British Journal of Pharmacology (1997) 121, 977–985

The mechanisms of the relaxation induced by vasoactive intestinal peptide in the porcine coronary artery

Junya Kawasaki, Sei Kobayashi, Yasushi Miyagi, Junji Nishimura, *Masatoshi Fujishima & [1]Hideo Kanaide

Division of Molecular Cardiology, Research Institute of Angiocardiology and *2nd Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 This study was designed to investigate the mechanism of the relaxation induced by vasoactive intestinal peptide (VIP) in medial strips of the porcine coronary artery, by determining the effect on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), the $[Ca^{2+}]_i$-force relation and the involvement of G-protein.

2 Front-surface fluorometry of fura-2 revealed that U46619, a thromboxane A_2 analogue, and the high K^+-depolarization induced increases in both the $[Ca^{2+}]_i$ and force of the medial strips. At a steady state of contraction, the extent of an increase in $[Ca^{2+}]_i$ induced by 100 nM U46619 was similar to that induced by 30 mM K^+-depolarization. VIP concentration-dependently (1 nM – 1 μM) induced transient decreases in both the $[Ca^{2+}]_i$ and force of the medial strips precontracted with 100 nM U46619. The decreases in the $[Ca^{2+}]_i$ and force induced by VIP during the contraction with U46619 were much greater than those with 30 mM K^+-depolarization.

3 The VIP-induced decreases in the $[Ca^{2+}]_i$ and force were attenuated by K^+ channel blockers such as tetrabutylammonium (TBA: non-selective K^+ channel blocker), charybdotoxin (large conductance Ca^{2+}-activated K^+ channel blocker), and 4-aminopyridine (4-AP: voltage-dependent K^+ channel blocker). However, neither glibenclamide (ATP-sensitive K^+ channel blocker) nor apamin (small conductance Ca^{2+}-activated K^+ channel blocker) had any significant inhibitory effect.

4 In the 30 mM K^+-depolarized strips, pretreatment with thapsigargin, a specific Ca^{2+}-ATPase inhibitor of the Ca^{2+} store sites, completely abolished the VIP-induced decrease in $[Ca^{2+}]_i$, but partially attenuated the VIP-induced decrease in force.

5 VIP shifted the $[Ca^{2+}]_i$-force relation of the U46619-induced contractions to the right in a concentration-dependent manner. In the α-toxin-permeabilized strips, VIP decreased the force development at a constant $[Ca^{2+}]_i$ level (pCa = 6.5) in a GTP-dependent manner, which was antagonized by guanosine-5′-O-(β-thiodiphosphate) (GDPβS).

6 We thus conclude that VIP relaxes the coronary artery via three mechanisms: (1) a decrease in $[Ca^{2+}]_i$ by inhibiting the Ca^{2+} influx presumably through the membrane hyperpolarization mediated by the activation of the large conductance Ca^{2+}-activated (charybdotoxin-sensitive) K^+ channels and voltage-dependent (4-AP-sensitive) K^+ channels; (2) a decrease in $[Ca^{2+}]_i$ by sequestrating cytosolic Ca^{2+} into thapsigargin-sensitive Ca^{2+} store sites; and (3) a decrease in the Ca^{2+}-sensitivity of the contractile apparatus through the activation of G-protein.

Keywords: Vasoactive intestinal peptide; fura-2; coronary artery; cytosolic Ca^{2+}-force relation; Ca^{2+}-sensitivity; K^+ channel

Introduction

Vasoactive intestinal peptide (VIP) was first isolated from the porcine small intestine and has been shown to possess various biological activities, including the regulation of cardiovascular and respiratory functions (Said & Mutt, 1970). VIP has a direct vasorelaxing effect on the cat cerebral artery (Larsson *et al*., 1976; Duckles & Said, 1982), dog carotid artery (D'Orleans *et al*., 1985), and rabbit mesenteric and hepatic arteries (Itoh *et al*., 1985; Brizzolara & Burnstock, 1991). Lee *et al*. (1984) and Edvinsson *et al*. (1985) have indicated that VIP-induced dilatation of the cerebral vessels is not related to the presence or absence of the endothelial cells. Immunocytochemical studies have revealed that VIP-containing nerves innervate various types of arteries (Edvinsson *et al*., 1980; Uddman *et al*., 1981; Lee *et al*., 1984; Ganz *et al*., 1986), including the human coronary vasculature (Gulbenkian *et al*., 1993). These results suggest that these nerves, by releasing VIP, may regulate vascular tone. However, the cellular mechanisms, especially the modulation of the $[Ca^{2+}]_i$ during the relaxation induced by VIP have yet to be elucidated.

The contraction of vascular smooth muscle is regulated not only by changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) but also by the agonist- and G-protein-mediated modulation of the $[Ca^{2+}]_i$-force relation (= the Ca^{2+} sensitivity of the contractile apparatus) (Somlyo & Somlyo, 1994). Our previous studies on the coronary artery showed that receptor-activating agonists and vasodilators modulate the $[Ca^{2+}]_i$-force relation (Hirano *et al*., 1990; Ushio-Fukai *et al*., 1993; Kuroiwa *et al*., 1995; Kureishi *et al*., 1995) and that G-protein mediates the increase and decrease in Ca^{2+} sensitivity of the contractile apparatus in membrane-permeabilized coronary arterial strips (Kuroiwa *et al*., 1993; Kureishi *et al*., 1995). However, whether or not VIP affects the levels of $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$-force relation and whether it modulates the Ca^{2+} sensitivity of the contractile apparatus in a G-protein-dependent manner remain unknown.

Standen *et al*. (1989) showed that VIP hyperpolarizes the surface membrane of vascular smooth muscle cells, which was inhibited by a K^+ channel blocker. The activation of K^+ channels by VIP was shown in *Xenopus* oocytes (Guillemare *et al*., 1994). These results support the role of K^+ channels in the biological function of VIP. However, the role of K^+ channels in the VIP-induced vasorelaxation and/or possible changes in $[Ca^{2+}]_i$ has yet to be clarified.

[1]Author for correspondence at: Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.

In the present study, we obtained the first successful recordings showing the effects of VIP on $[Ca^{2+}]_i$ and force in fura-2-loaded intact arteries and the effects on force at a constant $[Ca^{2+}]_i$ in membrane-permeabilized arteries. We found that in the pig coronary artery: (1) VIP induces transient decreases in $[Ca^{2+}]_i$ not only through inhibition of the Ca^{2+} influx, presumably by K^+ channel-mediated hyperpolarization, but also through the sequestration of cytosolic Ca^{2+} into the thapsigargin-sensitive Ca^{2+} store sites; and (2) VIP decreases the Ca^{2+} sensitivity of the contractile apparatus through the activation of G-protein.

Methods

Tissue preparation

Left circumflex coronary arteries (2–3 cm from the origin) were isolated from fresh porcine hearts at a local slaughterhouse immediately after they had been killed. All tissue specimens were placed in ice-cold normal physiological salt solution (PSS) and brought to our laboratory. After the segments were cut open longitudinally, the adventitia was trimmed away. The inner surface of the arteries was rubbed off by cotton swabs in order to remove the endothelium. Each media was cut into equal size strips ($1 \times 5 \times 0.1$ mm). The complete removal of the endothelium was confirmed by the lack of any relaxing response of the strips to 1 μM bradykinin.

Fura-2 loading

Coronary arterial media strips were loaded with the $[Ca^{2+}]_i$ indicator dye, fura-2, by incubating in oxygenated (a mixture of 95% O_2 and 5% CO_2) Dulbecco's modified Eagle medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 5% foetal bovine serum for 4 h at 37°C. After being loaded with fura-2, the media strips were incubated in normal PSS for at least 1 h at 37°C before the measurements were started, in order to remove the dye in the extracellular space and also for purposes of equilibration. Loading the medial strips with fura-2, *per se*, did not affect the contractility, as previously described (Abe *et al*., 1990; Hirano *et al*., 1990).

