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Inhibitory effect of nitrovasodilators and cyclic GMP on ET-1-activated Ca^{2+}-permeable nonselective cation channel in rat aortic smooth muscle cells

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1 In single vascular smooth muscle cells (VSMCs) isolated from the aortae of male Wistar rats, we examined the effects of nitric oxide (NO) donors such as sodium nitroprusside (SNP) and S-nitroso-N-acetyl-DL-penicillamine (SNAP), and 8-bromo-guanosine-3′:5′-cyclic monophosphate (8-bromo-cyclic GMP) on endothelin-1 (ET-1)-activated Ca^{2+}-permeable nonselective cation channel by use of whole-cell recordings of patch-clamp technique and monitoring of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura-2 real-time digital microfluorometry.

2 ET-1 evoked an initial transient peak and a subsequent sustained elevation in $[Ca^{2+}]_i$. After removal of extracellular Ca^{2+}, ET-1 evoked only an initial transient peak without a sustained phase. Nifedipine (1 μM), a specific blocker of the L-type voltage-operated Ca^{2+} channel (VOC), reduced the sustained phase to about 40% of the control level. The remaining part of the sustained phase was abolished by 30 μM SK&F 96365, a blocker of nonselective cation channels.

3 The nifedipine-resistant sustained elevation in $[Ca^{2+}]_i$ was abolished by 100 μM SNP, 10 μM SNAP and 300 μM 8-bromo-cyclic GMP. Neither SNP, SNAP nor 8-bromo-cyclic GMP significantly affected the basal level of $[Ca^{2+}]_i$.

4 In a VSMC clamped at a holding potential of -60 mV with K^+ in the pipette solution replaced by Cs^+, application of 10^{-8} M ET-1 induced an inward current with an increase in baseline fluctuation. With fluctuation analysis, unit conductance of the ET-1-induced current was calculated to be about 21 pS. The ET-1-induced current was linearly related to the membrane potentials with its reversal potential of -5.5 mV.

5 The ET-1-induced current was reversibly and completely inhibited by 30 μM SK&F 96365 or 500 μM Cd^{2+}. The current inhibited by SK&F 96365 or Cd^{2+} was linearly related to membrane potential with a reversal potential of about -5 mV.

6 The ET-1-induced current was reversibly and completely inhibited by 100 μM SNP, 10 μM SNAP and 300 μM 8-bromo-cyclic GMP. The current inhibited by SNP, SNAP or 8-bromo-cyclic GMP showed linear voltage-dependence and reversed at about -5 mV.

7 In a bath solution in which all cations were replaced by 30 mM Ca^{2+} and 100 mM nonpermeant cation N-methyl-D-glucamine (NMDG), ET-1 evoked a current with a reversal potential of -11 mV, from which $P_{Ca^{2+}}/P_{Cs^+}$ was calculated to be 2.1. This Ca^{2+} current was also abolished by 100 μM SNP, 10 μM SNAP and 300 μM 8-bromo-cyclic GMP. The current inhibited by SNP, SNAP or 8-bromo-cyclic GMP showed linear voltage-dependence and reversed at about -11 mV.

8 These results taken together indicate that NO through a cyclic GMP signalling pathway inhibits ET-1-activated Ca^{2+}-permeable nonselective cation channels, thereby suppressing the sustained increase in $[Ca^{2+}]_i$. Thus, the present study indicates that this Ca^{2+}-permeable nonselective cation channel is an important target for nitrovasodilators.

Keywords: Endothelin-1; nonselective cation channel; nitric oxide; sodium nitroprusside; S-nitroso-N-acetyl-DL-penicillamine (SNAP); cyclic GMP; vascular smooth muscle; rat aorta; patch-clamp

Introduction

Vascular contraction induced by agonists such as endothelin-1 (ET-1) requires entry of extracellular Ca^{2+} through the plasma membrane (Rubanyi & Polokoff, 1994). The voltage-operated Ca^{2+} channel (VOC) is a well-known Ca^{2+} entry channel activated by ET-1 (Goto *et al*., 1989; Inoue *et al*., 1990), but involvement of other channels permeable to Ca^{2+} has been implicated (Huang *et al*., 1990; Inoue *et al*., 1990; Simpson *et al*., 1990). In this context, several studies have shown that a Ca^{2+}-permeable nonselective cation channel is activated by ET-1 in vascular smooth muscle cells (VSMCs) (Van Renterghem *et al*., 1988; Chen & Wagoner, 1991; Enoki *et al*., 1995). Furthermore, we have recently shown that cloned ET_A receptors are functionally coupled to Ca^{2+}-permeable nonselective cation channels, when expressed in Ltk^- cells, a mouse fibroblast cell line (Enoki *et al*., 1995).

Likewise, the mechanisms of action of nitrovasodilators and nitric oxide (NO) are not totally understood, but their actions are considered to be mediated by activation of guanylate cyclase and the resultant increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content in VSMCs (Ignarro *et al*., 1986). Cyclic GMP is known to exert its effect by reducing the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through several mechanisms: (1) activation of K^+ channels, leading to hyperpolarization of the membrane and subsequent inhibition of VOCs (Archer *et al*., 1994), (2) direct

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inhibition of VOCs (Lorenz *et al*., 1994), (3) activation of the Ca^{2+}-pump in the plasma membrane (Rashatwar *et al*., 1987; Yosida *et al*., 1991) and the endoplasmic reticulum (Cornwell *et al*., 1991).

Several lines of evidence indicate that although NO or cyclic GMP completely inhibit both agonist-induced vasoconstriction and increase in $[Ca^{2+}]_i$ (Karaki *et al*., 1988; Collins *et al*., 1988; Magliola & Jones, 1990; Blayney *et al*., 1991; Salomone *et al*., 1995), a complete block of VOC by dihydropyridines has only partial effects on these parameters (Blayney *et al*., 1991; Salomone *et al*., 1995). Based on these results, we hypothesized that the Ca^{2+}-permeable nonselective cation channel is another important target for the cyclic GMP signalling system.

To verify this point, we examined the effects of NO donors such as sodium nitroprusside (SNP) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) and 8-bromo-guanosine-3′:5′-cyclic monophosphate (8-bromo-cyclic GMP) on ET-1-activated Ca^{2+}-permeable nonselective cation channels by use of whole-cell recordings of patch-clamp technique and monitoring of $[Ca^{2+}]_i$ with fura-2 real-time digital microfluorometry in single, freshly dispersed VSMCs.

