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ENDOTHELIN AUGMENTS UNITARY CALCIUM CHANNEL CURRENTS ON THE SMOOTH MUSCLE CELL MEMBRANE OF GUINEA-PIG PORTAL VEIN

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SUMMARY

1. The effects of endothelin (ET) on the Ca^{2+} channel current in smooth muscle cells of the guinea-pig portal vein were investigated using the patch-clamp technique with whole-cell and cell-attached configurations.

2. ET augmented the macroscopic Ba^{2+} current in a dose-dependent manner; this effect was inhibited by nifedipine or Cd^{2+}. Augmentation of the inward current by ET did not depend on the amplitude of the depolarizing pulse. Further, when the membrane potential was held at −60 mV, ET increased the amplitude of the Ba^{2+} inward current measured at the peak and end of the depolarizing pulse to the same extent.

3. By contrast, when the membrane potential was held at −80 mV, depolarizing pulses to potentials more negative than 0 mV produced greater augmentation of the inward current than did those more positive than 0 mV. Moreover, when a depolarizing pulse to below 0 mV was applied, ET increased the peak amplitude of the inward current more than the amplitude measured at the end of pulse.

4. Using the patch-clamp technique with cell-attached configuration, two types of unitary Ba^{2+} current with conductances of 22 and 12 pS were obtained in 50 mM-Ba^{2+} solution. Nifedipine inhibited both types of unitary channel current, but the sensitivity of the 22 pS Ca^{2+}channel to nifedipine was 20-fold higher than the 12 pS Ca^{2+}channel.

5. Bath application of ET prolonged the mean open time, reduced the number of sweeps in which no Ca^{2+} channel was opened ('blank' sweep), and increased the number of channel openings evoked by each depolarizing pulse without changes of conductance. As a consequence, ET increased the open probability of both channels.

6. Augmentation of the 12 pS channels by ET was seen only in the early phase of a depolarizing pulse (57 ms from the onset of 170 ms pulse), while augmentation of the 22 pS channels was seen during the entire period of a depolarizing pulse.

7. When ET was added to the pipette solution, the activity of both Ca^{2+} channels was increased. However, this effect was less frequently observed than when ET was applied in the bath.

8. These results suggest that ET augments both the nifedipine-sensitive and

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resistant Ca^{2+} channels in the smooth muscle cell membrane of the guinea-pig portal vein, but in different ways. Presumably, ET acts indirectly on the voltage-dependent Ca^{2+} channel.

INTRODUCTION

Furchgott & Zawadzki (1980) reported that the vasodilatation induced by acetylcholine is due to a substance released from the endothelium. Since then, the role of endothelium in the regulation of vascular tone has been under extensive investigation and several endothelium-derived relaxing and contractile factors have been identified. Concerning the contractile factors, DeMey & Vanhoutte (1983) reported that anoxia augmented contractile responses induced in the canine femoral artery by noradrenaline and that this anoxic augmentation of the mechanical responses was abolished on removal of the endothelium. Furthermore, some investigators have reported that conditioned-media of cultured endothelial cells contain vasoconstrictor peptide (bovine aorta: Hickey, Rubanyi & Highsmith, 1985; rabbit aorta: Gillespie, Owasoyo, McMurtry & O'Brien, 1985; bovine pulmonary artery: O'Brien, Robbins & McMurtry, 1987).

Recently Yanagisawa, Kurihara, Kimura, Tomobe, Kobayashi, Mitsui, Yazaki, Goto & Masaki (1988) isolated an endothelium-derived vasoconstrictor peptide containing twenty-one amino acid residues, endothelin (ET), from the conditioned-media of porcine aortic endothelial cells, and showed that this peptide is one of the most potent vasoconstrictors known. They suggested that ET is an endogenous modulator of voltage-dependent Ca^{2+} channels. Silberberg, Poder & Lacerda (1989) and Goto, Kasuya, Matsuki, Takuwa, Kurihara, Ishikawa, Kimura, Yanagisawa & Masaki (1989) reported that ET activates the Ca^{2+} channel that is sensitive to Ca^{2+} antagonists. On the other hand, Auguet, Delaflotts, Chabrier, Pirotzky, Clostre & Braquet (1988) reported that ET produces contraction and that this response is insensitive to dihydropyridine Ca^{2+} antagonists. These results may suggest heterogenous sites of actions for ET. It has been reported that there are two types of Ca^{2+} channel (L- and T-types) in some smooth muscle cells (Worley, Deitmer & Nelson, 1986; Yatami, Seidel, Allen & Brown, 1987; Benham, Hess & Tsien, 1987; Yoshino, Someya, Nishio & Yabu, 1988). Therefore, it is of interest to study the action of ET on these two types of Ca^{2+} channel.

The present experiments were designed to observe the effects of ET on the two types of voltage-dependent Ca^{2+} channel of smooth muscle cells using the whole-cell and cell-attached configuration of the voltage-clamp technique. Some of the results were reported at the 62nd general meeting of the Japanese Pharmacological Society (Inoue, Nakao, Kitamura & Kuriyama, 1989*a*).

METHODS

Single-cell dispersion. Guinea-pigs of either sex was stunned and bled. The portal vein was excised, and freed from the adventitia, using fine scissors. The procedures used to disperse the smooth muscle cells were similar to those reported previously (Inoue, Kitamura & Kuriyama, 1987; Momose & Gomi, 1980). Briefly, small segments of the tissue were incubated at 35 ± 1 °C in Ca^{2+}-free PSS (physiological salt solution) containing 0·2% collagenase (clostridio peptidase A;

Boehringer-Mannheim, Mannheim, FRG). After completion of digestion, single cells were dispersed by gentle agitation with a glass pipette. The cell suspension was filtered through a fine nylon mesh, and centrifuged at 900 r.p.m. for 1 min. The cell sediment was resuspended in fresh PSS containing 0·1% trypsin inhibitor (type II S), and 0·1% bovine serum albumin (BSA, essentially fatty acid free; Sigma Chemical Co., St Louis, MO, USA), and was stored at 10 °C. All experiments were started within 1 h after cell harvest, and performed at room temperature.

Electrical recording. The dispersed cells were put into a small chamber (0·2 ml) on the stage of a microscope (TMD-Diaphoto; Nikon Co., Tokyo, Japan). The electrodes were allowed to advance towards a cell by using a three-dimensional micromanipulator (Leitz GmbH, Wetzlar, FRG). Suction pipettes, made of Pyrex glass capillary tubes, were prepared using a double stepped electrode puller (PP-83; Narishige Sci. Instrum. Lab., Tokyo, Japan), and the tips of the electrodes were heat-polished with a microforge (MF-83; Narishige Sci. Instrum. Lab., Tokyo, Japan). Whole-cell voltage-clamp recording was carried out with a path electrode (2–5 MΩ) through a patch-clamp amplifier (EPC-7; List Medical Electronics, Darmstadt, FRG) as described by Inoue, Xiong, Kitamura & Kuriyama (1989*b*). Unitary Ba^{2+} currents were recorded in the cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Electrical responses were monitored by a high-gain storage oscilloscope and thermo-writing pen recorder (VC-10 and RJG-4124; Nihon Kohden Co., Tokyo, Japan), and were stored on an FM tape-recorder (DC–2·5 kHz) (A-65; SONY-Magnescale Co., Tokyo, Japan). Stored records were displayed on a digital storage oscilloscope (4094B; Nicolet Instrum Co., Madison, WI, USA) and were then read out on an *X–Y* plotter (7440B; Hewlett–Packard Co., San Diego, CA, USA) for demonstration of the data. The data were analysed using a microcomputer (ATAC-450; Nihon Kohden Co., Tokyo, Japan). The methods for subtraction of the leak and capacitive currents were similar to those reported previously (Inoue *et al.* 1989*b*). The open probability (P) in each sweep was calculated from the total period of channel opening divided by the duration of the pulse.