Front-surface fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored simultaneously with force development, by use of a front-surface fluorometer specifically designed for fura-2 fluorometry (CAM-OF3, Japan Spectroscopic Co., Tokyo, Japan), as previously described (Ushio-Fukai *et al*., 1993). In brief, the ratio of the fluorescence (500 nm) intensities at alternating 340 nm and 380 nm excitation light was monitored. The fluorescence ratio was expressed as a percentage, by assigning the values at rest in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ of the vascular strips were calculated according to the method of Grynkiewicz *et al*. (1985) with the K_d (apparent dissociation constant) of the fura-2-Ca^{2+} complex of 225 nM (at 37°C). The absolute values of $[Ca^{2+}]_i$ for 0% and 100% levels were determined in separate measurements and were 108 ± 27 nM ($n = 10$) and 715 ± 103 nM ($n = 10$), respectively. The $[Ca^{2+}]_i$ values obtained were considered to be an approximation to the true $[Ca^{2+}]_i$ value, and the calibration of the absolute levels of $[Ca^{2+}]_i$ at the end of such experiments is liable to be uncertain (Miyagi *et al*., 1995). Therefore, a statistical analysis of the $[Ca^{2+}]_i$ signal was performed by use of the % ratio.

Measurement of force development

Coronary arterial media strips were mounted vertically in a quartz organ bath, and then the isometric tension was measured, as described previously (Ushio-Fukai *et al*., 1993; Kuroiwa *et al*., 1995; Kureishi *et al*., 1995). In brief, during the fura-2 equilibration period (1 h), the strips were stimulated with 118 mM K^+ PSS every 15 min and the resting load was adjusted to an optimal resting tension, about 300 mg. At the beginning of each protocol, the responsiveness of each strip to 118 mM K^+ PSS was recorded. The developed force was expressed as a percentage, by assigning the values at rest in normal PSS (5.9 mM K^+) to be 0%, and those at a steady state of contraction in 118 mM K^+ PSS to be 100%.

Membrane permeabilization of coronary arterial strips

Small strips ($50-100$ $\mu m \times 1.0$ mm) of the pig left circumflex coronary arterial smooth muscle were permeabilized with *Staphylococcus aureus* α-toxin while the isometric tension was measured in a well on a 'bubble' plate, as previously described (Kobayashi *et al*., 1989; Kuroiwa *et al*., 1993; Kureishi *et al*., 1995). In brief, each strip was incubated in normal relaxing solution (Ca^{2+} free, 1 mM ethyleneglycol-bis-(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)) for 5–10 min and then permeabilized by addition of 5000 units ml^{-1} *Staphylococcus aureus* α-toxin in a relaxing solution for 60 min at 25°C. To deplete the calcium of the Ca^{2+} store sites, the permeabilized strips were treated with the calcium ionophore A23187 (10 μM) in the relaxing solution for at least 10 min. This procedure abolishes the intracellular Ca^{2+} release induced by caffeine, inositol trisphosphate (IP_3), or agonists, without affecting either the Ca^{2+} sensitivity of the contractile apparatus in permeabilized strips or its modulation by agonists (Kobayashi *et al*., 1991).

Drugs and solutions

The composition of normal PSS for fura-2 studies was as follows (mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. High K^+ PSS was prepared by replacing NaCl with equimolar KCl. PSS was bubbled with a mixture of 95% O_2 and 5% CO_2, the resulting pH was 7.4. The relaxing solution for the permeabilized strips was (mM): potassium methanesulphonate 74.1, magnesium methanesulphonate 2, MgATP 4.5, EGTA 1, creatinine phosphate 10 and PIPES 30 (pH 7.1 with KOH at 25°C). In the activating solution (pCa 6.5) for the permeabilized strips, 10 mM EGTA was used and a specified amount of calcium methanesulphonate was added to obtain the desired concentration of free Ca^{2+} ions. Fura-2/AM and *Staphylococcus aureus* α-toxin were purchased from Dojindo Laboratories (Kumamoto, Japan). Bovine serum albumin (BSA) and thapsigargin were purchased from Sigma (St. Louis, MO, U.S.A.). VIP, charybdotoxin and apamin were purchased from the Peptide Institute, Inc. (Osaka, Japan). U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ was purchased from Funakoshi (Tokyo, Japan). Guanosine-5′-triphosphate (GTP) and guanosine-5′-O-(β-thiodiphosphate) (GDPβS) were purchased from Boehringer Mannheim (Germany). All other chemicals were from Wako (Osaka, Japan).

Data analysis

The values are expressed as the mean ± s.e.mean. Student's *t* test was used to determine statistical significance between the two groups, and an analysis of variance was used to determine the dose-dependent effect of VIP on the $[Ca^{2+}]_i$ and force. Bonferroni/Dunn's analysis was used to determine statistical significance between the six groups in Figure 4. An analysis of variance was used to determine the dose-dependent effect of VIP on the $[Ca^{2+}]_i$ and force. An analysis of covariance was used to determine the significance of the shift of the $[Ca^{2+}]_i$-force relationship from the control curve. *P* values of less than 0.05 were considered to have statistical significance. All data were collected by use of a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, and Macintosh; Apple Computer, U.S.A.).

Results

Effect of VIP on the $[Ca^{2+}]_i$ and force of coronary arterial medial strips during U46619-induced contraction

Figure 1a and b show representative recordings of changes in the $[Ca^{2+}]_i$ and force induced by 100 nM VIP of porcine coronary arterial medial strips precontracted with 100 nM U46619 (thromboxane A_2 analogue). When the bathing medium was changed from normal PSS (5.9 mM K^+) to 1178 mM K^+ PSS, both the $[Ca^{2+}]_i$ and force rapidly increased and reached the plateau phases within 10 min. The values at the resting and the plateau phases were designated to be 0% and 100% for both the $[Ca^{2+}]_i$ and force, respectively. The application of U46619 induced a rapid increase in $[Ca^{2+}]_i$, which reached a peak level in 5 min and then decreased to a plateau level within 10 min ($64.3 \pm 2.9\%$, $n=40$). The force also rapidly increased and reached a plateau level within 10 min ($92.7 \pm 2.0\%$, $n=40$). Therefore, VIP was applied after the increase in the $[Ca^{2+}]_i$ and force induced by U46619 reached the plateau level, namely, 10–15 min after the application of U46619. VIP induced a transient decrease in the $[Ca^{2+}]_i$ and force, which thereafter recovered to the level before the application of VIP. The levels of the $[Ca^{2+}]_i$ and force before and 15 min after the application of VIP were not significantly different ($P>0.05$). Neither the second application (Figure 1a) nor the additional application (Figure 1b) of 100 nM VIP, following the first application of 100 nM VIP, affected the levels of the $[Ca^{2+}]_i$ and force. In addition, prior application of VIP had no significant effect on the steady-state elevations of the $[Ca^{2+}]_i$ and force induced by 100 nM U46619 (Figure 1c) ($P>0.05$). These results thus suggest that the transient effects of VIP may be due to the desensitization to VIP, as previously observed in several types of vasculature (Miao & Lee, 1992; Huang *et al*., 1993).

