ELSEVIER

European Journal of Pharmacology 324 (1997) 277–282

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Activation of BK_{Ca} channel via endothelin ET_A receptors in porcine coronary artery smooth muscle cells

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Received 29 August 1996; revised 2 December 1996; accepted 24 January 1997

Abstract

It has been demonstrated previously that endothelin-1 stimulates the Ca^{2+}-activated K^+ (BK_{Ca}) channel activity in porcine coronary artery smooth muscle cells. The purpose of the present study was to delineate the endothelin receptor subtype involved in this action. In receptor binding studies, [^{125}I]endothelin-1 was shown to bind to the homogenate of porcine primary coronary artery smooth muscle cells in a single class of binding sites with K_D and B_{max} values of 73 pM and 99 fmol/mg protein, respectively. Furthermore, endothelin-1 and endothelin-3 displaced the binding of [^{125}I]endothelin-1 to these cells with respective IC_{50} values of 70 and 17 000 pM, a 240-fold difference in potency. The effects of endothelin-3 on the activity of the BK_{Ca} channel in porcine coronary artery smooth muscle cells were examined using the cell-attached patch-clamp technique. Similar to endothelin-1, endothelin-3 also exhibited a bell-shaped concentration-response curve. A maximal increase of 95% in channel open-state probability (P_o) was induced by 100 nM endothelin-3 as compared with the 320% increase in P_o by 1 nM endothelin-1. Thus, endothelin-1 was about 100-fold more potent and 3.4-fold more efficacious than endothelin-3 in this action. Both the receptor binding and the electrophysiological results suggest that the effects of endothelins on the BK_{Ca} channel are mediated through the endothelin ET_A receptor subtype.

Keywords: K^+ channel, Ca^{2+}-activated; Endothelin-1; Endothelin-3; Endothelin ET_A receptor; Patch-clamp

1. Introduction

The endothelin family of peptides are potent vasoconstrictors originally isolated from conditioned medium of porcine aortic endothelial cells (Yanagisawa et al., 1988). To date, three human endothelin isoforms have been identified, i.e., endothelin-1, endothelin-2, and endothelin-3 (Inoue et al., 1989), and extensive pharmacological and biochemical studies have led to the classification and molecular cloning of two receptor subtypes: endothelin ET_A and ET_B (Arai et al., 1990; Sakurai et al., 1990; Masaki et al., 1992). These two receptor subtypes have shown characteristic differences in binding affinities for the endothelin isoforms. While the endothelin ET_A receptors have higher affinity for endothelin-1 and endothelin-2 than endothelin-3, the endothelin ET_B receptors are nonselective for these three peptides (Masaki et al., 1992). Furthermore, earlier investigation on the cellular localization has revealed a distinct distribution of the two receptor subtypes: the endothelin ET_A receptors are mainly found in smooth muscle cells whereas endothelial cells only express the endothelin ET_B receptor subtype (Yanagisawa and Masaki, 1989; Hosoda et al., 1991; Lin et al., 1991). Subsequent studies, however, have identified an endothelin ET_B receptor subtype in the smooth muscle cells as well (Levin, 1995; Sokolovsky, 1995).

Mediated through the endothelin ET_A and/or ET_B receptors, exogenous endothelin-1 exerts many biological effects and causes a variety of cardiovascular and renal dysfunction such as hypertension, myocardial infarction and renal failure (Rubanyi and Polokoff, 1994; Ferro and Webb, 1996). An immediate event upon endothelin-1 binding to its receptors in smooth muscle cells is the stimulation of phospholipase-C-mediated metabolism of inositol phosphates which, in turn, raises the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) through mobilization of intracellular Ca^{2+} stores by inositol (1,4,5)trisphosphate (Van

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0014-2999/97/$17.00
PII S0014-2999(97)00079-4

Renterghem et al., 1988; Araki et al., 1989; Marsden et al., 1989; Huggins et al., 1993). Endothelin-1 also affects the activity of a number of ion channels, including stimulation of a non-selective cation channel and a Ca^{2+} channel in vascular smooth muscle cells (Van Renterghem et al., 1988; Chen and Wagoner, 1991; Silberberg et al., 1989), activation of chloride currents in coronary artery smooth muscle cells (Klöckner and Isenberg, 1991), and inhibition of the ATP-sensitive K^+ currents in smooth muscle cells (Miyoshi et al., 1992). In addition, endothelin-1 has been shown to exhibit a bell-shaped dose-response curve on the stimulation of Ca^{2+}-activated K^+ (BK_{Ca}) channel in coronary artery smooth muscle cells, with a maximal activation occurred at 1 nM (Hu et al., 1991).

