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Mechanical and electrophysiological effects of endothelin-1 on guinea-pig isolated lower oesophageal sphincter circular smooth muscle

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1 The effects of endothelin-1 (ET-1) on guinea-pig lower oesophageal sphincter (LOS) circular smooth muscle were investigated by using intracellular microelectrodes and isometric tension recording techniques.

2 ET-1 produced biphasic mechanical responses; an initial transient relaxation followed by a sustained contraction. The initial relaxation was not inhibited by either tetrodotoxin (TTX, 1 μM) or L-N^G-nitroarginine (L-NOARG, 100 μM). The sustained contraction was greatly attenuated by nifedipine (1 μM).

3 ET-1 (1–30 nM) induced a concentration-dependent hyperpolarisation that was unaffected by TTX or L-NOARG. The ET_A receptor antagonist, BQ123 (0.3 μM) abolished the ET-1-induced hyperpolarisation, whereas the ET_B receptor antagonist, BQ788 (0.3 μM) had no detectable effect. Sarafotoxin S6c (10 nM) did not change the membrane potential.

4 The ET-1-induced hyperpolarisation was abolished by apamin (0.1 μM). Interestingly, apamin abolished the ET-1-induced transient relaxation but potentiated the sustained contraction.

5 In Ca^{2+}-free Krebs solution, the ET-1-induced hyperpolarisation was greatly attenuated and returned to the control value when the tissue was reperfused with Krebs solution containing Ca^{2+}. The ET-1-induced hyperpolarisation was insensitive to nifedipine but was attenuated by SK&F 96365 (1-{β-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride, 50 μM), an inhibitor of receptor-mediated Ca^{2+} entry. The residual component of the ET-1-induced hyperpolarisation was sensitive to thapsigargin (1 μM).

6 These results demonstrate that, in guinea-pig LOS circular smooth muscle, ET-1 hyperpolarizes the membrane by activating apamin-sensitive K^+ channels, mainly as a result of receptor-mediated Ca^{2+} entry and partly by Ca^{2+} release from intracellular stores. The hyperpolarisation triggers the initial transient relaxation, which acts to oppose the sustained contraction.

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Abbreviations: 4-AP, 4-aminopyridine; ATP, adenosine triphosphate; BK_{Ca} channel, large conductance Ca^{2+}-activated K^+ channel; DMSO, dimethyl sulphoxide; ET, endothelin; ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; IP_3, inositol trisphosphate; K_{ATP} channel, ATP-sensitive K^+ channel; K_V channel, delayed rectifying K^+ channel; L-NOARG, L-N^G-nitroarginine; LOS, lower oesophageal sphincter, NA, noradrenaline NO, nitric oxide; NSCC, non-selective cation channel; RMCE, receptor-mediated Ca^{2+} entry; SK_{Ca} channel, small conductance Ca^{2+}-activated K^+ channel; SK&F 96365, 1-(β-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride; SOCC, store-operated Ca^{2+} channel; TEA, tetraethylammonium; TTX, tetrodotoxin

Introduction

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor composed of 21-amino acids and is one of a family of three isopeptides, namely ET-1, ET-2 and ET-3 (Yanagisawa *et al*., 1988; Inoue *et al*., 1989). The functional effects of endothelins are mediated through at least two distinct subtypes of receptors, the ET_A and ET_B receptors (Arai *et al*., 1990; Sakurai *et al*., 1990). It is generally accepted that in smooth muscle, ET-1 increases intracellular free Ca^{2+} by triggering Ca^{2+} influx and Ca^{2+} release from intracellular stores (Rubanyi & Polokoff, 1994). Voltage-dependent Ca^{2+} channels (Goto *et al*., 1989; Inoue *et al*., 1990) and voltage-independent Ca^{2+} channels (Simpson *et al*., 1990; Chen & Wagoner, 1991; Enoki *et al*., 1995; Minowa *et al*., 1997; Iwamuro *et al*., 1998; 1999) have been proposed as Ca^{2+} influx pathways.