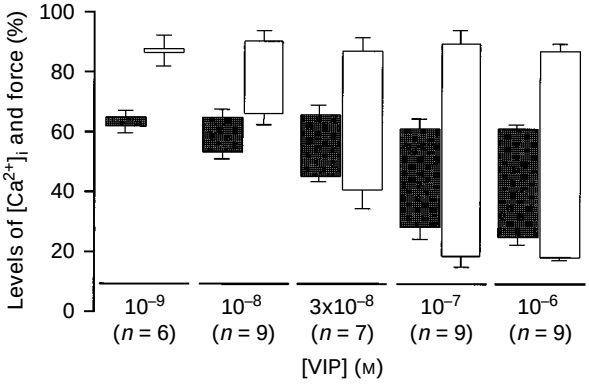


Figure 2 Concentration-dependent effects of VIP on the $[Ca^{2+}]_i$ (filled columns) and force (open columns) in coronary arterial strips precontracted with 100 nM U46619. Each point was obtained from repeated experiments ($n=6-9$) which were carried out according to the protocol shown in Figure 1a. The top and the bottom of each column represent the level of the $[Ca^{2+}]_i$ and force before and after the application of VIP, respectively. As the decreases in $[Ca^{2+}]_i$ and force were transient, the bottom levels were plotted at the lowest point of the force. Means with s.e.mean (shown by vertical lines) are depicted.

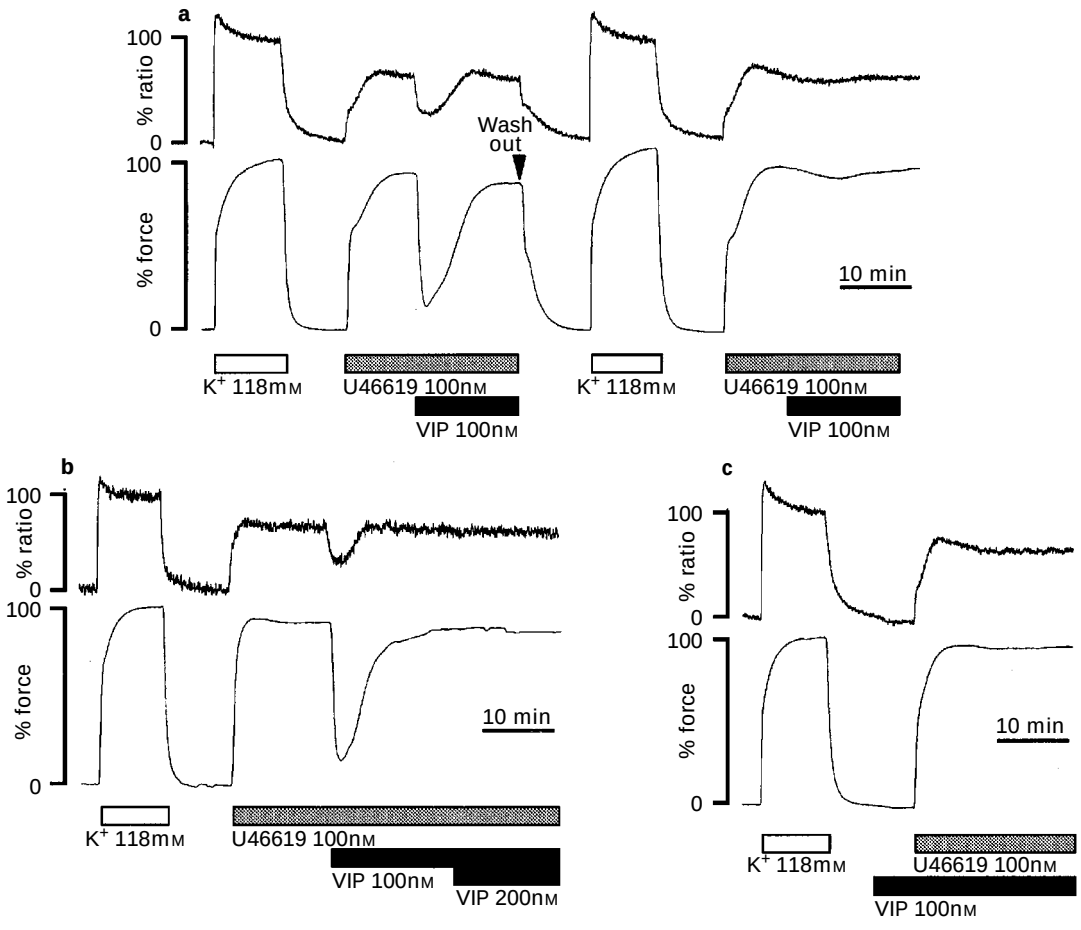


Figure 1 Representative recordings showing the effects of 100 nM VIP on the U46619-induced elevations of $[Ca^{2+}]_i$ and force in porcine coronary arterial strips. Before the measurements were started the responses of the fluorescence ratio and force to 118 mM K^+ were determined to give 100% levels. VIP was applied 10 min before (c) and 10–15 min after (a and b) the application of 100 nM U46619. In (a), after the first application of VIP, all of the drugs were washed out thoroughly with normal PSS, and then stimulated with 118 mM K^+ and subsequently with U46619. At 10 min after the application of U46619, the second application of VIP was performed. In (b), VIP was applied cumulatively to obtain concentrations of 100 nM and 200 nM.

As shown in Figure 2, both the effects of VIP on the $[Ca^{2+}]_i$ and force were dependent on the concentration of VIP ($P<0.05$ by an analysis of variance). The minimum concentration to induce the maximal effects was 100 nM. Therefore, 100 nM VIP was used in all of the experiments of the present study, except for the dose-response experiments shown in Figures 2 and 6c.

Effect of VIP on the $[Ca^{2+}]_i$ and force of coronary arterial medial strips during 30 mM K^+-induced depolarization

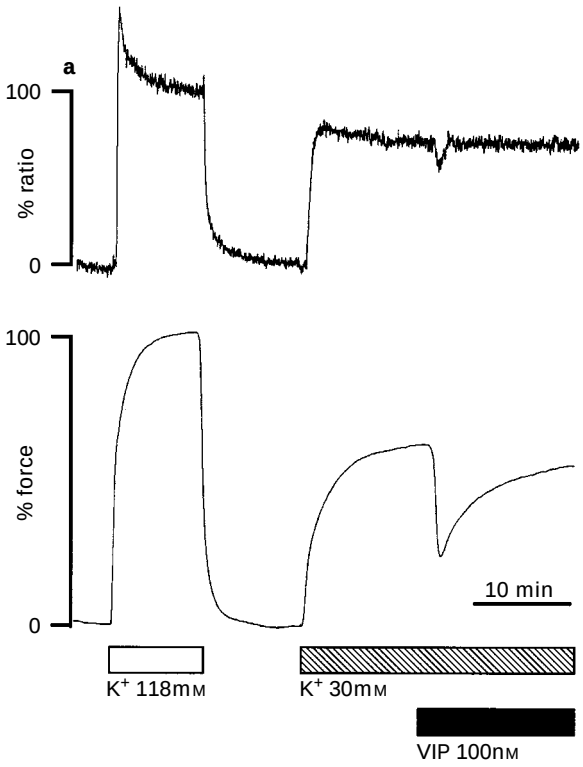
In order to examine the possible involvement of membrane hyperpolarization in the VIP-induced decrease in the $[Ca^{2+}]_i$ and force, the effect of VIP was observed during 30 mM K^+-induced depolarization, which can inhibit membrane hyperpolarization (Figure 3). When the medial strips were stimulated with 30 mM K^+, the $[Ca^{2+}]_i$ and force rapidly increased to reach plateau levels (Figure 3a). There was no significant difference in the $[Ca^{2+}]_i$ values between this level ($69.9 \pm 1.63\%$, $n=5$) and the plateau levels observed during the contraction induced by 100 nM U46619 (Figure 3b). VIP was applied at this level. The extent of the VIP-induced decrease in the $[Ca^{2+}]_i$ and force during the 30 mM K^+-induced depolarization was much smaller than that during stimulation with 100 nM U46619 (Figure 3) ($P<0.05$). Therefore, it was suggested that VIP may decrease the $[Ca^{2+}]_i$ and force, at least in part, by membrane hyperpolarization.