Solutions. The pipette was filled with Cs^+–TEA^+ (tetraethylammonium) solution in the whole-cell voltage-clamp experiments. The ionic composition of the Cs^+–TEA^+ solution was (mM): CsCl, 120; ATP (adenosine triphosphate) disodium (Kohjin Co. Ltd, Tokyo, Japan), 5; $MgCl_2$, 4; TEA-Cl, 20; and EGTA (ethyleneglycol-bis-*N,N,N',N'*-tetraacetic acid; Dojin kagaku, Kumamoto, Japan), 4. The bath solution contained (mM): $BaCl_2$, 10; TEA-Cl, 135; glucose, 10. For recording of unitary Ba^{2+} current, the pipette was filled with 50 mM-Ba^{2+} solution ($BaCl_2$, 50: TEA-Cl, 75; glucose, 10), and high-K^+ solution was used as a bath solution (KCl, 142; glucose, 12; EGTA, 4) in order to make the membrane potential of the single smooth muscle cells near 0 mV except at the patch membrane. The pH of the solutions was adjusted to 7·2–7·3 using 10 mM-*N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES; Dojin Kagaku, Kumamoto, Japan) titrated with tris-(hydroxymethyl)ethane (Tris). The solution in the chamber was rapidly exchanged by flushing with 5–10 ml of the test solutions, and the over-flowing solution was siphoned off by a water pump. Exchange of the solution was carried out within 1 min.

Drugs. Endothelin-1 (ET) was purchased from the Peptide Institute (Osaka, Japan) and dissolved in phosphate buffer solution containing 0·05% BSA. Nifedipine was dissolved in 50% propyleneglycol. The final concentration of both drugs was obtained by dilution with bath solution or pipette solution.

Intrapipette perfusion. In some cell-attached patch-clamp experiments, the pipette solution was exchanged. A fine polyethylene tube, filled with exchange solution and connected to a reservoir, was inserted into the suction electrode. The tip of the inner tube was allowed to approach to within 100–300 μm of the tip of the suction electrode. Intrapipette perfusion was performed by gradually applying a negative pressure (10–15 mmH_2O) to the suction pipette and a hydrostatic positive pressure created by lifting the reservoir (20 cm). By using Toluidine Blue, it was confirmed that the pipette solution was exchanged in 5–10 min.

Statistics. The values recorded were expressed as mean ± S.D. Statistical significances were determined using Student's t test. Probabilities of less than 5% ($P < 0·05$) were considered significant.

RESULTS

Effects of endothelin on the voltage-dependent Ba^{2+} current recorded in the smooth muscle cells of guinea-pig portal vein

In whole-cell voltage-clamp experiments, a 10 mM-Ba^{2+} solution was used as the bath solution, because in guinea-pig portal vein, $Ba^{2+}$is more permeable than Ca^{2+}in

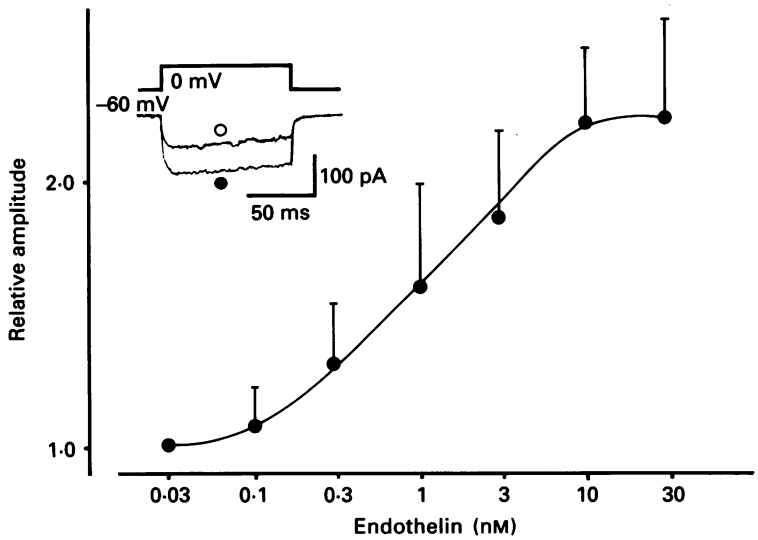

Fig. 1. The concentration–response relationship of bath-applied ET on the peak inward current evoked by a depolarizing pulse to 0 mV from the holding potential of −60 mV. The pipette was filled with Cs^+–TEA^+ solution and the bath was superfused with 10 mM-Ba^{2+} solution. The peak amplitude of the inward current was measured after 10 min perfusion of a test solution containing each concentration of ET (●), and was normalized to the control (○). Data were expressed as mean ± S.D. ($n = 5$). Inset, superimposed currents recorded before and after application of 10 nM-ET (holding potential, −60 mV; and the command pulse, 0 mV).

the Ca^{2+} channel. When the inward current was recorded by application of a depolarizing pulse to 0 mV (100 ms in duration) from the holding potential of −60 mV, addition of 10 nM-ET to the bath solution augmented the inward current (inset of Fig. 1). This augmentation occurred slowly, and to produce the maximum response more than 5 min was required. Therefore in this experiment, effects of ET were observed 10 min after application of ET. This effect of ET was sustained for over 30 min. Furthermore, it was not possible to abolish the ET-induced augmentation by washing out the bath. Figure 1 shows the concentration–response relationship for the action of ET on the inward current evoked by a depolarizing pulse to 0 mV from the holding potential of −60 mV. The current was measured at the peak amplitude. Endothelin (> 0·3 nM) augmented the inward current in a concentration-dependent manner, and 10 nM-ET produced the maximum augmentation of the inward current. The concentration to produce half of the maximal response (ED_{50}) was 0·6 nM ($n = 5$).

Figure 2*Aa* and *b* shows the current–voltage relationships in the absence and

presence of ET at the holding potentials of −60 and −80 mV, respectively. In both conditions, ET augmented the peak amplitude of Ba^{2+} inward current at any depolarizing pulse without changing the threshold (−40 mV). When the holding potential was kept at −60 mV, the maximum amplitude of current was observed by a depolarizing pulse to 0 mV both before and after application of 10 nM-ET (Fig. 2*Aa*). However, when the holding potential was kept at −80 mV, the maximum amplitude of the inward current was shifted from 0 to −10 mV by application of ET (Fig. 2*Ab*). Figure 2*B* shows the relationships between relative amplitudes of the inward current in the presence of 10 nM-ET and the amplitudes of the depolarizing pulse at the holding potential of −60 and −80 mV. When the holding potential was kept at −60 mV, the relative amplitude of the inward current was augmented almost 2-fold by ET at any given depolarizing potential. However, when the holding potential was kept at −80 mV, more enhancement of the inward current was observed in ranges of between −30 and −10 mV (about 2·5-fold) than was observed by application of depolarizing pulses more positive than 0 mV.

Figure 3 shows examples of the Ba^{2+} inward current evoked by depolarizing pulses to −20 and +20 mV (500 ms in duration) from the holding potential of −60 mV (*A*) and also of −80 mV (*B*) in the absence and presence of 10 nM-ET. The decay of the inward current observed in the absence and presence of ET was also compared by matching the amplitudes at their peak. When the holding potential was kept at −60 mV, ET (10 nM) increased the amplitude of inward current to twice that of the control but did not change the decay of the inward current evoked by depolarizing pulses (Fig. 3*A*). However, when the holding potential was kept at −80 mV, the decay was accelerated at −20 mV (Fig. 3*Ba*), but not changed at +20 mV (Fig. 3*Bb*). When the holding potential was kept at −60 mV, nifedipine (0·3 μM) or Cd^{2+} (20 μM) almost completely blocked the inward current recorded at any given depolarizing pulse both in the absence and presence of ET. The inward current was also inhibited but not completely by both drugs at the holding potential of −80 mV, and the small inward current still remained with a peak at −10 mV in the current–voltage relationship (data not shown). These findings suggest that the smooth muscle cell of the guinea-pig portal vein possessed two types of Ca^{2+} channel, and ET augmented both types of channel.

Effects of nifedipine on the unitary currents recorded from the guinea-pig portal vein

In the patch-clamp experiments with cell-attached configuration, 50 mM-Ba^{2+} solution was used as the pipette solution, and high-K^+ solution as the bath solution (see Methods). When the patch membrane was depolarized to above −30 mV from the holding potential of −80 mV, two types of unitary inward current were observed judging from differences in the amplitude (Fig. 4*Aa*). The amplitudes of both types of unitary current were increased with applications of less-negative depolarizing pulses. However, at the holding potential of −60 mV, only one type of unitary current, with large amplitude, could be seen (Fig. 4*Ab*). The channel with a large conductance could be observed even when the holding potential was more positive than −60 mV (e.g. −40 mV). The current–voltage (*I–V*) relationships of both types of channel are shown in Fig. 4*B*. Linear *I–V* relationships were obtained with a slope conductance of the unitary Ba^{2+} channel of 12 pS (the 12 pS channel) and

22 pS (the 22 pS channel). From these observations, it was confirmed that smooth muscle cells of the guinea-pig portal vein possess two types of Ca^{2+} channel, and that the 12 pS channel is inactivated at less-positive holding potentials than the 22 pS channel.

