A pertussis toxin-sensitive mechanism of endothelin action in porcine coronary artery smooth muscle

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1 Endothelin-1 (ET-1)-induced contraction of porcine coronary artery strips may be mediated via at least two intracellular signalling mechanisms, the activation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels and the stimulation of phosphoinositide breakdown. Here we have investigated the possible involvement of pertussis toxin (PT)-sensitive guanosine-5'-triphosphate (GTP)-binding proteins (G-proteins) in ET-1-induced activation of these two signalling pathways in porcine coronary artery smooth muscle.

2 Increase in extracellular K^+ concentration (10, 15 mM) shifted the dose-response relationship for the ET-1-induced contraction to the left.

3 The dihydropyridine Ca^{2+} channel blocker, nifedipine (10^{-8} M), induced a rightward shift in the dose-response curve for ET-1. Pretreatment of the arterial strips with PT ($0.1 \mu\text{g ml}^{-1}$) induced a similar rightward shift of the ET-1 dose-response curve but not of the KCl response. Nifedipine (10^{-8} M) did not further attenuate the ET-1-induced contraction in the PT-pretreated strips.

4 The pretreatment with PT significantly reduced $^{45}\text{Ca}^{2+}$ uptake of the arterial strips stimulated by ET-1, but had no effect on ET-1-induced production of inositol phosphates.

5 The contractile response of the arterial strips to phorbol dibutyrate, an active phorbol ester, was not significantly affected by 10^{-8} M nifedipine.

6 We confirmed that the pretreatment of the tissue with PT induced ADP-ribosylation of a 41 kDa membrane protein.

7 These findings indicate that activation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels by ET-1 in this tissue is mediated via a PT-sensitive G-protein in a manner apparently independent of the ET-1-induced activation of protein kinase C. It is concluded that the action of ET-1 in porcine coronary artery is mediated via two distinct signal transduction pathways, which are coupled to PT-sensitive and PT-insensitive GTP-binding proteins, respectively.

Keywords: GTP-binding proteins; dihydropyridines; phosphoinositides turnover; phorbol esters

Introduction

Endothelin-1 (ET-1) provokes a strong and sustained contraction of isolated vascular smooth muscle from different species (Kasuya *et al.*, 1989a; Saitoh *et al.*, 1989). Previous studies have demonstrated that the mechanisms of ET-1-induced vasoconstriction may involve both the activation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (Goto *et al.*, 1989; Kasuya *et al.*, 1989a) and inositol phospholipid hydrolysis (Kasuya *et al.*, 1989b). However, it is still unknown whether these two signal transduction mechanisms are independent of each other.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) act as transducers between membrane receptors for extracellular signals and intracellular effectors controlling the concentration of cytosolic signalling molecules such as adenosine 3':5'-cyclic monophosphate (cyclic AMP), Ca^{2+} , and inositol phosphates. It has been established that receptor activation of numerous hormones and neurotransmitters is coupled to phospholipase C activation via G-proteins in various cells (Smith *et al.*, 1985; Martin *et al.*, 1986). It has also been demonstrated that extracellular ligands directly (Yatani *et al.*, 1987; Toselli *et al.*, 1989) or indirectly (Osterrieder *et al.*, 1982; Shearman *et al.*, 1989)

regulate plasma membrane channels for K^+ or Ca^{2+} through G-proteins. We and others have recently demonstrated that ET-1 receptor is coupled to phospholipase C via a pertussis toxin (PT)-insensitive G-protein in cultured vascular smooth muscle cells (Bobik *et al.*, 1990; Takuwa *et al.*, 1990).

In this study, in order to understand the mechanism for activation by ET-1 of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels, we investigated the effect of PT on ET-1-induced contraction and Ca^{2+} influx in isolated coronary artery smooth muscle of the pig. The results suggested that ET-1-induced activation of the voltage-dependent Ca^{2+} channels is mediated by a PT-sensitive G-protein.