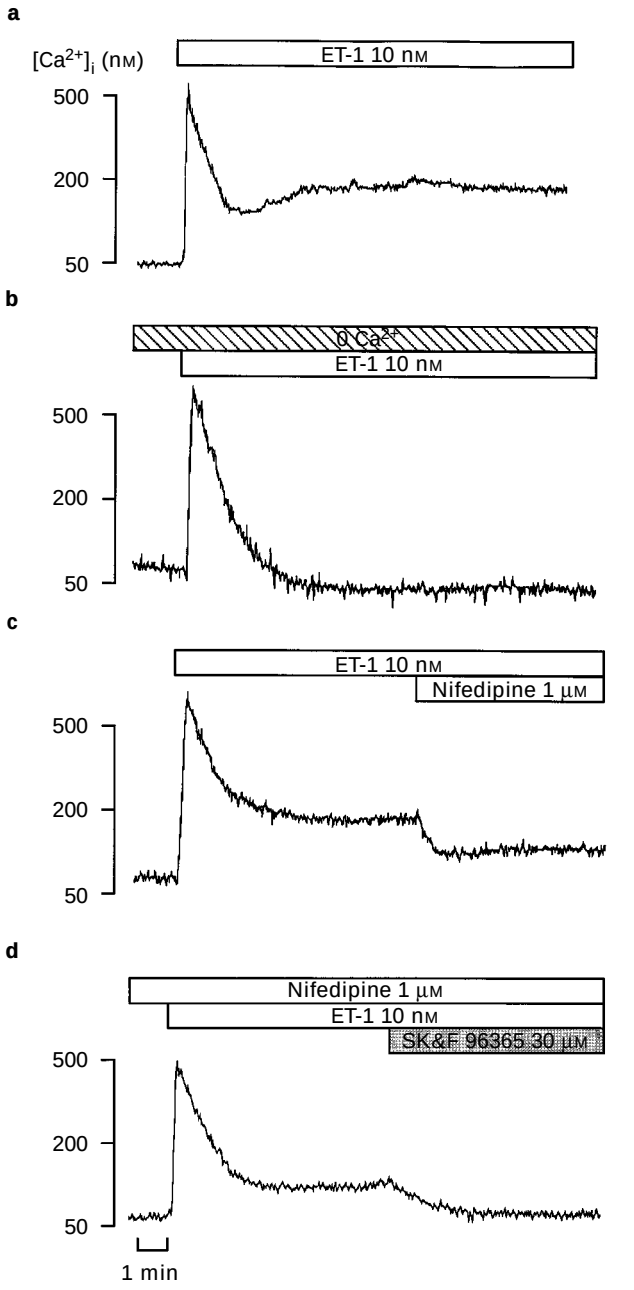

Figure 1 Typical tracings showing the effects of removal of extracellular Ca^{2+} (b), nifedipine (c) and nifedipine followed by SK&F 96365 (d) on endothelin-1 (ET-1)-induced elevations in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single vascular smooth muscle cells (VSMCs) from the rat thoracic aorta. The VSMCs were enzymatically dispersed from the rat thoracic aorta and cultured in 35 mm glass-bottomed plastic dishes as described in the Methods section. The cultured cell was loaded with a Ca^{2+} indicator fura-2 and subjected to microfluorometry with excitation wavelengths at 334 nm and 380 nm and an emission wavelength at 520 nm. The experiments were performed in normal Krebs-HEPES (a, c, d) or Ca^{2+}-free Krebs-HEPES solution containing 1 mM EGTA (b). At the beginning of each bar, ET-1, nifedipine or SK&F 96365 was added to the culture dish at a final concentration of 10 nM, 1 μM and 30 μM, respectively.

Methods

Preparation and primary culture of VSMCs for whole-cell recordings and measurement of $[Ca^{2+}]_i$

Isolated VSMCs were prepared from rat thoracic aortae as described previously (Inoue & Kuriyama, 1993; Enoki *et al*., 1995). Briefly, male Wistar rats (180–200 g) were anaesthetized with diethylether and exsanguinated. The thoracic aorta was removed, cleaned of surrounding tissues, dissected into small strips (2 mm × 5 mm) and kept in Ca^{2+}-free Krebs-HEPES solution containing (in mM): NaCl 140, KCl 3, $MgCl_2$ 1, glucose 11 and HEPES 10 (pH 7.3, adjusted with NaOH). The strips were incubated overnight (12–24 h) at 4°C in Ca^{2+}-free Krebs-HEPES solution containing papain (0.2–0.3 mg ml^{-1}) and 0.5 mM dithiothreitol. Thereafter, the strips were resuspended and incubated in Ca^{2+}-free Krebs-HEPES solution containing collagenase (0.25–0.5 mg ml^{-1}) at 35°C for 10 min. The digested strips were cut into pieces with fine scissors and triturated with a blunt-tipped pipette until a sufficient number of single cells was released. The freshly dispersed cells were used for electrophysiological experiments.

For measurement of $[Ca^{2+}]_i$ by use of fura-2 combined with real-time digital microfluorometry, dispersed VSMCs were seeded on 35 mm glass-bottomed plastic dishes (Meridian Instruments, MI, U.S.A.) and grown in Dulbecco's modified

Table 1 Effects of nifedipine, NO donors and 8-bromo-cyclic GMP on the ET-1-induced elevations in $[Ca^{2+}]_i$ in a single VSMC

Treatment	*ET-1-induced change in $[Ca^{2+}]_i$ level (%)*	n
No drug	96.6 ± 1.4	20
Nifedipine	41.2 ± 4.9††	28
Nifedipine + SNP	6.3 ± 3.4**	11
Nifedipine + SNAP	4.5 ± 3.8**	11
Nifedipine + 8-bromo-cyclic GMP	4.4 ± 4.4**	15

Preparation of and continuous monitoring of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a single cultured vascular smooth muscle cell (VSMC) were performed as described in the legend to Figure 1. In all experiments, endothelin-1 (ET-1) was added to culture dishes at a final concentration of 10^{-8} M and an increase in $[Ca^{2+}]_i$ was induced. Eight minutes after application of ET-1, nifedipine (1 μM) alone or in combination with sodium nitroprusside (SNP; 100 μM), S-nitroso-N-acetyl-DL-penicillamine (SNAP; 10 μM) or 8-bromo-cyclic GMP (300 μM) was added to culture dishes. Three minutes later, the values of $[Ca^{2+}]_i$ were determined and represented as percentages of those values at the time of each treatment. Significant difference compared to no drug group is shown as ††$P < 0.01$, and significant differences compared to nifedipine alone are shown as **$P < 0.01$.