Effect of K^+ channel blockers on VIP-induced decrease in the $[Ca^{2+}]_i$ and force

In order to determine which K^+ channels are responsible for the VIP-induced membrane hyperpolarization and decrease in the $[Ca^{2+}]_i$ and force, 10 min before and during the application of 100 nM U46619, arterial strips were pretreated with various types of K^+ channel blockers such as 1 mM tetrabutylammonium (TBA, non-specific K^+ channel blocker), 100 nM charybdotoxin (large conductance Ca^{2+}-activated K^+ channel blocker), 30 μM 4-aminopyridine (4-AP, voltage-dependent K^+ channel blocker), 3 μM glibenclamide (ATP-sensitive K^+ channel blocker) and 1 μM apamin (small conductance Ca^{2+}-activated K^+ channel blocker). At 10–15 min after the application of U46619, 100 nM VIP was applied. The concentrations of the K^+ channel blockers used were the maximum levels which did not affect the increase in $[Ca^{2+}]_i$ and force induced by U46619. Higher concentrations of the K^+ channel blockers, such as 3 mM TBA, 300 nM charybdotoxin and 100 μM 4-AP, potentiated the levels of the $[Ca^{2+}]_i$ and force induced by U46619, whereas 10 μM glibenclamide and 3 μM apamin decreased them (data, not shown). As shown in Figure 4, 1 mM TBA, 100 nM charybdotoxin and 30 μM 4-AP, partially inhibited VIP-induced decrease in the $[Ca^{2+}]_i$ and force. However, 3 μM glibenclamide and 1 μM apamin had no significant blocking effect on the transient decrease in $[Ca^{2+}]_i$ and force induced by VIP. Thus, the K^+ channels sensitive to TBA, charybdotoxin and 4-AP, but not to glibenclamide and apamin, seem to be involved in the VIP-induced decrease in $[Ca^{2+}]_i$ and force.

Effect of VIP on the $[Ca^{2+}]_i$ and force of coronary arterial medial strips during 30 mM K^+-induced depolarization in the presence of thapsigargin

In order to clarify the role of Ca^{2+} store sites in the VIP-induced decrease in $[Ca^{2+}]_i$ and force, the function of the Ca^{2+} store sites was disrupted by depleting the stored Ca^{2+} with a selective inhibitor for the Ca^{2+}-ATPase of the sarcoplasmic reticulum, thapsigargin (Thastrup *et al*., 1990). The decrease in $[Ca^{2+}]_i$ induced by VIP during 30 mM K^+-induced depolarization was completely abolished by pretreatment with thapsigargin, while the associated relaxation was only partially inhibited (Figure 5). These results suggest



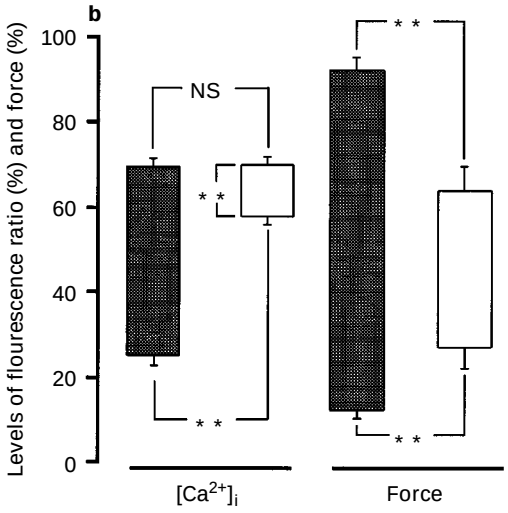


Figure 3 The effect of 100 nM VIP on $[Ca^{2+}]_i$ and force in coronary arterial strips during the stimulation with 30 mM K^+. (a) Representative recordings showing the effect of 100 nM VIP on the $[Ca^{2+}]_i$ and force in coronary arterial strips precontracted with 30 mM K^+. After the response (100%) to 118 mM K^+ had been recorded, the strip was stimulated with 30 mM K^+. VIP was applied when the $[Ca^{2+}]_i$ and force had reached plateau levels (at 15 min) during the stimulation with 30 mM K^+. (b) The effects of 100 nM VIP on $[Ca^{2+}]_i$ and force during stimulation with 100 nM U46619 (filled columns; $n=5$) and 30 mM K^+ (open columns; $n=5$). The top and the bottom levels of each column represent the levels of $[Ca^{2+}]_i$ and force before and after the application of VIP, respectively. The bottom levels were plotted at the lowest point of the force. Means with s.e.mean (shown by vertical lines) are depicted. **, $P<0.01$; NS, not significant.

that VIP decreases $[Ca^{2+}]_i$, at least in part, by sequestering cytosolic Ca^{2+} into the thapsigargin-sensitive Ca^{2+} store sites. The finding that VIP induced relaxation without changing the level of $[Ca^{2+}]_i$ during high K^+-depolarization

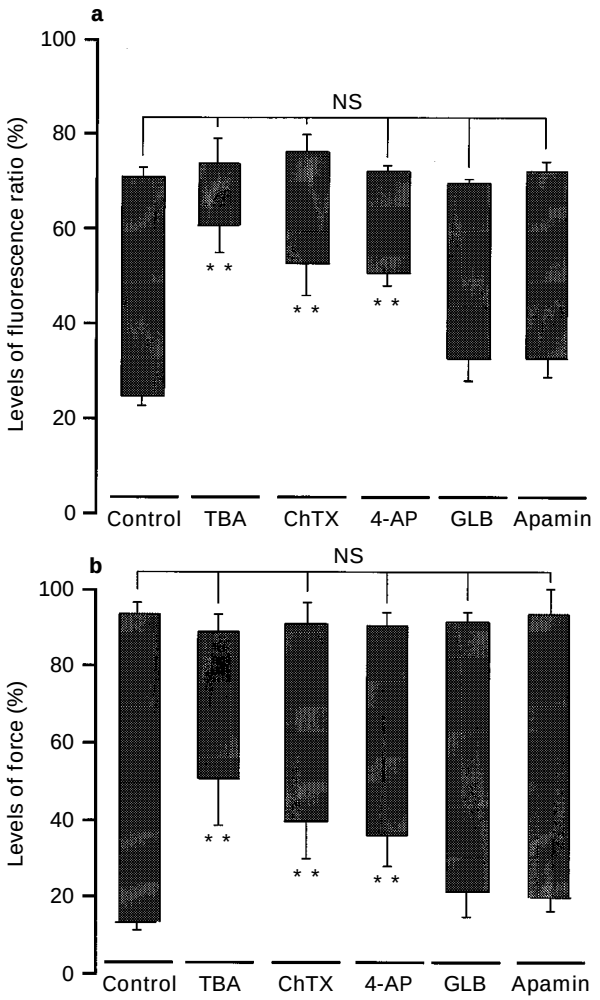


Figure 4 The effects of K^+ channel blockers on the VIP-induced decrease in $[Ca^{2+}]_i$ (a) and force (b), during stimulation with 100 nM U46619. The top and the bottom of each column represent the value of $[Ca^{2+}]_i$ and force before and after the application of 100 nM VIP, respectively. As the decreases in $[Ca^{2+}]_i$ and force were transient, the bottom levels were plotted at the lowest point of the force. Before the experiments were started, the responses of $[Ca^{2+}]_i$ and force to 118 mM K^+ were determined to give 100% levels. The strips were pretreated, 10 min before and during the application of 100 nM U46619, with 1 mM tetrabutylammonium (TBA, $n=5$), 100 nM charybdotoxin (ChTX, $n=5$), 30 μM 4-aminopyridine(4-AP, $n=5$), 3 μM glibenclamide (GLB, $n=5$) and 1 μM apamin ($n=5$). The control ($n=5$) represents the data from strips not pretreated with a K^+ channel blocker. VIP was applied 10–15 min after the application of U46619. The K^+ channel blockers, *per se*, did not affect the levels of the $[Ca^{2+}]_i$ and force of the strips stimulated with U46619 before the application of VIP ($P>0.05$; NS, not significant by ANOVA). Means with the s.e.mean (shown by vertical lines) are depicted. **, $P<0.0033$ according to Bonferroni/Dunn's analysis compared with the control.

