearance of this IP_3 -evoked K_{Ca} current (fig. 5), suggesting that CPA inhibits the Ca^{2+} -pump required for filling of IP_3 sensitive stores. Heparin, an ER IP_3 receptor blocker (Ghosh et al., 1988; Irvine, 1992), was used to prevent Ca^{2+} release from ER. The action of heparin (present in the patch pipette) was estimated by the suppression of K_{Ca} current evoked by bradykinin which is known to produce IP_3 in endothelial cells (Adams et al., 1989; Colden-Stanfield et al., 1987) (fig. 6). Moreover, after exposing cells first to heparin for several minutes and then to bradykinin, the agonist was not able to activate this K_{Ca} current. However, despite the presence of heparin in the pipette, CPA was still able to evoke K_{Ca} (fig. 7). This suggests that CPA (i) acts independently of IP_3

receptors and (ii) increases $[Ca^{2+}]_i$ by inhibiting ER Ca^{2+} -pump activity.

In conclusion our study shows that inhibition of the ER Ca^{2+} -pump alone, apparently without activation of any mechanism leading to IP_3 formation, causes elevation of $[Ca^{2+}]_i$ near K^+ channels. This $[Ca^{2+}]_i$ elevation induced by CPA could be caused by a continuous leak of Ca^{2+} from the ER stores which is normally balanced by ER uptake. This study also shows that inhibition of Ca^{2+} uptake by the ER leads to additional changes promoting Ca^{2+} entry. CPA activates the influx of extracellular Ca^{2+} through nonselective cation channels. CPA may provide a system with positive feedback by Ca^{2+} entry into endothelial cells.

All these events appear to occur secondary to inhibition of the ER Ca^{2+} -pump and are unrelated to opening of receptor-operated Ca^{2+} channels by receptor occupation. Our study showed that CPA effects to raise $[Ca^{2+}]_i$ near the plasma membrane bypassed the IP_3 -mediated, heparin-inhibited release system. Moreover, CPA pretreatment blocked IP_3 -mediated activation of K_{Ca} channels, suggesting that CPA emptied the store of Ca^{2+} in ER which had IP_3 -mediated release.

Acknowledgements

Supported by the Medical Research Council and Pharmaceutical Manufacturers Association of Canada.

References

- Adams, D.J., J. Barakeh, R. Laskey and C. van Breeman, 1989, Ion channels and regulation of intracellular calcium in vascular endothelial cells, *FASEB J* 3, 2389.
- Colden-Stanfield, M., W.P. Schilling, A.K. Ritchie, S.G. Eskin, L.T. Navarro and D.L. Kunze, 1987, Bradykinin-induced increases in cytosolic calcium and ionic currents in culture bovine aortic endothelial cells, *Circ. Res.* 61, 632.
- Dolor, R.J., L.M. Hurwitz, Z. Mirza, H.C. Strauss and G.R. Whorton, 1992, Regulation of extracellular calcium entry in endothelial cells: role of intracellular calcium pool, *Am. J. Physiol. (Cell Physiol.)* 262, C171.
- Freay, A., A. Johns, D.J. Adams, U.S. Ryan and C. van Breeman, 1989, Bradykinin and inositol 1,4,5-trisphosphate-stimulated calcium release from intracellular stores in cultured bovine endothelial cells, *Pflug. Arch.* 414, 377.
- Gericke, M., G. Droogmans and B. Nilius, 1993, Thapsigargin discharges intracellular calcium stores and induces transmembrane currents in human endothelial cells, *Pflug. Arch.* 422, 552.
- Ghosh, T.K., P.S. Eis, J.M. Mullaney, C.L. Ebert and D.L. Gill, 1988, Competitive, reversible, and potent antagonism of 1,4,5-trisphosphate-activated calcium release by heparin, *J. Biol. Chem.* 263, 11075.
- Goeger, D.E. and R.T. Riley, 1989, Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effect on Ca^{2+} binding and Ca^{2+} permeability, *Biochem. Pharmacol.* 38, 3995.
- Goeger, D.E., R.T. Riley, J.W. Dorner and R.J. Cole, 1988, Cyclopiazonic acid inhibition of the Ca^{2+} -transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles, *Biochem. Pharmacol.* 37, 978.
- Hagiwara, H., Y. Ohtsu, M. Shimonaka and Y. Inada, 1983, Ca^{2+} - or Mg^{2+} -dependent ATPase in plasma membrane of cultured endothelial cells from bovine carotid artery, *Biochim. Biophys. Acta* 734, 133.
- Hamill, O.P., A. Marty, E. Neher, B. Sakman and F.J. Singworth, 1981, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflu. Arch.* 391, 85.
- Inazu, M., H. Zhang and E.E. Daniel, 1993, LP-805, a releaser of endothelium-derived nitric oxide, activates an endothelial Ca^{2+} permeable non-specific cation channel, *Life Scie.*, submitted.

- Irvine, R.F., 1992, Inositol phosphates and Ca^{2+} entry: toward a proliferation or a simplification. *FASEB J.* 6, 3085.
- Johns, A., T.W. Lategan, N.J. Lodge, U.S. Ryan, C. van Breemen and D.J. Adams, 1987, Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells, *Tissue Cell* 19, 733.
- Kramer, R. and I.B. Levitan, 1988, Calcium-dependent inactivation of potassium current in the *Aplysia* neuron R15, *J. Neurosci.* 8(5), 1796.
- Kurebayashi, N. and Y. Ogawa, 1991, Discrimination of Ca^{2+} -ATPase activity of the sarcoplasmic reticulum from acto-myosine-type ATPase activity of myofibrils in skinned mammalian skeletal muscle fibres: distinct effects of cyclopiazonic acid on the two ATPase activities, *J. Muscle Res. Cell Motil.* 12, 355.
- Lansman, J.B., T.J. Hallam and T.J. Rink, 1987, Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* 325, 811.
- Laskey, R.E., D.J. Adams, A. Johns, G.M. Rubanyi and C. van Breemen, 1990, Membrane potential and Na^+ - K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria, *J. Biol. Chem.* 265, 2613.
- Luckhoff, A. and R. Busse, 1990a, Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential, *Pflugers Arch.* 416, 305.
- Luckhoff, A. and R. Busse, 1990b, Activators of potassium channels enhance calcium influx into endothelial cells as a consequence of potassium currents, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 342, 94.
- Mason, M.J., C. Garcia-Rodriguez and S. Grinstein, 1991, Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane: comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes, *J. Biol. Chem.* 266, 20856.
- Miller, C., E. Moczydlowski, R. Latorre and M. Phillips, 1985, Charybdotoxin, a protein inhibitor of single Ca^{2+} -activated K^+ channels from mammalian skeletal muscle, *Nature* 313, 316.
- Mukai, M., I. Kyogoku and M. Kuno, 1992, Calcium-dependent inactivation of inwardly rectifying K^+ channel in a tumor mast cell line, *Am. J. Physiol.* 262, C84.
- Nilius, B., 1991, Regulation of transmembrane calcium fluxes in endothelium NIPS

6, 110.

Partridge, L.O. and D. Swandulla, 1988, Calcium-activated nonspecific cation channels, *Trends Neurosci.* 11, 69.

Putney, J. W., Jr., 1990, Capacitative calcium-entry revisited, *Cell Calcium* 11, 611.

Schilling, W.P., A.K. Ritchie, L.T. Navarro and S.G. Eskin, 1988, Bradykinin-stimulated calcium influx in cultured bovine aortic endothelial cells, *Am. J. Physiol.* 255, H219.

Schlichter, L.C., 1992, Nonselective cation channels in intact human T lymphocytes, *Can. J. Physiol. Pharmacol.* 70, 247.

Seidler, N.W., I. Jona., M. Vegh and A. Martonosi, 1989, Cyclopiazonic acid is a specific inhibitor of the Ca^{2+} -ATPase of sarcoplasmic reticulum, *J. Biol. Chem.* 264, 17816.

Takeda, K., V. Shini and H. Stoeckel, 1987, Voltage-activated potassium, but not calcium currents in cultured bovine aortic endothelial cells, *Pflugers Arch.* 410, 385.

Thuringer, D. and R. Sauve, 1992, A patch-clamp study of the Ca^{2+} mobilization from internal stores in bovine aortic endothelial cells. II. Effects of thapsigargin on the cellular Ca^{2+} homeostasis, *J. Membrane Biol.* 130, 139.

Uyama, Y., Imaizumi, Y. and M. Watanabe, 1992, Effects of cyclopiazonic acid, a novel Ca^{2+} -ATPase inhibitor, on contractile responses in skinned ileal smooth muscle, *Br. J. Pharmacol.* 106, 208.

Vanderberg, C.A., 1987, Inward rectification of potassium channel in cardiac ventricular cells depends on internal magnesium ions, *Proc. Natl. Acad. USA* 84, 2560.

Zheng, X.F., C.Y. Kwan and E.E. Daniel, 1993, Role of intracellular Ca^{2+} in EDRF release in rat aorta, *J. Vasc. Res.*, submitted.

Figure Legends

Figure 1

A. CPA (10 μM) evoked an outward whole-cell current in endothelial cells. Command voltages were given in 20 mV steps in the range -60 to 80 mV from the holding potential of -60 mV. The intracellular solution contained (in mM): 140 KCl, 1 MgCl_2 , 0.5 CaCl_2 , 0.3 EGTA, 10 HEPES, and 4 Na_2ATP . This figure is representative of 6 experiments.

B. Instantaneous I-V curves for the currents shown in A.

C. Time course of the fluctuations evoked by CPA (10 μM). The changes were recorded at the voltage step to +80 mV from the holding potential of -60 mV. CPA was applied directly to the bath to get a final concentration as indicated.

Figure 2

A. ChTx-sensitive K_{Ca} whole-cell currents and their instantaneous I-V relationships. ChTx (50 nM) reduced the outward currents. Time-independent leakage was subtracted. Command voltages protocol was as illustrated. Different cell than in B or C, but the same command voltages were applied. This figure is representative of 3 experiments.

B. Time course of the changes in the amplitude of the outward K_{Ca} current exposed to charybdotoxin. ChTx (50 nM) prevented the CPA (10 μM) effect. After wash-out, addition of CPA (10 μM) resulted in oscillations of the current. The intracellular

solution contained (in mM): 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES and 4 Na₂ATP. This figure is representative of 3 experiments.

C. Instantaneous current-voltage relationship for the currents (i) when ChTx and CPA were present in the bath, (ii) when CPA was added after 'wash 1' and (iii) after second wash-out ('wash 2').

Figure 3

Effect of lanthanum (La) on the cyclopiazonic acid (CPA)-activated cell response. The external solution contained (in mM): 140 TEA.Cl, 20 TBA.Cl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 5 HEPES (pH = 7.4 with Tris). The internal solution contained (in mM): 132 K-gluconate, 2.2 MgCl₂, 0.3 CaCl₂, 2.2 EGTA, 10 glucose and 5 HEPES (pH = 7.4 with Tris).

A. Control currents evoked by voltage pulses given every 2 sec in 20 mV steps over the range -160 to +60 mV from the holding potential of -60 mV.

B. Currents after addition of CPA (10 μM).

C. Currents in the presence of CPA and La (50 μM).

D. The current-voltage relations for instantaneous currents. This figure is representative of 8 experiments.

Figure 4

A. Effect of IP₃ on whole-cell outward currents in an endothelial cell. IP₃ (10 μM) causes steady increase in an outward current with a maximum response at 4 - 5 min,

followed by the amplitude decrease. Instantaneous I-V curve relationships for the currents shown in the inset. Inset: Currents evoked by command voltages given in 20 mV steps in the range -60 to 80 mV from the holding potential of -60 mV. The pipette solution contained: IP₃ (10 μM), and (in mM): 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES and Na₂ATP. This figure is representative of 7 experiments.