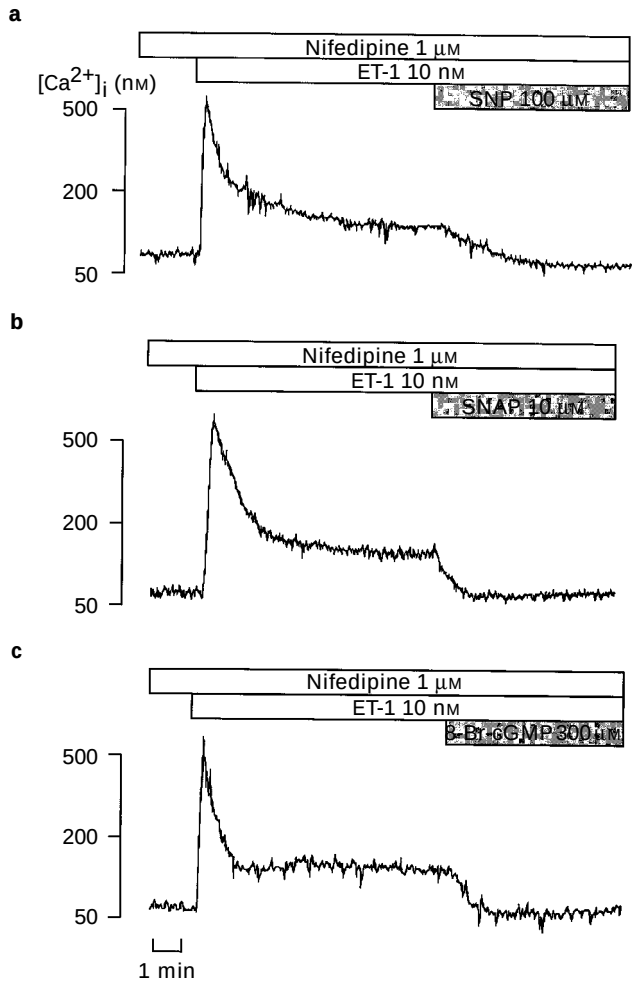

Figure 2 Typical tracings showing the effects of sodium nitroprusside (SNP) (a) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) (b) and 8-bromo-cyclic GMP (c) on nifedipine-resistant endothelin-1 (ET-1)-induced elevations in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single vascular smooth muscle cells (VSMCs) from the rat thoracic aorta. Preparation of and monitoring of $[Ca^{2+}]_i$ in a single cultured VSMC was performed as described in the legend to Figure 1. In all experiments, nifedipine was added to culture dishes at a final concentration of 1 μM, 3 min before application of ET-1 (final concentration, 10 nM). Approximately 8 min after application of ET-1, SNP, SNAP or 8-bromo-cyclic GMP was added to culture dishes at final concentrations of 100 μM, 10 μM and 300 μM, respectively.

Eagle's medium containing 10% foetal bovine serum supplemented with 100 u ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin for 24 h. After being deprived of nutrients in serum-free medium for further 24 h, they were used for the experiments.

Microfluorometry of fura-2

For loading of fura-2, the cultured VSMCs were incubated in Ca^{2+}-free Krebs-HEPES solution containing 5 μM fura-2/AM (acetoxymethyl ester) for 60 min at 37°C (Sakamoto *et al*., 1993; Itoh *et al*., 1994). After washing with normal Krebs-HEPES solution (2 mM $CaCl_2$ was added to Ca^{2+}-free Krebs-HEPES solution), they were kept in fresh Krebs-HEPES solution at 25°C for at least 30 min. For experiments in the absence of extracellular Ca^{2+}, the solution in the culture dish was replaced with Ca^{2+}-free Krebs-HEPES solution containing 1 mM EGTA immediately before $[Ca^{2+}]_i$ measurements. Fura-2 microfluorometry was done at 25°C by an Attofluor Ratio-Vision real-time digital fluorescence analyser (Atto Instruments, Potomac, MD, U.S.A.), based on a Carl-Zeiss Axiovert-100 inverted epifluorescent microscope. A 100-W mercury burner served as the source of excitation. In measurements of $[Ca^{2+}]_i$ fura-2 was excited at two wavelengths, 334 nm and 380 nm, by using 10 nm bandpass interference filters, which were alternately selected by a computer-controlled excitation and shutter control unit. A 520 nm long-pass emission filter was used to select fluorescence emission. $[Ca^{2+}]_i$ was calculated from the ratio of fura-2 fluorescence at 334 nm to that at 380 nm by use of external standards (De Erausquin *et al*., 1990).

Electrophysiology

Freshly dispersed VSMCs were perfused with Krebs-HEPES solution visualized with Nomarski optics (Carl-Zeiss Axioscope) and whole-cell recordings were made with thin-wall borosilicate glass patch pipettes (resistance, 3–5 MΩ) as described previously (Kobayashi & Takahashi, 1993; Enoki *et al*., 1995). Pipettes were filled with Cs-aspartate solution containing (in mM): Cs-aspartate 120, CsCl 20, $MgCl_2$ 2, HEPES 10, EGTA 10 (pH 7.3, adjusted with CsOH). EGTA was added to the pipette solution at a final concentration of 10 mM, a concentration having enough buffering capacity for Ca^{2+} to prevent a transient increase in $[Ca^{2+}]_i$ (Neher, 1988), and the concentration of Ca^{2+} in the solution was maintained at 100 nM by adding an amount of $CaCl_2$ calculated as described by Van Heeswijk *et al*. (1984). Tight seal whole-cell currents were recorded with an EPC7 patch-clamp amplifier (List, Darmstadt, Germany) and analysed with the pClamp software package (Axon Instruments, Burlingame, CA, U.S.A.). Perfusion rate was maintained at 2.2–2.5 ml min^{-1} and the bath volume was ~1.0 ml. All experiments were done under voltage-clamp at a holding potential of −60 mV at room temperature (22–24°C). To test the permeability of Ca^{2+} through the cation channel, the bath solution was switched from Krebs-HEPES to 30 mM Ca^{2+}/100 mM N-methyl-D-glucamine (NMDG) solution which contained (in mM): $CaCl_2$ 30, NMDG chloride 100, $MgCl_2$ 1, glucose 11, HEPES 10 (pH 7.4, adjusted with Tris). In all experiments, the bath solution was supplemented with 1 μM nifedipine to block Ca^{2+} entry through VOC. Current-voltage relationships were obtained by applying voltage steps ranging from −100 to +80 mV in 20 mV increments before and after application of drugs. The drug-induced currents at each membrane potential were determined by subtracting currents before application of the drug from currents after its application.

