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Endothelin increases single-channel calcium currents in coronary arterial smooth muscle cells

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Endothelin (ET), a newly identified vasoconstrictor peptide produced by endothelial cells, depends on extracellular calcium for its action [(1988) Nature 332, 411–415]. It is not yet known whether the increase in calcium influx induced by ET results from a direct effect on the Ca^{2+} channels or is secondary to a reduction in membrane potential. To address this question, we studied the effects of ET on single-channel calcium currents of freshly dissociated porcine coronary artery smooth muscle cells using the cell-attached mode of the patch-clamp technique. We show that ET increases Ca^{2+}-channel activity with no effect on channel open time or conductance. The ability of bath-applied ET to increase single-channel calcium currents in the cell-attached mode is evidence that the peptide acts via a second messenger system.

Endothelin; Ca^{2+} channel; (Vascular smooth muscle, Coronary artery)

1. INTRODUCTION

In recent years, a number of investigators have shown that endothelial cells produce and release a stable, slow-acting vasoconstrictor substance [2–4]. One such vasoconstrictor has been identified as a 21-amino-acid peptide named endothelin (ET) [1]. ET-induced vasoconstriction depends on extracellular calcium and can be attenuated by the Ca^{2+}-channel blocker nicardipine [1]. These observations suggest that ET increases calcium influx through dihydropyridine (DHP) sensitive, high-threshold Ca^{2+} channels. This influx may result from a change in the Ca^{2+} channel properties or may reflect a reduction in the smooth muscle cells membrane potential. We have utilized the patch-clamp technique [5] to control membrane potential and to investigate the effects of ET on the Ca^{2+} channels of freshly dissociated porcine coronary artery smooth muscle cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of single smooth muscle cells*

The procedure for preparation of single smooth muscle cells was developed in collaboration with Dr Ligia Toro and will be described in detail elsewhere. In short, adult porcine hearts were obtained from a local slaughterhouse within 30 min after death. Smooth muscle cells from the proximal right coronary artery were loosened enzymatically with papain, elastase and collagenase and dispersed by gentle aspiration with a wide-bore pipette. Cells, typically 100–200 μm long and 5–10 μm in diameter, were used within 10 h of preparation.

2.2. *Arterial ring preparation*

Segments of the proximal ring coronary artery were cleaned of adhering connective tissue and the intima rubbed off in cold physiological salt solution (PSS) containing (in mM): 113.8 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 5 D-glucose. Rings (3 mm in width) were suspended between a fixed support and an isometric force transducer (F-60, Narco Bio-Systems, Houston, TX) in an organ bath containing 4 ml PSS at 37°C, gassed with 95% O_2/5% CO_2. Tension was recorded on a Brush recorder (model 220, Gould, Cleveland, OH). The vessels were equilibrated for 1 h at a passive tension of 2 g and challenged 6 times in the next 2 h with 80 mM K^+. Equilibration was achieved when two successive exposures to 80 mM K^+ resulted in the same active tension and the vessels relaxed to the same passive tension. A concentration-effect curve was constructed from recordings of the contractile

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responses to the cumulative addition of ET. The vessels were exposed to each concentration for 25 min.

2.3. *Electrophysiology*

Cells were placed in a 400 l chamber, at room temperature and depolarized to approx. 0 mV with a bath solution containing (in mM): 140 K-aspartate, 10 EGTA, 10 Hepes, pH 7.4, adjusted with *N*-methyl-D-glucamine. Single Ca^{2+} channel currents were recorded using the gigaseal patch-clamp technique in the cell-attached mode [5] with a List EPC-7 patch-clamp amplifier. Patch electrodes (2–4 MΩ) were fabricated from soft glass (Garner no.8161). Pipette solution contained (in mM): 150 NaCl, 10 EDTA, 10 Hepes, 0.001 tetrodotoxin (pH 7.4). Under these experimental conditions, the Ca^{2+} channels conduct Na^+ [6]. The patch of membrane under the pipette was voltage clamped to −100 mV and stepped to −50 or to −30 mV for 700 ms, every 3 s. The currents were stored on a videocassette tape (PCM1, Medical Systems, Greenvale, NY) for subsequent analysis. Single-channel records were low-pass filtered at 1 kHz (−3 dB) and digitized at 5 kHz using a laboratory computer (PDP 11/73 Digital Equipment, Marlboro, MA). The initial 30 ms of the depolarization step was excluded. Channel openings were detected and idealized by an algorithm which uses both amplitude and slope information or by an automated, interactive threshold detection program. Analysis was performed on a MicroVax II (Digital Equipment), using custom-made analysis software. Histograms of amplitude and open time were constructed from the idealized events. Amplitude histograms were fitted to Gaussian distributions. Open time histograms were fitted to exponential probability density functions.