B. Time course of the whole-cell current changes shown in A, and when TEA (5 mM) was added to the bath solution (different cell than in A, but the same pipette solution and command voltage protocol was used). The figure with TEA is representative of 3 cells.

Figure 5

Time course of the whole-cell current changes when (i) ('control') currents were recorded under control conditions with standard intracellular solution in the pipette (in mM: 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES and 4 Na₂ATP), (ii) ('11 mM EGTA, IP₃') pipette solution contained high concentration of EGTA and IP₃ (in mM: 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES, 4 Na₂ATP and 10 μM IP₃), and (iii) ('CPA/IP₃') cell was pretreated with CPA (10 μM) added to the bath for 6-8 min and recorded with the presence of IP₃ in the pipette solution (in mM: 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP, and 10 μM IP₃). Each trace is representative of 4 experiments.

Figure 6

Effect of heparin on a whole-cell outward K_{Ca} current in an endothelial cell. The cell was prestimulated with bradykinin (50 nM). Heparin (10 μ g/ml) reduced the outward current. Steady-state current-voltage relationships for the currents shown in the inset. Inset: Currents recorded at different time after command voltages given every 20 mV in the range between -60 to 80 mV from the holding potential of -60 mV. The pipette solution contained: heparin (10 μ g/ml) and (in mM): 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP. This figure is representative of 5 experiments.

Figure 7

A. Effect of CPA on a whole-cell outward K_{Ca} current in the presence of heparin in an endothelial cell. The pipette contained: heparin (10 μ g/ml) and (in mM): 140 KCl, 1 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP. CPA (10 μ M) was added to the bath. Command voltages were given every 20 mV in the range -60 to +80 mV from the holding potential of -60 mV. This figure is representative of 7 experiments.

B. Instantaneous current-voltage relationships for the currents shown in A.

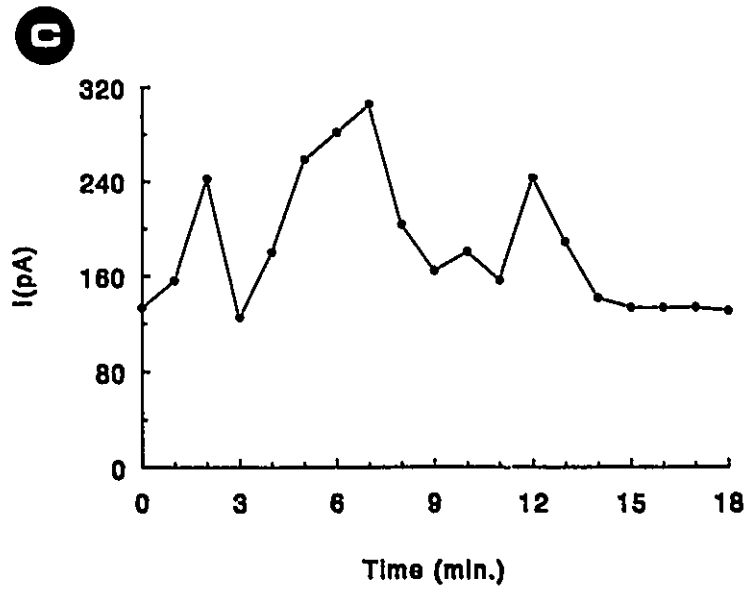
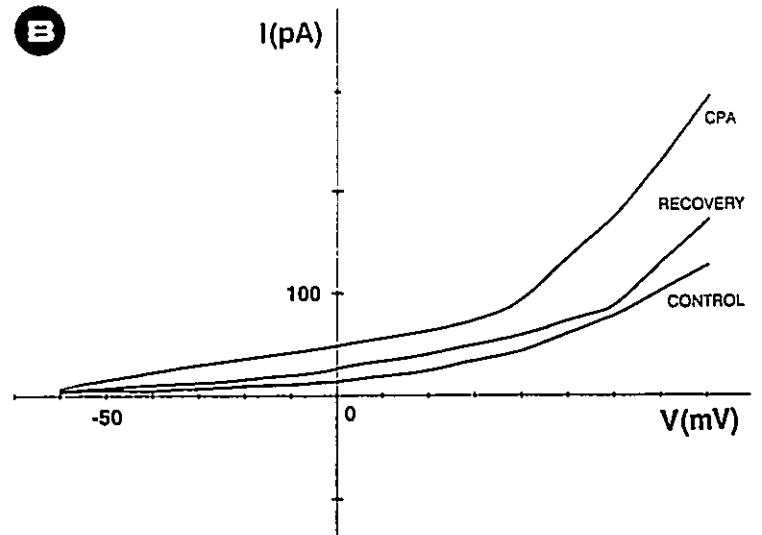
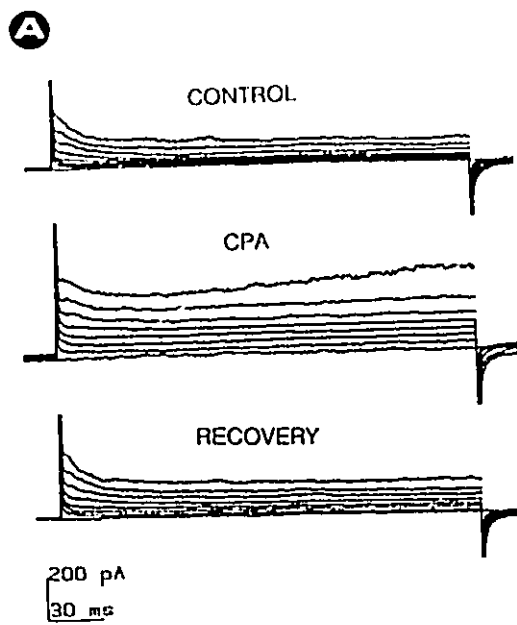


Figure 1

A

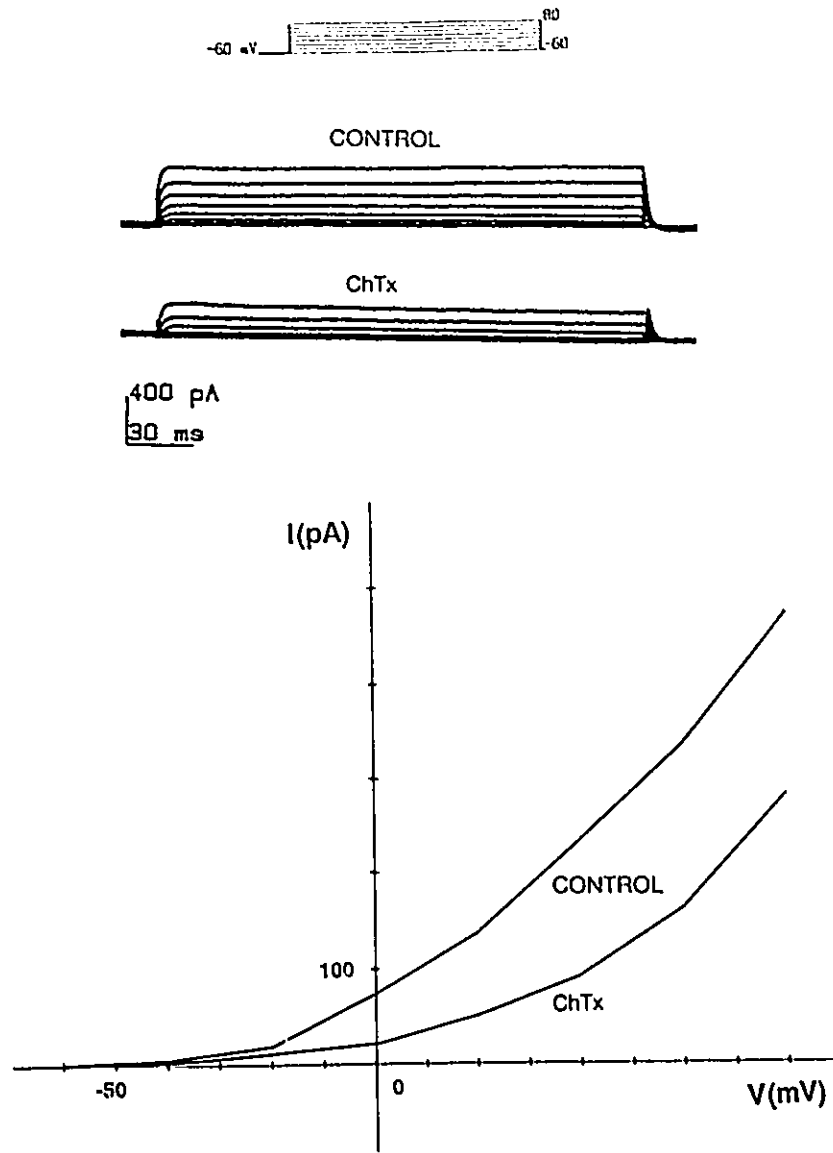


Figure 2

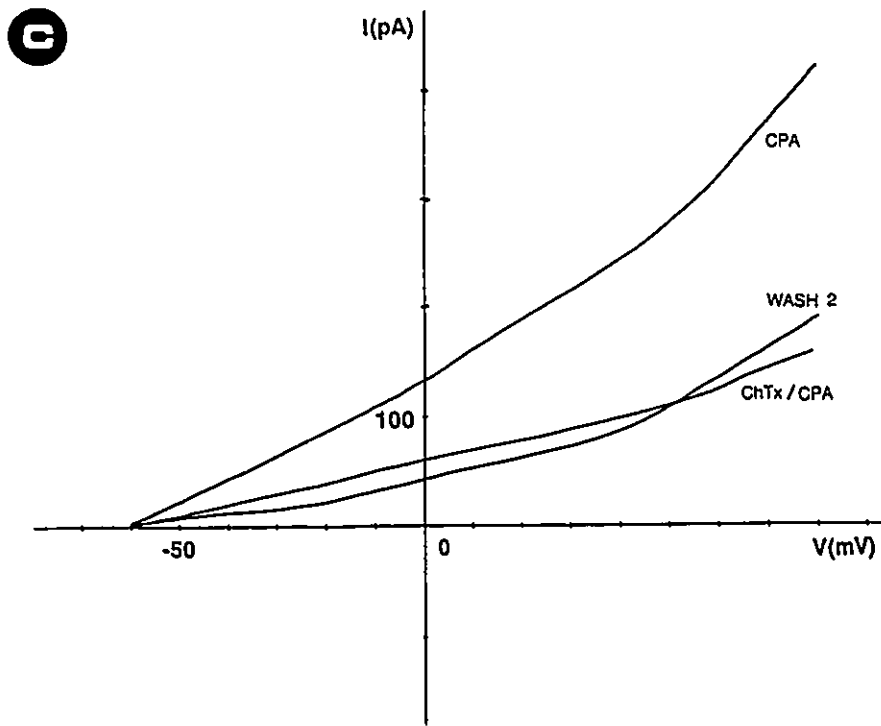
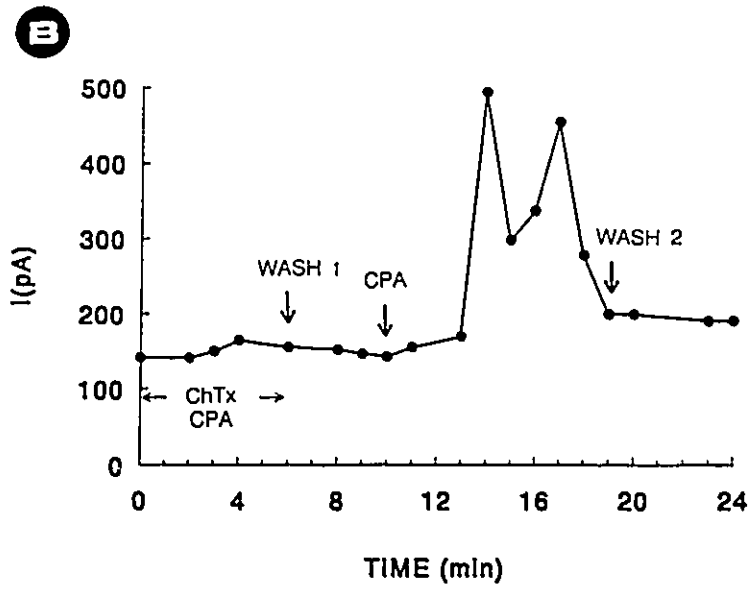