In some experiments, fluctuation analysis was performed to obtain unit conductance according to Sigworth (1980). For this purpose, current was low-pass-filtered at 1 kHz. The variance (σ^2) and mean (I) of the current were calculated every 1 s, and drawn in a variance-mean plot. Regression curve was obtained according to the equation,

$$\sigma^2 = iI - I^2/N$$

where i is a size of single-channel currents and N is the total number of channels. Single-channel conductance (γ) was calculated from the equation,

$$\gamma = i/(E - E_{rev})$$

where E is holding potential and E_{rev} is reversal potential.

Statistical analysis

All results are expressed as mean ± s.e.mean. The data were subjected to a two-way analysis of variance, and when significant F values were encountered, Newman-Keuls' multiple-range test was used to test for significant differences between treatment means. A probability level of $P<0.05$ was considered statistically significant.

Drugs

ET-1 was purchased from Peptide Institute (Osaka, Japan). Fura-2/AM and EGTA were from Dojin Chemicals (Tokyo, Japan). SNP and SNAP and collagenase were from Wako Pure Chemicals (Osaka, Japan). Nifedipine, 8-bromo-cyclic GMP and papain were from Sigma. SK&F 96365 was from Biomol (Plymouth Meeting, PA, U.S.A.). Nifedipine was dissolved in dimethyl sulphoxide (DMSO) and the final concentration of DMSO was 0.1%.

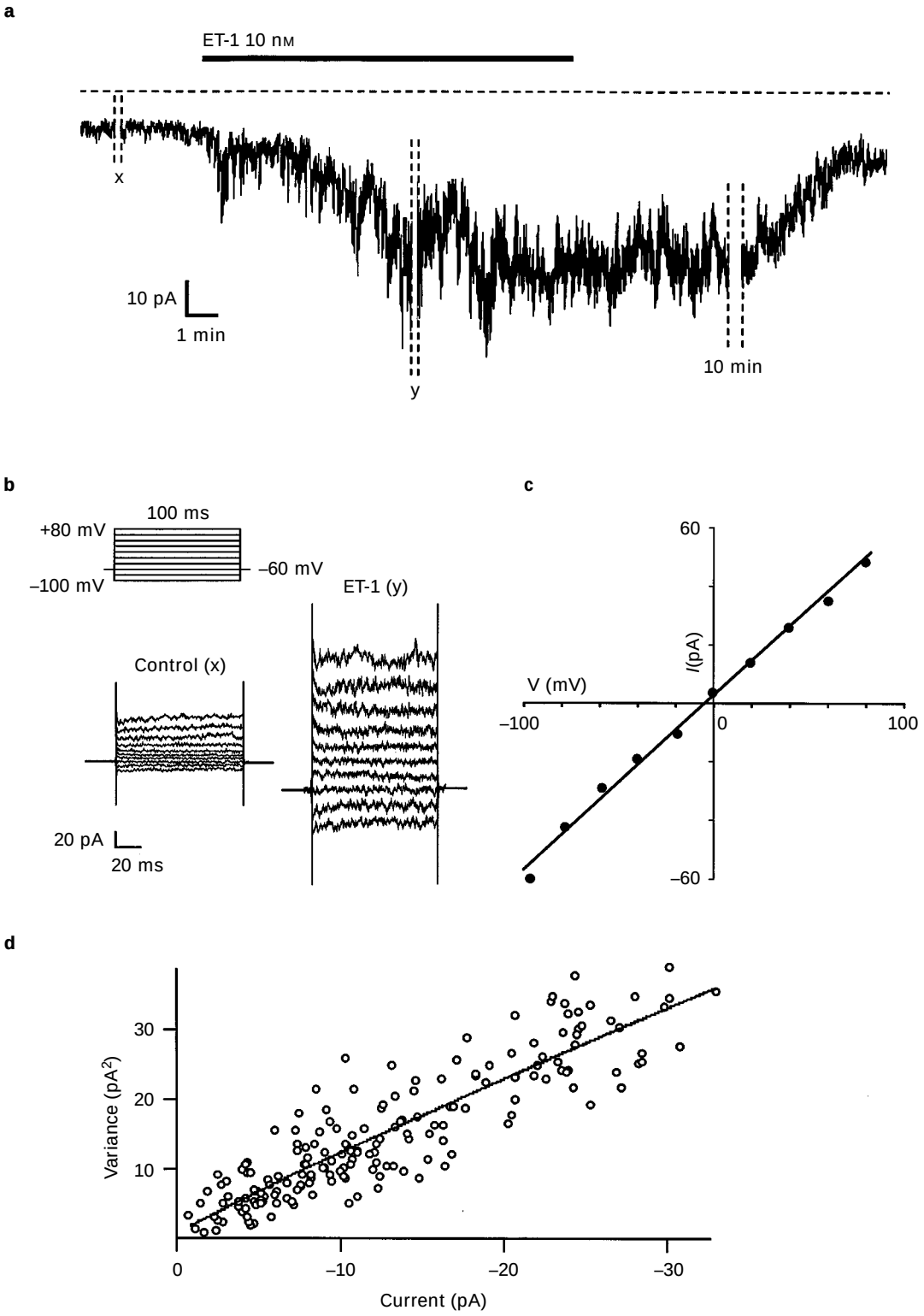

Figure 3 Whole-cell recordings of endothelin-1 (ET-1)-induced inward current in vascular smooth muscle cells (VSMCs) freshly dispersed from the rat thoracic aorta. (a) A trace showing the long-lasting inward current induced by ET-1. ET-1 (10 nM) was added to the bath solution during the time interval indicated by a horizontal bar. The zero-current level was indicated by a dashed line. (b) A family of currents elicited by command pulses (upper left) from a holding potential of −60 mV before (lower left, corresponding to x in (a)) and after (lower right, corresponding to y in (a)) the application of ET-1 in the same cell as in (a). (c) A current-voltage relationship for the ET-1-induced current. The ET-1-induced currents at each membrane potential were determined by subtracting currents before application of ET-1 (x in (a)) from currents after its application (y in (a)). (d) Fluctuation analysis of the ET-1-induced current. Variances were plotted against mean currents of the data in (a).