In this communication, we further delineated the endothelin receptor subtype involved in the action of endothelin-1 on the BK_{Ca} channel activity in porcine coronary artery smooth muscle cells. Displacement of [^{125}I]endothelin-1 binding by both endothelin-1 and endothelin-3 was performed, and the results were compared with the effects of these two peptides on the BK_{Ca} channel activities of these cells using the patch-clamp technique.

2. Materials and methods

2.1. Materials

[^{125}I]Endothelin-1 was purchased from New England Nuclear (Boston, MA, USA). Endothelin-1 and endothelin-3 were obtained from American Peptide (Sunnyvale, CA, USA). Collagenase, papain, and DL-dithiothreitol were products of Sigma (St. Louis, MO, USA).

2.2. Preparation of single porcine coronary artery smooth muscle cells

Porcine hearts were obtained from a local slaughterhouse. The left circumflex and left anterior descending coronary arteries were isolated and excess fat and connective tissue were removed. Single smooth muscle cells were prepared by enzymatic dissociation. Briefly, the vessels were cut open, and the endothelium was removed from the tissue with a cotton swab. The media intima were incubated in a saline solution (135 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 5 mM glucose and 10 mM HEPES, pH 7.3) containing 2 mg/ml collagenase, 2 mg/ml papain and 0.4 mg/ml DL-dithiothreitol for 60 min at 37°C (Hu et al., 1995). The smooth muscle cells released after treatment were used in the receptor binding as well as patch-clamp experiments.

2.3. Binding experiments

Freshly isolated smooth muscle cells from porcine coronary artery were pelleted in a table-top centrifuge and washed with 5 ml of cold phosphate-buffered saline. The cells were pelleted again, resuspended in 1 ml of 50 mM Tris buffer, pH 7.0, sonicated and homogenized. Cell debris were removed by brief centrifugation, and the supernatant was used in binding assays. Protein was determined by dye-binding (Bradford, 1976), using bovine γ-globulin as standard.

[^{125}I]Endothelin-1 binding experiments were performed according to a published protocol (Jeng et al., 1991) with minor modification. The saturation binding of [^{125}I]endothelin-1 to the homogenate of porcine coronary artery smooth muscle cells was conducted by incubating various concentrations of the radioligand with 20 μg of protein in 100 μl of binding buffer containing 2 mg/ml bovine serum albumin, 0.002% (v/v) Triton X-100 and 0.2 mg/ml NaN_3 in 50 mM Tris, pH 7.0, at 37°C for 1 h. In general, nine concentrations of the radioligand ranging from 1 pM to 1 nM were chosen. Following incubation, the mixture was spun in a microfuge centrifuge at 12 000 rpm for 30 min. An aliquot (90 μl) of the supernatant was removed, and the radioactivity was counted to determine the concentration of free ligand. The pellet was washed with 400 μl of cold binding buffer and was used to quantitate the bound ligand. Non-specific binding was measured in the presence of 1 μM endothelin-1. Specific binding was the difference between the total and non-specific binding. Scatchard plots were utilized to analyze the binding data (Scatchard, 1949).

Competition binding experiments were carried out in a manner similar to saturation binding, except that 60–70 pM [^{125}I]endothelin-1 was incubated with 20 μg of protein and various concentrations of non-radioactive endothelin-1 or endothelin-3. An IBM-compatible version of ALLFIT was used to fit data to a one-site model.

