MECHANISMS INVOLVED IN ENDOTHELIN-1-INDUCED VASOCONSTRICTION IN THE RABBIT PULMONARY ARTERY

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ABSTRACT

We investigated the mechanism involved in the endothelin-1 (ET-1)-induced contraction in small branches of rabbit pulmonary artery by use of isometric tension recording method. ET-1 (>0.1 nM) produced a slow developing and sustained contraction in a concentration-dependent manner. BQ123 and BQ788, receptor antagonists for ET_A and ET_B receptors respectively, only partially suppressed the amplitude of ET-1-induced contraction, but simultaneous application of BQ123 and BQ788 completely abolished it. Nicardipine, applied during the sustained contraction induced by ET-1, partially attenuated the amplitude of the contraction, and subsequent removal of extracellular Ca²⁺ completely abolished the sustained contraction. In EGTA-containing Ca²⁺-free Krebs solution, ET-1 evoked small transient contraction in the presence of BQ123 or BQ788. Pretreatment of the tissues with Calphostin C, a selective protein kinase C (PKC) inhibitor, suppressed the amplitude of ET-1-induced contraction, and additional application of BQ788 completely abolished it. The present results show that in smooth muscle cells of rabbit pulmonary artery, ET-1 activates both ET_A and ET_B receptor subtypes to enhance the Ca²⁺ influx and to release Ca²⁺ from the intracellular storage sites, and that stimulation of ET_A receptor subtype by ET-1 induces contraction mainly through activation of PKC.

Endothelin is a family of peptide comprising of endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 and sarafotoxin. ET-1 is one of the most potent vasoconstrictors originally isolated from cultured porcine endothelial cells (22). Two receptor subtypes for endothelin are known; ET_A receptor which is selective to ET-1 and ET-2, and non-selective ET_B receptor (1, 18). Although it had been originally considered that ET_A and ET_B receptors mediate vasoconstriction and vasodilation, respectively, recent studies indicate that ET_B receptor subtype mediates contraction of smooth muscle in some tissues (7, 14). Furthermore, contraction induced by stimulation of both ET_A and ET_B receptors is reported only in some tissues including rabbit pulmonary artery or rat trachea (6, 10). In vas-

cular smooth muscle from various tissues, ET-1 is reported to induce Ca²⁺ influx through Ca channel (2, 8), to release Ca²⁺ from intracellular storage site (9, 17), and to activate protein kinase C (PKC) (12, 16, 19).

Although there is a considerable literature on the action of endothelin on pulmonary artery, little is known about the cellular mechanisms in relation to receptor subtypes of endothelin. Therefore, it is of interest to examine the effects of ET-1 on the pulmonary artery in relation to the receptor subtypes and the cellular mechanisms involved, since both ET_A and ET_B receptors are found in this tissue.

In the present study, we used small branches of rabbit pulmonary artery with diameter of about 300 μ m; this is the typical 'resistance vessels' in the pulmonary vasculature which may contribute to the progression of pulmonary hypertension. The

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Fig. 1 Effects of 1 nM ET-1 in the presence or absence of BQ123 (1 μM) or BQ788 (1 μM) on smooth muscle strips of rabbit pulmonary artery. a-c: Actual traces of force induced by 128 mM K⁺, ET-1 in the presence or absence of BQ123 (1 μM) and/or BQ788 (1 μM). Each agent was applied as indicated by the horizontal bar. d: Mean values of relative amplitude of ET-1-induced contraction in the presence (closed column) or absence (open column) of BQ123, BQ788 or both of the drugs. The maximum contraction induced by 128 mM K⁺ was taken as a relative amplitude of 100%. e: Dose-response curve of ET-1 in the presence (triangle) or absence (circle) of BQ788. Each column and symbol represents the mean value of 3–6 observations. Asterisks indicate significant difference (***P* < 0.01, ****P* < 0.001) and vertical bars indicate SE.

effects of nicardipine, removal of Ca²⁺ from extracellular solution and Calphostin C, a selective PKC inhibitor, were observed to characterize the source of Ca²⁺ mobilized and the possible role of PKC in the contraction induced by ET-1.

MATERIALS AND METHODS

Materials

Male albino rabbits, weighing 1.8–2.1 kg, were anaesthetized with pentobarbitone sodium (50 mg/kg, i.v.), and then exsanguinated. Lungs were excised immediately into chamber filled with Krebs solution. The peripheral branches with diameter around 300 μm were subsequently dissected free of parenchyma from both lower lobes, and cleaned by removal of connective tissue.