Methods

Measurement of contraction of arterial strips

Right coronary arteries were isolated from fresh adult porcine hearts obtained from a local slaughterhouse. Arterial segments were cut into 2×15 mm helical strips with endothelial cells removed by rubbing the intimal surface with a cotton swab, and suspended in 5 ml of siliconized glass organ chambers filled with a Krebs-Ringer solution of the following composition (in mM): NaCl 113, KCl 4.8, CaCl_2 2.2, KH_2PO_4 1.2, MgCl_2 1.2, NaHCO_3 25 and glucose 5.5. The solution was maintained at 37°C and gassed with 5% $\text{CO}_2/95\%$ O_2 .

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Arterial strips were equilibrated at a passive tension of 1.25 g until the contractile tension induced by 50 mM KCl attained a steady state. Isometric contraction was measured as previously described (Kasuya *et al.*, 1989a).

Treatment with pertussis toxin

After equilibration by repetitive application of 50 mM KCl, arterial strips were incubated for 15 h in the Krebs-Ringer solution containing $0.1 \mu\text{g ml}^{-1}$ PT. During the incubation period, the solution was changed every 3 h with fresh solution of the same composition, and maintained at 37°C and gassed with 5% $\text{CO}_2/95\% \text{O}_2$. Arterial strips showed comparable contractile responses to 50 mM KCl before and after the treatment with PT. Control muscle strips were similarly incubated in the Krebs-Ringer solution without PT for 15 h.

$^{45}\text{Ca}^{2+}$ -uptake

Arterial strips were cut into small segments and equilibrated for 2 h in the Krebs-Ringer solution gassed with 5% $\text{CO}_2/95\% \text{O}_2$ at 37°C . After the equilibration period, the segments weighing 15 mg were transferred into a N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES)-buffered solution of the following composition (in mM): NaCl 140, KCl 5.9, CaCl_2 1.5, glucose 11.0 and 5 HEPES-tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4). After further 30 min equilibration at 37°C , the incubation with human ET-1 (Peptide Institute, Osaka, Japan) or 50 mM KCl for 30 min was started. $^{45}\text{CaCl}_2$ (74 MBq ml^{-1} , $740 \text{ GBq mmol}^{-1}$; Amersham) was added 10 min before the end of the stimulation period. The segments were briefly washed three times with ice-cold, calcium-free HEPES-buffered solution containing 2 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). After the final washing, the segments were blotted between two sheets of filter paper (Whatman 3MM) and homogenized with a glass-glass homogenizer in scintillation solution (ACS II, Amersham). The radioactivity was then determined by a Beckman liquid scintillation counter. The Ca^{2+} uptake of the tissues was calculated as nmol Ca^{2+} per g wet tissue.

Measurement of inositol phosphate production

Arterial strips were cut into small segments and labelled by incubation in the aerated Krebs-Ringer solution containing 9.25 MBq ml^{-1} myo-[1,2- ^3H]-inositol ($1.66\sim 2.96 \text{ TBq mmol}^{-1}$, New England Nuclear) at 37°C for 6 h. After washing three times with [^3H]-inositol-free Krebs-Ringer solution, the segments (30 mg) were preincubated at 37°C in 1 ml of the Krebs-Ringer solution containing 10 mM LiCl for 10 min and then stimulated with ET-1 for 15 min. The reaction was terminated by adding ice-cold perchloric acid to a final concentration of 10% (v/v), and the segments were homogenized with a glass-glass homogenizer. The supernatants of the homogenates were neutralized and applied to a Dowex 1X-8 anion exchange column for the separation of inositol phosphates as described previously (Kasuya *et al.*, 1989b).

In vitro ADP-ribosylation of arterial smooth muscle cell membrane

To estimate the effectiveness of PT-treatment under our experimental conditions, *in vitro* ADP-ribosylation was performed in the presence of ^{32}P NAD, using the membranes prepared from arterial strips, either untreated or treated with PT as described above. Arterial strips, either untreated or treated with PT, were washed twice with phosphate buffered saline (PBS), minced to small pieces, and homogenized in buffer of the following composition (in mM): Tris-HCl (pH 7.4) 10, dithiothreitol 1, and ethylene diamine N,N,N',N'-tetraacetic acid (EDTA) 5. The homogenates were sonicated for 2 min and centrifuged at 500 g for 5 min. The resulting