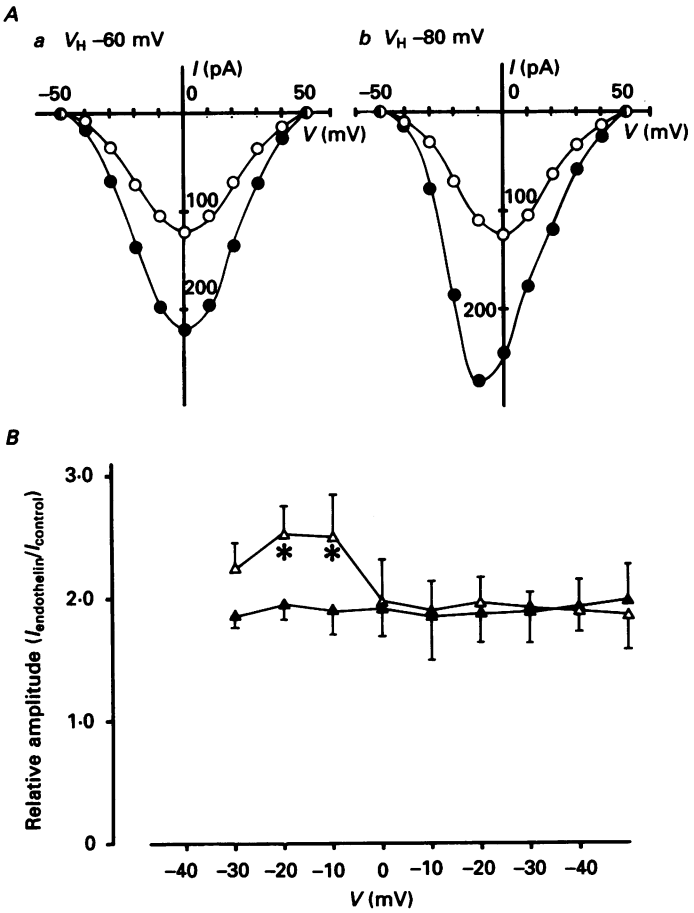

Fig. 2. The effect of ET (10 nM) on the Ba^{2+} inward current evoked in 10 mM-Ba^{2+} solution. *Aa*, the current–voltage relationship observed in the absence (○) or presence (●) of 10 nM-ET at the holding potential of −60 mV. Depolarizing pulses were applied every 30 s and pulse duration was 100 ms. The peak height of the inward current was plotted against the amplitude of the depolarizing pulse. The current–voltage relationships in the presence of ET were obtained 10 min after application of ET. *Ab*, the current–voltage relationship observed in the absence (○) and presence (●) of 10 nM-ET at the holding potential of −80 mV. *B*, the relationship between the rate of augmentation of inward current by 10 nM-ET and the amplitude of depolarizing pulse at the holding potential (V_H) of −60 (▲) or −80 mV (△) ($n = 5$). Each amplitude was normalized to control. * Indicates $P < 0{\cdot}05$.

To investigate the properties of both channels, the effects of nifedipine on the unitary Ba^{2+} channel current were observed. When a depolarizing pulse to −20 mV was applied every 10 s, from the holding potential of −80 mV (150 ms in duration) in control conditions, both types of Ca^{2+} channel could be recorded (Fig. 5*Aa*). The 12 pS channel and 22 pS channel (Fig. 5*Aa*) were observed in fifty-one sweeps and forty-three sweeps out of total number of seventy-three depolarizing pulses. Bath

application of 0·3 μM-nifedipine almost completely inhibited the 22 pS channel (it was observed in only two out of eighty-two sweeps). On the other hand, the 12 pS channel was still observed in the presence of 0·3 μM-nifedipine (Fig. 5*Ab*); in this patch membrane, the 12 pS channel was seen in fifty-one out of a total of seventy-

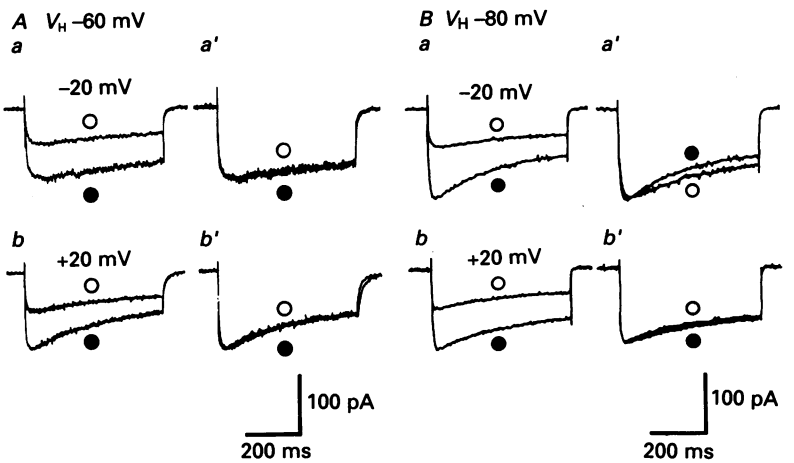

Fig. 3. Traces of inward currents evoked by a depolarizing pulse to −20 and +20 mV, from the holding potential of −60 mV (*A*) and −80 mV (*B*). Duration of the pulse was 500 ms. Traces recorded in the absence (○) and presence (●) of 10 nM-ET are superimposed (*a* and *b*). Traces in *a'* and *b'* were obtained by matching the peak of the inward current in the absence of ET to that in the presence of ET. The data of *A* and *B* were obtained from different cells.

three sweeps under control conditions, and out of a total of eighty-two sweeps in the presence of 0·3 μM-nifedipine in the bath. Figure 5*B* shows the relationships between the concentration of nifedipine and relative value of the averaged open probability of both types of Ca^{2+}channels. The mean open probability per sweep was obtained by the division of total open time of each channel by the total period of the depolarizing pulse (150 ms × number of depolarizing pulse), and that observed in the absence of nifedipine was registered as 1·0. The 22 pS channel was inhibited to half the control during application of 0·03 μM-nifedipine, and almost completely inhibited during application of 0·1 μM-nifedipine (Fig. 5*B*). By contrast, the above concentrations of nifedipine (0·03–0·1 μM) did not inhibit the 12 pS channel (Fig. 5*B*). While a higher concentration of nifedipine (> 0·3 μM) inhibited the 12 pS channel, and 3 μM-nifedipine blocked it completely. The IC_{50} values observed from these relationships were 50 pM for the 22 pS channel and 1 μM for the 12 pS channel.

Effects of endothelin on the unitary currents of the 22 pS channel recorded from the smooth muscle cells of guinea-pig portal vein

Figure 6*A* shows the change in the open probability of the 22 pS channel recorded from smooth muscle cells of the guinea-pig portal vein by application of successive depolarizing pulses. In this cell, 53 out of 101 depolarizing pulses evoked unitary currents but most of the sweeps showed open probabilities of less than 0·1. The mean open probability of the unitary current of the 22 pS channel calculated from the total sweeps was 0·04, and when this value was calculated only from the sweeps in which

the channel was opened ('non-blank' sweeps), it increased to 0·06. Upon addition of ET (30 nM) to the bath, the open probability of individual sweeps was increased and the number of 'blank' sweeps was decreased. Thus, to obtain fifty-three 'non-blank' sweeps, eighty-one sweeps were required. The mean open probability of the 22 pS