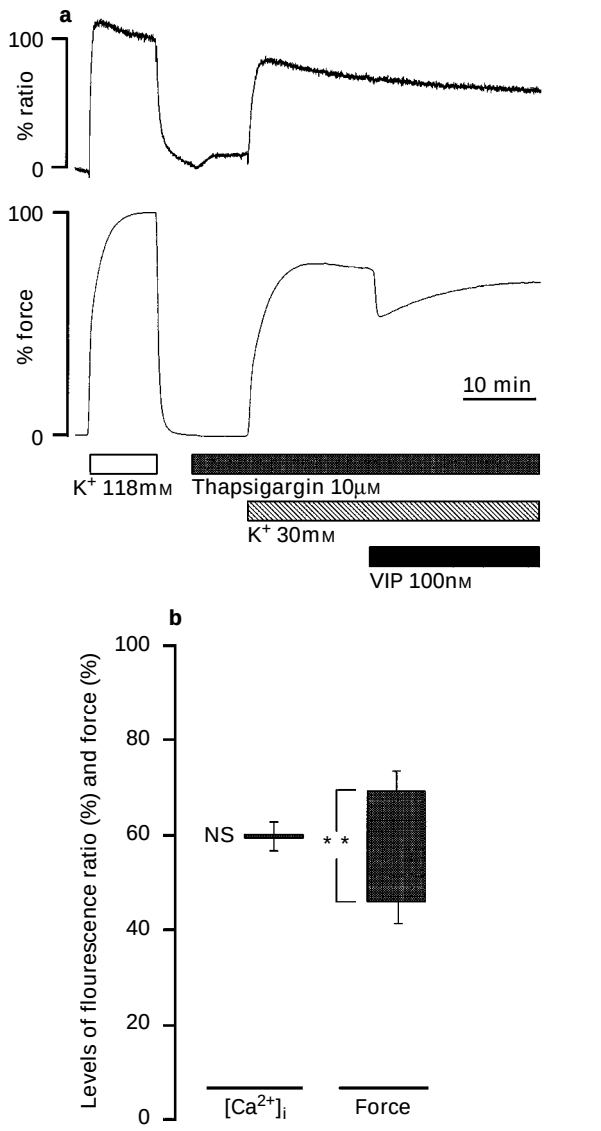


Figure 5 Effects of thapsigargin on the changes in $[Ca^{2+}]_i$ and force of coronary artery medial strips induced by VIP during 30 mM K^+-induced depolarization. (a) Representative recordings showing the effect of 10 μM thapsigargin on the 100 nM VIP-induced decrease in $[Ca^{2+}]_i$ and force during 30 mM K^+-induced depolarization. After the response (100%) to 118 mM K^+ was recorded, thapsigargin was applied 10 min before and during the 30 mM K^+-induced depolarization. VIP was applied 15 min after stimulation with 30 mM K^+. (b) A summary of the repeated experiments ($n=5$) carried out according to the protocol shown in (a). The top and the bottom of each column represent the level of the $[Ca^{2+}]_i$ and force before and after the application of VIP, respectively. Means with s.e.mean (shown by vertical lines) are depicted. ** $P<0.01$.

in the presence of thapsigargin, is compatible with the notion that VIP may decrease the Ca^{2+}-sensitivity of the contractile apparatus.

Effects of VIP on the $[Ca^{2+}]_i$-force relationships

Figure 6a and b shows representative recordings of the control (without VIP) $[Ca^{2+}]_i$-force relationship of contractions during membrane depolarization with 118 mM K^+ and in the presence of 100 nM U46619, respectively. Extracellular Ca^{2+} was applied cumulatively to increase the final concentration from 0 to 1.25 mM. As shown in Figure 6b, when U46619 was applied in Ca^{2+}-free PSS, there was only a transient and a small rise in $[Ca^{2+}]_i$ mediated by the release of intracellular Ca^{2+}. Note that the sustained elevation of $[Ca^{2+}]_i$ observed in the presence of extracellular $[Ca^{2+}]_i$ (Figure 1) was abolished in the absence of extracellular Ca^{2+}. The control $[Ca^{2+}]_i$-force relationship curves obtained from Figure 6a and b are shown in Figure 6c. The $[Ca^{2+}]_i$-force relationship of the VIP-induced relaxation during stimulation with U46619, obtained from the results shown in Figure 2, are also plotted in Figure 6c. Compared with the control (without VIP) curve in the presence of 100 nM U46619, VIP ($\geqslant 3\times10^{-8}$ M) shifted the $[Ca^{2+}]_i$-force relationship to the right ($P<0.05$) closer to the curve obtained during membrane depolarization with 118 mM K^+. However, the lower concentrations (10^{-9} and 10^{-8} M) of VIP did not have any such effect ($P>0.05$). As shown in Figure 6c, the extent of the rightward shift of the $[Ca^{2+}]_i$-force relationship induced by VIP depended on the concentration of VIP.