supernatants were centrifuged at 10,000 g for 5 min. The resulting precipitates were resuspended in the homogenization buffer and used as membrane samples. The membranes ($30 \mu\text{g}$ protein/sample) were incubated with $25 \mu\text{g ml}^{-1}$ PT for 75 min at 25°C in a reaction buffer of the following composition (in mM): Tris-HCl (pH 7.5) 20, thymidine 10, [adenylate- ^{32}P]-nicotinamide adenine dinucleotide 0.04, (NAD; $740 \text{ GBq mmol}^{-1}$, New England Nuclear), ATP 1, EDTA 0.5, dithiothreitol 20, and MgCl_2 1. The reaction was terminated by adding perchloric acid (PCA) to a final concentration of 10% (v/v), and the membranes were washed once with 10 mM Tris-HCl (pH 7.5). The washed membranes were solubilized in sodium dodecyl sulphate (SDS)-sampling buffer and analyzed by SDS/9% polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

Drugs

The following drugs were used: nifedipine and phorbol 12,13-dibutyrate were from Sigma Chemical Co. Ltd. Endothelin-1 was obtained from Peptide Institutes (Osaka, Japan). Pertussis toxin (islet-activating protein: IAP) was from Funakoshi Co. Ltd. (Tokyo, Japan).

Statistical analysis of data

Values are expressed as means $\pm$ s.e. Comparisons were made by a one-way analysis of variance followed by the Bonferroni method (Figure 1, Table 1), or Student's *t* test for unpaired values (Figures 3 and 4). The level of statistically significant difference was $P < 0.05$.

Results

Effect of membrane depolarization on endothelin-1-induced contraction

Our previous studies demonstrated that ET-1 activates the dihydropyridine-sensitive voltage-dependent Ca^{2+} channels in porcine coronary artery smooth muscle. In order to confirm whether the voltage-dependent process actually constitutes a part of the mechanisms by which ET-1 induces contraction, we investigated the effect of membrane depolarization on the dose-response relationships for ET-1-induced contraction. The elevation of extracellular K^+ concentration induced a small contraction in the absence of ET-1 (Figure 1a); the contractile responses to 5 mM, 10 mM and 15 mM K^+ expressed as percentages of tension generated by 50 mM KCl were $0 \pm 0\%$, $1.7 \pm 0.1\%$ and $17.3 \pm 2.0\%$, respectively. The cumulative addition of ET-1 superimposed a slowly-developing, dose-dependent contraction which attained a steady state after 15–20 min (Figure 1a). Increased extracellular K^+ significantly augmented the plateau tension induced by a low concentration of ET-1, whereas the maximum contraction induced by 10^{-7} M ET-1 was not significantly different (Figure 1b).

Effect of pertussis toxin-pretreatment on the endothelin-1-induced contraction

To elucidate whether a PT-sensitive G-protein is involved in the action of ET-1, we treated porcine coronary artery strips with PT as described under Methods. To verify the effectiveness of the PT-pretreatment, we incubated plasma membranes prepared from the PT-treated and control strips with PT *in vitro* in the presence of [^{32}P]-NAD. The labelled membrane proteins were separated by SDS-PAGE and visualized by autoradiography. In the control strips, a single 41 kDa species of the membrane protein was ADP-ribosylated (Figure 2). The pretreatment of intact strips with PT greatly reduced the additional ADP-ribosylation *in vitro* of the 41 kDa membrane protein, indicating that the pretreatment

Figure 1 (a) Typical contractile response of porcine coronary artery strips to cumulatively applied endothelin-1 (ET-1) in high-K⁺ Krebs Ringer solutions. (b) Dose-response relationships for vasoconstriction superimposed by cumulatively added ET-1 under conditions of elevated extracellular K⁺: 15 mM (■); 10 mM (▲); control (○). Contractile responses induced by ET-1 are expressed as percentages of maximum tension induced by 50 mM KCl; since increase of extracellular KCl induced a small vasoconstriction, the steady-state contractile tension induced by KCl alone was subtracted when measuring ET-1-induced tensions. Points represent means with s.e. shown by vertical bars ($n=4$). *Significantly different from values with normal KCl; **significantly different from values with 10 mM KCl ($P<0.05$, ANOVA with Bonferroni method).

with PT effectively ADP-ribosylated this protein in the intact tissue.