Porcine ET, purchased from Peninsula Labs (Belmont, CA), was suspended, on arrival, in distilled water (at 100 μM), divided into aliquots and placed in siliconized tubes and frozen for future use.

3. RESULTS AND DISCUSSION

The sensitivity of porcine right coronary artery rings to ET was tested to determine the concentration required for the electrophysiological experiments. The concentration of ET resulting in half-maximal contraction (EC_{50}) was calculated to be 50 nM, using nonlinear regression analysis (fig.1A). Dose-dependent constriction and the response to 80 mM K^+ (K^+) are exemplified in fig.1B. The EC_{50} obtained in these experiments is two orders of magnitude larger than that reported by Yanagisawa et al. [1]. The cause of this discrepancy is unknown but may be due to differences in the preparative procedure. In this study, prior to the application of ET, the rings were conditioned by several exposures to a high potassium solution (see section 2) which has been shown to be required for priming and stabilizing pharmacological responses in the coronary artery ring preparation [7]. Alternatively, it may be due to partial degradation of the peptide [8], despite the precautions taken (see section 2).

Single-channel currents were identified as high-threshold (L-type) Ca^{2+} channel currents by a number of criteria: The voltage-dependence and kinetic characteristics of the channels were as expected for high-threshold Ca^{2+} channels in the absence of extracellular divalent ions [6]. The channels activated at approx. −60 mV, channel activity proceeded throughout the 700 ms depolarization pulse, records with openings were clustered and summed single-channel records did not show a transient component. Moreover, the channels were DHP sensitive; the DHP agonist Bay K 8644 at 50 nM increased mean open time.

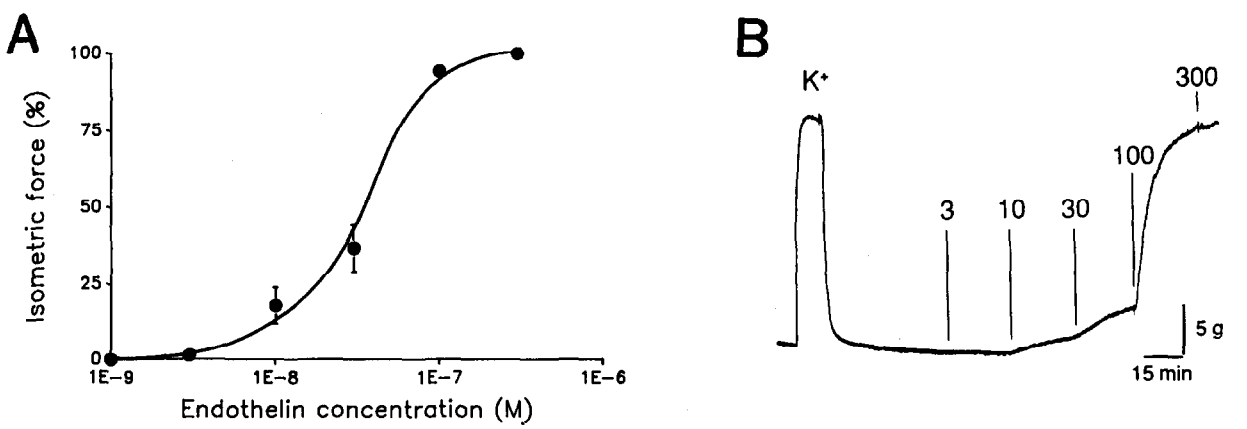

Fig.1. Dose-dependent vasoconstriction by ET of porcine right coronary artery rings. (A) Average cumulative concentration-effect curve (n = 7) expressed as percent of maximal contraction induced by 300 nM ET. Bars represent one SE. (B) Representative cumulative force recording. Horizontal lines indicate time of ET application. Numbers are ET concentration in nM. The last of the control applications of 80 mM K^+ solution (see methods) is shown for comparison (K^+).