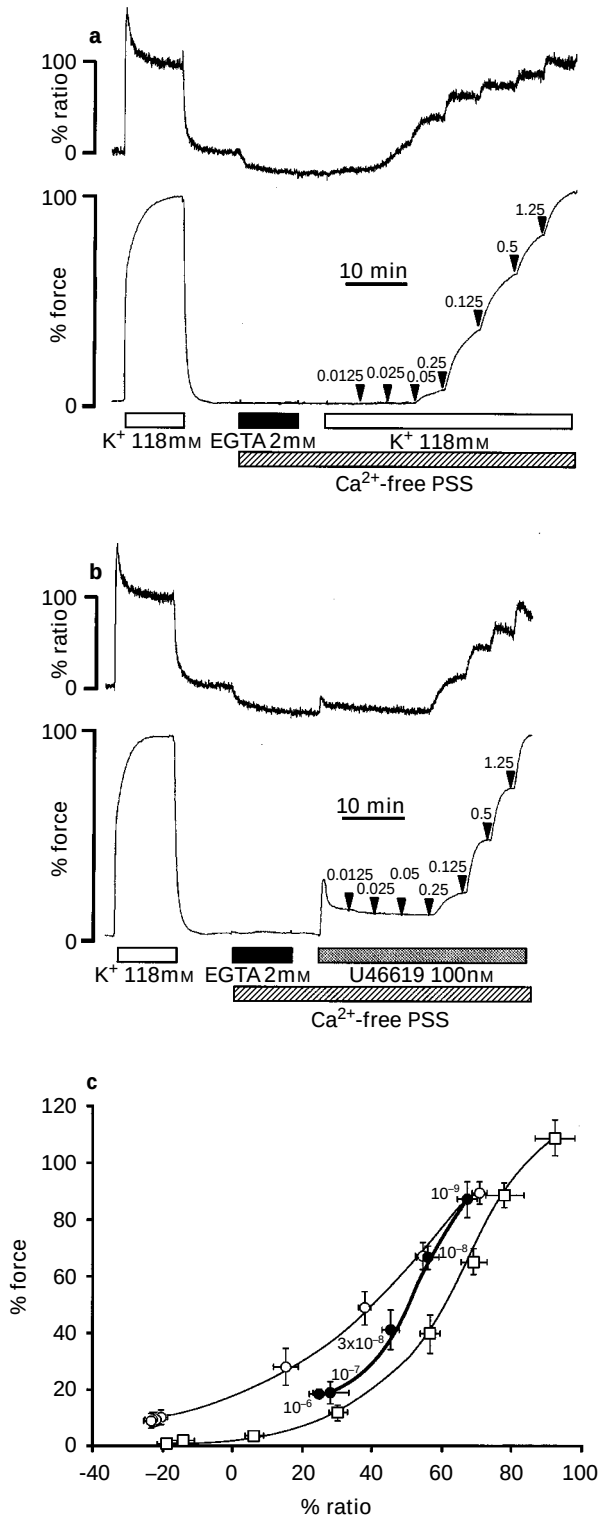


Figure 6 The effects of VIP on $[Ca^{2+}]_i$-force relationships in porcine coronary arterial strips. Representative recordings showing the effects of cumulative applications of extracellular Ca^{2+} (0 mM – 1.25 mM) on the $[Ca^{2+}]_i$ and force in coronary arterial strips during the depolarization with 118 mM K^+ (a) and during the stimulation with 100 nM U46619 (b). The numbers shown in the figure by an arrow (0.0125 mM – 1.25 mM, ▼) represent the final concentration of extracellular Ca^{2+}. (c) The $[Ca^{2+}]_i$ (abscissa scale)-force (ordinate scale) relationship obtained at the maximum point of the VIP-induced relaxation during stimulation with 100 nM U46619 (●, with the number representing the molar concentration of VIP). The data were obtained from the results shown in Figure 2. The control $[Ca^{2+}]_i$-force relationship curves (without VIP) were obtained at plateau levels of the contractions induced by the cumulative applications of extracellular Ca^{2+} (0 mM – 1.25 mM) during depolarization with 118 mM K^+ (□, n = 5) or during the stimulation with 100 nM U46619 (○, $n = 5$) in Ca^{2+}-free PSS, as shown in (a) and (b), respectively.

Contribution of G-protein to the VIP-induced modulation of Ca^{2+} sensitivity of the contractile apparatus in arterial strips

In order to determine whether or not VIP decreases the Ca^{2+}-sensitivity of the contractile apparatus through the activation of G-protein, coronary arterial strips were permeabilized with *Staphylococcus aureus* α-toxin. To maintain a constant $[Ca^{2+}]_i$, without affecting the Ca^{2+}-sensitivity of the contractile apparatus, the sarcoplasmic reticulum was depleted of calcium by A23187 (10 μM) and $[Ca^{2+}]_i$ was buffered with 10 mM EGTA to maintain pCa 6.5. VIP (100 nM) induced relaxation at a constant $[Ca^{2+}]_i$ (pCa 6.5) in the presence of 10 μM GTP (Figure 7a), but not in its absence (Figure 7c). This GTP-dependent relaxation induced by VIP was abolished by 1 mM GDPβS, a non-hydrolysable GDP analogue (Figure 6b). These results suggest that VIP decreases the Ca^{2+} sensitivity of the contractile apparatus in a G-protein-dependent manner.

Discussion

In the present study, by using the simultaneous measurement of the $[Ca^{2+}]_i$ and force and a receptor-coupled membrane permeabilization, we clarified for the first time that: (1) the effects of VIP on $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$-force relationship and the

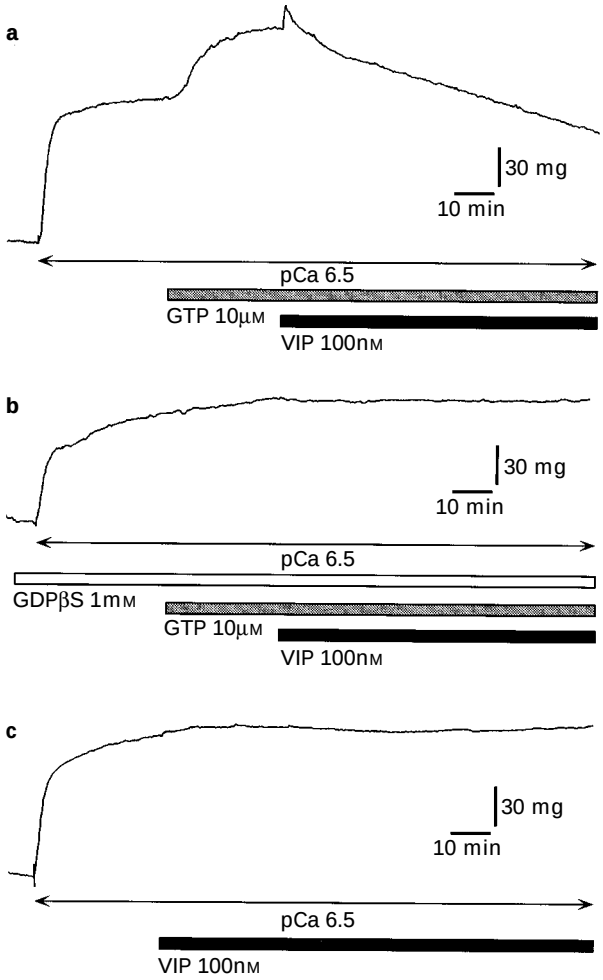


Figure 7 The effects of guanosine nucleotides on the VIP-induced relaxation at a constant $[Ca^{2+}]_i$ (pCa 6.5) in α-toxin-permeabilized coronary arterial smooth muscle. The sarcoplasmic reticulum was depleted of calcium by treatment with 10 μM A23187. During the contraction induced by constant Ca^{2+} (pCa 6.5, buffered with 10 mM EGTA), 100 nM VIP was applied in the presence (a, b) and absence (c) of 10 μM guanosine-5′-triphosphate (GTP). In (b), no responses to GTP or VIP were observed in the presence of 1 mM guanosine-5′-O-(β-thiodiphosphate) (GDPβS).

Ca^{2+} sensitivity of the contractile apparatus in the vascular strips; and (2) the role of intracellular Ca^{2+} sequestration, K^+ channels and G-protein in the VIP-induced vasorelaxation.

The effects of VIP on the levels of $[Ca^{2+}]_i$ in arterial smooth muscle

One of the novel findings of the present study is that the VIP-induced vasorelaxation is associated with a reduction of $[Ca^{2+}]_i$. VIP concentration-dependently inhibited the steady-state elevations of $[Ca^{2+}]_i$ induced by agonist (U46619) stimulation in vascular smooth muscle (Figures 1 and 2). Since the steady-state elevation of $[Ca^{2+}]_i$ induced by U46619 was completely abolished by the removal of extracellular Ca^{2+} (Figure 6b), it is likely that VIP inhibits extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$ (=presumably the influx of extracellular Ca^{2+}) induced by U46619. This VIP-induced reduction of $[Ca^{2+}]_i$ observed during U46619-contraction was much greater than that observed during depolarization with high K^+ PSS (Figure 3). This suggests that the VIP-induced decrease in $[Ca^{2+}]_i$ consists of two components: one which is inhibited by high K^+-induced depolarization (sensitive to high K^+-induced depolarization), and another which is not (resistant to high K^+-induced depolarization). Since the VIP-induced membrane hyperpolarization, if any, was eliminated in high K^+ PSS, the one component which is sensitive to high K^+-depolarization may be mediated by the membrane hyperpolarization. Therefore, these results support the idea that VIP may inhibit the extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$ (= the Ca^{2+} influx), at least in part, through membrane hyperpolarization. Although VIP-induced hyperpolarization of the membrane has been found in various types of tissues and cells, including vascular smooth muscle (Standen *et al*., 1989; Guillemare *et al*., 1994), further studies on the membrane potential are still needed to determine the involvement of membrane hyperpolarization in the VIP-induced reduction of $[Ca^{2+}]_i$ in arterial smooth muscle. The other component of the VIP-induced decrease in $[Ca^{2+}]_i$, which was resistant to high K^+-induced depolarization (Figure 3), is mainly mediated by the uptake of cytosolic Ca^{2+} into the intracellular store sites, because this component was completely abolished by pretreatment with thapsigargin, a selective blocker of the Ca^{2+}-pump of the store sites (Thastrup *et al*., 1990), as shown in Figure 5. Since it has been shown that VIP elevates the cytosolic concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in vascular smooth muscles (Edvinsson *et al*., 1985; Itoh *et al*., 1985; Ganz *et al*., 1986; Ignarro *et al*., 1987) and that cyclic AMP stimuates Ca^{2+} uptake into the sarcoplasmic reticulum through activation of the Ca^{2+}-pump ATPase (Raeymaekers *et al*., 1990), our results are compatible with the notion that VIP stimulates the uptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum through the elevation of cyclic AMP in arterial smooth muscle. Our previous study on the fura-2 fluorometry of porcine coronary artery revealed that isoprenaline, a β-adrenoceptor agonist which is well known to increase cytosolic cyclic AMP, similarly stimulates the uptake of Ca^{2+} into the ryanodine-sensitive storage sites (Ushio-Fukai *et al*., 1993).