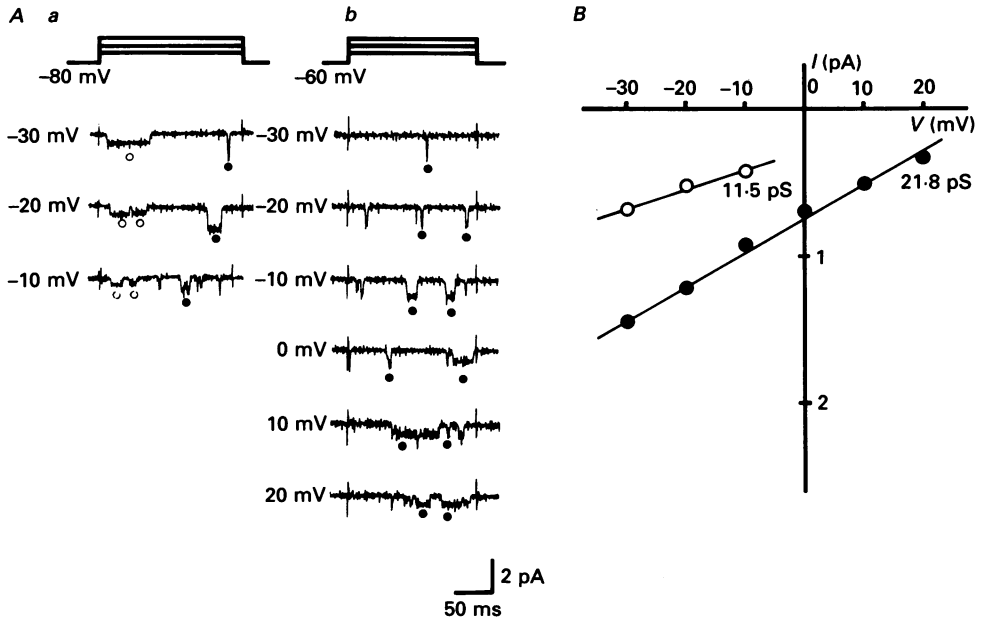

Fig. 4. *A*, The unitary Ba^{2+} currents observed at the holding potential of −80 mV (*A a*) and −60 mV (*A b*). The pipette was filled with 50 mM-Ba^{2+} solution, and the bath was superfused with a high-K^+ solution. Depolarizing pulses of 150 ms in duration to various membrane potentials were applied. Open and closed circles indicate typical channel openings of the 12 pS channel and 22 pS channel, respectively. The data of *A a* and *A b* were obtained from the same cell. *B*, the current–voltage relationship in 50 mM-Ba^{2+} solution of two types of unitary Ba^{2+} channel current, the unitary slope conductances being 11·5 pS (○) and 21·8 pS (●).

channel calculated from total sweeps in the presence of ET was increased to 0·11 (control, 0·04), and the mean open probability calculated from only 'non-blank' sweeps was increased to 0·16 (control, 0·06). The amplitude of the 22 pS channel was not changed in the presence and absence of ET (0·67 *vs.* 0·68 pA). Similar results were obtained from two other membrane patches; the mean open probabilities in the absence and presence of ET calculated from total sweeps were $0{\cdot}04 \pm 0{\cdot}01$ (control) and $0{\cdot}12 \pm 0{\cdot}04$ (30 nM-ET), respectively ($n = 3, P < 0{\cdot}05$), while the values calculated from 'non-blank' sweeps only in the absence and presence of ET were $0{\cdot}07 \pm 0{\cdot}03$ (control) and $0{\cdot}19 \pm 0{\cdot}05$ (30 nM-ET), respectively ($n = P < 0{\cdot}05$). The percentage of 'blank' sweeps amongst total sweeps decreased from 46·5 to 34·6% in this cell, and the averages of the percentage of 'blank' sweeps in the absence and presence of ET were $48{\cdot}7 \pm 3{\cdot}5$ and $34{\cdot}6 \pm 5{\cdot}5$%, respectively ($n = 3$, $P < 0{\cdot}05$). On the other hand, the number of channel openings per single 'non-blank' sweep was not changed by 30 nM-ET (in the absence of ET, 4·1; in the presence of ET, 5·3) and the averages of

this value in the absence and presence of ET were $3{\cdot}9 \pm 0{\cdot}3$ and $4{\cdot}3 \pm 0{\cdot}7$, respectively ($n = 3$). The bottom traces in Fig. 6*Ab* and *Bb* show the summated unitary current of the 22 pS channel in the absence and presence of 30 nM-ET (fifty-three 'non-blank' sweeps). In the control the summated unitary current of the 22 pS channel did not show decay, and ET increased the amplitude of summated current about 2-fold without changing decay.

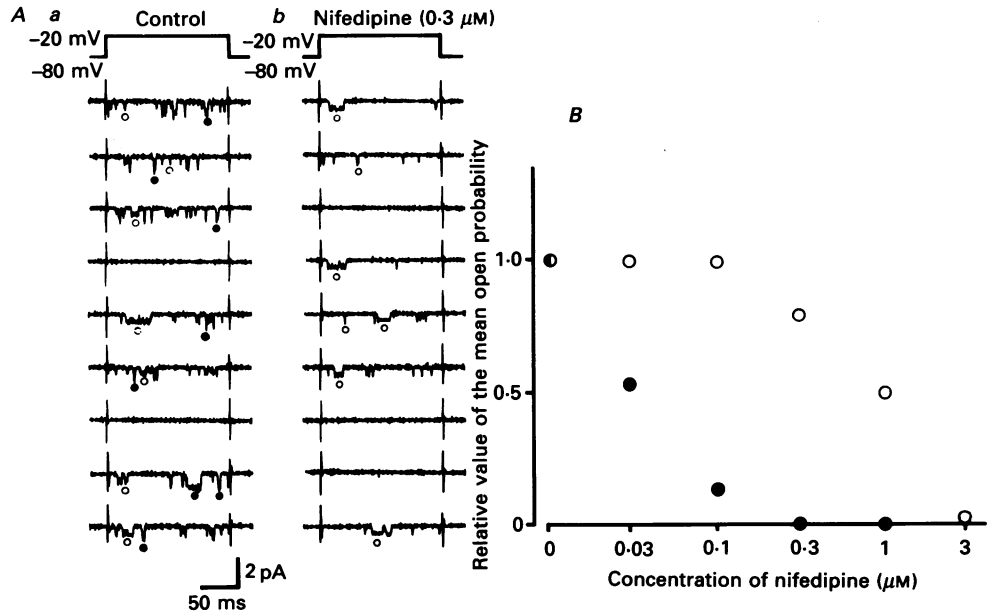

Fig. 5. Effect of nifedipine on the activity of two types of unitary Ba^{2+} channel current. *A*, the unitary Ba^{2+} current evoked by a depolarizing pulse (duration 150 ms) to −20 mV from the holding potential of −80 mV in the absence (*Aa*) and presence (*Ab*) of 0·3 μM-nifedipine. Depolarizing pulses were applied every 10 s, and sequential traces were recorded. The data were obtained from the same cell. Open and closed circles indicate the typical channel openings of the 12 and 22 pS channel, respectively. *B*, the relationship between the open probability of two types of unitary Ba^{2+} channel and the concentration of nifedipine. The open probability at each concentration of nifedipine was calculated from the total sweeps (see text). Data were obtained only from the cells which showed activity of both types of unitary Ba^{2+} channel during a depolarizing pulse of −20 mV at the holding potential of −80 mV in the control condition ($n = 3$–5).

Figure 7 shows histograms of the open and closed times of the 22 pS channel in the absence (*A*) and presence (*B*) of 30 nM-ET. The histograms of open time could be fitted with single exponential curves, and ET prolonged the time constant of the open time from 5·1 to 9·3 ms. The mean open times in the absence and presence of ET were $4{\cdot}6 \pm 1{\cdot}5$ and $9{\cdot}3 \pm 2{\cdot}3$ ms, respectively ($n = 3$, $P < 0{\cdot}05$). On the other hand, the closed-time histograms could be fitted with double exponential curves. ET slightly shortened the time constant of the slow component from 28·5 to 23·4 ms, but the fast component was not modified (control, 4·4 ms; 30 nM-ET, 4·3 ms; Fig. 7*Ab* and *Bb*). Averages of the time constants of the fast and slow components of the closed time in the absence and presence of ET were not significantly modified (slow

component: control, 29·8 ± 1·2 ms; 30 nM-ET, 25·8 ± 3·9 ms; fast component: control; 4·0 ± 0·8 ms; 30 nM-ET; 3·9 ± 0·9 ms; $n = 3$).