Single Ca^{2+} channel activity was observed in 15 of 47 patches of which only 3 remained intact for more than 30 min, the time required to complete an experiment (10 min control followed by at least 20 min in ET). In these three patches, ET increased the probability of channel opening (p_o). The increase in channel activity developed with a delay of several minutes and is consistent with the gradual development of tension observed in coronary rings following exposure to ET. A diary of the number of channel openings per trace demonstrates the slow augmentation in channel activity induced by ET (fig.2A). Channel activity rose progressively throughout the experiment, which terminated with the loss of the gigaseal. Fig.2B compares the cumulative number of openings per trace during a 10 min period of control (con) to the cumulative activity, 11–21 min after ET application (endo). Individual consecutive traces prior to and 23 min following ET application are depicted in the inset to fig.2B. The increase in frequency of channel opening, 20 min after ET application, varied from 22 to 585% (table 1); a more pronounced effect was observed on the integral of current per unit time (70 to 735%), probably because missed closures do not affect this measurement. Open time and amplitude distributions were not significantly altered by ET (fig.3). Amplitude histograms were fitted by the sum of two normal distributions. A slight shift of the amplitude distribution in the experiment presented in fig.3 was not reproduced in the other experiments.

Table 1

Activation of single-channel Ca^{2+} currents by endothelin

Parameter	Expt no.	Control	Endothelin	Endothelin/ control
Number of openings per trace	1	5.5	37.7	6.85
	2	17.5	30.1	1.72
	3	12.4	15.1	1.22
Integral of current (pA/ms)	1	0.26	2.17	8.35
	2	0.72	1.62	2.25
	3	0.50	0.85	1.70

Average channel activity was calculated for 100 consecutive depolarization steps (630 ms in duration) prior to (control) and 17.5–22.5 min after ET application (endothelin). The average integral of current per trace was estimated from the idealized channel openings. Cell-attached membrane patches were stepped to −50 mV (expt 1) or to −30 mV (expts 2,3) from a holding potential of −100 mV. The large increase in channel activity at −50 mV may be due to a more pronounced effect of ET at threshold potentials

A direct effect of ET on Ca^{2+} channels has been suggested [1,9]. However, the ability of bath-applied ET to activate Ca^{2+} channels recorded in the cell-attached mode of the patch clamp technique is strong evidence that the peptide acts via a second messenger system. A specific ET receptor not in direct association with the Ca^{2+} channel could explain the lack of interaction between the recently characterized ET-binding site and nicardipine [9]. Ca^{2+} channel open probability has been shown to be regulated by a number of second messenger

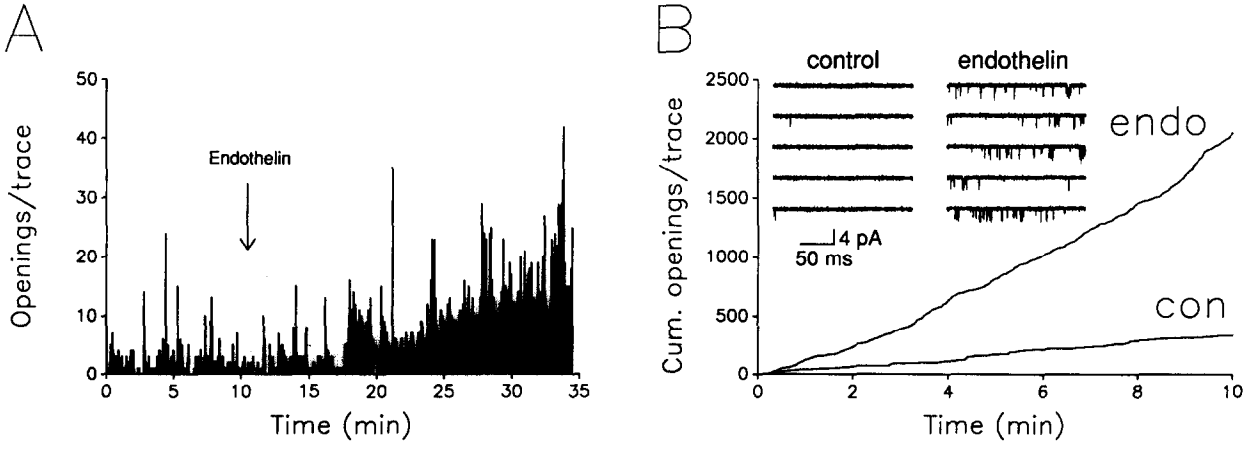

Fig.2. Increase in Ca^{2+} channel activity induced by ET (expt 1). (A) Number of channel openings per 200 ms of each depolarization step, starting 30 ms after the onset of the step. (B) Cumulative number of channel openings as a function of time.