As for the VIP receptor subtypes, there are at least 2 receptor subtypes, namely, pituitary adenylate cyclase activating peptide (PACAP)/VIP type I and PACAP/VIP type II receptors, based on pharmacological binding studies (Arimura, 1992; Harmar & Lutz, 1994). PACAP/VIP type I receptor has a higher affinity for PACAP 27 and PACAP 38 than VIP, while type II receptor has an equal affinity for PACAP 27, PACAP 38 and VIP. In vascular smooth muscle, Huang *et al*. (1993) have shown that VIP is as potent as PACAP 27 and PACAP 38 in the porcine coronary arterial medial strips. It is thus likely that VIP-induced relaxation of the porcine coronary artery is mediated by PACAP/VIP type II receptors. However, further investigations are required to determine definitely which receptor subtype is responsible for the VIP-induced relaxation of the vascular smooth muscle cells.

The role of K^+ channels in the VIP-induced vasorelaxation and decrease in $[Ca^{2+}]_i$

We discovered the important role of some types of K^+ channels in the VIP-induced decrease in the $[Ca^{2+}]_i$ and force. During the stimulation with 30 mM K^+, which can abolish the effect of membrane hyperpolarization, the effects of VIP on the $[Ca^{2+}]_i$ and force were both attenuated, as compared with the effects of VIP during the U46619 stimulation (Figure 3b). These results suggest that membrane hyperpolarization is involved in the decrease in $[Ca^{2+}]_i$ and force induced by VIP. Recent evidence also suggests that vascular tone and membrane potential are regulated by several types of K^+ channels, including Ca^{2+}-activated K^+ channels, voltage-dependent K^+ channels and ATP-sensitive K^+ channels (Nelson & Quayle, 1995). Therefore, in the present study we investigated the effects of selective blockers for these K^+ channels on the VIP-induced decrease in the $[Ca^{2+}]_i$ and force during stimulation with U46619. Comparative studies with these selective K^+ channel blockers, as shown in Figure 4, suggested that the activation of a large conductance Ca^{2+}-activated K^+ channel and voltage-dependent K^+ channels may contribute to the VIP-induced decrease in the $[Ca^{2+}]_i$ and force, while, in contrast, ATP-sensitive K^+ channels and small conductance Ca^{2+}-activated K^+ channels may not. In agreement with the present results, Kishi *et al*. (1996) suggested that charybdotoxin (a large conductance Ca^{2+}-activated K^+ channel blocker) but not apamin (a small conductance Ca^{2+}-activated K^+ channel blocker) inhibited VIP-induced relaxation and hyperpolarization in rat colon, respectively. Hattori *et al*. (1992) have suggested that glibenclamide does not inhibit the VIP-induced relaxation of the rabbit mesenteric artery by a tension study. On the other hand, Standen *et al*. (1989) showed that VIP induces membrane hyperpolarization which is antagonized by glibenclamide, a blocker of the ATP-sensitive K^+ channels, in the rabbit cerebral artery. This discrepancy might be due to species and tissue differences.

The effects of VIP on the $[Ca^{2+}]_i$-force relationship and on the Ca^{2+} sensitivity of the contractile apparatus and the role of G-protein in these effects

In the present study, we found that VIP shifted the $[Ca^{2+}]_i$-force relationship to the right in the porcine coronary artery (Figure 6). A similar rightwards shift of the $[Ca^{2+}]_i$-force relationship in the porcine coronary artery was also produced by other cyclic AMP-elevating agonists, such as isoprenaline (Ushio *et al*., 1993), adrenomedullin (Kureishi *et al*., 1995) and calcitonin gene-related peptide (Fukuizumi *et al*., 1996). Therefore, together with the previously described elevation of the cyclic AMP levels induced by VIP in arterial smooth muscles (Edvinsson *et al*., 1985; Itoh *et al*., 1985; Ganz *et al*., 1986; Ignarro *et al*., 1987), these findings suggest that the effect of VIP on the $[Ca^{2+}]_i$-force relationship may be mediated by an elevation of cyclic AMP. Since we recently obtained evidence of a G-protein-mediated decrease in the Ca^{2+} sensitivity of the contractile apparatus (Kureishi *et al*., 1995), in the present study we examined whether VIP also decreases the Ca^{2+} sensitivity of the contractile apparatus through the activation of G-protein, by use of membrane permeabilization with α-toxin. The GTP-dependent relaxation induced by VIP at a constant $[Ca^{2+}]_i$ and its complete blockade by GDPβS (Figure 7) indicate that G-protein mediates the VIP-induced decrease in Ca^{2+} sensitivity of the contractile apparatus. The decrease in the myofilament Ca^{2+} sensitivity induced by the activation of a cyclic AMP-mediating pathway was first described in saponin-permeabilized arterial strips (Kerrick & Hoar, 1981; Itoh *et al*., 1982). Since a similar phenomenon was also produced in the α-toxin-permeabilized arterial strip (Nishimura & van Breemen, 1989), our results with the α-toxin-permeabilized coronary artery support the hypothesis that VIP may decrease the Ca^{2+} sensitivity of the contractile apparatus in the porcine coronary artery through

the activation of G-protein and a cyclic AMP-mediated pathway. However, it is also possible that VIP might antagonize the agonist-induced G-protein-mediated increase in Ca^{2+} sensitivity based on the following observations. As (Figure 6), the application of increasing concentrations of VIP shifted the relationship between the $[Ca^{2+}]_i$ and force in agonist-stimulated muscle towards that of high-K stimulation. In Figure 7a, the application of VIP appeared to reverse the potentiation of force caused by GTP in the permeabilized preparation.

In summary, by using front-surface fluorometry of fura-2 and membrane permeabilization with α-toxin, we demonstrated that VIP relaxes the coronary artery by decreasing not only $[Ca^{2+}]_i$ but also the Ca^{2+} sensitivity of the contractile apparatus. It seems that VIP may decrease $[Ca^{2+}]_i$ by two mechanisms: (1) the inhibition of the Ca^{2+} influx presumably through membrane hyperpolarization induced by the activation of K^+ channels; and (2) stimulation of the uptake of Ca^{2+} into thapsigargin-sensitive store sites. Finally, it was also observed that VIP decreases myofilament Ca^{2+} sensitivity through the activation of G-protein.

This study was supported in part by Grants-in-Aid for Developmental Scientific Research (No.06557045), for General Scientific Research (No.07407022, 07833008) and for Creative Basic Research Studies of Intracellular Signaling Network from the Ministry of Education, Science, Sports and Culture, Japan, and also by the Kimura Memorial Heart Foundation Research Grant for 1995.