Effects of endothelin on the unitary current of the 12 pS channel recorded from the smooth muscle cells of guinea-pig portal vein

Figure 8*Aa* shows the change in the open probability of the 12 pS channel of individual depolarizing pulses. These data were obtained from a cell which showed

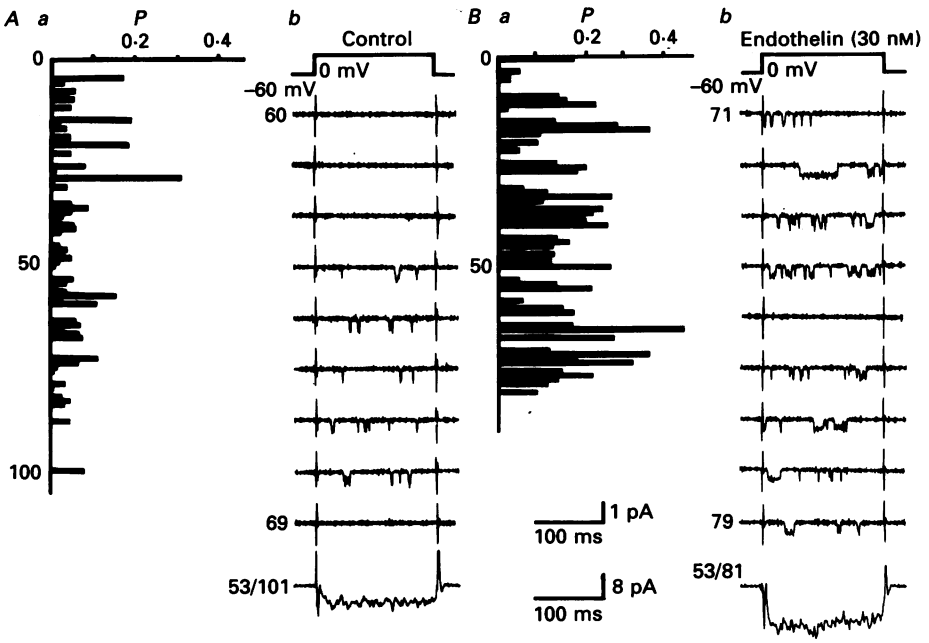

Fig. 6. Effects of ET on the unitary 22 pS channel current evoked by depolarizing pulses of 170 ms in duration from the holding potential of −60 mV. *A*, control; *B*, 10 min after application of 30 nM-ET in the bath. *a*, changes in the open probability (*P*) on sequential current sweeps. *b*, samples of sequential sweeps (60th to 69th in control, and 71st to 79th in the presence of ET) of the unitary Ba^{2+} current in the absence and presence of ET. Bottom traces shows the summated Ba^{2+} currents of 101 sweeps (*A*) and 81 sweeps (*B*); 53/101 and 53/81 indicate the number of sweeps in which unitary Ba^{2+} channel currents appeared. *A* and *B* were obtained from the same cell.

only a few openings of the 22 pS channel. The mean open probabilities of the 12 pS channel in this cell calculated from total sweeps and from 'non-blank' sweeps only in the absence of ET were 0·04 and 0·07, respectively, and in the presence of 30 nM-ET they were 0·07 and 0·13, respectively. The mean open probabilities calculated from total sweeps were 0·04 ± 0·03 (control) and 0·09 ± 0·03 (30 nM-ET; $n = 3$, $P < 0·05$), respectively, and the averages from 'non-blank' sweeps only were 0·08 ± 0·01 (control) and 0·15 ± 0·03 (30 nM-ET; $n = 3$, $P < 0·05$), respectively. ET reduced the percentage of 'blank' sweeps in total sweeps from 53·2 ± 5·5 to 40·2 ± 5·3% ($n = 3, P < 0·05$). The amplitude of the 12 pS channel was not changed in the presence and absence of ET (0·51 *vs.* 0·50 pA). The bottom sweeps in Fig. 8*Aa* and *Ba* show the summated unitary currents of fifty-two and fifty-four 'non-blank' sweeps in the absence and presence of ET, respectively. As can be seen, ET augmented the

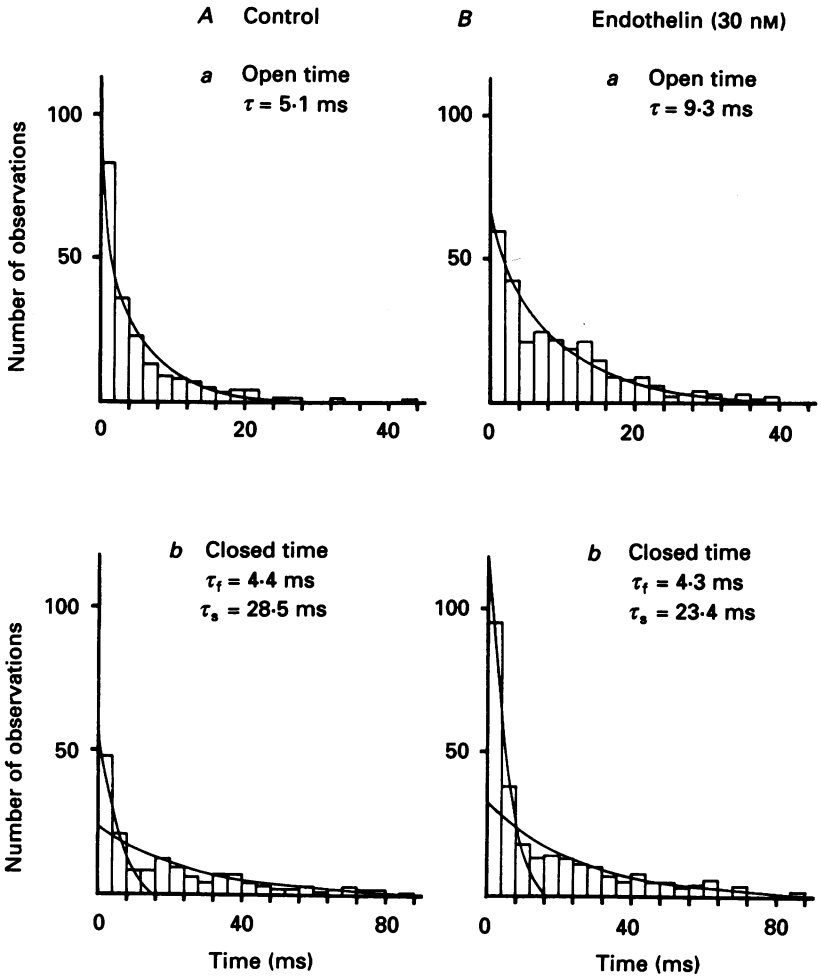

Fig. 7. Effects of ET (30 nM) on the open and closed time of the unitary 22 pS channel. Data were obtained from Fig. 6. Curves in the open-time histograms were drawn by fitting data to the following equation using a non-linear least-squares method: $n(t) = a \exp(-t/\tau)$, where $n(t)$, a and τ are the number of observations of channel opening at t ms, maximum number of observations, and the time constant. Closed-time histograms were fitted with double exponential curves: $n(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(t/\tau_2)$. Subscripts 1 and 2 indicate the 1st and 2nd components of maximum number of observations (a_1 and a_2) and the time constants (τ_1 and τ_2), respectively.

summated current at peak to about 4 times control, and then the summated current decayed. At the end of the pulse (170 ms) there was no enhancement of the amplitude of the summated current by ET. Therefore, the decay of the summated current in the presence of ET was steeper than that observed in the control. This finding was consistent with the acceleration of the current decay induced by ET in the whole-cell experiments (Fig. 3).