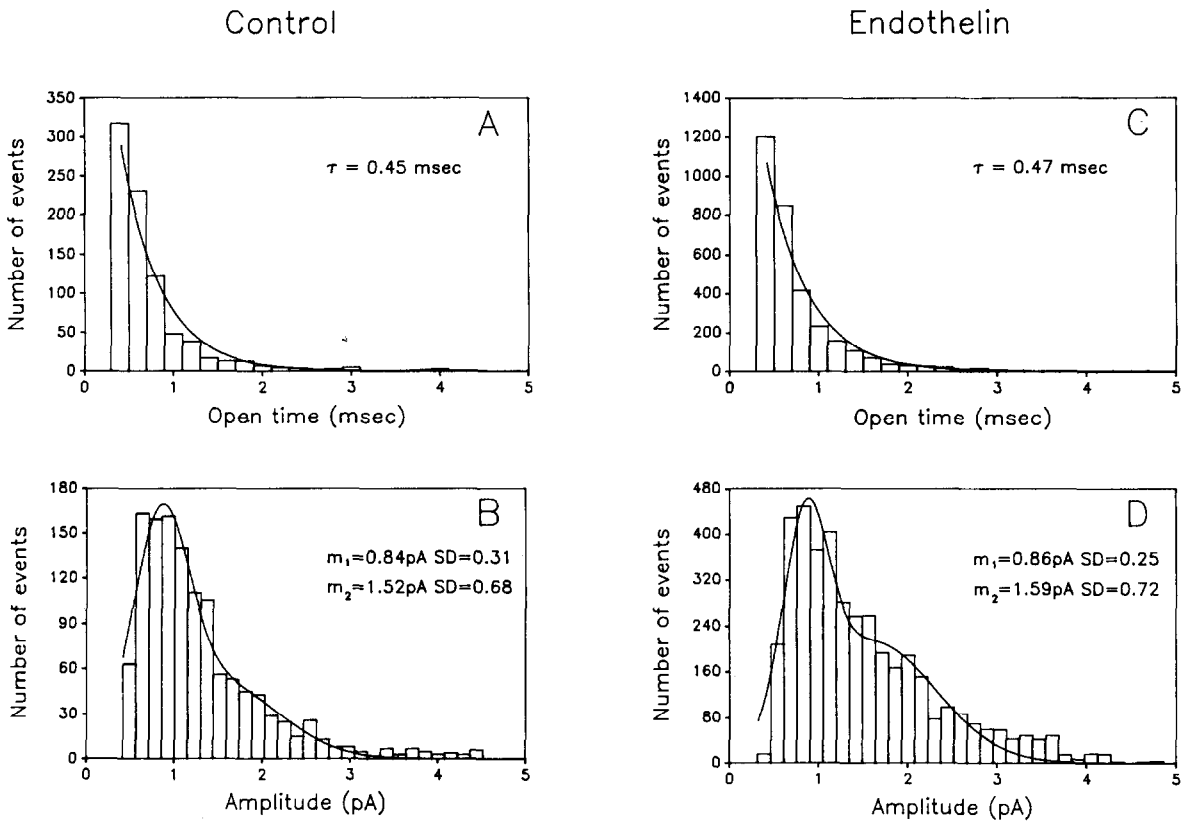

Fig.3. Open time and amplitude histograms of single-channel currents prior to (A,B) and following ET application (C,D) from the same experiment presented in fig.2. Time constants of open time distributions and estimates of the means and standard deviations of the amplitude distributions are presented in each figure. 16 (B) and 20 (D) channel openings which arose from overlapping events (larger than 5 pA) were excluded.

systems. cAMP-dependent phosphorylation increases p_o of neuronal [10], skeletal muscle [11] and cardiac muscle [12] L-type Ca^{2+} channels, which are also stimulated by activators of protein kinase C (PKC) [13]. cGMP-dependent protein kinase has been shown to enhance Ca^{2+} currents in snail neurons [14], however, a direct effect of this enzyme on Ca^{2+} channels has not yet been demonstrated. In vascular smooth muscle, cAMP is thought to mediate relaxation induced by β-adrenergic stimulation while cGMP mediates the vasodilatory effects of EDRF and nitrovasodilators. Hence, a stimulatory role for cAMP or cGMP and their kinases by activation of Ca^{2+} channels seems unlikely. In contrast, PKC has been shown to increase the L-type Ca^{2+} current of a vascular smooth muscle cell line [15] and may therefore be the as yet unidentified second messenger activated by ET.

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