References

ABE, S., KANAIDE, H. & NAKAMURA, M. (1990). Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and on tension in the coronary artery of the pig. *Br. J. Pharmacol.*, **101,** 545–552.

ARIMURA, A. (1992). Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. (Review). *Regul. Peptides*, **37,** 287–303.

BRIZZOLARA, A.L. & BURNSTOCK, G. (1991). Endothelium-dependent and endothelium-independent vasodilatation of the hepatic artery of the rabbit. *Br. J. Pharmacol.*, **103,** 1206–1212.

D'ORLÈANS-JUSTE, P., DION, S., MIZRAHI, J. & REGOLI, D. (1985). Effects of peptides and non-peptides on isolated arterial smooth muscles: role of endothelium. *Eur. J. Pharmacol.*, **114,** 9–21.

DUCKLES, S.P. & SAID, S.I. (1982). Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation. *Eur. J. Pharmacol.*, **78,** 371–374.

EDVINSSON, L., FAHRENKRUG, J., HANKO, J., OWMAN, C., SUNDLER, F. & UDDMAN, R. (1980). VIP (vasoactive intestinal polypeptide)-containing nerves of intracranial arteries in mammals. *Cell Tissue Res.*, **208,** 135–142.

EDVINSSON, L., FREDHOLM, B.B., HAMEL, E., JANSEN, I. & VERRECCHIA, C. (1985). Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci. Letts.*, **58,** 213–217.

FUKUIZUMI, Y., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1996). The effect of calcitonin gene-related peptide on the cytosolic calcium concentration and force in the porcine coronary artery. *J. Pharmacol. Exp. Ther.*, **278,** 220–231.

GANZ, P., SANDROCK, A.W., LANDIS, S.C., LEOPOLD, J., GIMBRONE, JR., M.A. & ALEXANDER, R.W. (1986). Vasoactive intestinal peptide: vasodilatation and cyclic AMP generation. *Am. J. Physiol.*, **250,** H755–760.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260,** 3440–3450.

GUILLEMARE, E., LAZDUNSKI, M. & HONORE, E. (1994). CGRP-induced activation of K_{ATP} channels in follicular Xenopus oocytes. *Pflügers Arch.*, **428,** 604–609.

GULBENKIAN, S., SAETRUM OPGAARD, O., EKMAN, R., COSTA ANDRADE, N., WHARTON, J., POLAK, J.M., QUEIROZ E MELO, J. & EDVINSSON, L. (1993). Peptidergic innervation of human epicardial coronary arteries. *Circ. Res.*, **73,** 579–588.

HARMAR, T. & LUTZ, E. (1994). Multiple receptors for PACAP and VIP. [Review]. *Trends. Pharmacol. Sci.*, **15,** 97–99.

HATTORI, Y., NAGASHIMA, M., ENDO, Y. & KANNO, M. (1992). Glibenclamide does not block arterial relaxation caused by vasoactive intestinal polypeptide. *Eur. J. Pharmacol.*, **213,** 147–150.

HIRANO, K., KANAIDE, H., ABE, S. & NAKAMURA, M. (1990). Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. *Br. J. Pharmacol.*, **101,** 273–280.

HUANG, M., SHIRAHASE, H. & RORSTAD, O.P. (1993). Comparative study of vascular relaxation and receptor binding by PACAP and VIP. *Peptides*, **14,** 755–762.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am. J. Physiol.*, **253,** H1074–1082.

ITOH, T., IZUMI, H. & KURIYAMA, H. (1982). Mechanisms of relaxation induced by activation of β-adrenoceptors in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **326,** 475–493.

ITOH, T., SASAGURI, T., MAKITA, Y., KAMNURA, Y. & KURIYAMA, H. (1985). Mechanisms of vasodilation induced by vasoactive intestinal polypeptide in rabbit mesenteric artery. *Am. J. Physiol.*, **249,** H231–240.

KERRICK, W.G.L. & HOAR, P.E. (1981). Inhibition of smooth muscle tension by cyclic AMP-dependent protein kinase. *Nature*, **292,** 253–255.

KISHI, M., TAKEICHI, T., SUTHAMNATPONG, N., ISHII, T., NISHIO, H., HATA, F. & TAKEWAKI, T. (1996). VIP- and PACAP-mediated nonadrenergic, noncholinergic inhibition in longitudinal muscle of rat distal colon: involvement of activation of charybdotoxin- and apamin-sensitive K^+ channels. *Br. J. Pharmacol.*, **119,** 623–630.

KOBAYASHI, S., GONG, M.C., SOMLYO, A.V. & SOMLYO, A.P. (1991). Ca^{2+} channel blockers distinguish between G protein-coupled pharmacomechanical Ca^{2+} release and Ca^{2+} sensitization. *Am. J. Physiol.*, **260,** C364–370.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A.V. & SOMLYO, A.P. (1989). Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. Physiological role of inositol 1,4,5-trisphosphate in pharmacomechanical coupling. *J. Biol. Chem.*, **264,** 17997–18004.

KUREISHI, Y., KOBAYASHI, S., NISHIMURA, J., NAKANO, T. & KANAIDE, H. (1995). Adrenomedullin decreases both cytosolic Ca^{2+} concentration and Ca^{2+}-sensitivity in pig coronary arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **212,** 572–579.

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1993). Role of GTP-protein and endothelium in contraction induced by ethanol in pig coronary artery. *J. Physiol.*, **470,** 521–537.

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1995). Mechanism of endothelium-dependent relaxation induced by substance P in the coronary artery of the pig. *Br. J. Pharmacol.*, **116,** 2040–2047.

LARSSON, L.I., EDVINSSON, L., FAHRENKRUG, J., HAKANSON, R., OWMAN, C., SCHAFFALITZKY, de, MUCKADELL, O. & SUNDLER, F. (1976). Immunohistochemical localization of a vasodilatory polypeptide (VIP) in cerebrovascular nerves. *Brain Res.*, **113,** 400–404.

LEE, T.J., SAITO, A. & BEREZIN, I. (1984). Vasoactive intestinal polypeptide-like substance: the potential transmitter for cerebral vasodilation. *Science*, **224,** 898–901.

MIAO, F.J. & LEE, T.J. (1992). Regional variation in acute vascular homologous tachyphylaxis. *J. Pharmacol. Exp. Ther.*, **260,** 644–651.

MIYAGI, Y., KOBAYASHI, S., NISHIMURA, J., FUKUI, M. & KANAIDE, H. (1995). Resting load regulates cytosolic calcium-force relationship of the contraction of bovine cerebrovascular smooth muscle. *J. Physiol.*, **484,** 123–137.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.*, **268,** C799–822.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochem. Biophys. Res. Commun.*, **163,** 929–935.

RAEYMAEKERS, L., EGGERMONT, J.A., WUYTACK, F. & CASTEELS, R. (1990). Effect of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca^{2+} pump of bovine pulmonary artery. *Cell Calcium*, **11,** 261–268.

SAID, S.I. & MUTT, V. (1970). Polypeptide with broad biological activity: isolation from small intestine. *Science*, **169,** 1217–1218.

SOMLYO, A.P. & SOMLYO, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature*, **372,** 231–236.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245,** 177–180.

THASTRUP, O., CULLEN, P.J., DRØBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2466–2470.

UDDMAN, R., ALUMETS, J., EDVINSSON, L., HAKANSON, R. & SUNDLER, F. (1981). VIP nerve fibres around peripheral blood vessels. *Acta Physiol. Scand.*, **112,** 65–70.

USHIO-FUKAI, M., ABE, S., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1993). Effects of isoprenaline on cytosolic calcium concentrations and on tension in the porcine coronary artery. *J. Physiol.*, **462,** 679–696.

(Received September 27, 1996
Revised March 11, 1997
Accepted March 24, 1997)