Figure 2

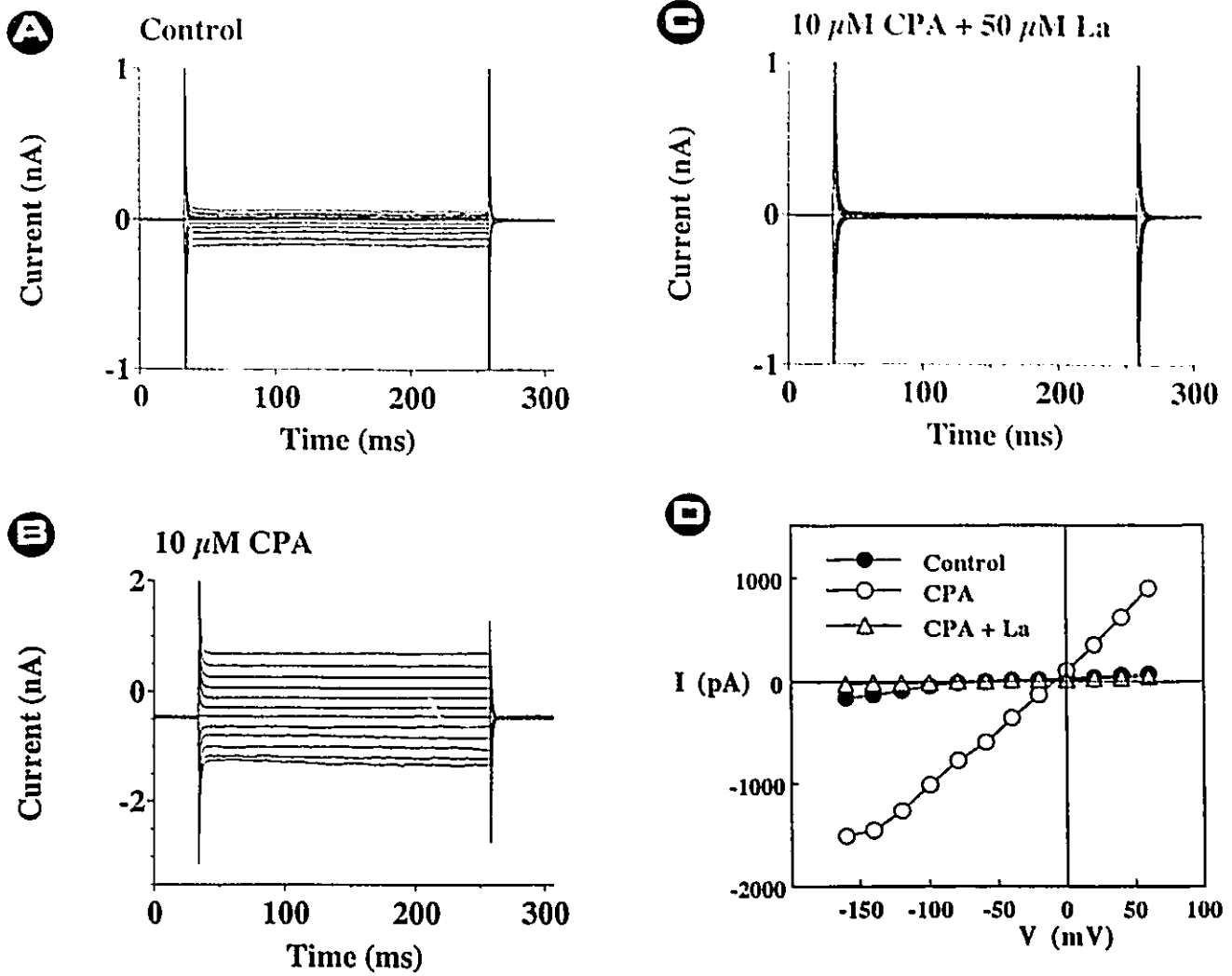


Figure 3

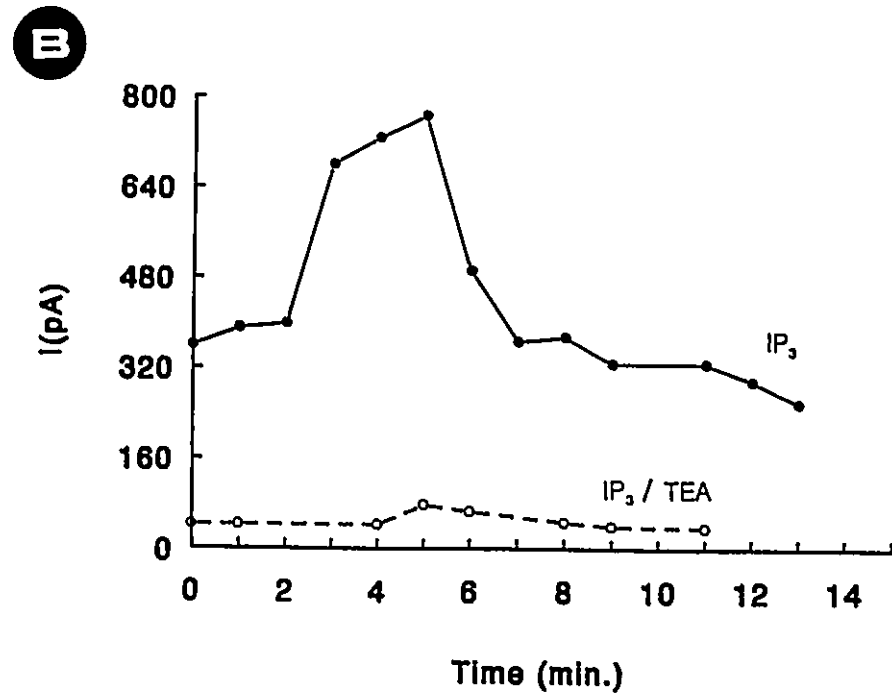
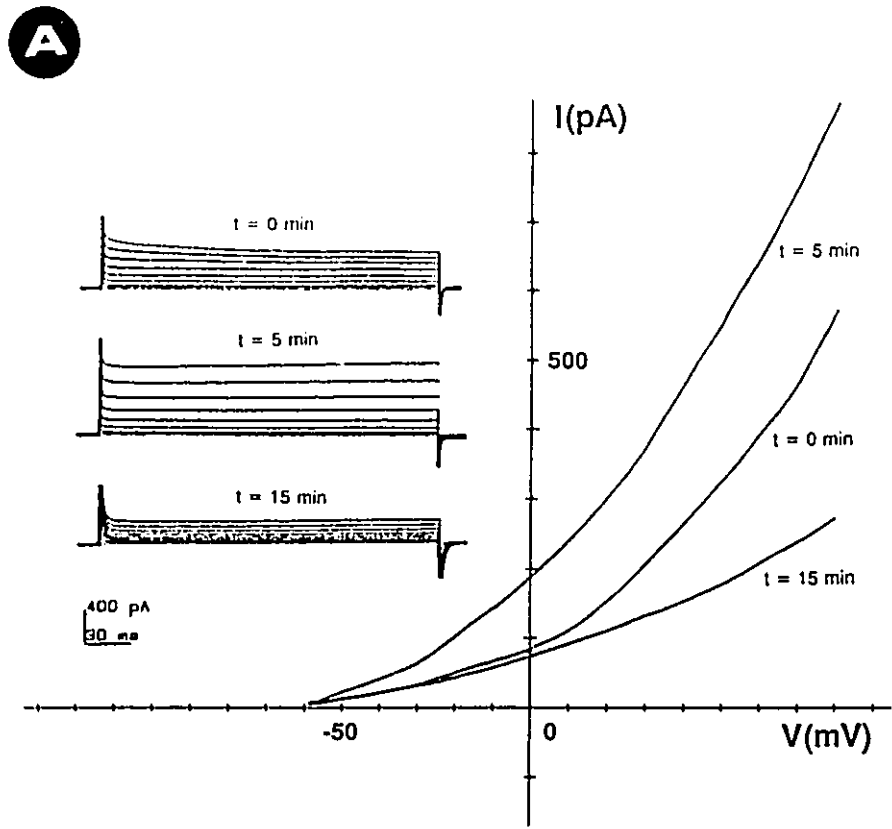