Pretreatment of arterial strips with PT did not significantly affect KCl-induced contraction (Figure 3a). In contrast, PT-pretreatment caused a significant reduction of ET-1-induced contraction. The extent of the attenuation of ET-1-induced contraction by pretreatment with PT was greater at lower doses of ET-1 (10^{-10} – 3×10^{-9} M), so that the cumulative dose-response curves apparently shifted to the right and steepened. The apparent EC₅₀ values were 5.3×10^{-9} M (95% confidence interval, 2.3×10^{-9} – 1.6×10^{-8} M) in PT-pretreated strips and 4.7×10^{-10} M (2.3×10^{-10} – 9.8×10^{-10} M) in control strips. A low dose (10^{-8} M) of nifedipine induced a similar rightward shift of the cumulative dose-response curve for ET-1-induced contraction in strips not pretreated with PT. In contrast, the same concentration of nifedipine did not significantly affect the dose-response curve for ET-1 in PT-pretreated strips (Figure 3b).

Effect of pertussis toxin-pretreatment on calcium influx induced by endothelin-1

We investigated whether the inhibitory effect of PT on ET-1-induced contraction is due to the inhibition of ET-1-induced Ca²⁺ influx, which plays an important role in ET-1-induced contraction of this smooth muscle. As shown in Table 1, 10^{-9} M ET-1 augmented Ca²⁺ uptake of the control coronary artery strips by $47 \pm 4.5\%$. We previously demonstrated that nifedipine inhibits this ET-1-stimulated Ca²⁺ uptake (Kasuya *et al.*, 1989a). Pretreatment of coronary artery strips with PT almost completely inhibited ET-1-stimulated Ca²⁺ uptake

Figure 2 *In vitro* ADP-ribosylation of a 41-kDa protein in plasma membranes prepared from porcine coronary artery smooth muscle. The coronary artery strips were incubated with or without pertussis toxin (PT) $0.1 \mu\text{g ml}^{-1}$ for 15 h and crude membrane fractions were prepared. The membranes were then incubated with PT $25 \mu\text{g ml}^{-1}$ in the presence of [³²P]-NAD. Membrane proteins were resolved by SDS-PAGE and autoradiography.

(Table 1). Elevation of extracellular K⁺ concentration (50 mM; equimolar substitution for Na⁺) also induced an increase in Ca²⁺ uptake by $29 \pm 9.1\%$. However, unlike the ET-1-induced Ca²⁺ uptake, the KCl-induced Ca²⁺ uptake was not significantly suppressed by PT-pretreatment.

Effect of pertussis toxin-pretreatment on inositol phosphates production induced by endothelin-1

We examined the effect of PT-treatment on ET-1-induced phosphoinositide hydrolysis, which is also thought to be a signalling pathway evoked by ET-1. As shown in Figure 4, ET-1 stimulated the production of inositol phosphates in the control coronary artery strips. The pretreatment with PT did not significantly affect the ET-1-induced production of total inositol phosphates.

Effect of nifedipine on contractile response to phorbol ester

In order to clarify whether the ET-1-induced activation of the voltage-dependent Ca²⁺ channels is secondary to the activation of protein kinase C evoked through the ET-1-induced phosphoinositide hydrolysis, we investigated the effect of nifedipine on phorbol ester-induced contraction. Phorbol 12,13-dibutyrate (PDBu), an active phorbol ester, induced contraction of porcine coronary artery strips in a dose-dependent manner (Figure 5a), whereas the biologically inactive enantiomer, 4a-phorbol 12,13-dibutyrate, did not (data not shown). In contrast to contraction induced by ET-1, nifedipine (10^{-8} M) did not significantly affect contrac-

tion induced by PDBu (Figure 5b). This indicates that the activation of the Ca^{2+} channel is not involved in phorbol ester-induced contraction of this particular smooth muscle tissue.