Isometric Tension Recording

For the isometric tension recording, fine circularly

cut strips of 0.2 mm width and 0.8–1.0 mm length were prepared as described previously (23). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels using small knives. The absence of endothelial cells was confirmed by the inability of 1 μM acetylcholine to cause relaxation as described previously. The strip was set up horizontally in a chamber with a capacity of 1 ml, through which the test solution was perfused rapidly by jetting from one end and by sucking off simultaneously with water pump from the other end. An isometric tension was recorded using a strain gauge transducer (U-gauge, Shiko, Japan). The resting force was adjusted so as to obtain a maximal contraction with 128 mM K⁺.

Calculation of Hill Coefficient

The slope of the concentration-response relationship for the effect of ET-1 on force is shown as the Hill coefficient (*N*) and mid-point position [pK =

($-\log K$), where K is the dissociation constant]. These were obtained by fitting the data points for each curve to equation (1) by a non-linear least-squares method,

$$F/F_0 = (C/K)^N / [1 + (C/K)^N] \quad (1)$$

where C represents the concentration of ET-1, and F_0 is the maximum response evoked by 30 nM ET-1 expressed as a relative force of 100%.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ , 137.5; K^+ , 5.9; Mg^{2+} , 1.2; Ca^{2+} , 2.6; HCO_3^- , 15.5; H_2PO_4^- , 11.3; Cl^- , 134.3 and glucose, 11.5. The contraction of K^+ was modified by replacing NaCl with KCl, isotonicity. Ca^{2+} -free Krebs solution was made by substituting an equimolar concentration of MgCl_2 and adding 2 mM EGTA. The solution was bubbled with 95% O_2 and 5% CO_2 , and the pH maintained at 7.3–7.4.

Drugs

Drugs used were ET-1 (Peptide Institute, Japan), EGTA (Dojin, Japan), PDBu (Wako Pure Chemical, Japan). BQ123 and BQ788 were kindly provided by Banyu Pharmaceutical (Japan) and Ciba-Geigy (Japan), respectively.

Statistics

The values recorded were expressed as mean $\pm$ SE and statistical significance determined using a paired or unpaired Student's t -test. Probabilities less than 5% ($P < 0.05$) were considered significant.

RESULTS

Effects of BQ123 or BQ788 on ET-1-Induced Contraction

ET-1 (>0.3 nM) induced contraction in a dose-dependent manner (Fig. 1e), and the maximum amplitude of contraction induced by ET-1 (30 nM) was $102.1 \pm 2.5\%$ of that induced by 128 mM K^+ . Fig. 1, a–c show typical contraction induced by 1 nM ET-1, which reaches its peak about 30 min after drug application. BQ123 (1 μM , a selective ET_A receptor antagonist) did not change the resting tension, and attenuated the ET-1-induced contraction significantly (Fig. 1, a and d). BQ788 (1 μM , a selective ET_B receptor antagonist) also

Fig. 2 Effects of nicardipine (1 μM) and EGTA-containing Ca^{2+} -free Krebs solution on the ET-1-induced contraction in the absence or presence of BQ788. a and b: 1 μM nicardipine was applied during the maintained contraction induced by ET-1 in the absence (a) or presence (b) of BQ788 and the extracellular solution was subsequently replaced with EGTA-containing Ca^{2+} -free Krebs solution. c: Mean values of relative amplitude of ET-1-induced contraction in the absence (open column) or presence (closed column) of 1 μM nicardipine obtained from 3–4 observations, where the maximum contraction induced by 128 mM K^+ was taken as a relative force of 100% for each strip. The hatched column indicates the difference between the amplitude of ET-1-induced contraction in the absence and presence of BQ788. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). N.S., not significantly different. Vertical bars indicate SE.

attenuated the contraction induced by ET-1 (Fig. 1, b and d), and in the presence of BQ123 and BQ788, 1 nM ET-1 did not produce contraction at all (Fig. 1c).

Fig. 1d shows mean values of the relative amplitude of the ET-1-induced contraction obtained from 3–6 strips in the presence or absence of ET receptor antagonists. BQ123 or BQ788 (1 μM), when applied alone, suppressed the amplitude of ET-1-induced contraction to approximately 50% of the control values.