2.4. Patch-clamp experiments

Single BK_{Ca} channel currents in porcine coronary artery smooth muscle cells were recorded using cell-attached configuration of the patch-clamp technique (Hamill et al., 1981). The recording set-up has been described previously (Hu et al., 1990). Single channel recordings were first low-pass filtered at 2 kHz by a 8-pole Bessel filter (frequency Devices 902LPF) and video-taped with a Toshiba Pulse-Code Modulation data recorder (DX-900). After each experiment, the recordings were played back through a window discriminator (AI2020, Axon Instruments) at a sampling rate of 4 kHz and stored on a Compaq DeskPro/66M microcomputer for later analysis. Data acquisition and analysis were made with software p-Clamp version 6.0. The open-state probability of BK_{Ca} channels (P_o) was calculated as the total open duration at the first level divided by the total duration of the recordings, which were typically 40 s or longer for all experiments. The resistance of the patch electrodes after fire polishing was about 5 MΩ. The solutions used in both the

bath and pipette were identcal, containing 140 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.3. All experiments were performed at room temperature.

3. Results

3.1. Binding experiments

[^{125}I]Endothelin-1 binding to its receptors in porcine coronary artery smooth muscle cells was performed in order to delineate the receptor subtype involved in the action of endothelin-1 in the electrophysiological studies (Hu et al., 1991). It was found that [^{125}I]endothelin-1 bound to smooth muscle cell homogenate in a high affinity and saturable manner. The results of a representative saturation binding experiment and the Scatchard plots are shown in Fig. 1. There was one class of binding sites for [^{125}I]endothelin-1; the K_d and B_{max} values were 73 ± 3 pM and 99 ± 28 fmol/mg protein (mean $\pm$ S.E.M., $n = 3$), respectively.

Competition of [^{125}I]endothelin-1 binding to the homogenate of porcine coronary artery smooth muscle cells by endothelin-1 and endothelin-3 also revealed that there was one class of binding sites for both the non-radioactive ligands (Fig. 2). Endothelin-1 and endothelin-3 displaced [^{125}I]endothelin-1 binding with IC_{50} values of 0.07 ± 0.01 nM ($n = 4$) and 17.0 ± 3.8 nM ($n = 6$), respectively. Thus, endothelin-1 was about 240-fold more potent than endothelin-3 in binding to their receptors.

3.2. Comparison of the effects of endothelin-1 and endothelin-3 on the activity of single BK_{Ca} channel

In a previous study, it was shown that endothelin-1 at 0.1 nM caused a slight increase in both the BK_{Ca} channel

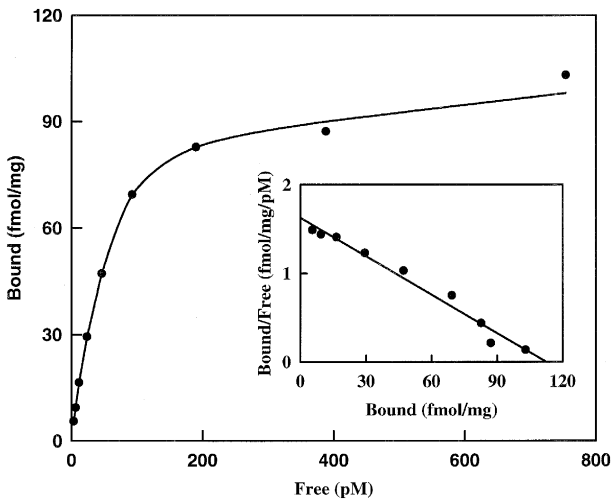

Fig. 1. Binding of [^{125}I]endothelin-1 to the membrane preparations of porcine coronary artery smooth muscle cells. Bound ligand was calculated by subtracting the non-specific binding from total binding. The inset shows Scatchard plots of the same data.

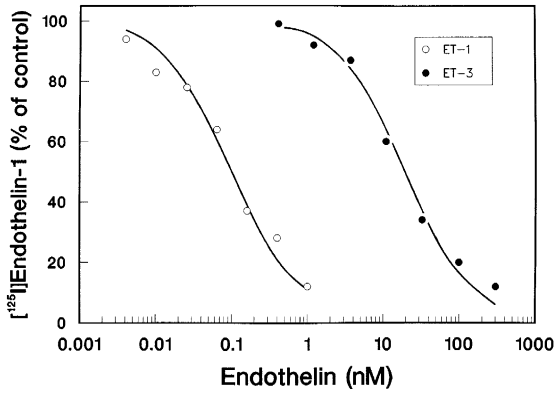

Fig. 2. Displacement of [^{125}I]endothelin-1 binding to the membrane preparations of porcine coronary artery smooth muscle cells by endothelin-1 and endothelin-3.