Figure 3 (a) Dose-response relationships for KCl-induced contraction of porcine coronary artery strips pretreated with pertussis toxin (PT, ●) or with vehicle (○). (b) Effects of PT pretreatment and of nifedipine on cumulative dose-response curves of endothelin-1 (ET-1)-induced contraction of porcine coronary artery strips. Dose-response relationships of intact vascular strips to cumulative ET-1 in the absence (○) and presence (●) of $10^{-8} M$ nifedipine are compared with those in the absence (▲) or presence (■) of $10^{-8} M$ nifedipine for the vascular strips pretreated with $0.1 \mu\text{g ml}^{-1}$ PT. Nifedipine was added 15 min before the first dose of ET-1. Contractile responses are expressed as percentages of maximum tension induced by 50 mM KCl. Bars represent $\pm$ s.e. ($n = 4-6$). *Significantly different from control levels with $P < 0.05$ (Student's t test for unpaired values).

Table 1 Effect of pretreatment with pertussis toxin (PT) on endothelin-1 (ET-1)-induced Ca^{2+} uptake in porcine coronary artery smooth muscle

	Ca^{2+} uptake (nmol g^{-1} tissue)	
	Intact	PT-treated
Unstimulated	992 ± 35	962 ± 22
KCl 50 mM	$1281 \pm 90^*$	$1192 \pm 90^*$
ET-1 $10^{-9} M$	$1462 \pm 45^*$	$1005 \pm 6^\dagger$

Data are means $\pm$ s.e. ($n = 3$).

*Significantly different from unstimulated values for intact tissues with $P < 0.05$ (ANOVA with Bonferroni method); † Significantly different from values for intact tissues stimulated with ET-1, $P < 0.05$ (Student's t test for unpaired values).

Figure 4 Stimulation by endothelin-1 (ET-1, $10^{-7} M$) of production of total $[^3\text{H}]$ -inositol phosphates (c.p.m. 30 mg^{-1} tissue) in porcine coronary artery strips preincubated with or without pertussis toxin (PT). Error bars represent $\pm$ s.e. ($n = 4$). *Significantly different with $P < 0.05$; NS, not significantly different (Student's t test for unpaired values).

Figure 5 (a) Typical contractile response of porcine coronary artery strips to cumulatively applied phorbol 12,13-dibutyrate (PDBu) in the absence or in the presence of $10^{-8} M$ nifedipine. (b) Effect of nifedipine on cumulative dose-response curves of PDBu-induced vasoconstriction of porcine coronary artery strips. Dose-response relationships for PDBu in the absence (○) and presence (●) of $10^{-8} M$ nifedipine are shown. Contractile responses are expressed as percentages of maximum tension induced by 50 mM KCl. Bars represent $\pm$ s.e. ($n = 4$).

Discussion

The activation of voltage-dependent Ca^{2+} channels by ET-1 has been demonstrated in various types of cells (Ishikawa *et al.*, 1989; Matsumura *et al.*, 1989; Saitoh *et al.*, 1989; Yoshizawa *et al.*, 1989; Nakao *et al.*, 1990). Our previous studies demonstrated that the Ca^{2+} influx through the dihydropyridine-sensitive voltage-dependent Ca^{2+} channels plays an important role for ET-1-induced contraction of porcine coronary artery (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989; Kasuya *et al.*, 1989a). We have shown in the present study that ET-1-induced contraction over low dose range (10^{-11} – 10^{-10} M) is greatly enhanced by membrane depolarization (Figure 1b). This is consistent with the notion that the activation of voltage-dependent Ca^{2+} channels is an important process for the action of low doses of ET-1 in this tissue, since, if the Ca^{2+} influx is assumed to be independent of the membrane potential, the ET-1-induced contraction should be rather inhibited by membrane depolarization because of a decreased driving force for Ca^{2+} influx. We previously demonstrated that ET-1-induced depolarization of the smooth muscle membrane alone is not sufficient to explain the activation of the Ca^{2+} channels by ET-1 (Kasuya *et al.*, 1989a), and that ET-1 has its specific receptor that is separate from the dihydropyridine receptor complex (Miyazaki *et al.*, 1990). Moreover, it has been reported that ET receptor subtypes belong to the superfamily of G protein-coupled receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). These observations raise the possibility that the putative receptor(s) for ET-1 may modulate the Ca^{2+} channel through a transducer molecule such as membrane G-proteins. ET-1 also activates phospholipase C with production of two second messengers, inositol trisphosphate and 1,2-diacylglycerol, leading to the mobilization of intracellular Ca^{2+} and activation of protein kinase C in porcine coronary artery. The ET-1-induced activation of phospholipase C is independent of Ca^{2+} influx (Kasuya *et al.*, 1989b). Further, it has previously been demonstrated that the activation of protein kinase C by active phorbol esters can enhance the inward Ca^{2+} current via the voltage-dependent Ca^{2+} channels in various types of cells (Dosemeci *et al.*, 1988; Vivaudou *et al.*, 1988). These findings suggest the possibility that the activation of voltage-dependent Ca^{2+} channels is secondary to the activation of phospholipase C by ET-1. We designed the present study to resolve these possibilities by dissecting the intracellular signalling mechanisms by virtue of PT.