Figure 9 shows histograms of the open and closed times of the 12 pS channel

obtained in the absence and presence of 30 nM-ET. The histograms of open time were fitted with single exponential curves with mean open times of 3·1 ms (control) and 6·5 ms (30 nM-ET), respectively. The average time constants of the open time in the absence and presence of 30 nM-ET were 3·2±0·2 and 5·8±0·5 ms, respectively

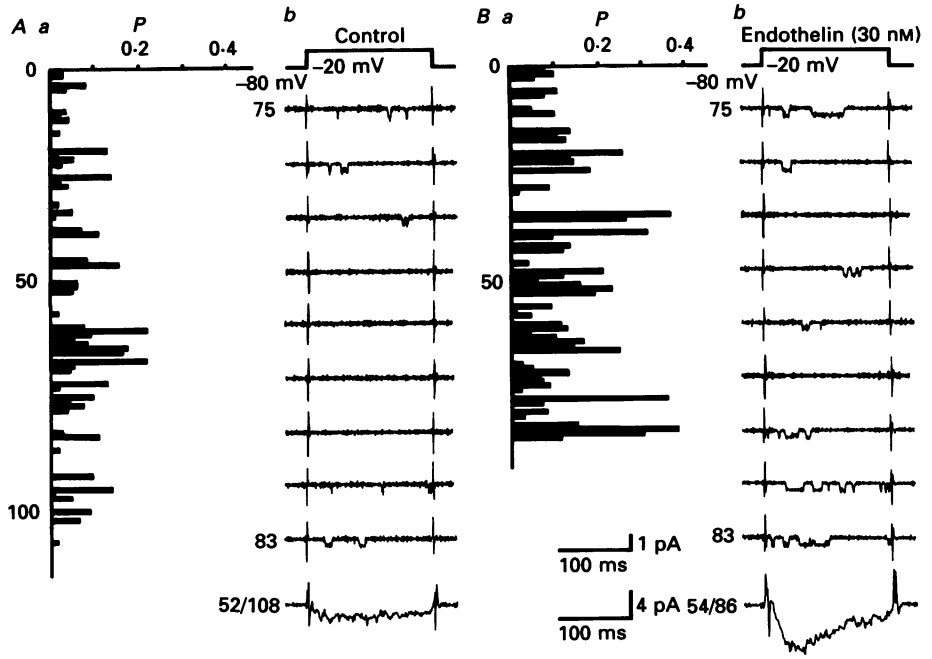

Fig. 8. Effects of ET on the unitary 12 pS channel current evoked by depolarizing pulses to −20 mV of 170 ms in duration from the holding potential of −80 mV. *A*, control; *B*, in the presence of 30 nM-ET. *a*, changes in the open probability of the unitary channel on sequential current sweeps. *b*, samples of sequential sweeps of the unitary 12 pS channel current (75th to 83rd). Bottom traces show the summated unitary current of 108 (control) and 86 (in the presence of ET) sweeps, the meaning of 52/108 and 54/86 being the same as in Fig. 6. Data were obtained from a cell which showed only a few openings of the 22 pS channel. *A* and *B* were obtained from the same cell.

($n = 3, P < 0·01$). Histograms of the time constants of closed time could be fitted with double exponential curves. The time constant of the slow component of the closed time was shortened by ET from 26·2 to 19·6 ms in this cell, and the mean values in the absence and presence of ET were 25·4±0·6 and 21·0±1·5 ms ($n = 3$, $P < 0·05$), respectively. By contrast, the fast component was not modified by ET (control, 4·3±1·3 ms; 30 nM-ET, 4·7±2·0 ms; $n = 3$).

As shown in the bottom sweeps of Fig. 8*Ab* and *Bb*, ET augmented the peak amplitude of the summated unitary current of the 12 pS channel. As the actions of ET on the 12 pS channel differed at the peak and end of the depolarizing pulse, we compared the effect of ET on the unitary current obtained during the early (the first one-third; 57 ms in duration) and late (the last one-third; 57 ms in duration) periods of 'non-blank' sweeps (pulse duration of 170 ms). The mean open time obtained in the early period in the absence of ET was 3·2±0·4 ms ($n = 3$) and in the late period was 3·3±0·5 ms ($n = 3$). The values of open probability and number of channel opening

per each 'non-blank' sweep were not significantly different in the early and late periods in the absence of ET. While 30 nM-ET increased the mean open time obtained in the early period (from $3{\cdot}2 \pm 0{\cdot}4$ to $6{\cdot}3 \pm 0{\cdot}8$ ms; $n = 3$; $P < 0{\cdot}01$), it did not change the value obtained at the late period (from $3{\cdot}3 \pm 0{\cdot}5$ to $3{\cdot}2 \pm 0{\cdot}4$ ms; $n = 3$). ET also

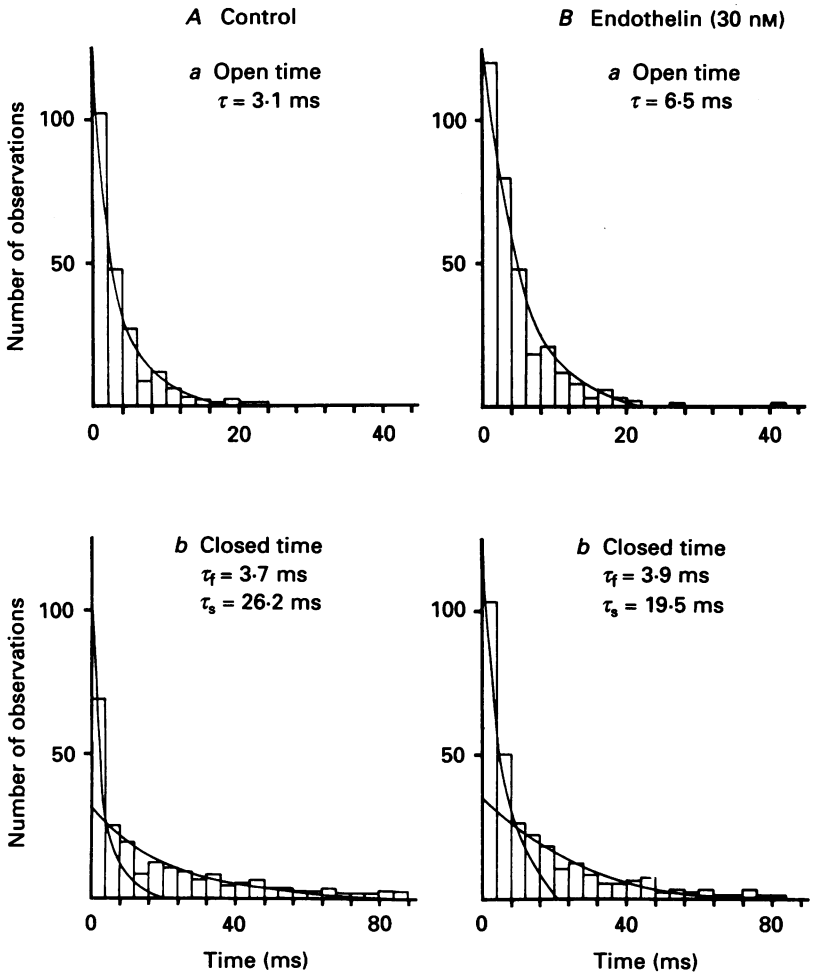

Fig. 9. Effects of ET (30 nM) on the open and closed time of the 12 pS channel. Data were obtained from Fig. 8. Curves in the histogram were fitted using the equations described in Fig. 7. *Aa* and *Ba*, open-time histograms in the absence and presence of ET, respectively. *Ab* and *Bb*, closed-time histograms in the absence and presence of ET, respectively.

increased the open probability in the early period (control, $0{\cdot}07 \pm 0{\cdot}01$; 30 nM-ET, $0{\cdot}25 \pm 0{\cdot}08$; $n = 3$; $P < 0{\cdot}05$) but produced no change during the late period (control, $0{\cdot}06 \pm 0{\cdot}01$; 30 nM-ET, $0{\cdot}07 \pm 0{\cdot}02$; $n = 3$). Although the number of channels openings per 'non-blank' sweep was not significantly increased by ET (control, $3{\cdot}3 \pm 0{\cdot}4$; 30 nM-ET, $4{\cdot}2 \pm 0{\cdot}6$; $n = 3$), the number of channel openings per 'non-blank' sweep in the early period was increased by ET from $1{\cdot}3 \pm 0{\cdot}3$ to $2{\cdot}2 \pm 0{\cdot}4$ ($n = 3$; $P < 0{\cdot}05$). On the other hand, those obtained in the late period were not changed (control,

1·0±0·2; 30 nM-ET, 1·0±0·2; $n = 3$). ET did not change the time between the last closure of the channel and the end of the depolarizing pulse (control, 44·2±4·3 ms; 30 nM-ET, 49·4±5·3 ms; $n = 3$). These results are summarized in Table 1. Thus, ET augmented the 12 pS channel, especially at the beginning of the depolarizing pulse, but this effect disappeared during application of a depolarizing pulse (170 ms in duration).