mean open-time and the frequency of channel opening when compared with those obtained from the same cell in the absence of the vasoconstrictor (Hu et al., 1991). These effects were more profound when the extracellular concentration of endothelin-1 was raised to 1 nM. At 10 nM endothelin-1, however, both the BK_{Ca} channel mean open-time and the frequency of channel opening were sharply decreased to values which were not significantly different from that obtained with 0.1 nM endothelin-1 (Hu et al., 1991).

The potency of endothelin-3 on the activity of BK_{Ca} channel was noticeably weaker when compared with that of endothelin-1; no significant effect was observed at 1 nM endothelin-3 (Fig. 3). Between the concentrations of 10 and 100 nM endothelin-3, both the BK_{Ca} channel mean open-time and the frequency of channel opening were markedly increased when compared with the control. Interestingly, endothelin-1 at 1 nM appeared to increase channel open frequency more preferentially than the mean open-time (Hu et al., 1991), while endothelin-3 at 10 or 100 nM increased P_o mainly by lengthening the mean open-time (Fig. 3).

The dose-response curve of endothelin-3-induced increases in the open-state probability of the BK_{Ca} channels in porcine coronary artery smooth muscle cells is shown in Fig. 4. It was shown previously that, when compared with the control cells, the calculated P_o values increased from 1.2- to 3.2-fold as the extracellular concentrations of endothelin-1 increased from 0.1 to 1 nM (Hu et al., 1991). Further increases in endothelin-1 concentrations resulted in decreased P_o values. At 100 nM endothelin-1, the P_o value was about one-half of that obtained in the control cells. Likewise, endothelin-3 also induced a bell-shaped concentration-response curve in P_o. In the presence of 10 and 100 nM endothelin-3, the calculated P_o values increased by 60% and 95%, respectively, over the control value. At concentrations greater than 100 nM endothelin-3, the P_o value decreased monotonically, but still remained

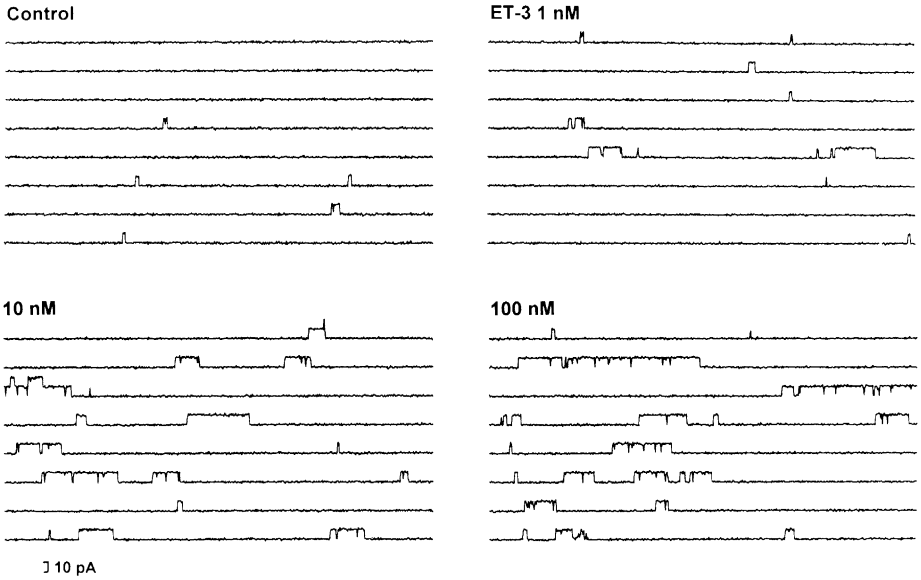

Fig. 3. Effect of endothelin-3 on the activity of single BK_{Ca} channel. The concentrations of endothelin-3 examined were 1, 10, and 100 nM. Upward deflections were outward channel openings. Each trace had a duration of 1 s. The scale of a 10 pA reference current is shown at the lower left corner of the figure. The channel recordings were made when the patch was held at +40 mV, and the data were filtered at 2 kHz.

above the control value at a concentration as high as 10 μM (Fig. 4). When compared with endothelin-1, endothelin-3 was about 100-fold less potent and 3.4-fold less efficacious in activating the BK_{Ca} channels in porcine coronary artery smooth muscle cells. The concentration required for half-maximal activation of the BK_{Ca} channels was about 100 pM for endothelin-1 and 10 nM for endothelin-3.