Results

Measurement of $[Ca^{2+}]_i$ in freshly dispersed VSMCs

As shown previously (Enoki *et al*., 1995), ET-1 at a concentration of 10^{-8} M evoked biphasic changes in $[Ca^{2+}]_i$ in freshly dispersed VSMCs in Krebs-HEPES solution: an initial transient peak and a subsequent sustained phase (Figure 1a). In the absence of external Ca^{2+}, ET-1 evoked only the initial peak without the sustained phase (Figure 1b).

In separate experiments, we attempted to determine the supramaximal concentration of nifedipine, a specific blocker of L-type VOCs, to inhibit completely L-type VOCs. For this purpose, we tested varying concentrations of nifedipine on an increase in $[Ca^{2+}]_i$ in VSMCs induced by high K^+ (50 mM) stimulation, which causes depolarization of the plasma membrane and subsequent activation of VOCs. Nifedipine completely suppressed high K^+-induced increase in $[Ca^{2+}]_i$ in VSMCs at concentrations higher than 10^{-7} M (data not shown). Therefore, in the following experiments, we added 1 μM nifedipine to the bath solution to block VOCs completely and focused on the nifedipine-resistant part of the sustained increase in $[Ca^{2+}]_i$.

When 1 μM nifedipine was added to the bath solution during the sustained elevation of $[Ca^{2+}]_i$ induced by 10^{-8} M ET-1, it suppressed the $[Ca^{2+}]_i$ to about 40% ($41.2 \pm 4.9\%$, $n=28$) of the control level (Figure 1c; Table 1).

In the presence of 1 μM nifedipine, SK&F 96365 which has been shown to be a blocker of nonselective cation channels (Merritt *et al*., 1990; Blayney *et al*., 1992) suppressed the sustained elevation of $[Ca^{2+}]_i$ induced by 10^{-8} M ET-1 in a concentration-dependent manner: the IC_{50} was 3×10^{-6} M (data not shown) and a complete inhibition was obtained at concentrations higher than 30 μM (Figure 1d).

Next, we investigated the effects of NO on the nifedipine-resistant part of the ET-1-induced increase in $[Ca^{2+}]_i$ in VSMCs, by use of the NO donors SNP and SNAP. SNP suppressed the nifedipine-resistant sustained elevation of $[Ca^{2+}]_i$ induced by 10^{-8} M ET-1 in a concentration-dependent manner with an IC_{50} of 10^{-6} M (data not shown) and complete inhibition was obtained at concentrations higher than 10^{-5} M (Figure 2a; Table 1). Similarly, SNAP suppressed the nifedipine-resistant sustained elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner with an IC_{50} of 10^{-7} M (data not shown) and complete inhibition was obtained at concentrations higher than 10^{-6} M (Figure 2b; Table 1).

To examine whether the inhibitory effect of NO donors was mediated by cyclic GMP, we determined the effect of 8-bromo-cyclic GMP on $[Ca^{2+}]_i$. Like SNP and SNAP, 8-bromo-cyclic GMP dose-dependently suppressed the nifedipine-resistant sustained elevation of $[Ca^{2+}]_i$ induced by 10^{-8} M ET-1: the IC_{50} was 3×10^{-5} M (data not shown) and complete inhibition was obtained at concentrations higher than 300 μM (Figure 2c; Table 1).

Neither SNP, SNAP nor 8-bromo-cyclic GMP significantly affected the basal level of $[Ca^{2+}]_i$ (data not shown). To investigate the mechanism of action of SNP, SNAP and 8-bromo-cyclic GMP on the nifedipine-resistant sustained increase in $[Ca^{2+}]_i$, we performed whole-cell recordings with the patch-clamp technique.

Whole-cell current recordings

In VSMCs clamped at a holding potential of -60 mV, application of 10^{-8} M ET-1 induced a slow inward current with an increase in baseline fluctuation (Figure 3a). In most cells, the responses continued for over 10 min even after washout of ET-1 (Figure 3a). The amplitude of the sustained inward current varied between cells, ranging from 5 pA to 40 pA. The ET-1-induced current was linearly related to membrane potential between -100 mV and $+80$ mV (Figures 3b and 3c). The reversal potential of the current was -5.5 ± 0.8 mV ($n=14$). The calculated equilibrium potential for Cl^- (E_{Cl}; -46.9 mV in normal Krebs-HEPES solution) was not close to the reversal potential, and the pipette solution contained excess EGTA to suppress completely the increase in