The apparent effect of the pretreatment of coronary artery strips with PT on ET-1-induced contraction closely mimicked the action of nifedipine, in that contraction to relatively low concentrations of ET-1 (10^{-10} – 3×10^{-9} M) was preferentially inhibited by the PT-pretreatment (Figure 3b). The effects of the PT-pretreatment and nifedipine were not additive; treatment with 10^{-8} M nifedipine of the PT-pretreated strips did not further attenuate the ET-1-induced contraction. This suggests that the nifedipine-sensitive component of ET-1-induced contraction may be selectively removed by the pretreatment with PT. Furthermore, the pretreatment of the coronary artery strips with PT also inhibits the Ca^{2+} uptake induced by a low concentration of ET-1 (10^{-9} M), which is previously demonstrated (Kasuya *et al.*, 1989a) to be completely inhibited by nifedipine (Table 1). These results suggest that ET-1-induced activation of the voltage-dependent Ca^{2+} channel is mediated by a PT-sensitive signalling pathway. In contrast, ET-1-induced production of inositol phosphates was not significantly affected by the pretreatment of the arterial strips with PT (Figure 4), consistent with our previous findings with rat A10 smooth muscle cells in culture (Takuwa *et al.*, 1990).

A possible pathway of the ET-1-induced activation of the Ca^{2+} channels would be via the stimulation of protein kinase C secondary to the ET-1-induced breakdown of phosphoinositides. A prerequisite of this hypothesis is that the activation of protein kinase C opens the Ca^{2+} channels. However, the

dose-response relationship for phorbol ester-induced contraction of porcine coronary artery was not significantly affected by nifedipine at the dose that reduced the ET-1-induced contraction (Figure 5). Moreover, the contractile response induced by PDBu at the dose which induces the maximum tension (10^{-6} M) was not significantly inhibited by the removal of extracellular Ca^{2+} (data not shown). These results indicate that the voltage-dependent Ca^{2+} channels are not actively involved in the contraction elicited by the activation of protein kinase C in this particular tissue, and hence, that the ET-1-induced activation of the Ca^{2+} channel is unlikely to be a secondary event following the activation of protein kinase C due to the stimulation of phosphoinositide turnover by ET-1. This idea is also consistent with the previous demonstration that the active phorbol ester, tetradecanoylphorbol 13-acetate, neither positively nor negatively affects the Ca^{2+} influx via the voltage-dependent Ca^{2+} channels in this particular tissue (Itoh *et al.*, 1986).

The possibility existed that the long incubation of the tissue in PT-containing solution might bring about a membrane hyperpolarization or a reduction in the density of ET receptors; the response of the arterial tissue to agonists might be attenuated through these 'indirect' effects of PT rather than through a specific inhibition of the ET-1-induced activation of the Ca^{2+} channels. We examined these possibilities as follows: (i) if the PT-pretreatment somehow caused a membrane hyperpolarization, responses of the tissue to raised concentrations of KCl would also be affected. However, the PT-treatment did not alter the dose-response relationships for KCl-induced contraction (Figure 3a). Furthermore, the arterial strips showed a similar KCl-induced Ca^{2+} uptake before and after the PT-treatment (Table 1). (ii) If the pretreatment with PT resulted in a reduction in the density of ET receptors, the PT-treatment would cause not only a decrease of ET-1-induced Ca^{2+} entry but also a suppression of ET-1-induced phosphoinositide hydrolysis. However, the PT-pretreatment did not affect ET-1-stimulated production of inositol phosphates (Figure 4). These findings support the view that the PT-treatment under the experimental conditions used in the present study selectively blocked the signal transduction pathway from ET receptors to voltage-dependent Ca^{2+} channels in porcine coronary artery smooth muscle.