Fig. 3 ET-1-induced contraction in EGTA-containing Ca²⁺-free Krebs solution in the absence or presence of BQ123 or BQ788. a-c: Actual traces of force induced by 1 nM ET-1 in the absence (a) or presence of BQ123 (1 μM; b) and BQ788 (1 μM; c) in EGTA-containing Ca²⁺-free Krebs solution, or in the normal Krebs solution containing 2.6 mM Ca²⁺. d: Mean values of relative amplitude of ET-1-induced contraction in the absence or presence of BQ123 (1 μM) or BQ788 (1 μM) in Ca²⁺-free Krebs solution (closed column). As control ET-1-induced contraction in the absence of BQ123 or BQ788 in normal Krebs solution (open column) are also shown, where the maximum contraction induced by 128 mM K⁺ was taken as a relative force of 100% for each strip. Three muscle strips were used. Asterisks indicate significant difference (***) $P \leq 0.001$. N.S., not significantly different. Vertical bars indicate SE.

Fig. 1e shows dose-response curves of ET-1 in the absence and presence of BQ788. BQ788 (1 μM) suppressed the maximum response to $63.9 \pm 4.7\%$ of that induced by 128 mM K⁺. ED₅₀ was 5.39×10^{-10} M and 1.40×10^{-9} M, in the absence and presence of 1 μM BQ788, respectively.

Effects of Nicardipine and Ca²⁺-Free EGTA-Containing Krebs Solution on ET-1-Induced Contraction

To investigate the possible role of Ca channels involved in the action of ET-1, we examined the effects of nicardipine and EGTA-containing Ca²⁺-free Krebs solution on the sustained contraction evoked by ET-1. Nicardipine (1 μM) applied during the sustained contraction evoked by ET-1, gradually and significantly suppressed the tension to $63.8 \pm 5.7\%$ of the control value. However, a substantial part of the force still remained, and the

remaining force was completely abolished by replacing the extracellular solution with 2 mM EGTA-containing Ca²⁺-free Krebs solution (Fig. 2a).

In the presence of BQ788, ET-1 induced a slow developing and maintained contraction and the amplitude of which was also partially attenuated by 1 μM nicardipine (Fig. 2b).

Fig. 2c shows the effect of nicardipine (1 μM) on the mean amplitude of ET-1-induced contraction in the presence and absence of BQ788. Nicardipine suppressed the amplitude to about 60% of the control values. This observation indicates that ET-1 opens dihydropyridine-sensitive Ca channels. Nicardipine suppressed the amplitude to a similar extent irrespective of the presence or absence of BQ788 (indicated as hatched columns in Fig. 2c), thereby indicating that ET_B receptor mainly open dihydropyridine-insensitive Ca channels.

To study the possible role of intracellularly

Fig. 4 Effects of Calphostin C ($1 \mu\text{M}$) on PDBu-induced enhancement of 128 mM K^+ -induced contraction. a: $0.1 \mu\text{M}$ PDBu was applied on the tonic phase of 128 mM K^+ -induced contraction. b: After the pretreatment of the tissue with Calphostin C ($1 \mu\text{M}$) for 120 min, 128 mM K^+ and $0.1 \mu\text{M}$ PDBu were applied as indicated by the horizontal bars. c: Summary of the effects of Calphostin C on PDBu-induced enhancement of 128 mM K^+ -induced contraction calculated from 3 observations. Asterisks indicate significant difference ($***P < 0.001$) from the control strips. Vertical bars indicate SE.

stored Ca^{2+} in ET-1-induced contraction, we examined the effects of ET-1 in EGTA-containing Ca^{2+} -free Krebs solution. When extracellular solution was replaced with EGTA-containing Ca^{2+} -free Krebs solution the resting tension was not affected, and application of ET-1 produced a small transient contraction (Fig. 3a). Upon the second application, ET-1 did not produce any force in EGTA-containing Ca^{2+} -free Krebs solution (data not shown), but ET-1 did evoke large contraction when the extracellular solution was replaced with normal Krebs solution containing 2.6 mM Ca^{2+} . As shown in Fig. 3, b and c, neither BQ123 nor BQ788 inhibited the ET-1-induced contraction in EGTA-containing Ca^{2+} -free Krebs solution.

Fig. 3d shows the mean amplitudes of force induced by ET-1 in the presence or absence of BQ123 or BQ788 after the removal of Ca^{2+} from

extracellular solution. There was no significant difference between the force induced by ET-1 in the presence or absence of BQ123 or BQ788 in Ca^{2+} -free Krebs solution.