TABLE 1. The effect of ET on the mean open time, open probability and number of channels open per sweep in the first and the last one-third of the depolarizing pulse (57 ms in duration for each during 170 ms pulse), and the time between the last closure of the channel and the end of the pulse

	Control	Endothelin (30 nM)
First one-third of the depolarizing pulse		
Mean open time	3·2±0·4	6·3±0·8**
Average open probability	0·07±0·01	0·25±0·08*
Number of openings/sweep	1·3±0·3	2·2±0·4*
Last one-third of the depolarizing pulse		
Mean open time	3·3±0·5	3·2±0·4
Average open probability	0·06±0·01	0·07±0·02
Number of openings/sweep	1·0±0·2	1·0±0·2
Time between the last closure of the channel and the end of depolarization	44·2±4·3	49·4±5·3

The open probability was calculated from total duration of channel opening divided by the duration of each part of the pulse (57 ms). Number of openings/sweep means the average of the number of channel openings in each one-third part (57 ms) per single sweep ($n = 3$). * and ** indicate significant difference from the control ($P < 0·05$ and $P < 0·01$, respectively).

Effects of endothelin on the unitary currents when applied via the pipette

The effects of ET (30 nM) on both types of unitary current were also observed by addition of ET via the pipette, using the intrapipette perfusion technique. As shown in Fig. 10, 30 nM-ET in the pipette augmented the activity of both types of Ca^{2+} channel; however, these effects of ET in the pipette were observed less frequently than those observed by bath application of ET. For the 22 pS channel, the effects of ET were observed in three out of four cells when it was applied in the bath, but only in two out of five cells when it was applied via the pipette. For the 12 pS channel, augmentation of channel activity induced by ET was seen in three out of four cells, and in three out of seven cells during bath and pipette application, respectively.

DISCUSSION

Properties of two types of Ca^{2+} channels in smooth muscle cells of the guinea-pig portal vein

The existence of two types of Ca^{2+} channel in smooth muscle cells has been reported in some tissues (rabbit mesenteric artery: Worley *et al.* 1986; dog saphenous

vein: Yatani *et al.* 1987; rabbit ear artery: Benham *et al.* 1987; guinea-pig taenia coli: Yoshino *et al.* 1988). On the other hand, only one type of Ca^{2+} channel was reported in some smooth muscle tissues (amphibian stomach and guinea-pig thoracic aorta: Caffrey, Josephson & Brown, 1986; rabbit ileum: Inoue *et al.* 1989*b*). In smooth

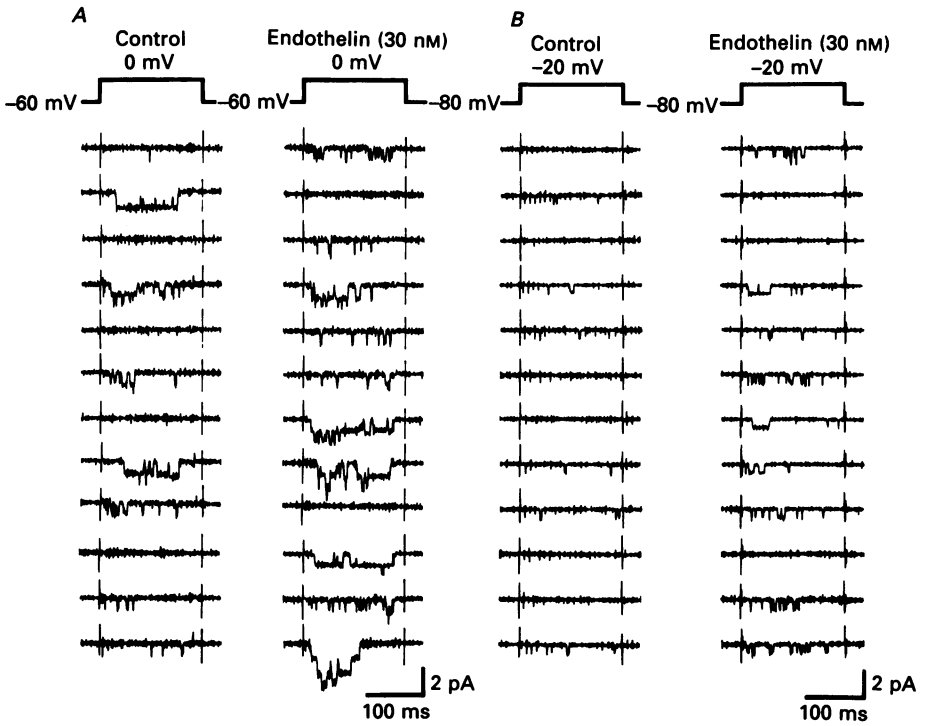

Fig. 10. Effects of ET (30 nM) applied in the pipette on both types of unitary Ba^{2+} currents. *A*, traces of the unitary 22 pS channel current evoked by the depolarizing pulse (170 ms in duration) to 0 mV from the holding potential of −60 mV in the absence (*a*) and presence (*b*) of ET. *B*, traces of the unitary 12 pS channel current evoked by a depolarizing pulse (170 ms in duration) to −20 mV from the holding potential of −80 mV in the absence (*a*) and presence (*b*) of 30 nM-ET.

muscle cells of the guinea-pig portal vein, we observed two types of Ca^{2+} channel, although these were difficult to recognize from the whole-cell voltage-clamp experiments, i.e. (1) there was no hump on the current–voltage relation curve in the range of the less-negative membrane potentials, (2) there was no shift of the threshold potential to produce inward current when the holding potential was changed, (3) there was no significant difference in the decay of inward currents recorded at two different holding potentials, and (4) nifedipine, at higher concentration ($> 3\ \mu$M), inhibited the inward current completely. However, the 22 pS channel persisted at more positive holding potentials (−40 to −60 mV), and was activated by a more positive depolarizing pulse than the 12 pS channel; it was also inactivated slowly and was highly sensitive to nifedipine. Judging from these properties, the 22 pS channel is the same as the L-type Ca^{2+} channel observed in cardiac cells and neurons (Nillius, Hess, Lansman & Tsien, 1985; Fox, Nowcky & Tsien, 1987). On the other hand, the 12 pS channel was activated by a less-positive

depolarizing pulse, and was inactivated at the holding potential of −60 mV. These properties of the 12 pS channel are similar to those reported as the T-type Ca^{2+} channel (Behnam *et al.* 1987; Yatani *et al.* 1987). However, the characteristics of the 12 pS channel in the guinea-pig portal vein were somewhat different from those of the T-type Ca^{2+} channel, namely: (1) The summated current of the 12 pS channel did not show the obvious rapid inactivation under the control condition. (2) Nifedipine inhibited the 12 pS channel 20 times less potently than the 22 pS channel. By contrast, the T-type Ca^{2+} channel of the smooth muscle membrane has been reported to be inactivated rapidly and to be insensitive to 1,4-dihydropyridine derivatives (Benham *et al.* 1987; Yatani *et al.* 1987). Wörley *et al.* (1986) reported that two types of Ca^{2+} channels with different conductances existed in the rabbit mesenteric artery, and that both types of channels were sensitive to nisoldipine. However, most investigations of the sensitivity of T-type Ca^{2+} channels to dihydropyridine derivatives were done with the application of a single concentration. Therefore, it is difficult to compare the sensitivity of the 12 pS channel in the guinea-pig portal vein to dihydropyridine derivatives with that of other T-type Ca^{2+} channels. (3) In the chick sensory neuron, Fox *et al.* (1987) reported that the mean open times of T-type and L-type Ca^{2+} channels were 0·53 and 1·19 ms using 110 mM-Ba^{2+} as a charge carrier. Carbone & Lux (1984) also obtained open time constants of 0·72 and 4·67 ms for the small and large Ca^{2+} channel, respectively, of chick dorsal root ganglion using 100 mM-Cs^{+} as a charge carrier. The mean open times of 12 and 22 pS channels in the guinea-pig portal vein were $3{\cdot}2 \pm 0{\cdot}2$ and $4{\cdot}6 \pm 1{\cdot}5$ ms, respectively, and the averages of the fast components of the closed time constants of the 12 and 22 pS channels were $4{\cdot}3 \pm 1{\cdot}3$ and $4{\cdot}0 \pm 0{\cdot}8$ ms, and of the slow components were $25{\cdot}4 \pm 0{\cdot}6$ and $29{\cdot}8 \pm 1{\cdot}2$ ms, respectively. These values were roughly the same in both channels. Thus the two channels could not be distinguished by differences in kinetics. It is difficult to compare these values obtained from other tissue of different species using different experimental conditions. However, the 12 pS channel of the guinea-pig portal vein obtained in the present experiment may have different features from the T-type Ca^{2+} channel previously reported.