Recently, it has been reported that ET-1 has diverse effects on vascular and non-vascular smooth muscle. Some studies indicate that ET-1 produces a transient vasodilation through the release of nitric oxide (NO) from endothelial cells (De Nucci *et al*., 1988; Higashi *et al*., 1997) or by the activation of ATP-sensitive K^+ (K_{ATP}) channels (Hasunuma *et al*., 1990; Eddahibi *et al*., 1993). It has also been reported that ET-1 can induce an endothelium-derived hyperpolarisation in rat mesenteric artery (Nakashima & Vanhoutte, 1993). In patch-

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clamp studies of smooth muscle cells isolated from guinea-pig mesenteric arterioles (Hill *et al*., 1997), it has been demonstrated that ET-1 can activate large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. In different regions of the gastrointestinal tract, ET-1 elicits a biphasic mechanical response consisting of a transient relaxation followed by sustained contraction in guinea-pig ileum (Lin & Lee, 1990; Miasiro & Paiva, 1990), rat stomach (Allcock *et al*., 1995), rat duodenum (Irie *et al*., 1995) and opossum internal anal sphincter (Chakder & Rattan, 1999). The activation of apamin-sensitive K^+ channels was suggested as the mechanism of the ET-1-induced transient relaxation in the guinea-pig ileum (Lin & Lee, 1992).

It is important to maintain a high level of tone in the lower oesophageal sphincter (LOS) to prevent gastro-oesophageal reflux; a transient relaxation of the LOS allows the passage of food and liquid into the stomach (Goyal & Paterson, 1989). Small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channels play an important role in the generation of the inhibitory junction potential evoked by intrinsic nerve stimulation in this region (Imaeda *et al*., 1998; Yuan *et al*., 1998). Since ET-1 causes both relaxation and contraction of gastrointestinal smooth muscle, ET-1 may be involved in the mechanism of the transient relaxation, or the sustained contraction, of LOS. However, the pharmacological properties of ET-1 in LOS have not been well characterized.

In the present inquiry, we have studied the effects of ET-1 on the circular smooth muscle of the guinea-pig LOS using both intracellular microelectrode recording and isometric contraction techniques. The aims of the study were to investigate the mechanisms responsible for the ET-1-induced biphasic mechanical responses and to elucidate the relationship between ET-1-induced changes in membrane potential and mechanical responses. A preliminary account of some of the data has been presented elsewhere (Imaeda *et al*., 2000).

Methods

Tissue preparation

Male guinea-pigs, weighing 250–400 g, were killed by stunning and cervical dislocation, a procedure approved by the Animal Ethics Committee of the University of Oxford. The stomach, including a portion of oesophagus, was quickly excised and placed in Krebs solution at room temperature. The oesophagus and stomach were cut open in the longitudinal direction along the greater curvature and pinned flat with the mucosal side up. The mucosa was removed with micro-scissors. Transverse strips (1–2 mm wide and 8–10 mm long) were cut from the area of the LOS, which was identified as a thickened region between the lower oesophagus and the fundus.

Intracellular recording

Preparations were immobilized by pinning them to the Sylgard covered base of a 3 ml Perspex organ bath, superfused at a rate of 3 ml min^{-1} with warmed (37°C) Krebs solution bubbled with 95% O_2–5% CO_2 to pH 7.4. The ionic composition of Krebs solution was (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 2.4, KCl 4.7, $MgCl_2$ 1.3, glucose 11.1. Ca^{2+}-free Krebs solution was prepared by simply omitting the $CaCl_2$. Conventional intracellular recording techniques were used to measure the changes in membrane potential of circular smooth muscle cells of the guinea-pig LOS. Changes in membrane potential were recorded using glass microelectrodes (outer diameter 1.5 mm, filled with 1 M KCl, resistances 80–150 MΩ) connected to the high input impedance headstage of an Axoclamp-2A (Axon Instruments) and displayed on a cathode-ray oscilloscope (2430A, Tektronix). After low-pass filtering (cut off frequency, 1 kHz), potential changes were digitized and stored on a Macintosh computer (MacLab, Chart version 3.3.3, ADI Instruments) for subsequent analysis. The criteria for an acceptable impalement of a smooth muscle cell were a rapid change in potential upon impalement and withdrawal, and a stable recording for at least 3 min prior to each experimental procedure.

Mechanical responses

In the majority of experiments, mechanical responses were recorded separately from membrane potential recordings. Each strip of LOS was tied at each end with fine polyester thread and set up in a 5 ml organ bath, containing warmed (37°C), gassed (95% O_2–5% CO_2) Krebs solution. One end of the strip was fixed at the bottom of the organ bath and the other was attached to a force transducer (TRI 201, Letica Scientific Instruments) for measurement of the isometric tension of circular smooth muscle. An initial passive tension of 9.8 mN was applied to each strip, which was then allowed to equilibrate for at least 1 h before starting experiments, with regular washings at 15 min intervals.

In some preparations, electrical and mechanical responses were recorded simultaneously. In these cases, one end of the preparation was attached to a lever of the mechano-transducer. The muscle was stretched gently (approximately 9.8 mN) and the membrane potentials of smooth muscle cells in the immobilized region measured.