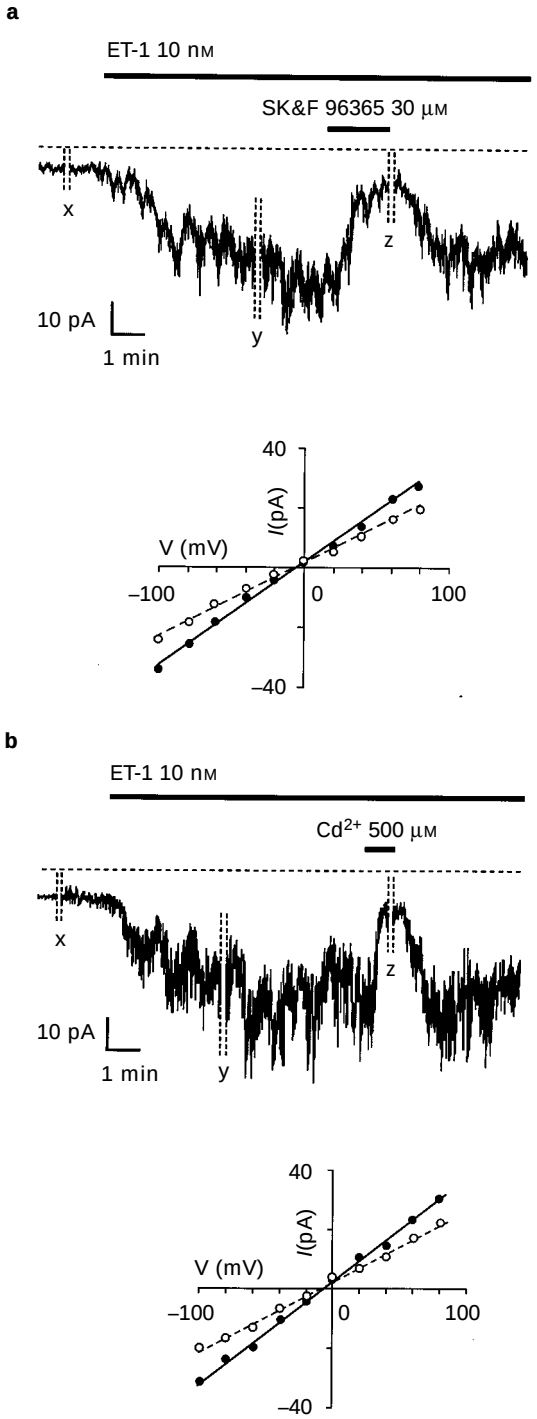

Figure 4 Typical tracings and current-voltage relationships showing the effects of SK&F 96365 (a) and Cd^{2+} (b) on the endothelin-1 (ET-1)-induced currents in vascular smooth muscle cells (VSMCs) freshly dispersed from the rat thoracic aortae. The cells were clamped at a holding potential of -60 mV with the whole-cell configuration and ET-1 was added to the bath solution at a final concentration of 10 nM during the time interval indicated by a horizontal bar. After the ET-1-induced inward current had reached a steady-state, SK&F 96365 or Cd^{2+} was added to the bath solution at a final concentration of 30 μM or 500 μM, respectively. At the time indicated by x, y and z, voltage steps ranging from -100 to $+80$ mV in 20 mV increments were applied. The current-voltage relationships (lower part) for the ET-1-induced current (●) and the current inhibited by SK&F 96365 (○) or Cd^{2+} (○) were calculated by subtracting currents before application of either drug from currents after its application at each membrane potential.

$[Ca^{2+}]_i$, which might trigger Ca^{2+}-activated Cl^- current. These results indicate that the ET-1-induced current is carried not through the Cl^- channel but through a cation channel, which is equally permeable to both extracellular Na^+ and intracellular Cs^+. In this sense, the channel is considered to be nonselective in nature.

To estimate the unit conductance of the ET-1-induced current, fluctuation analysis was performed. Variance and mean of the current were calculated every 1 s after stimulation with ET-1 (Figure 3d). From this calculation, unit conductance of the current was estimated to be 21.2 ± 0.8 pS ($n=14$).

The ET-1-induced inward current was reversibly inhibited by 30 μM SK&F 96365 (Figure 4a). Like the ET-1-induced current, the current inhibited by SK&F 96365 was linear against the membrane potential (between -100 mV and $+80$ mV) and its reversal potential was close to -5.5 mV (Figure 4a). Similarly, the ET-1-induced currents were reversibly and completely inhibited by 500 μM Cd^{2+} (Figure 4b), which has been shown to be a blocker of nonselective cation channels (Krautwurst *et al*., 1994). The current inhibited by Cd^{2+} was also linear with the reversal potential close to -5.5 mV (Figure 4b).

Notably, the ET-1-induced current was reversibly inhibited by NO donors such as 100 μM SNP (Figure 5a) and 10 μM SNAP (Figure 5b). At a holding potential of -60 mV, 100 μM SNP and 10 μM SNAP inhibited the currents by $88.2 \pm 6.4\%$ ($n=6$) and $85.1 \pm 5.4\%$ ($n=6$), respectively. The currents inhibited by SNP and SNAP showed a linear voltage-dependence with reversal potentials of -5.5 ± 2.3 mV ($n=6$) and -4.3 ± 3.1 mV ($n=6$), respectively. These values were not significantly different from that of the ET-1-induced current (see above).

Furthermore, the ET-1-induced current was reversibly inhibited by 300 μM 8-bromo-cyclic GMP (Figure 5c). At a holding potential of -60 mV, the current was inhibited by $84.7 \pm 5.6\%$ ($n=6$). The current inhibited by 8-bromo-cyclic GMP showed a linear voltage dependence with a reversal potential of -5.7 ± 1.5 mV ($n=6$): this value was not significantly different from that of the ET-1-induced current (see above). These data indicate that cyclic GMP and NO possess the same action in terms of nonselective cation current.

To examine the permeability of Ca^{2+}, all cations in the bath solution were replaced by Ca^{2+} and the nonpermeant cation NMDG. Even under this condition, ET-1 evoked an inward current at a holding potential of -60 mV (Figure 6), indicating that the current is carried by Ca^{2+}. The current showed linear voltage-dependence and the reversal potential was -10.9 ± 2.1 mV ($n=9$). From this reversal potential, the relative permeability of Ca^{2+} to Cs^+ ($P_{Ca^{2+}}/P_{C^+}$) was calculated according to the modified Goldman-Hodgkin-Katz equation of Lewis (1979). For the calculation of relative permeabilities, activities rather than concentrations were used: the assumed activity coefficients for Cs^+ and Ca^{2+} were 0.75 and 0.45, respectively. The relative permeability ($P_{Ca^{2+}}/P_{Cs^+}$) was calculated to be 2.1.

The ET-1-induced Ca^{2+} current in 30 mM Ca^{2+}/100 mM NMDG solution was reversibly and completely inhibited by the NO donors SNAP (10 μM) (Figure 6a) and 8-bromo-cyclic GMP (300 μM) (Figure 6b). At a holding potential of -60 mV, 10 μM SNAP and 300 μM 8-bromo-cyclic GMP inhibited the current by $82.3 \pm 7.7\%$ ($n=4$) and $83.5 \pm 8.2\%$ ($n=5$), respectively. The Ca^{2+} currents inhibited by SNAP and 8-bromo-cyclic GMP showed linear voltage-dependence and their reversal potentials in 30 mM Ca^{2+}/100 mM NMDG solution were -11.7 ± 3.3 mV ($n=4$) and -11.1 ± 3.1 mV