Figure 4

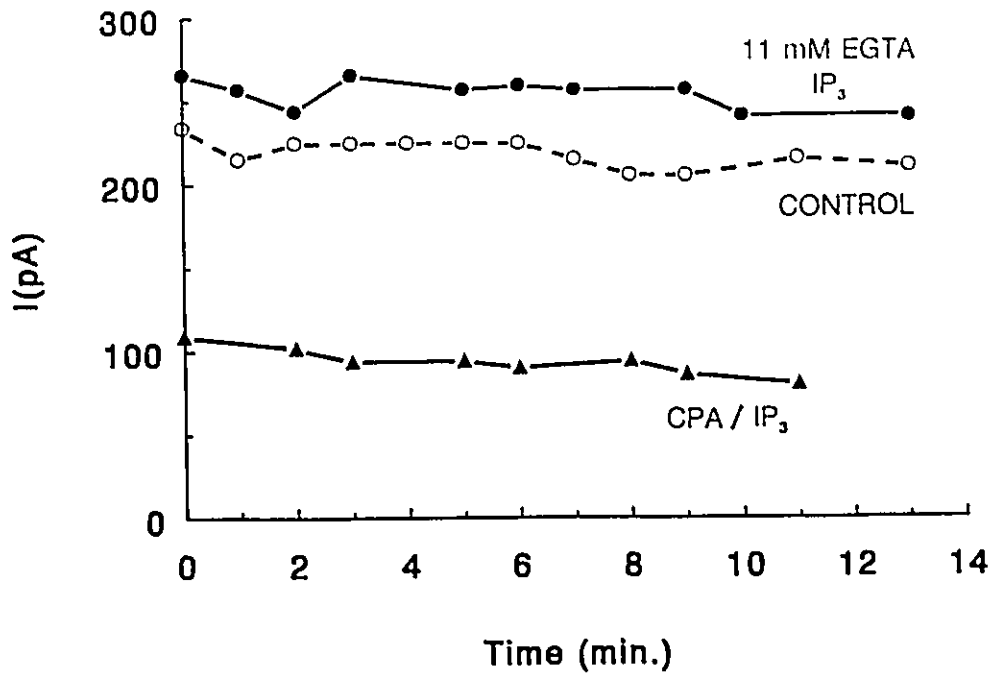


Figure 5

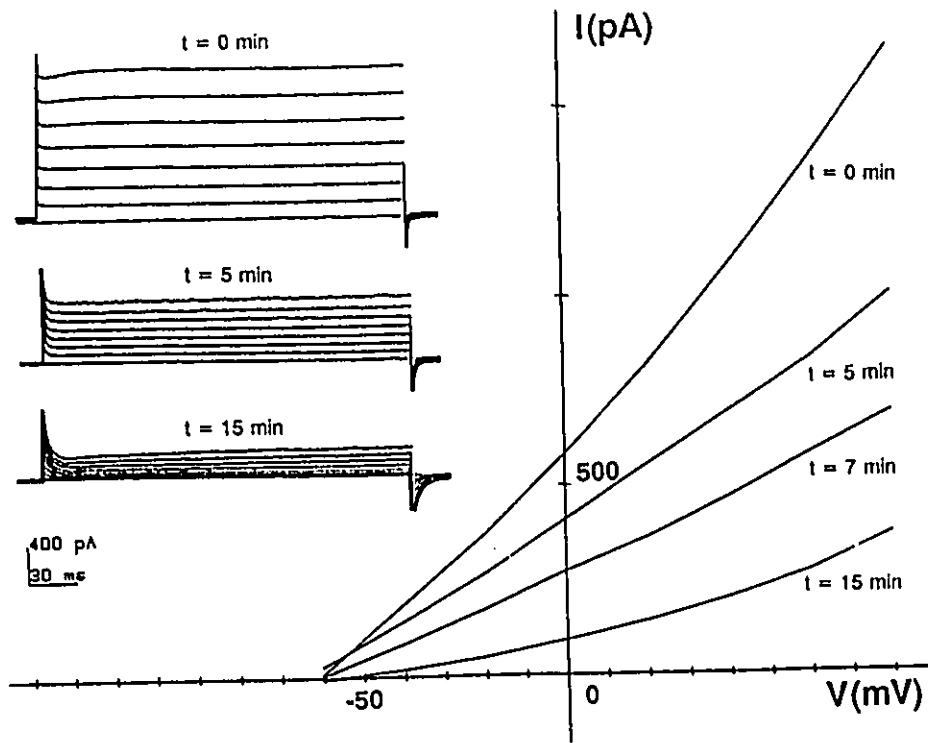


Figure 6

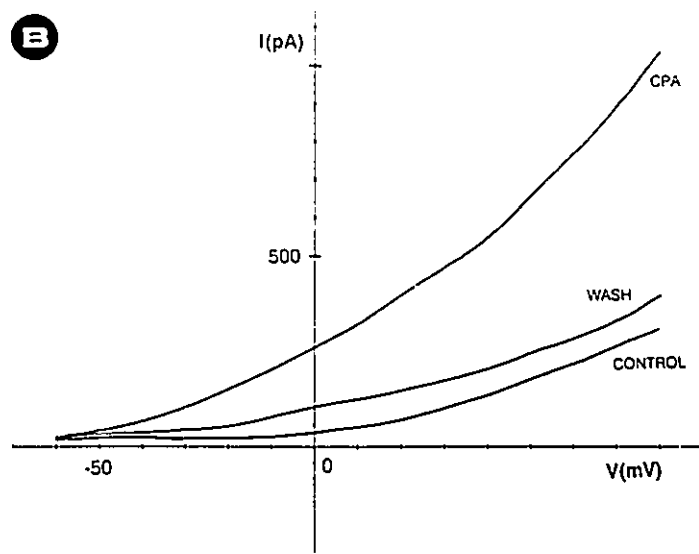
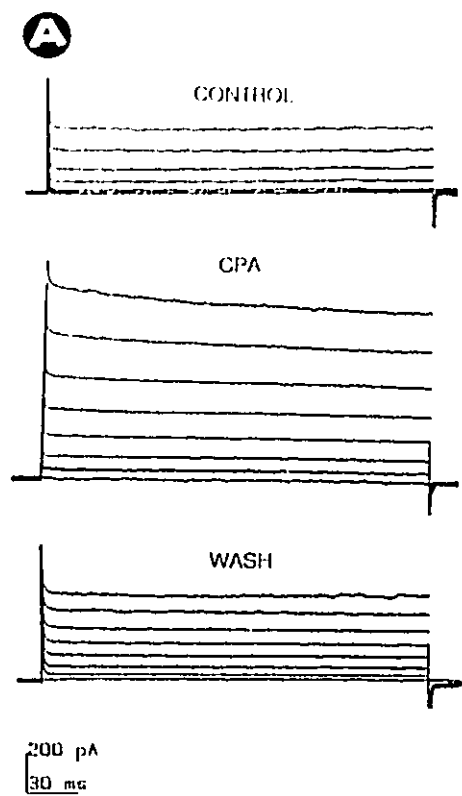


Figure 7

CHAPTER IV

DISCUSSION

1. The Effects of VIP on Ion Channels in Endothelial Cells

This study showed for the first time that endothelial cells contain a high density of functional VIP receptors. These receptors had been studied by ligand binding (Pasyk et al., 1992), but the objective of this work was to demonstrate that these receptors are functional. Using patch-clamp techniques it was demonstrated that VIP modulates plasma membrane K^+ channels in bovine pulmonary endothelial cells. Stimulation of these receptors inhibited activity of inwardly rectifying K^+ channels ($I_{K_{in}}$) and activated opening of the Ca^{2+} -dependent K^+ channels (K_{Ca}). Both K^+ channels seems to be regulated by intracellular free Ca^{2+} .

The notion of an outward K^+ current evoked by depolarization or an agonist which depends on $[Ca^{2+}]_i$ level is not new. Since VIP-evoked outward current revealed K^+ selectivity and Ca^{2+} dependence (see Paper No. 3), this allows me to classify this current as a Ca^{2+} -activated K^+ current. The Ca^{2+} -dependent K^+ channels are present in many endothelial cells types, where they are activated by various vasoactive agonists, bradykinin or acetylcholine (Colden-Stanfield et al., 1990; Sakai, 1990). The efflux of K^+ through these channels hyperpolarizes the endothelial cell plasma membrane (Daut et al., 1989; Busse et al., 1988). This transient hyperpolarization has special significance in endothelial cell physiology. Since endothelial cells do not have voltage-operated Ca^{2+} channels, Ca^{2+} enters the cell through nonselective cation channels (Adams et al., 1989). This Ca^{2+} entry is enhanced when the driving force for Ca^{2+} influx is increased by hyperpolarization.

It is likely that by activating Ca^{2+} -dependent K^+ currents, VIP contributes to endothelial cell membrane hyperpolarization and promotes an influx of extracellular Ca^{2+} which is necessary for the synthesis and release of EDRF.

The inwardly rectifying K^+ current activated by hyperpolarization consists of a K^+ influx at membrane potentials more negative than E_{K} and a K^+ efflux at membrane potentials more positive than E_{K} . This study showed that VIP inhibits K^+ influx through the I_{Kin} channels. This inhibition of I_{Kin} channel activity by VIP was demonstrated at both the whole-cell and single channel levels. By inhibiting the I_{Kin} channel activity, VIP reduced the influx of K^+ . This reduced K^+ influx tends to shift the membrane potential back towards more negative values, which may create the driving force for the influx of extracellular Ca^{2+} . The interaction between VIP receptor occupation, modulation of K^+ channels and Ca^{2+} fluxes in endothelial cells requires further investigation (see Further Directions).

The role of intracellular free Ca^{2+} in the regulation of I_{Kin} channel activity remains unclear. An additional and novel aspect of this study was an observation that K^+ influx through the I_{Kin} channel in bovine pulmonary artery endothelial cells was decreased with elevated intracellular Ca^{2+} level. Similar observations were already reported in mast cells (Mukai et al., 1992), cardiac muscle (Vandenberg, 1987) and *Aplysia* neurons (Kramer and Levitan, 1988). In contrast with these reports is an effect of bradykinin on I_{Kin} channel activity (Colden-Stanfield et al., 1990). Colden-Stanfield et al. (1990) reported that bradykinin, known for mobilization of Ca^{2+} from internal stores through the IP_3 system followed by external Ca^{2+} influx, activated

rather than inhibited $I_{K_{in}}$ channel opening in bovine aortic endothelial cells. In bovine pulmonary artery endothelial cells, under our experimental conditions, however, bradykinin (50-100 nM) reduced whole-cell $I_{K_{in}}$ currents evoked by hyperpolarization (Pasyk and Daniel, an unpublished observation).

Since VIP exerted opposite effects on the regulation of the two K^+ conductances, $I_{K_{in}}$ and K_{Ca} , one may ask whether there is a relationship between these two events. Similar results with distinct effects on these K^+ conductances were also obtained with other agents, GTP- γ -S and CPA (see Paper No. 3 and 4). It is likely that these channels are two distinct entities. However, more detailed studies elucidating this question were not performed in our and other endothelial cell types. More evidence, suggesting that $I_{K_{in}}$ and outward K^+ channels constitute two different molecular entities comes from the work of McCloskey and Cahalan (1990) in rat mast cells. This study demonstrated that some activators of a G protein (e.g. GTP- γ -S) inhibited the $I_{K_{in}}$ conductance and induced the appearance of an outward K^+ current. The time course of this $I_{K_{in}}$ decay and appearance of outward K^+ conductances were different in two different cells. The lack of correlation in the time courses between these two K^+ conductances suggests that the $I_{K_{in}}$ channel is not converted into the outward K^+ channel. Furthermore, these two channels revealed different unitary conductances (8 pS for an outward K^+ conductance and 26 pS for $I_{K_{in}}$), different sensitivities to inhibition by Ba^{2+} (100 μ M Ba^{2+} blocked $I_{K_{in}}$ completely, whereas 20 mM Ba^{2+} did not completely inhibit an outward K^+ conductance) and different effects with pertussis toxin (the toxin has no effect on $I_{K_{in}}$

current and inhibited induction of the outward K^+ current). These results suggest that $I_{K_{in}}$ and outward K^+ channels are two distinct proteins present in the membrane of mast cells. Furthermore, recent cloning and sequencing of the $I_{K_{in}}$ channel of macrophage cell revealed that the molecular structure of $I_{K_{in}}$ channel polypeptide is distinct from the basic structure of voltage operated K^+ channels (Kubo et al. 1993).

2. The Role of a G Protein

We examined the effects of various G protein activators (a GTP analog, cholera and pertussis toxins) on K^+ channels of bovine pulmonary artery endothelial cells. We demonstrated that activation of these cells by GTP- γ -S, a non-hydrolysable GTP analog and cholera toxin led to the inhibition of $I_{K_{in}}$ channel opening. The activity of the $I_{K_{in}}$ channels were pertussis toxin insensitive.

Since GTP- γ -S and cholera toxin mimicked the effects of VIP on $I_{K_{in}}$, this suggests that the G protein couples the VIP receptor to the $I_{K_{in}}$ channel. In general, the regulation of an ion channel by a G protein can be either direct or indirect with a second messenger being a mediator (Brown, 1991; Olate and Allende, 1991; Yatani et al., 1987). Our evidence suggest that this coupling between the G protein and $I_{K_{in}}$ channel is direct in its nature. VIP and GTP- γ -S reduced the $I_{K_{in}}$ channel activity in isolated patches (outside-out and inside-out, respectively), where the second messenger system is not operating. Thus, the G protein, activated after VIP receptor

stimulation, apparently couples directly to the $I_{K_{in}}$ channel. Such a direct coupling between a G protein and $I_{K_{in}}$ channel has been reported in many other cell types with various agonists (Fargon et al., 1990; Hoyer et al., 1991; McCloskey and Cahalan, 1990; Nakajima et al., 1988).