Drugs

Drugs used were 4-aminopyridine (4-AP), atropine sulphate, apamin, endothelin-1 (ET-1), glibenclamide, guanethidine sulphate, L-N^G-nitroarginine (L-NOARG), nifedipine, phentolamine mesylate, propranolol hydrochloride, suramin, tetraethylammonium chloride (TEA chloride), tetrodotoxin (TTX), thapsigargin (all from Sigma), BQ123, BQ788 (RBI), sarafotoxin S6c, SK&F 96365 (1-{β-[3-(4-methoxy-phenyl)-propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride) (TOCRIS, Bristol, U.K.). Individual drugs were dissolved in distilled water or dimethyl sulphoxide (DMSO; Sigma), at concentrations at least 1000 times higher than used in the experiments and serially diluted in Krebs solution to the required final bath concentration.

Statistical analysis

Measured values were expressed as means ± s.e.mean, unless stated otherwise. The n value refers to the number of preparations. Statistical significance was determined using a paired or unpaired Student's t-test. Probabilities of less than 5% ($P<0.05$) were considered significant.

Results

Mechanical responses produced by ET-1

Smooth muscle strips of the guinea-pig LOS maintained their resting tone at 4.0±1.7 mN (mean±s.d., $n=6$) and showed oscillatory contractions, with an amplitude of 4.5±1.5 mN (mean±s.d., $n=6$) and a frequency of 0.4±0.1 Hz (mean±s.d., $n=6$) (Figure 1A). These oscillatory contractions were insensitive to TTX (1 μM; $n=5$), atropine (1 μM; $n=3$) and guanethidine (5 μM; $n=3$), suggesting that they were myogenic in origin. Nifedipine, an L-type Ca^{2+} channel blocker (1 μM) abolished them ($n=5$) and reduced the maintained tone from 3.8±0.5 mN ($n=5$) to 1.1±0.2 mN ($n=5$, $P<0.05$) (Figure 1B). ET-1 (10 nM) abolished the oscillatory contractions and induced a biphasic mechanical response composed of an initial transient relaxation (1.7±0.8 mN, mean±s.d., $n=9$) followed by a sustained contraction (9.0±2.6 mN, mean±s.d., $n=9$) (Figure 1C). The initial transient relaxation was not inhibited by TTX (1 μM; control, 1.9±0.2 mN; TTX, 2.2±0.1 mN, $n=5$, $P>0.1$), L-NOARG, an inhibitor of nitric oxide synthase (100 μM; control, 2.0±0.2 mN; L-NOARG, 2.6±0.2 mN, $n=5$, $P>0.1$) or guanethidine (5 μM; control, 2.0±0.4 mN; guanethidine, 2.0±0.2 mN, $n=3$, $P>0.1$). Nifedipine (1 μM) significantly attenuated the ET-1-induced sustained contraction (control, 8.7±1.4 mN; nifedipine, 1.0±0.2 mN, $n=5$, $P<0.05$) (Figure 1D). After 1.5–2 h of washing with normal Krebs solution, the oscillatory contraction and the ET-1-induced biphasic mechanical responses returned (data not shown).

Membrane potential changes produced by ET-1

At rest, it was noted that the membrane potential underwent spontaneous fluctuations (about ⩽5 mV, seven out of 24 preparations) at a frequency of 2.0±0.2 Hz, (mean±s.d., $n=7$). The spontaneous fluctuations were nifedipine sensitive (1 μM; $n=3$) (Figure 2A,B). As spontaneous electrical and mechanical activities were inhibited by nifedipine, it was tempting to speculate that both were related phenomena. In separate experiments, simultaneous recordings of electrical and mechanical activities showed a dissociation between these