We demonstrated in the present study that the treatment of the coronary artery strips with PT efficiently ADP-ribosylated a 41 kDa membrane protein, which most likely represents the α -subunit of a G-protein (Figure 2). This further substantiates the idea that a certain G-protein, sensitive to PT, mediates the activation of the voltage-dependent Ca^{2+} channels by ET-1. In accordance with this notion, it has recently been demonstrated that *in vivo* pressor action of ET-3 in rats is apparently mediated by Ca^{2+} influx evoked via a PT-sensitive G-protein (Tabrizchi & Triggle, 1990). It has also been demonstrated that ET-1-induced Ca^{2+} influx is mediated through a PT-sensitive G-protein in striatal astrocytes (Martin *et al.*, 1991). PT prevents functional coupling of activated receptors and certain classes of G-proteins such as G_i and G_o by specifically ADP-ribosylating them. In favour of this argument, recent studies have demonstrated that the voltage-dependent Ca^{2+} channels are directly modulated by a number of G-proteins (Rosenthal *et al.*, 1988a). For example, a G_o -protein directly mediates the ligand-induced inhibition of voltage-dependent Ca^{2+} channels in various neuronal cells (Holz *et al.*, 1986; Hescheler *et al.*, 1987; Wanke *et al.*, 1987; Bergamaschi *et al.*, 1988). In addition, a G_i -protein and a G_s -protein directly mediate the stimulation of the voltage-dependent Ca^{2+} channel by hormones in endocrine cells (Hescheler *et al.*, 1988; Rosenthal *et al.*, 1988b) and cardiac cells (Yatani *et al.*, 1987), respectively. At present, however, it cannot be concluded unequivocally whether the PT-sensitive G-protein(s) that are coupled to ET receptor directly interact with the voltage-dependent Ca^{2+} channels or whether they affect the Ca^{2+} channel via an indirect mechanism.

The observations presented in this and previous studies (Goto *et al.*, 1989; Kasuya *et al.*, 1989a,b) indicate that ET-1 may evoke two independent pathways of intracellular signal transduction in porcine coronary artery smooth muscle: one pathway leads to the activation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels via a PT-sensitive G-protein, and the other involves the stimulation of phosphoinositides turnover through a PT-insensitive mechanism. Further study is required to examine whether these two signalling systems are coupled to different subtypes of the ET receptor or to a single class of ET receptor. Two distinct subtypes of endothelin receptor, name ET_A and ET_B receptors, have recently been cloned and pharmacologically characterized (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Hosoda *et al.*, 1991); ET_A receptors have much higher affinity for ET-1 and ET-2 as compared with ET-3, while ET_B receptors almost equally accept all three isopeptides of endothelin. However, radioligand binding studies and affinity cross-linking studies have detected only a single class of specific binding sites for ET-1 on the membrane fraction from porcine coronary artery smooth muscle, with pharmacological profiles consistent with the ET_A receptors (unpublished observation). Although the possibility remains that there might exist more than one sub-subclass of ET_A receptors, no

positive evidence suggesting ET_A receptor sub-subtypes has been presented thus far. It is therefore tempting to suggest that two distinct signalling systems of ET-1 in porcine coronary artery smooth muscle cells are coupled to a single ET-1 receptor. In this context, it is worth noting that both M_1 and M_4 subtypes of muscarinic acetylcholine receptors may each be coupled to two different classes of G-proteins that are distinguishable by sensitivity to PT (Ashkenazi *et al.*, 1989). Nichols *et al.* (1989) showed that, in a rat *in vivo* model, the pressor response mediated by an apparently single subclass of α_1 -adrenoceptors may be coupled to both phospholipase C and dihydropyridine-sensitive Ca^{2+} channels via PT-insensitive and PT-sensitive G-proteins, respectively. Besides the phosphoinositides turnover response, a concomitant activation of the voltage-dependent Ca^{2+} channels via a PT-sensitive pathway may represent a newly recognized signalling mechanism for various G-protein-coupled agonists, including the endothelins, in a number of vascular smooth muscle tissues.

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