3. Cyclic AMP is *Not* a Second Messenger for VIP Transduction Pathway

The major finding of this study was an observation that cAMP is not a second messenger for VIP action in endothelial cells. Since VIP activates adenylate cyclase and production of cAMP in many cell types (Hirata et al., 1985; Nabika et al., 1985; Sata et al., 1988), it was expected that cAMP may also serve as a second messenger for VIP transduction pathway in endothelial cells. Our study provide both direct and indirect evidence allowing me to exclude that VIP stimulated cAMP elevation in bovine pulmonary artery endothelial cells. Direct evidence comes from the measurement of cAMP levels in our cell line after stimulation with isoproterenol and VIP. Isoproterenol is known to raise cAMP in many endothelial cell types (McEwan et al., 1990). Our cAMP level measurements in cultured bovine pulmonary endothelial cells confirmed this mode of isoproterenol action. However, stimulation with VIP did not elevate cAMP level in these cells.

Indirect evidence excluding VIP-activated cAMP elevation in bovine pulmonary artery endothelial cells comes from electrophysiological experiments. The effects of isoproterenol and VIP on $I_{K_{in}}$ channels were significantly different. VIP strongly

reduced or abolished $I_{K_{in}}$ channel activity, whereas the inhibitory effect of isoproterenol on this channel was very weak (about 20% of the VIP effect). The magnitude of the effects evoked by some activators of the cAMP cascade (forskolin, cAMP analogs) had intermediate values. These results suggest that the mechanisms of inhibition of the $I_{K_{in}}$ channel activity by VIP and isoproterenol are distinct.

It is likely that very weak inhibitory effect of isoproterenol and relatively weak inhibitory effects of forskolin and cAMP analogs on the $I_{K_{in}}$ channel activity can be explained by cAMP-dependent phosphorylation of the $I_{K_{in}}$ channel. It has been demonstrated in many cell types that cAMP-dependent protein kinase phosphorylates the K^+ channel and this process changes activity of the channel (Ewald et al., 1985). In this case, the channel is not modulated by the changes in intracellular Ca^{2+} level, but probably by increased sensitivity of the channel to Ca^{2+} (Kebabian, 1992). In many tissues, elevation in the cAMP level leads to the decrease of $[Ca^{2+}]_i$ (Exton, 1987; Hall et al., 1989; Suppattapone et al., 1988). The interaction between the cAMP and intracellular Ca^{2+} was not studied in our endothelial cell line.

Another piece of evidence allowing me to eliminate cAMP as a second messenger for VIP transduction pathway is the effect of VIP on the $I_{K_{in}}$ activity in the isolated patches. Although in isolated patches a second messenger system is considered not to operate, VIP was still able to inhibit $I_{K_{in}}$ channel opening in outside-out patches. This effect was mediated by an unidentified G protein that most likely couples directly to the channel. As discussed above, this G protein revealed GTP- γ -S and cholera toxin sensitivity and resistance to pertussis toxin. Here, it is important to

mention that presence of a putative membrane- delimited second messenger in excised patches of endothelial cells can not be completely ruled out.

The only evidence suggesting that VIP may act through the second messenger system in bovine pulmonary artery endothelial cells comes from the experiment in cell-attached mode. Applied to the bath, VIP was also able to inhibit the single $I_{K_{in}}$ channel activity in the cell-attached patch. The characteristic feature of this inhibition was reduced amplitude of the channel opening, with no decrease in probability of the channel opening. In the outside-out patch, VIP mainly reduced the open probability of the $I_{K_{in}}$ channel. This suggests involvement of two different modes of VIP action on the single channel activity in cell-attached and outside-out patches. An alternative explanation of the mode of the VIP action in cell-attached patches is that VIP could depolarize the membrane and as a consequence diminish the unitary current amplitude.

Furthermore, our experiments do not exclude existence of a putative VIP-activated second messenger other than cAMP. Recent studies suggest that VIP and homologous with VIP peptide, pituitary adenylate cyclase activating polypeptide (PACAP) not only activate adenylate cyclase and increase intracellular cAMP levels in various tissues, but they also stimulate the phosphatidylinositol cascade and mobilize intracellular Ca^{2+} (Canny et al., 1992; Deutsch and Sun, 1992; Tatsuno et al., 1992; Watanabe et al., 1992).

4. Calcium Signalling

Since Ca^{2+} plays a central role in regulating ion channels and production and secretion of EDRF in endothelial cells, we also studied Ca^{2+} signalling in cultured bovine pulmonary endothelial cells. We have used cyclopiazonic acid (CPA), an inhibitor of SR/ER Ca^{2+} pump, as a tool to examine Ca^{2+} fluxes (Goeger et al., 1988; Seidler et al., 1989; Uyama et al., 1992; Mason et al., 1991). Parallel studies in our laboratory revealed that CPA relaxed rat aorta in an endothelium-dependent manner, suggesting CPA-induced release of EDRF (Zheng et al., 1993a).

We have studied effects of CPA on K^+ channels and Ca^{2+} entrance in our endothelial cell line. CPA activated Ca^{2+} -dependent K^+ currents, presumably as a consequence of the unbalanced spontaneous leakage of Ca^{2+} from internal stores followed by Ca^{2+} entrance across the cell membrane. CPA also reduced the whole-cell I_{Kin} currents. An expected consequence of these changes is hyperpolarization of the cell membrane and an increased driving force for Ca^{2+} entry. In other studies we have found that CPA also enhanced activity of nonselective cation channels (permeable for Na^+ , K^+ , Ca^{2+}) (Zhang et al., 1993). Influx of Ca^{2+} through these channels could help maintain $[\text{Ca}^{2+}]_i$ elevation and EDRF release.

Inositol 1,4,5-trisphosphate (IP_3) in the patch pipette also produced an increase in an outward current which revealed K^+ selectivity and dependence on intracellular Ca^{2+} . Heparin, a selective blocker of ER IP_3 receptor (Ghosh et al., 1988), reversed the effect of IP_3 in endothelial cells (see Paper No. 4). Heparin, however, was not

able to prevent CPA-induced Ca^{2+} -activated K^+ currents. This suggests that CPA acts to activate Ca^{2+} -dependent K^+ currents in endothelial cells by a mechanism independent of IP_3 .

It is still unclear what activates influx of external Ca^{2+} in endothelial cells. Some authors have reported that intracellular Ca^{2+} and IP_4 control Ca^{2+} influx (Luckhoff and Claphan, 1992). Other studies, however, indicate that empty ER stores are a trigger for Ca^{2+} influx (Dolor et al., 1992; Schilling et al., 1992). Further studies are necessary to elucidate what are the activators of Ca^{2+} influx (see below).

5. Further Directions

The research on the mode of action of VIP in endothelial cells is by no means completed. The newly begun studies create interesting questions in endothelial cell physiology. One of the central question is whether VIP does or does not activate any second messenger system in endothelial cells. Our study allows me to exclude cAMP as an expected second messenger for VIP transduction pathway in endothelial cells and showed that VIP directly inhibits endothelial I_{Kin} channel activity. These results do not, however, exclude the possibility that VIP activates second messenger other than cAMP. Intracellular Ca^{2+} may be another putative second messenger for VIP transduction system. It should be evaluated whether VIP affects IP_3 production in endothelial cells. Also intracellular Ca^{2+} can be measured using a Ca^{2+} sensitive dye, fura-2, after stimulation of endothelial cells with VIP. Furthermore, measurements

of the other cyclic nucleotide, cGMP level after stimulation with VIP should be accomplished. Production of EDRF is associated with an increase in cGMP level in endothelial cells (Martin et al., 1988; Marczin et al., 1992).

Another important aspect of research on the mode of VIP action in endothelial cells would be direct measurement of a possible VIP-stimulated nitric oxide production. This could be accomplished at the tissue level (e.g. organ bath studies, 'sandwich' preparation), as well as, at the level of a single cell. Carbon fibre electrodes sensitive to electrochemical substances (e.g. nitric oxide) enable direct measurement of the secretory product of a single endothelial cell. As a strong oxidizing agent, nitric oxide generates the current at the surface of such an electrode. Cellular voltammetry enables study of ionic channel activity and measurement of a secretory product of the cell at the same time (Amato, 1992; Chow et al., 1992).

A real challenge in endothelial cell physiology is elucidation of the pathways of Ca^{2+} entry. It has been demonstrated that Ca^{2+} enters an endothelial cell through nonselective cation channels activated by an agonist or mechanical stimuli (stretch, shear stress). Due to lack of selective antagonists, function of the nonselective cation channels activated by an agonist is poorly understood. A novel inhibitor of these channels SK&F 96365 may bring more information on the action of these class of channels (Graier et al., 1992a; Merritt et al., 1990). SK&F 96365 should be tested in endothelial cells in patch-clamp experiments with VIP.

Mechanosensitive nonselective cation channels in endothelial cells are another fascinating but least understood pathways for Ca^{2+} entry. A difficulty in studying this

class of channels has been again the relative lack of pharmacological agents to manipulate it. Recently, amiloride has been shown to be a potent and selective blocker of mechanosensitive channels (Hamill et al., 1992). Another mechanosensitive channel blocker, gadolinium, may lack channel specificity (Yang and Sachs, 1989). The action of amiloride and its analog, bromo-hexamethyleneamiloride (BrHMA) (Lane et al., 1991), should be tested on stretch-activated nonselective cation channels in bovine pulmonary artery endothelial cells. Since the effects generated by mechanical stimuli in a variety of endothelial cells are similar to the second messenger responses resulting from agonist-receptor coupling: changes in intracellular Ca^{2+} , production of cAMP or IP_3 (Shen et al., 1992; Watson, 1991; Nollert et al., 1990), the mode of action of mechanosensitive channels requires further elucidation.

It remains still unclear whether the nonselective cation channels in bovine pulmonary artery endothelial cells are controlled by intracellular calcium, as reported in other cell types (Partridge and Swandulla, 1988), or an agonist exerts direct effect on these channels. If calcium ionophore A23187 opens these channels (without emptying calcium stores by an agonist or CPA), it is likely that these channels are calcium sensitive. Any agonist that elevates intracellular calcium may then open this channel, but empty Ca^{2+} ER stores may still play a role.

REFERENCES

- Adams, D.J, Barakeh, J., Laskey R. and C. Van Breeman, 1989, Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.* 3, 2389.
- Amato, I., 1992, Analytical chemists push the cellular envelope, *Science* 255, 925.
- Barish, M.E., 1983, A transient calcium-dependent chloride currents in the immature *Xenopus* oocyte, *J. Physiol.* 342, 309.
- Beavo, J.A. and D.H. Reifsnyder, 1990, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors, *TIPS* 11, 150.
- Berridge, M.J., 1986, Inositol phosphate as second messenger, *Receptor Biochem. Methodology* 7, 25.
- Birnbaumer, L. and A.M. Brown, 1990, G proteins and the mechanism of action of hormones, neurotransmitters and autocrine and paracrine regulatory factors, *Am. Rev. Resp. Dis.* 141, S106.
- Bissonnette, B.M, M.J. Collen, H. Adachi, R.T. Jensen and J.D. Gardner, 1984, Receptors for vasoactive intestinal peptide and secretin on rat pancreatic acini, *Am. J. Physiol.* 246, G710.
- Bossu, J.L., A. Feltz and F. Tanzi, 1989, Voltage-dependent calcium currents in freshly dissociated capillary endothelial cells, *FEBS Lett.* 255, 377.
- Bregestovski, P., A. Bakhramov, S. Danilov, A. Moldobaeva and K. Takeda, 1988, Histamine-induced inward currents in cultured endothelial cells from human umbilical vein, *Br. J. Pharmacol.* 95, 429.
- Bregestovski, P.D. and U.S. Ryan, 1989, Voltage-gated and receptor-mediated ionic currents in the membrane of endothelial cells, *J. Mol. Cell Cardiol.* 21 (Suppl. 1), 103.
- Brock, T.A., P.A. Dennis, K.K. Griendling, T.S. Diehl and P.F. Davis, 1988, GTP γ S loading of endothelial cells stimulates phospholipase C and uncouples ATP receptor, *Am. J. Physiol.* 255, C667.
- Brown, A.M., 1991, Ion channels as G protein effectors, *NIPS* 6, 158.
- Buchan K.W. and W. Martin, 1991, Modulation of agonist-induced calcium mobilization in bovine aortic endothelial cells by phorbol myristate acetate and cyclic AMP but not cyclic GMP, *Br. J. Pharmacol.* 104, 361.