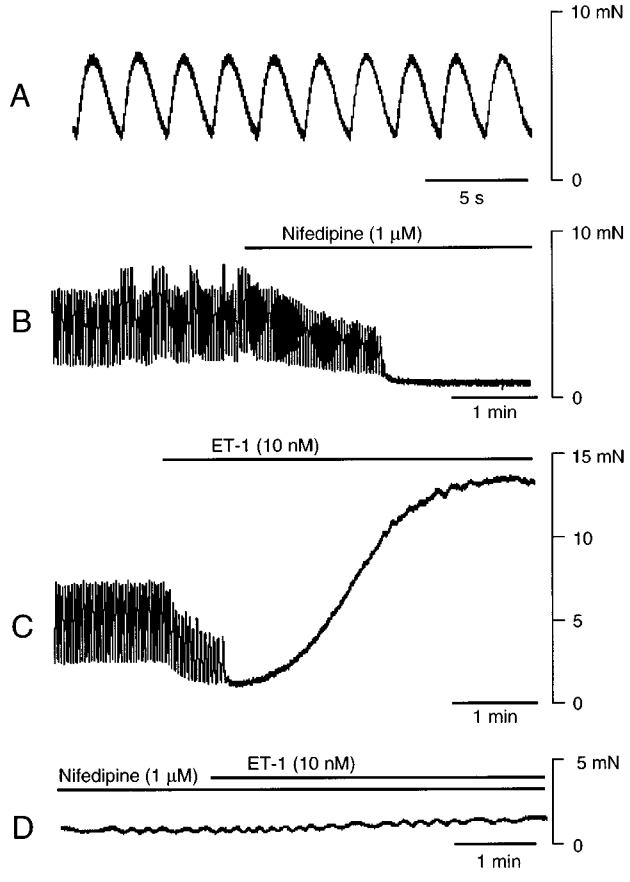

Figure 1 Effects of nifedipine and ET-1 on mechanical responses of the guinea-pig LOS circular smooth muscle. (A) Representative trace of spontaneous oscillatory contractions. The resting tone of smooth muscle was 2.8 mN. (B) Representative trace of the effect of nifedipine (1 μM) on the oscillatory contractions. The resting tone was 2.8 mN in the absence of nifedipine. The tone in the presence of nifedipine was 1.3 mN. (C) Representative trace of biphasic mechanical responses produced by ET-1 (10 nM). The resting tone was 3.1 mN. (D) Representative trace of mechanical responses produced by ET-1 (10 nM) in the presence of nifedipine (1 μM). The tone in the presence of nifedipine was 1.1 mN.

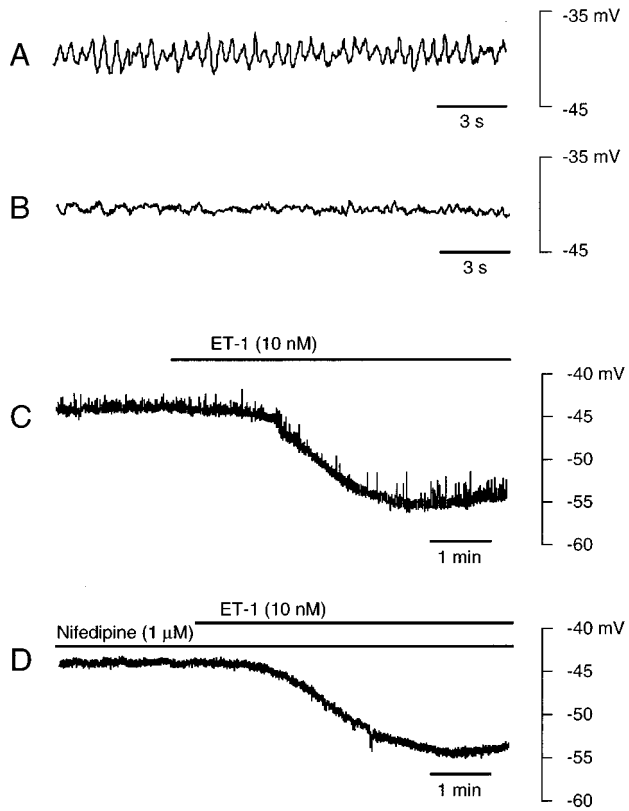

Figure 2 Effects of nifedipine and ET-1 on the membrane potential of the guinea-pig LOS circular smooth muscle. (A) Representative trace showing spontaneous irregular fluctuations in membrane potential. The resting membrane potential was −41 mV. (B) Representative trace showing quiescence of the membrane potential in the presence of nifedipine (1 μM). The resting membrane potential was −41 mV. (C) Example of the hyperpolarisation produced by ET-1 (10 nM). The resting membrane potential was −44 mV. (D) Effects of nifedipine (1 μM) on the ET-1-induced (10 nM) hyperpolarisation. The resting membrane potential was −45 mV.