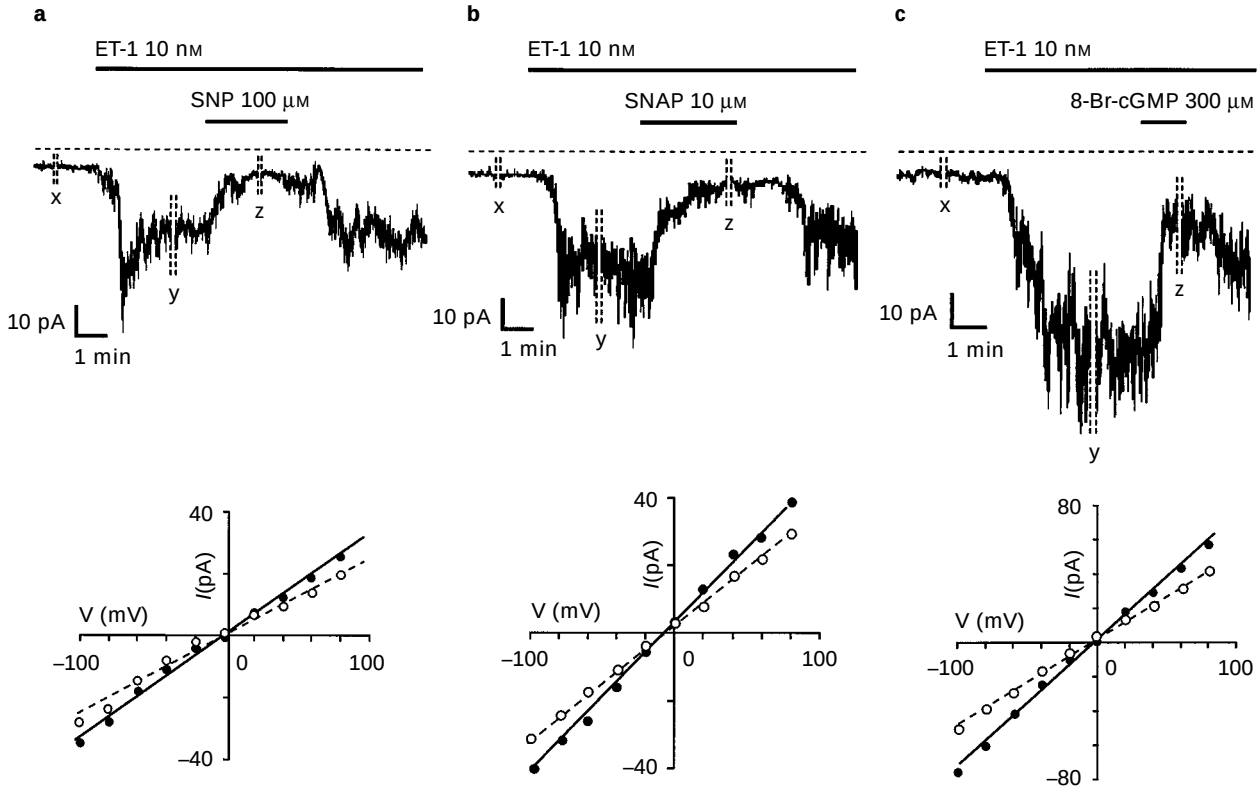

Figure 5 Typical tracings and current-voltage relationships showing the effects of sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine (SNAP) and 8-bromo-cyclic GMP on endothelin-1 (ET-1)-induced currents in normal Krebs-HEPES solution in vascular smooth muscle cells (VSMCs) freshly dispersed from rat thoracic aortae. The cells were clamped at a holding potential of -60 mV with the whole-cell configuration in normal Krebs-HEPES solution. ET-1 was added to the bath solution at a final concentration of 10 nM during the time interval indicated by a horizontal bar. After the ET-1-induced inward current had reached a steady-state, SNP (a), SNAP (b) or 8-bromo-cyclic GMP (c) was added to the bath solution at final concentrations of 100 μM, 10 μM and 300 μM, respectively. At the time indicated by x, y and z, voltage steps ranging from -100 to $+80$ mV in 20 mV increments were applied, and the current-voltage relationships (lower part) for the ET-1-induced current (●) and the current inhibited by SNP, SNAP or 8-bromo-cyclic GMP (○) were calculated.

($n=5$), respectively. These values were not significantly different from that of the ET-1-induced current in 30 mM Ca^{2+}/100 mM NMDG solution (see above). Essentially similar results were obtained with 100 μM SNP (data not shown). These results suggest that NO and cyclic GMP inhibit the ET-1-activated nonselective cation channel which is permeable to Ca^{2+}.