- Busse, R., H. Fichtner, A. Luckhoff and M. Kohlhardt, 1988, Hyperpolarization and increased free calcium in acetylcholine-stimulated endothelial cells, *Am. J. Physiol.* 255, H965.
- Canny B.J., S.R. Rawlings and D.A. Leong, 1992, Pituitary adenylate cyclase activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes, *Endocrinology* 130, 211.
- Chow, R.H., L. von Ruden and E. Neher, 1992, Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells, *Nature* 356, 60.
- Clark, R.B., T. Nakajima, W. Giles, K. Kanai, Y. Momose and G. Szabo, 1990, Two distinct types of inwardly rectifying K⁺ channels in bull-frog atrial myocytes, *J. Physiol.* 424, 229.
- Colden-Stanfield, M., W.P. Schilling, A.K. Ritchie, S.G. Eskin, L.T. Navarro and D.L. Kunze, 1987, Bradykinin-induced increases in cytosolic calcium and ionic currents in culture bovine aortic endothelial cells, *Circ. Res.* 61, 632.
- Colden-Stanfield, M., W.P. Schilling, L.D. Possani and D.L. Kunze, 1990, Bradykinin-induced potassium current in cultured bovine aortic endothelial cells, *J. Membrane Biol.* 116, 227.
- Cooper, K., J.L. Rae and J. Dewey, 1991, Inwardly rectifying potassium current in mammalian lens epithelial cells, *Am. J. Physiol.* 261, C115.
- Couvineau, A. and M. Laburthe, 1985, The rat liver vasoactive intestinal peptide binding site. Molecular characterization by covalent cross-linking and evidence for differences from the intestinal receptor, *Biochem. J.* 225, 473.
- Daut, J., A. Dischner and G. Mehrke, 1989, Bradykinin induces a transient hyperpolarization of cultured guinea-pig coronary endothelial cells, *J. Physiol.* 410, 48P.
- Davies, J.M. and K.I. Williams, 1984, Endothelial-dependent relaxant effects of vasoactive intestinal polypeptide and arachidonic acid in rat aortic strips, *Prostaglandins* 27, 195.
- Davies P.F. and S.C. Tripathi, 1993, Mechanical stress mechanisms and the cell. An endothelial paradigm, *Circul. Res.* 72, 239.
- Deutsch, P.J. and Y. Sun, 1992, The 38 amino acid form of pituitary adenylate cyclase activating polypeptide stimulates dual signalling cascades in PC12 cells and promotes

neurite outgrowth, *J. Biol. Chem.* 267, 5108.

Dockray, G.J., 1987, Physiology of enteric nervous system. In: *PHYSIOLOGY OF THE GASTROINTESTINAL TRACT*. Ed. L.R. Johnson. Raven Press, N.Y., p 41.

Dolor, R.J., L.M. Hurwitz, Z. Mirza, H.C. Strauss and G.R. Whorton, 1992, Regulation of extracellular calcium entry in endothelial cells: role of intracellular calcium pool, *Am. J. Physiol. (Cell Physiol.)* 262, C171.

Duckles, S.P. and S.I. Said, 1982, Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation, *Eur. J. Pharmacol.* 78, 371.

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

Exton, J.H., 1987, Calcium signalling in cells, molecular mechanisms, *Kidney Int.* 32 (Suppl. 23), S68.

Fargon, F., P.A. McNaughton and F.V. Sepuleveda, 1990, Possible involvement of GTP-binding proteins in the deactivation of an inwardly rectifying K^{+} current in enterocytes isolated from guinea-pig small intestine, *Pflugers Arch.* 417, 240.

Frangos, J.A., S.G. Eskin, L.V. McIntire and C.L. Ives, 1985, Flow effects on prostacyclin production by cultured human endothelial cells, *Science* 227, 1477.

Freay, A., A. Johns, D.J. Adams, U.S. Ryan and C. van Breeman, 1989, Bradykinin and inositol 1,4,5-trisphosphate-stimulated calcium release from intracellular stores in cultured bovine endothelial cells, *Pflugers Arch.* 414, 377.

Furchgott, R.F. and J.V. Zawadzki, 1980, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, *Nature* 299, 373.

Ganz, P., A.W. Sandrock, S.C. Landis, J. Leopold, M.A. Gimbrone Jr. and R.W. Alexander, 1986, Vasoactive intestinal peptide vasodilation and cyclic AMP generation, *Am. J. Physiol.* 250, H755.

Ghosh, T.K., P.S. Eis, J.M. Mullaney, C.L. Ebert and D.L. Gill, 1988, Competitive, reversible, and potent antagonism of 1,4,5-trisphosphate-activated calcium release by heparin, *J. Biol. Chem.* 263, 11075.

Goeger, D.E., R.T. Riley, J.W. Dorner and R.J. Cole, 1988, Cyclopiazonic acid inhibition of the Ca^{2+} -transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles, *Biochem. Pharmacol.* 37, 978.

- Graier, W. F., K. Groschner, K. Schmidt and W.R. Kukovetz, 1992a, SK&F 96365 inhibits histamine-induced formation of endothelium-derived relaxing factor in human endothelial cells, *Biochem. Biophys. Res. Commun.* 186, 1539.
- Graier, W., K. Groschner, K. Schmidt and W.R. Kukovetz, 1992b, Increases in endothelial cyclic AMP levels amplify agonist-induced formation of endothelium-derived relaxing factor (EDRF), *Biochem J.* 288, 345.
- Gray, D.W. and I. Marshall, 1992, Novel signal transduction pathway mediating endothelium-dependent β -adrenoceptor vasorelaxation in rat thoracic aorta, *Br. J. Pharmacol.* 107, 684.
- Guggino, S.E., B.A. Suarez-Isla, W.B. Guggino and B. Sacktor, 1985, Forskolin and antidiuretic hormone stimulate a Ca^{2+} -activated K^+ channel in cultured kidney cells, *Am. J. Physiol.* 249, F448.
- Hagiwara, S. and K. Takahashi, 1974, The anomalous rectification and cation selectivity of the membrane of a starfish egg cell, *J. Membrane Biol.* 18, 61.
- Hall, I.P., J. Donaldson and S.J. Hill, 1989, Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP level in bovine tracheal smooth muscle, *Br. J. Pharmacol.* 97, 603.
- Hallam, T.J., R. Jacob and J.E. Merritt, 1988, Evidence that agonists stimulate bivalent-cation influx into human endothelial cells, *Biochem. J.* 255, 179.
- Hamill, O.P., J.W. Lane and D.W. McBride Jr., 1992, Amiloride: a molecular probe for mechanosensitive channels, *TIPS Oct.* 13, 373.
- Himmel, H.M., A.R. Whorton and H.C. Strauss, 1993, Intracellular calcium, currents and stimulus-response coupling in endothelial cells, *Hypertension* 21, 112.
- Hirata, Y., M. Tomita, S. Takata and T. Fujita, 1985, Functional receptors for vasoactive intestinal peptide in cultured vascular smooth muscle cells from rat aorta, *Biochem. Biophys. Res. Commun.* 132, 1079.
- Ho, K., C.G. Nicholas, W. J. Lederer, J. Lytton, P. M. Vassilev, M. V. Kanazirska and S.C. Hebert, 1993, Cloning and expression of an inwardly rectifying ATP-regulated potassium channel, *Nature* 362, 31.
- Hoyer, J., R. Popp, J. Meyer, H.J. Galla and H. Gogelein, 1991, Angiotensin II, vasopressin and GTP[γ -S] inhibit inward-rectifying K^+ channels in porcine cerebral capillary endothelial cells, *J. Membr. Biol.* 123, 55.

Ignarro, L.J., 1990, Biosynthesis and metabolism of endothelium-derived nitric oxide, *Annu. Rev. Pharmacol. Toxicol.* 30, 535.

Ignarro, L.J., H. Lipton, J.C. Edwards, W.H. Baricos, A.L. Hyman et al., 1981, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates, *J. Pharmacol. Exp. Ther.* 218, 739.

Itoh, T., T. Sasaguri, Y. Makita, Y. Kanmura and H. Kuriyama, 1985, Mechanisms of vasodilation induced by vasoactive intestinal polypeptide in rabbit mesenteric artery, *Am. J. Physiol.* 249, H231.

Jacob, R., J.E. Merritt, T.J. Halman and T.J. Rink, 1988, Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells, *Nature* 335, 40.

Johns, A., T.W. Lategan, N.J. Lodge, U.S. Ryan, C. van Breemen and D.J. Adams, 1987, Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells, *Tissue Cell* 19, 733.

Katz, B., 1949, Les constantes electriques de la membrane du muscle, *Arch. Sci. Physiol.* 3, 285.

Kebabian, J., 1992, The cyclic AMP cascade: A signal transduction system, *Neurotransmissions* 2, 1.

Knowles, R.G. and S. Moncada, 1992, Nitric oxide as a signal in blood vessel, *TIBS* 17, 398.

Kramer, R. and I.B. Levitan, 1988, Calcium-dependent inactivation of potassium current in the *Aplysia* neuron R15, *J. Neurosci.* 8(5), 1796.

Krupinski, J., 1991, *Mol. Cell Biochem.* 104, 73.

Kubo, Y., T. J. Baldwin, Y.N. Jan and L.Y. Jan, 1993, Primary structure and functional expression of a mouse inward rectifier potassium channel, *Nature* 362, 127.

Kuhn, M., A. Otten, J.C. Froelich and U. Foerstermann, 1991, Endothelial cyclic GMP and cyclic AMP do not regulate the release of endothelium-derived relaxing factor/nitric oxide from bovine aortic endothelial cells, *J. Pharmacol. Exp. Ther.* 256, 677.

Laburthe, M., B. Breant and C. Rouyer-Fessard, 1984, Molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium by covalent

cross-linking. Evidence for two classes of binding sites with different structural and functional properties, *Eur. J. Biochem.* 139, 181.

Lane, J.W., D.W. McBride and O.P. Hamill, 1991, Amiloride block of the mechanosensitive cation channel in *Xenopus* oocytes, *J. Physiol.* 441, 347.

Lansman, J.B., T.J. Hallam and T.J. Rink, 1987, Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* 325, 811.

Laskey, R.E., D.J. Adams, A. Johns, G.M. Rubanyi and C. van Breemen, 1990, Membrane potential and Na^+ - K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria, *J. Biol. Chem.* 265, 2613.

Laskey, R.E., D.J. Adams, S. Purkerson and C. van Breemen, 1991, Cytosolic calcium ion regulation in cultured endothelial cells. In: *Regulation of Smooth Muscle Contraction*, Ed. R.S. Moreland, Plenum Press, N.Y., p 257.

Laurenza, A., E.M. Sutkowski and K.B. Seamon, 1989, Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *TIPS* 10, 442.