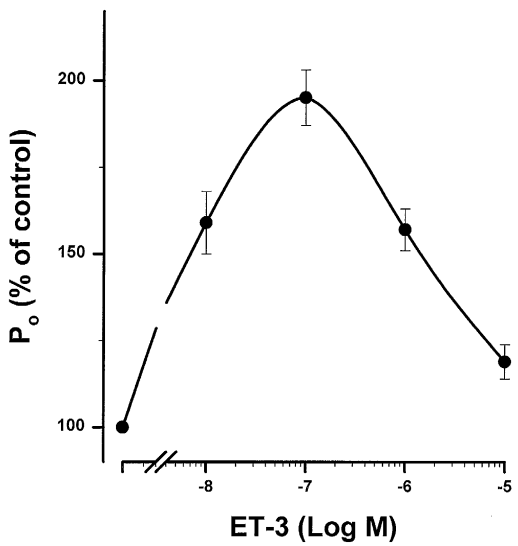

Fig. 4. Concentration–response curve of endothelin-3 on BK_{Ca} channel activity. The ordinate shows the normalized open-state probability P_o with respect to the control (100%) determined in the absence of endothelin-3. Each data point represents mean ± S.E.M. ($n = 6$).

4. Discussion

At the beginning of its discovery, endothelin-1 was identified as the most potent peptidic vasoconstrictor, about 10-fold more potent than angiotensin II in causing constriction in porcine coronary arterial strips (Yanagisawa et al., 1988). Subsequent pharmacological studies have revealed that this peptide constricts almost all of the vascular and non-vascular tissues from various species examined (Randall, 1991). Surprisingly, endothelin-1 produces dose-dependent depressor responses prior to its vasoconstriction effects in anesthetized rats (De Nucci et al., 1988). Similar results of vasorelaxation have also been documented in tissue preparations with intact endothelium, and the observed effects have been shown to be due to stimulated release of prostacyclin and endothelium-derived relaxing factor by endothelin-1 (De Nucci et al., 1988). In contrast, a direct, endothelin-1-induced relaxation in vascular tissues without endothelium has not been demonstrated. It is well established that the smooth muscle cells in these tissues contain BK_{Ca} channels (Latorre et al., 1989; Wilde and Lee, 1989; Buljubasic et al., 1992; Leblanc et al., 1994; Gollasch et al., 1996). Our results show that both endothelin-1 and endothelin-3 can stimulate the BK_{Ca} channel activity in isolated porcine coronary artery smooth muscle cells (Figs. 3 and 4; Hu et al., 1991), and activation of these channels is expected to result in cell membrane hyperpolarization and smooth muscle cell relaxation. Consistent with these findings, Hadj-Kaddour et al. (1995) have recently found that both endothelin-1 and endothelin-3 induce a dose-dependent relaxation of guinea pig trachea preparations pre-contracted with 1 μM carbachol, and this

relaxation could be attenuated by the BK_{Ca} channel blocker charybdotoxin. These results indicate that, under appropriate experimental designs, activation of the activity of BK_{Ca} channel may also be shown to contribute to the vasodilatory effects induced by the endothelin family of peptides.