Effects of Calphostin C on ET-1-Induced Contraction

To investigate the possible role of PKC in ET-1-induced contraction, we used Calphostin C. PDBu ($0.1 \mu\text{M}$, an active phorbol ester) enhanced the amplitude of 128 mM K^+ -induced contraction by $111.5 \pm 13.4\%$, but after pretreatment of the tissue with $1 \mu\text{M}$ Calphostin C for 120 min, $0.1 \mu\text{M}$ PDBu did not amplify the amplitude of the contraction induced by 128 mM K^+ (Fig. 4). Pretreatment with 0.01% DMSO, the solvent for Calphostin C, showed no inhibitory effects on the stimulatory action of PDBu on 128 mM K^+ -induced contraction (data not shown).

Fig. 5, a and b shows the effects of DMSO and Calphostin C on ET-1-induced contraction in the presence and absence of BQ788. Pretreatment of the tissue with DMSO showed no effects on ET-1-induced contraction in the presence and absence of BQ788 (Fig. 5a). On the other hand, in muscle strips pretreated with Calphostin C, ET-1 did not produce any contraction at all in the presence of BQ788. Furthermore, ET-1 induced only small and sustained contraction after washing out of BQ788 (Fig. 5b). The effects of pretreatment of the tissue with DMSO or Calphostin C are summarized in Fig. 5c. BQ788-sensitive component of ET-1-induced contraction (the group of columns indicated as '1)–2)', whose values are calculated as difference of the amplitudes induced by ET-1 between in the absence and presence of BQ788) was significantly smaller in Calphostin C-treated strips than in control or DMSO-treated strips.

DISCUSSION

ET Receptor Subtypes

In the present study, ET-1 induced contraction at concentrations ranging from 0.3 nM to 30 nM in a dose-dependent manner, which is consistent with previous reports in rabbit pulmonary artery (10), and slightly less potent than in the rabbit mesenteric artery (23).

ET-1-induced contraction was partially suppressed by BQ123 (a selective ET_A receptor antagonist) or BQ788 (a selective ET_B receptor antagonist), and was completely abolished by simulta-

Fig. 5 Effects of Calphostin C (1 μ M) on the contraction induced by 1 nM ET-1 in the presence or absence of BQ788. a and b: Actual traces of force induced by ET-1 in the presence or absence of BQ788 (1 μ M) of the strips pretreated with Calphostin C (1 μ M; a) or DMSO (0.01%; b). After Calphostin C or DMSO were washed out, ET-1 was applied in the presence of BQ788 (1 μ M). c: Mean values of relative amplitude of ET-1-induced contraction in the presence or absence of BQ788 (1 μ M) in control (without pretreatment; open column), DMSO-pretreated (hatched column) and Calphostin C-pretreated (closed column) strips obtained from 3–6 observations. Asterisks indicate significant difference (***) $P < 0.001$. N.S., not significantly different from the control strips. Vertical bars indicate SE.

neous application of BQ123 and BQ788 (1 μ M each). These results suggest that both ET_A and ET_B receptors are involved in the ET-1-induced contraction of rabbit pulmonary artery, and that there are no ET receptor subtypes other than ET_A and ET_B in mediating the ET-1-induced contraction. These findings are consistent with recent finding observed in the same tissue (10).

Possible Role of Intracellular and Extracellular Ca²⁺ Pools in ET-1-Induced Contraction

It was reported that ET-1 increases intracellular Ca²⁺ concentration ([Ca²⁺]_i) in vascular smooth muscle cells by activating dihydropyridine-sensitive (22) or -insensitive (2, 8) Ca channels in tissues such as guinea-pig portal vein or porcine coronary artery. It has also been reported that ET-1 activates phosphatidyl inositol turnover, which in turn re-

lease Ca²⁺ from intracellular storage sites in smooth muscle cells (9, 17). In addition, recent experimental evidence suggests that ET-1 enhances Ca²⁺ influx and releases Ca²⁺ from intracellular storage sites in the same tissue (5, 6, 12).