In the present study the values of the unitary channel conductances of 22 and 12 pS (50 mM-Ba^{2+} as the pipette solution) were similar to those reported in guinea-pig taenia coli (25 and 12 pS: Yoshino *et al.* 1988), but larger than those reported in the mesenteric arteries (15 and 8 pS; Worley *et al.* 1986). In the dog saphenous vein and rabbit ileum, nifedipine-sensitive unitary currents were reported with similar conductance values to these found in the present experiments (Yatani *et al.* 1987; Inoue *et al.* 1989*b*). However, in rabbit ileal smooth muscle cells, a lack of T-type Ca^{2+} channels was reported (Inoue *et al.* 1989*b*) and in the dog saphenous vein, the unitary current conductance of the T-type Ca^{2+} channel was smaller than that of the guinea-pig portal vein (8 *vs.* 12 pS). These findings also suggested the heterogeneity of the Ca^{2+} channel distribution in smooth muscle cells among species and tissues.

Effects of endothelin on unitary currents in smooth muscle cells of the guinea-pig portal vein

It has been reported that ET has a variety of actions: for example, an inotropic action on the cardiac myocyte, inhibitory effects on renin release, augmentation of

the release of atrial natriuretic peptide, and stimulation of deoxyribonucleic acid (DNA) synthesis (Ishikawa, Yanagisawa, Kimura, Goto & Masaki, 1988; Rakugi, Nakamura, Saito, Higaki & Ogihara, 1988; Takagi, Matsuoka, Atarashi & Yagi, 1988; Fukuda, Hirata, Yoshimi, Kojima, Kobayashi Yanagisawa & Masaki, 1988: Nakaki, Nakayama, Yamamoto & Kato, 1989). All of these actions depended on the extracellular Ca^{2+} concentration, and were inhibited by Ca^{2+} antagonists. These results support the hypothesis that ET activates the voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels. On the other hand, it was reported that the main site of action of ET in aorta is on the intracellular store of Ca^{2+} (Auguet *et al.* 1988; Miasiro, Yamamoto, Kanaide & Nakamura, 1988; Van Renterghem, Vigne, Barhanin, Schmid-Alliana, Frelin & Lazdunski, 1988; Resink, Scott-Burden & Buhler, 1988; Kai, Kanaide & Nakamura, 1989; Marsden, Danthuluri, Brenner, Ballermann & Brock, 1989; Sugiura, Inagami, Hara & Johns, 1989). In the present study, ET augmented the Ba^{2+} inward current due to activation of both types of Ca^{2+} channel by increasing the open probability without changing the amplitude of the unitary current conductance. The action of ET on the 22 pS channel was concluded to be due to prolongation of the open time (by about 2-fold) and to reduction of the 'blank' sweeps from $48{\cdot}7 \pm 3{\cdot}5$ to $34{\cdot}6 \pm 5{\cdot}5\%$. However, ET did not change the number of channel openings per sweep ($3{\cdot}9 \pm 0{\cdot}3$ to $4{\cdot}3 \pm 0{\cdot}7$).

Silberberg *et al.* (1989) reported that in porcine coronary artery, addition of ET to the bath increased the number of openings of the unitary L-type Ca^{2+} channel but had no effect on the mean open time or conductance. Thus the responses of the unitary L-type Ca^{2+} channel of the porcine coronary artery to ET resemble those of the 12 pS channel rather than those of the 22 pS channel. Silberberg *et al.* (1989) considered that this channel was of the L-type because this ET-sensitive Ca^{2+} channel was sensitive to dihydropyridine and because the summated current did not show a transient component. In the present experiments, the 12 pS channel was also sensitive to nifedipine, and did not show inactivation during a period of depolarization. Moreover, the amplitude of the unitary current of the L-type Ca^{2+} channel in the porcine coronary artery was smaller than in cardiac cells (0·86 *vs.* about 6 pA respectively) when recorded under the same conditions (150 mM-Na^+ in the pipette, depolarization to -50 mV; Hess, Lansman & Tsien, 1986; Silberberg *et al.* 1989). Therefore, in some respects the properties of L-type channels of porcine coronary artery resemble those of the 12 pS channel rather than the 22 pS channel of the guinea-pig portal vein. However, there are still discrepancies in the voltage dependence and the mean open time of the unitary channel current.

ET augmented the 12 pS Ca^{2+} channel recorded during the early part of the depolarizing pulse, but not during the late part of the pulse. Therefore the decay of the inward current in the presence of ET was steeper than in the absence of ET. In cardiac muscle cells, Bay K 8644 and nitrendipine are known to have agonistic and antagonistic actions on the L-type Ca^{2+} channel: they increased the peak amplitude of the inward current and accelerated the decay of the inward current (Bean, Sturek & Hermsmeyer, 1986; Brown, Kunze & Yatani, 1986). By contrast, in the present experiments no evidence was obtained that ET has an antagonistic action because the mean open time, the open probability and the number of channel openings measured during the late period of each sweep were not changed by ET. Furthermore,

ET did not prolong the time between the last closure of the channel and the end of the depolarizing pulse, suggesting that ET does not prevent reopening of the channel during depolarization. Although the precise mechanism of action of ET on the 12 ps channel is not clear, ET may act on the 12 pS channel in the resting state and/or in the open state, while during a depolarizing pulse ET may be freed from the binding site or prevented from connecting with the activating process of the receptor–12 pS channel pathway. Such a phenomenon may not be due to changes in the characteristics of the channel itself because these parameters did not change in the early and late period of sweeps under the control condition. One of the properties of the T-type Ca^{2+} channels is a rapid decay of the current (Carbone & Lux, 1984; Nillius *et al.* 1985; Benham *et al.* 1987; Fox *et al.* 1987; Yatani *et al.* 1987). On the other hand, in the guinea-pig portal vein no significant decay of the summated unitary current of the 12 pS channel was seen in the control. However, it could not be excluded that an intrinsic mechanism is involved in the decay of summated unitary current of the 12 pS channel, such as the current-induced inactivation mechanism observed in L-type Ca^{2+}channels (Ca^{2+}-induced inactivation of the Ca^{2+} current; Ohya, Kitamura & Kuriyama, 1988).

In the present experiments, to produce the maximum augmentation of the Ba^{2+} inward current more than 5 min was required after the application of ET in the bath, and this response was not easily reversed by wash-out. Similar results were also reported in the porcine coronary artery (Silberberg *et al.* 1989). Furthermore, it is of interest that the augmentation induced by ET was observed more frequently upon bath application than pipette application. These results suggest that this peptide does not act directly on the voltage-dependent Ca^{2+} channels, but acts through intrinsic products, such as second messengers. Recently, there have been many reports which indicate that ET stimulates phospholipase C, or mobilizes the release of Ca^{2+} from the intracellular store (Miasiro *et al.* 1988; Van Renterghem *et al.* 1988; Resnik *et al.* 1988; Kai *et al.* 1989; Marsden *et al.* 1989; Sugiura *et al.* 1989). In particular, Kai *et al.* (1989) and Hirata, Yoshimi, Takata, Watanabe, Kumagai, Nakayama & Sakakibara (1988) reported that ET induced a sustained elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, and a transient elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in Quin-2-loaded culture aortic media. Since the sustained component was markedly inhibited by the Ca^{2+} antagonist diltiazem, and was not observed in Ca^{2+}-free media, they postulated that this component was likely to be mediated by Ca^{2+} influx. Hirata *et al.* (1988) also reported that a specific receptor for ET existed in cultured rat aortic smooth muscle cells, which was affected by neither neurotoxins nor Ca^{2+} antagonists. A contribution of cyclic AMP, cyclic GMP and inositol phosphate to the action of ET upon the Ca^{2+} channel may be ruled out, because these substrates did not activate the voltage-dependent Ca^{2+} channel in the smooth muscle cell (Klöckner & Isenberg, 1985; Ohya, Kitamura & Kuriyama, 1987; Ohya, Terada, Yamaguchi, Inoue, Okabe, Kitamura, Hirata & Kuriyama, 1988). Fish, Sperti, Colucci & Clapham (1988) postulated that the Ca^{2+} channel is activated through the stimulation of protein kinase C. Further experiments are required to elucidate the second messenger involved in the vasoconstrictor action of ET on smooth muscle cells.

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