The mechanism by which endothelin-1 exerts its effect on the BK_{Ca} channel activity has been investigated by Van Renterghem et al. (1988) using rat aortic smooth muscle cells from the A7r5 cell line. These authors have shown that, upon binding to its receptors, endothelin-1 stimulates phospholipase C-mediated metabolism of inositol phosphates and mobilizes intracellular Ca^{2+}, resulting in a transient increase in the BK_{Ca} currents. In our studies, the observed activation of BK_{Ca} channels by endothelin-1 and endothelin-3 in the primary cells of porcine coronary artery smooth muscle possessed different characteristics when compared with that observed in A7r5 cells. For example, the effect was more sustained in coronary artery smooth muscle cells than that in A7r5 cells (results not shown). In addition, the endothelin-1-stimulated BK_{Ca} channel activity was almost entirely abolished by dihydropyridine-sensitive Ca^{2+} antagonists such as PN 200-110 (Hu et al., 1991). These results suggest that the increased $[Ca^{2+}]_i$ through the dihydropyridine-sensitive Ca^{2+} channel may play a predominant role in the activation of the BK_{Ca} channel activity reported here, although the contribution of increased $[Ca^{2+}]_i$ due to mobilization of intracellular Ca^{2+} by inositol (1,4,5)trisphosphate cannot be ignored.

Both endothelin-1 and endothelin-3 showed bell-shaped concentration-response curves in the stimulation of BK_{Ca} channel activity in porcine coronary artery smooth muscle cells (Fig. 4; Hu et al., 1991). Although the descending part of the curves might be explained by desensitization of the endothelin receptors or the transient nature of BK_{Ca} channel activity at high concentrations of the vasoconstrictors, the precise mechanism leading to the inhibition of this channel is not clear at present. On the other hand, since endothelin-1 has been shown to stimulate the activity of phospholipase C which in turn produces diacylglycerols to activate protein kinase C (Nishizuka, 1986), the activity of the BK_{Ca} channel may be subjected to a positive or negative control by protein kinase C. Indeed, Minami et al. (1993a) have reported that activation of protein kinase C inhibits the BK_{Ca} channel of cultured porcine coronary artery smooth muscle cells. However, in a separate report these authors have demonstrated that staurosporine, a protein kinase C inhibitor, did not have significant effects on the inhibition of the BK_{Ca} channel by endothelin-1 and concluded that the inhibitory effects of endothelin-1 on these channels might be mediated through a protein kinase C-independent pathway (Minami et al., 1995). In addition to $[Ca^{2+}]_i$, an elevated level of intracellular cAMP is also capable of activating the BK_{Ca} channel in porcine coronary artery smooth muscle cells (Minami et al., 1993b). Unfortunately, the role of endothelin-1 in modulating the levels of intracellular cAMP remains controversial. While endothelin-1 increases the levels of cAMP in the rat epididymus epithelial cells and mesangial cells, it decreases the production of this second messenger in rat brain microvessel endothelial cells and rat cardiomyocytes (Huggins et al., 1993). Thus, whether the endothelin-1-induced inhibition of BK_{Ca} channel activity is via the cAMP-dependent or another yet undefined pathway awaits further investigation.

The endothelin receptor subtypes involved in mediating the effects of endothelin-1 on various types of K^+ channels appear to be different. In *Xenopus* ooctye co-transfected with cDNA encoding the delayed rectifier K^+ channel from rat heart and the cDNA encoding either endothelin ET_A or endothelin ET_B receptors, similar results of endothelin-1-induced inhibition of channel openings were obtained (Ishii et al., 1992). These results show that a non-selective endothelin ET_B receptor subtype may be involved in the action of endothelin in this system. In contrast, endothelin-3 was more potent than endothelin-1 in activating an inwardly rectifying K^+ current in the atrial cells of newborn rats (Kim, 1991), suggesting the possible involvement of a third endothelin ET_C receptor subtype (Masaki et al., 1992). In the present study, endothelin-1 and endothelin-3 displaced [^{125}I]endothelin-1 binding to porcine primary coronary artery smooth muscle cells with IC_{50} values of 70 pM and 17 nM, respectively (Fig. 2). Furthermore, both endothelin-1 and endothelin-3 showed bell-shaped concentration-response curves on the BK_{Ca} channel activity in these cells. Endothelin-1 was about 100-fold more potent than endothelin-3 in these responses; the respective concentrations for half-maximal activation were about 100 pM and 10 nM (Fig. 4; Hu et al., 1991). The close correlation between the binding affinities and the results obtained in the electrophysiological studies suggests that the effects of endothelin-1 on the BK_{Ca} channel involve the activation of the endothelin ET_A receptor subtype.

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