Lechleiter, J.D., D.A. Dartt and P. Brehm, 1988, Vasoactive intestinal peptide activates Ca^{2+} -dependent K^+ channels through a cAMP pathway in mouse lacrimal cells, *Neuron* 1, 227.

Lee, T.J.F., A. Saito and I. Berezin, 1984, Vasoactive intestinal polypeptide-like substance: the potential transmitter for cerebral vasodilation, *Science* 224, 898.

Lewis, D.L., J.D. Lechleiter, D. Kim, C. Nanavti and D.E. Clapham, 1990, Intracellular regulation of ion channels in cell membranes, *Mayo Clin. Proc.* 65, 1127.

Lincoln J. and G. Burnstock, 1990, Neural-endothelial interactions in control of local blood flow. In: *THE ENDOTHELIUM: An Introduction to Current Research*. Ed. J.B. Warren, Wiley-Liss, Inc., p 21.

Ling, B.N. and W.C. O'Neill, 1992, Ca^{2+} -dependent and Ca^{2+} -permeable ion channels in aortic endothelial cells, *Am. J. Physiol.* 263, H1827.

Lodge, N.J., U.S. Ryan, C. van Breemen and D.J. Adams, 1988, Bradykinin and thrombin activated cation channels in cultured endothelial cells from bovine pulmonary artery, *Biophys. J.* 53, 265a.

Luckhoff, A. and R. Busse, 1990, Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential,

Pflugers Arch. 416, 305.

Luckhoff, A., A. Mulsch and R. Busse, 1990, cAMP attenuates autacoid release from endothelial cells: relation to internal calcium, *Am. J. Physiol.* 258, H960.

Luckhoff, A. and D.E. Clapham, 1992, Inositol 1,3,4,5-tetrakis-phosphate activates an endothelial Ca^{2+} -permeable channel, *Nature* 355, 356.

Marczin, N., U.S. Ryan and J.D. Catravas, 1992, Endothelial cGMP does not regulate basal release of endothelium-derived relaxing factor in culture, *Am. J. Physiol.* 263, L113.

Martin, W., D.G. White and A.H. Henderson, 1988, Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells, *Br. J. Pharmacol.* 93, 229.

Mason, M.J., C. Garcia-Rodriguez and S. Grinstein, 1991, Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane: comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes, *J. Biol. Chem.* 266, 20856.

Matsuda, H., A. Saigusa and H. Irisawa, 1987, Ohmic conductance through the inwardly rectifying K^+ channel and blocking by internal Mg^{2+} , *Nature* 325, 156.

Matsuda, H., 1988, Open-state substructure of inwardly rectifying potassium channels revealed by magnesium block in guinea-pig heart cells, *J. Physiol.* 397, 237.

McCloskey, M.A. and M.D. Cahalan, 1990, G protein control of potassium channel activity in a mast cell line, *J. Gen. Physiol.* 95, 205.

McEwan, J.R., H. Parsaee, D.C. Lefroy and J. MacDermont, 1990, Receptors linked to adenylate cyclase on endothelial cells, In: *THE ENDOTHELIUM: An Introduction to Current Research*. Wiley-Liss, Inc., New York, p 45.

Merritt, J.E., W.P. Armstrong, C.D. Benham, T.J. Hallam, R. Jacob et al., 1990, SK&F 96365, a novel inhibitor of receptor-mediated calcium entry, *Biochem. J.* 271, 515.

Mo M., S.G. Eskin and W.P. Schilling, 1991, Flow-induced changes in Ca^{2+} signalling of vascular endothelial cells: Effect of shear stress and ATP, *Am. J. Physiol.* 260, H1698.

Moncada, S. A.G. Herman, E.A. Higgs and J.R. Vane, 1977, Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the

anti-thrombic properties of vascular endothelium, *Throm. Res.* 11, 323.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology and pharmacology, *Pharmacol. Rev.* 43, 109.

Mukai, M., I. Kyogoku and M. Kuno, 1992, Calcium-dependent inactivation of inwardly rectifying K^+ channel in a tumor mast cell line, *Am. J. Physiol.* 262, C84.

Nabika, T., Y. Nara, Y. Yamori, W. Lovenberg and J. Endo, 1985, Angiotensin II and phorbol ester enhance isoproterenol and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells, *Biochem. Biophys. Res. Commun.* 131, 30.

Nakajima, Y., S. Nakajima and M. Inoue, 1988, Pertussis toxin-insensitive G protein mediates substance P-induced inhibition of potassium channels in brain neurons, *Proc. Natl. Acad. Sci.* 85, 3643.

Nilius, B., 1991, Regulation of transmembrane calcium fluxes in endothelium, *NIPS* 6, 110.

Nollert M.U., S.G. Eskin and L.V. McIntire, 1990, Shear stress increases inositol triphosphate levels in human endothelial cells, *Biochem. Biophys. Res. Commun.* 170, 281.

Northup, J.K., 1985, In: *Molecular mechanisms of transmembrane signalling*. P. Cohen and M. Houslay eds., p 91.

Olate, J. and J.E. Allende, 1991, Structure and function of G proteins, *Pharmac. Ther.* Vol. 51, 403.

Olesen, S.P., D.E. Clapham and P.F. Davis, 1988, Hemodynamic shear stress activates a K^+ current in vascular endothelial cells, *Nature* 331, 168.

Pallotta, B.S., K.L. Magleby and J.N. Barrett, 1981, Single channel recordings of Ca^{2+} -activated K^+ currents in rat muscle cell culture, *Nature* 293, 471.

Palmer, R.M.J., A.G. Ferrige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, *Nature* 327, 524.

Palmer, R.M.J., D.S. Ashton and S. Moncada, 1988, Vascular endothelial cells synthesize nitric oxide from L-arginine, *Nature* 333, 664.

Partridge, L.O. and D. Swandulla, 1988, Calcium-activated nonspecific cation channels, *Trends Neurosci.* 11, 69.

- Pasyk, E., Y.K. Mao, S. Ahmad, S.H. Shen and E.E. Daniel, 1992, An endothelial cell-line contains functional VIP receptors: They control inwardly-rectifying K⁺ channels, *Eur. J. Pharmacol.* 212, 209.
- Rasmussen, H. et al., 1987, Protein kinase C in the regulation of smooth muscle contraction, *FASEB J.* 1, 177.
- Reseine, T. and S.F. Law, 1992, *Methods in Neurosciences* 8, 358.
- Rubanyi, G.M., C.J. Romero, P.M. Vanhoutte, 1986, Flow-induced release of endothelium-derived relaxing factor, *Am. J. Physiol.* 250, H1145.
- Sakai, T., 1990, Acetylcholine induces Ca-dependent K currents in rabbit endothelial cells, *Japan J. Pharmacol.* 53, 235.
- Sata, T., J. Linden, L.W. Liu, E. Kubota and S.I. Said, 1988, Vasoactive intestinal peptide evokes endothelium-dependent relaxation and cyclic AMP accumulation in rat aorta, *Peptides* 9, 854.
- Schilling, W.P., O.A. Cabello and L. Rajan, 1992, Depletion of the inositol 1,4,5-trisphosphate-sensitive intracellular Ca²⁺ store in vascular endothelial cells activates the agonist-sensitive Ca²⁺-influx pathway, *Biochem J.* 284, 521.
- Schilling, W.P., A.K. Ritchie, L.T. Navarro and S.G. Eskin, 1988, Bradykinin-stimulated calcium influx in cultured bovine aortic endothelial cells, *Am. J. Physiol.* 255, H219.
- Schoeffer, P. and J.C. Stoclet, 1985, Effect of vasoactive intestinal polypeptide (VIP) on cyclic AMP level and relaxation in rat isolated aorta, *Eur. J. Pharmacol.* 109, 275.
- Seamon, K.B., 1985, Activation of hormone sensitive adenylate cyclase by forskolin. In: *Proceedings of the International Symposium on Forskolin: Its Chemical, Biological and Medical Potential*, p. 51, Hoechst Centre for Basic Research, Bombay.
- Seidler, N.W., I. Jona, M. Vegh and A. Martonosi, 1989, Cyclopiazonic acid is a specific inhibitor of the Ca²⁺-ATPase of sarcoplasmic reticulum, *J. Biol. Chem.* 264, 17816.
- Shen J., F.W. Lusinskas, A. Connolly, C.F. Dewey and M.A. Gimbrone, 1992, Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells, *Am. J. Physiol.* 262, C384.
- Silver, M.R. and T.E. DeCoursey, 1990, Intrinsic gating of inward rectifier in bovine pulmonary artery endothelial cells in the presence or absence of internal Mg²⁺, *J.*

Gen. Physiol. 96, 109.

Suppattapone, S., S.K. Danoff, A. Thiebert, S.K. Joseph, J. Steiner and S.H. Snyder, 1988, Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium, Proc. Natl. Acad. Sci. USA 85, 8747.

Takeda, K., V. Shini and H. Stoeckel, 1987, Voltage-activated potassium, but not calcium currents in cultured bovine aortic endothelial cells, Pflu. Arch. 410, 385.

Takeda, K. and M. Klepper, 1990, Voltage-dependent and agonist-activated ionic currents in vascular endothelial cells: A review, Blood Vessels 27, 169.

Tatsuno, I., T. Yada, S. Vigh, H. Hidaka and A. Arimura, 1992, Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) increase cytosolic free calcium concentration in cultured rat hippocampal neurons, Endocrinology 131, 73.

Thom, S., A. Hughes, G. Martin and P.S. Sever, 1987, Endothelium-dependent relaxation in isolated human arteries and veins, Clin. Sci. 73, 547.

Trube, G. and J. Hescheler, 1984, Inward-rectifying channels in isolated patches of the heart cell membrane: ATP-dependence and comparison with cell-attached patches, Pflugers Arch. 401, 178.

Uyama, Y., Y. Imaizumi and M. Watanabe, 1992, Effects of cyclopiazonic acid, a novel Ca^{2+} -ATPase inhibitor, on contractile responses in skinned ileal smooth muscle, Br. J. Pharmacol. 106, 208.

Vandenberg, C.A., 1987, Inward rectification of potassium channel in cardiac ventricular cells depends on internal magnesium ions, Proc. Natl. Acad. USA 84, 2560.

Watanabe, T., Y. Masuo, H. Matsumoto, et al., 1992, Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline, Biochem. Biophys. Res. Commun. 173, 252.

Watson, P.A., 1991, Function follows form: Generation of intracellular signals by the deformation, FASEB J. 5, 2013.

Yanagisawa M., H. Kurihara, S. Kimura, G. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature (London) 332, 411.

Yang, X.C. and F. Sachs, 1989, Block of stretch-activated ion channels in *Xenopus*

oocytes by gadolinium and calcium ions, *Science* 243, 1068.