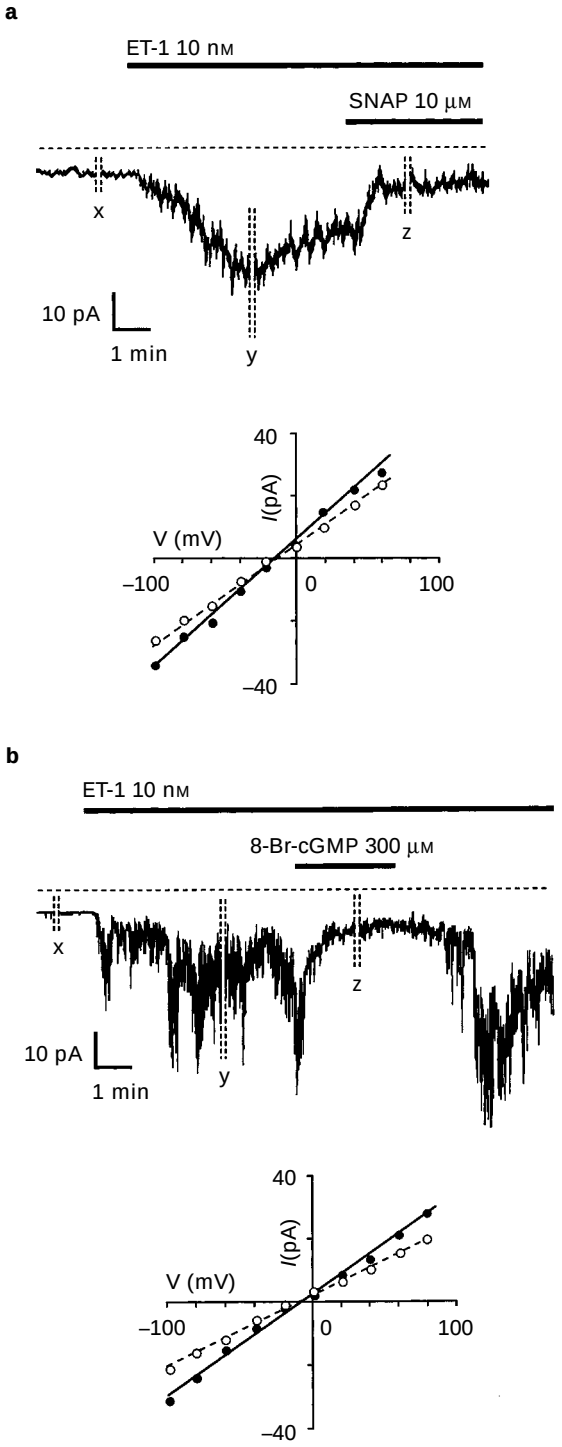

Figure 6 Typical tracings and current-voltage relationships showing the effects of S-nitroso-N-acetyl-DL-penicillamine (SNAP) (a) and 8-bromo-cyclic GMP (b) on endothelin-1 (ET-1)-induced currents in 30 mM Ca^{2+}/100 mM N-methyl-D-glucamine (NMDG) solution in vascular smooth muscle cells (VSMCs) freshly dispersed from rat thoracic aortae. The cells were clamped at a holding potential of −60 mV with the whole-cell configuration in 30 mM Ca^{2+}/100 mM NMDG solution. ET-1 was added to the bath solution at a final concentration of 10 nM during the time interval indicated by a horizontal bar. After the ET-1-induced inward current had reached a steady-state, SNAP (a) or 8-bromo-cyclic GMP (b) was added to the bath solution at final concentrations of 10 μM and 300 μM, respectively. At the time indicated by x, y and z, voltage steps ranging from −100 to +80 mV in 20 mV increments were applied, and the current-voltage relationships (lower part) for the ET-1-induced Ca^{2+} current (●) and the current inhibited by SNAP or 8-

Discussion

Measurement of $[Ca^{2+}]_i$ in freshly dispersed VSMCs

As shown previously (Enoki *et al*., 1995), ET-1 induced an increase in $[Ca^{2+}]_i$ consisting of two components: a rapid initial transient phase and a sustained phase (Figure 1a). From its dependence on extracellular Ca^{2+} (Figures 1a and b), the initial transient phase is considered to be the result of mobilization of Ca^{2+} from intracellular stores via increased formation of inositol 3-phosphate (IP_3), whereas the sustained phase is the result of transmembrane Ca^{2+} influx.

Half of the sustained phase seems to be the result of Ca^{2+} entry through VOCs, based on its sensitivity to an inhibitor of L-type VOCs nifedipine (Figure 1c; Table 1). The remaining half (nifedipine-resistant part) of the sustained phase is probably the results of Ca^{2+} entry through nonselective cation channels, because it was sensitive to blockers of nonselective cation channels such as mefenamic acid (Enoki *et al*., 1995) and SK&F 96365 (Figure 1d).

The nifedipine-resistant part of the ET-1-induced increase in $[Ca^{2+}]_i$ was inhibited by NO donors such as SNP and SNAP and also by 8-bromo-cyclic GMP (Figure 2; Table 1). These results indicate that both NO and cyclic GMP act on a nonselective cation channel in addition to VOCs.

Whole-cell current recordings

As shown recently by Enoki *et al*. (1995), whole-cell recordings of patch-clamp technique showed that ET-1 activates nonselective cation channels in freshly dispersed VSMCs, based on the linear current-voltage relationship, reversal potential and sensitivity to blockers of nonselective cation channel such as SK&F 96365 (Merritt *et al*., 1990; Blayney *et al*., 1992) and Cd^{2+} (Krautwurst *et al*., 1994) (Figures 3 and 4). Unit conductance of the ET-1-activated current was estimated to be approximately 21 pS by fluctuation analysis of the whole-cell recording data. This value is similar to those of nonselective cation channels activated by noradrenaline and acetylcholine in VSMCs (Inoue & Kuriyama, 1993; Wang *et al*., 1993), and that of Ca^{2+}-permeable cation channels activated by adenosine 5′-triphosphate (ATP) in VSMCs (Benham & Tsien, 1987). Characteristically, activation of the channel persisted even after washout of ET-1, and it was independent of an elevation of $[Ca^{2+}]_i$, because the current was induced in the presence of excessive EGTA (10 mM) in the patch pipette which prevents an elevation of $[Ca^{2+}]_i$ (Enoki *et al*., 1995).

More importantly, this channel is permeable to Ca^{2+} (Enoki *et al*., 1995), because ET-1 induced a current in the bath solution which contained only Ca^{2+} as a diffusible cation and nifedipine to block L-type VOCs (Figure 6). In this sense, this channel can be regarded as a receptor-operated Ca^{2+} channel (ROC) (Bolton, 1979). To assess the contribution of Ca^{2+} entry through this channel to an increase in $[Ca^{2+}]_i$, we calculated the increase in $[Ca^{2+}]_i$ resulting from opening of this channel. We assumed that in a model cell held at −60 mV, ET-1 induces a constant inward Ca^{2+} current of 10 pA (cf. Figure 6b) and that the volume of a model cell is 10 pl. Without Ca^{2+} buffering, such a Ca^{2+} current can induce an increase of 300 μM $[Ca^{2+}]_i$ per minute, which is large enough to explain the ET-1-induced increase in $[Ca^{2+}]_i$.

Both NO donors and 8-bromo-cyclic GMP reversibly inhibited the cation current (Figure 5) and Ca^{2+} current (Figure 6) induced by ET-1. Previously, it has been shown that both NO and cyclic GMP induce outward currents by activating charybdotoxin-sensitive K^+ channels (Archer *et al*., 1994).

However, the inhibition of ET-1-induced currents by NO donors and 8-bromo-cyclic GMP in the present study is not due to activation of the K^+ channel but due to inhibition of a nonselective cation channel, because the *I*-V relationship for the current component inhibited by NO donors or 8-bromo-cyclic GMP was essentially similar to that for the current induced by ET-1, in terms of its linearity and its reversal potential (Figures 5 and 6), and K^+ in the pipette solution was replaced by Cs^+ to inhibit conductance due to K^+ channels. These results indicate that both NO donors and 8-bromo-cyclic GMP reduce the ET-1-induced inward current by inhibiting the activity of a nonselective cation channel. Conversely, these data indicate that NO generated from NO donors acts through a cyclic GMP signalling system, although it is at present unclear whether cyclic GMP acts directly or indirectly through activation of cyclic GMP-dependent protein kinase.

Taken together with data from the Ca^{2+} microfluorometry study, these results indicate that NO, through cyclic GMP-dependent signalling pathways, inhibits ET-1-activated Ca^{2+}-permeable nonselective cation channels. This is associated with suppression of the sustained increase in $[Ca^{2+}]_i$. Thus, the present study indicates that this Ca^{2+}-permeable channel may be an important target for nitrovasodilators in aortic smooth muscle cells.

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