In the present study we found that nicardipine only partially suppressed the ET-1-induced sustained contraction and removal of Ca²⁺ from extracellular solution completely abolished it, indicating that ET-1 enhances Ca²⁺ influx through dihydropyridine-sensitive and -insensitive Ca channels. Furthermore, present study indicates that activation of ET_A receptor by ET-1 opens both dihydropyridine-sensitive and -insensitive Ca channels, because contraction induced by ET-1 in the presence of BQ788 was suppressed only partially by nicardipine. We did not study the function of ET_B receptor directly for the following reason: ET-1 induced only transient but not the sustained contraction in

the presence of BQ123, and therefore it was practically not feasible to observe the effects of nicardipine on the sustained contraction induced by ET-1. It seems reasonable to assume that ET_B receptor mainly activates dihydropyridine-insensitive Ca channel, since in the presence of nicardipine, BQ788 significantly suppressed the amplitude of sustained contraction induced by ET-1, and since BQ788 did not alter significantly the extent of suppression by nicardipine on the amplitude of ET-1-induced contraction.

ET-1 releases Ca²⁺ from intracellular storage sites through activation of both ET_A and ET_B receptor subtypes, since ET-1 induced transient contractions in EGTA-containing Ca²⁺-free Krebs solution in the presence or absence of BQ123 or BQ788. However, in guinea-pig trachea, it has been reported that ET_A receptor releases Ca²⁺ from intracellular storage sites and that ET_B receptor activates Ca channels (6), indicating species and tissue specificities.

Possible Role of Protein Kinase C in ET-1-Induced Contraction

ET-1 has also been reported to activate PKC by producing diacylglycerol (DAG) through a breakdown of membrane phospholipid in smooth muscle cells of rat, bovine or rabbit aorta (4, 11). Several studies suggest that activation of PKC plays a major role in force development induced by ET-1 (12, 16); however, there are reports which suggest only partial or no contribution of PKC in generating the contraction (13, 15, 19, 23). In the present study, pretreatment with selective PKC inhibitor Calphostin C (1 µM, 120 min) abolished the enhancing action of PDBu on 128 mM K⁺-induced contraction without any effects on contraction induced by 128 mM K⁺ itself, suggesting that Calphostin C specifically and completely blocked the activity of PKC.

In the presence of BQ788, ET-1-induced contraction was completely abolished by Calphostin C, indicating that PKC plays a primary role in vasoconstriction through the activation of ET_A receptor in this tissue. We did not examine the function of ET_B receptor alone directly, since after the application of BQ123 the baseline became unstable in the Calphostin C-treated strips, the reason of which was not clear. Under the assumption that there are no ET receptor subtypes other than ET_A and ET_B, the amplitude of contraction induced by ET_B can be estimated from the difference in the amplitude

of contraction induced by ET-1 in the absence and presence of BQ788 (Fig. 5c). PKC activation is suggested to play only a partial role in the vasoconstriction through the ET_B receptor, because the estimated amplitude of contraction induced by ET_B receptor was partially but significantly suppressed by pretreatment with Calphostin C.

The different roles of ET_A and ET_B receptor subtypes observed in pulmonary artery can be summarized as follows; 1) ET_A receptor activates both dihydropyridine-sensitive and -insensitive Ca channels; on the other hand ET_B receptor mainly activates dihydropyridine-insensitive Ca channels. 2) While PKC has a primary role on contraction mediated by ET_A receptor stimulation, PKC plays only a partial role in contraction through activation of ET_B receptor. On the other hand, we have previously reported that in rabbit mesenteric artery muscle contraction induced by ET-1 is mediated by ET_A receptor subtype alone and that PKC activation contributes to only a part of force development (23). Therefore, difference between tissues may exist in the intracellular signal transduction system activated by ET_A receptor subtype stimulation.

In some pathophysiological state such as pulmonary hypertension or chronic heart failure, expression of mRNA and plasma concentration of ET-1 were reported to increase (3, 20, 21). Although plasma concentration of ET-1 is much lower than that to induce vasoconstriction, local concentration around vascular smooth muscle may reach a sufficient level to produce contraction. Thus, it is possible that this peptide may contribute to the pathogenesis of pulmonary hypertension.

In conclusion, in smooth muscle of rabbit pulmonary artery, ET-1 activates ET_A and ET_B receptor subtypes, and both receptors activate Ca²⁺ influx through the membrane and Ca²⁺ release from intracellular storage sites. Furthermore, activation of PKC is suggested to contribute to the development of ET-1-induced vasoconstriction. Especially in ET_A receptor stimulation, activation of this kinase seems necessary to the force generation.

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