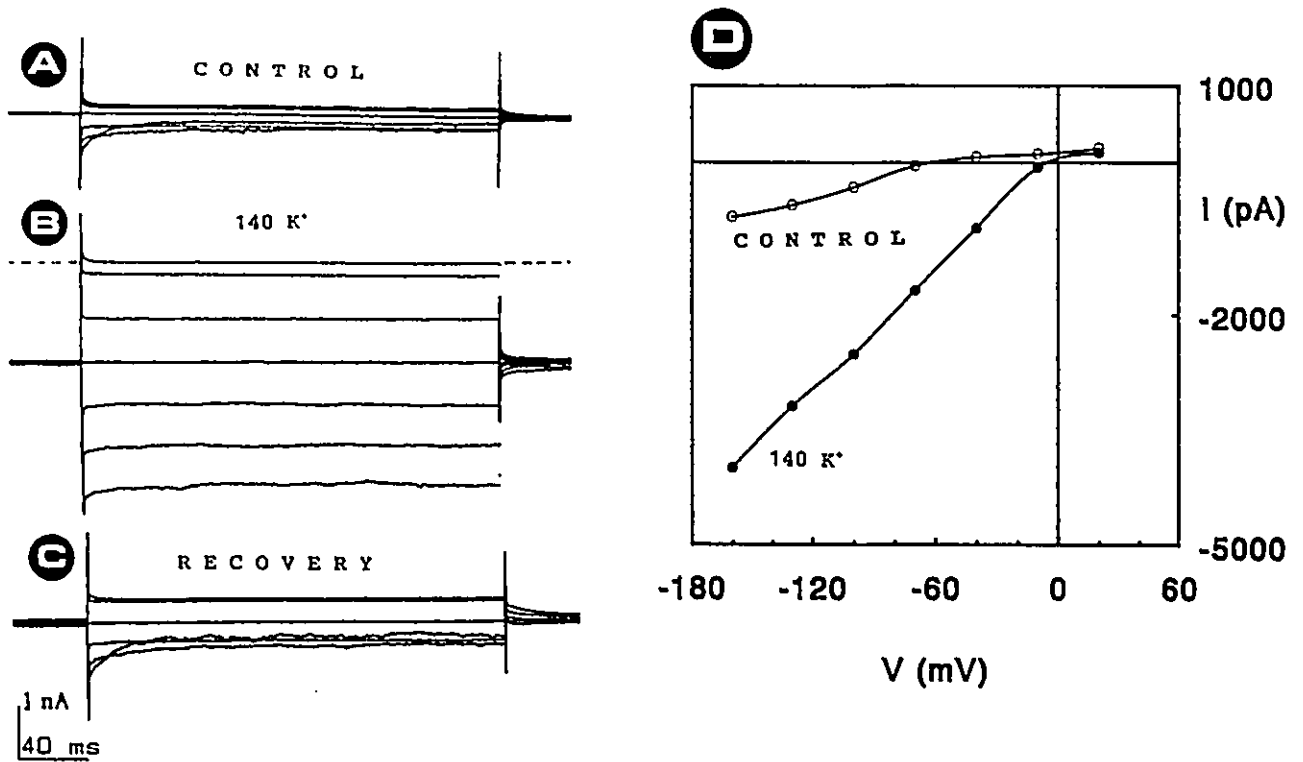
Yatani, A., J. Codina, Y. Imoto, J.P. Reeves, L. Birnbaumer and A.M. Brown, 1987, A G protein directly regulates mammalian cardiac calcium channels, *Science* 235, 207.

Zhang, H., M. Inazu, B. Weir, M.R. Buchanan and E.E. Daniel, 1993, Cyclopiazonic acid stimulates calcium influx through non-specific cation channels in endothelial cells, *Eur. J. Pharmacol.*, submitted.

Zheng, X.F., C.Y. Kwan and E.E. Daniel, 1993a, Role of intracellular Ca^{2+} in EDRF release in rat aorta, *J. Vasc. Res.*, submitted.

Zheng, X.F., C.Y. Kwan and E.E. Daniel, 1993b, Role of β -adrenoceptor activation in EDRF release in rat aorta, *Can. J. Physiol. Pharmacol.*, submitted.

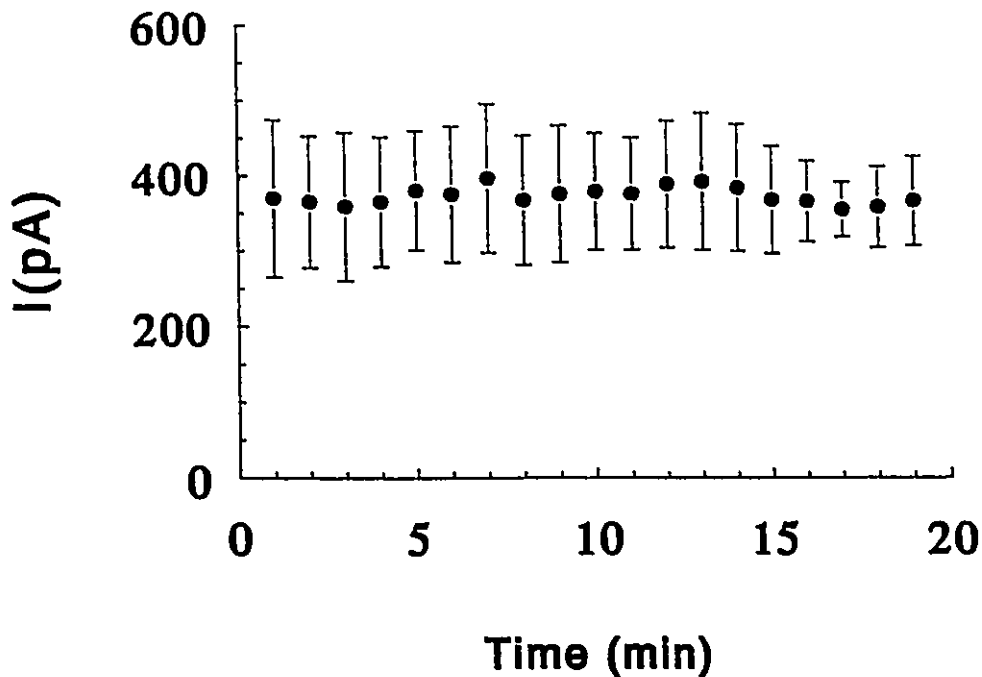
APPENDIX I



Whole-cell inward rectifier K^+ currents from a single endothelial cells. A. Currents recorded in normal external solution for command pulses given every 3 s in 30 mV steps over the range -160 to +20 mV from the holding potential of -70 mV. B. Currents recorded in high K^+ (140 mM K^+) external solution (in exchange for Na^+). Dotted line indicates 0 current level. C. Recovery from the high K^+ effect. D. Currents for I-V curve were measured at 10th ms after the beginning of the voltage protocol.

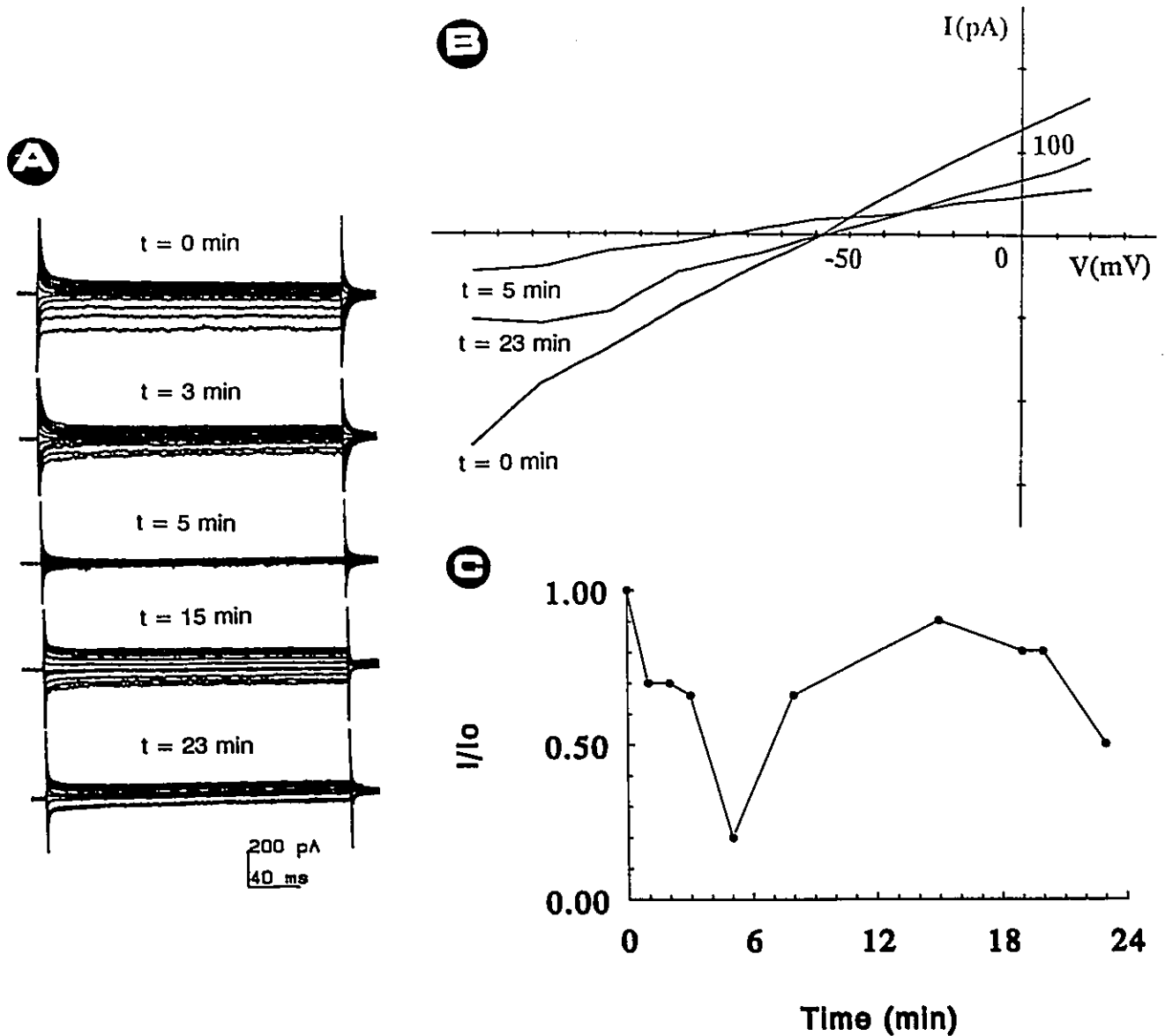
APPENDIX II

Stability of the whole-cell $I_{K_{in}}$ currents



Time course of changes of mean values of the whole-cell $I_{K_{in}}$ control currents ($n = 4$ cells). Cells were recorded continuously for 19 min; no run-down was observed. Currents were evoked by command voltages given every 3 s in 20 mV steps in the range -160 to +40 mV from the holding potential of -60 mV. The amplitudes of the currents were measured at -140 mV at 20th ms after the beginning of the voltage protocol. (•) represents mean \pm SE pA ($n = 4$). Intracellular solution contained (in mM): 135 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 20 HEPES and 0.5 ATP (pH = 7.3 with KOH). External bath solution contained (in mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose, 10 HEPES (pH = 7.3 with KOH).

APPENDIX III



CPA-evoked amplitude fluctuations of the $I_{K_{in}}$ whole-cell currents. **A**. Currents were evoked by command voltages given every 3 s in 20 mV steps in the range between -160 to +40 mV from the holding potential of -60 mV. CPA (10 μ M) was applied directly to the bath between 0 and 1st min; t = 0 min is the time of the control recording. **B**. Current-voltage relationships for the currents shown in **A**. Currents for I-V curve were measured at 30th ms after the beginning of the voltage pulses. **C**. Time course of current amplitude changes evoked by CPA. The currents were measured at -140 mV at 30 ms after the beginning of the voltage protocol. I/I_0 means the value of the current amplitude in the relation to the control current. Intracellular solution contained (in mM): 140 KCl, 2 MgCl₂, 0.5 CaCl₂, 0.3 EGTA, 10 HEPES, 4 Na₂ATP (pH = 7.3 with KOH). Extracellular solution contained (in mM): 135 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 11 glucose and 10 HEPES (pH = 7.3 with NaOH). This figure is representative of 2 experiments.