two parameters ($n=4$). The control resting membrane potential ranged between -38 to -52 mV and was not significantly altered by nifedipine (control, -44.8 ± 0.5 mV, $n=24$; nifedipine, -44.2 ± 0.4 mV, $n=52$, $P>0.1$). ET-1 (10 nM) induced a sustained hyperpolarisation with an amplitude of 12.3 ± 1.1 mV ($n=6$) (Figure 2C). The amplitude of the ET-1 induced hyperpolarisation was not affected by 1 μM nifedipine (12.3 ± 0.7 mV, $n=6$, $P>0.1$) (Figure 2D). Therefore, intracellular membrane potential recordings were made in the presence of nifedipine (1 μM) to prevent contraction. The ET-1-induced hyperpolarisation was also unaffected by TTX (1 μM; $n=3$), atropine (1 μM; $n=4$), guanethidine (5 μM; $n=3$) and suramin (100 μM; $n=3$), suggesting that acetylcholine, NA and ATP respectively were not involved. L-NOARG (100 μM) did not inhibit the ET-1-induced hyperpolarisation (control, 11.8 ± 1.0 mV; L-NOARG, 11.3 ± 1.3 mV, $n=4$, $P>0.1$), suggesting that NO is not involved in the generation of the membrane hyperpolarisation either. The results in Figure 3 clearly demonstrate that ET-1 produced a concentration-dependent (1–30 nM) hyperpolarisation of the smooth muscle membrane ($n=3-9$). Interestingly, simultaneous recordings of membrane potential and mechanical responses showed that ET-1 (10 nM) hyperpolarized the membrane and produced a sustained contraction following an initial brief relaxation. The sustained contractions were increased in the repolarizing phase after ET-1 was removed ($n=4$, data not shown).

ET receptors

The effects of BQ123, a selective ET_A receptor antagonist and BQ788, a selective ET_B receptor antagonist, on membrane potential were also investigated. BQ123 (0.3 μM) did not alter the resting membrane potential (control, -45.5 ± 2.0 mV; BQ123, -45.3 ± 1.1 mV, $n=4$, $P>0.1$). Similar results were obtained with BQ788 (0.3 μM) (control, -44.8 ± 2.2 mV, BQ788, -45.3 ± 1.3 mV, $n=4$, $P>0.1$). However, BQ123 (0.3 μM) abolished the ET-1-induced hyperpolarisation (Figure 4Aa;B). Interestingly, BQ788 (0.3 μM) prolonged the time to reach the maximum hyperpolarisation (control, 3.9 ± 0.4 min; BQ788, 7.1 ± 0.6 min, $n=4$, $P<0.05$), but did not attenuate the amplitude of the hyperpolarisation (Figure 4Ab;B). Sarafotoxin S6c (10 nM), a selective ET_B receptor agonist, had no detectable effect on the resting membrane potential (control, -46.3 ± 0.3 mV; sarafotoxin S6c, -46.0 ± 0.6 mV, $n=3$, $P>0.1$).

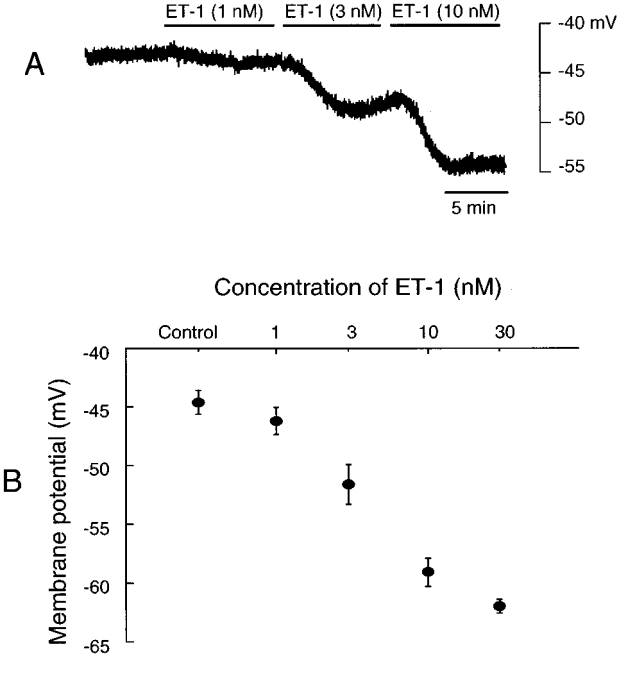

Figure 3 Concentration-dependence of the ET-1-induced hyperpolarisation. (A) Representative trace showing cumulative application of increasing concentrations (1–10 nM) of ET-1. Nifedipine (1 μM) was present throughout the recording. The resting membrane potential was -43 mV. (B) Mean membrane potential in the presence of ET-1 (1–30 nM). Values are mean$\pm$s.e.mean ($n=3-9$).

Effect of K^+ channel blockers on the ET-1-induced hyperpolarisation

Under control conditions, the amplitude of the ET-1-induced hyperpolarisation was 12.0 ± 0.8 mV ($n=6$). When apamin, an inhibitor of SK_{Ca} channel (0.1 μM) was applied alone, a small but significant membrane depolarization was recorded (control; -44.1 ± 1.1 mV, apamin; -41.0 ± 0.9 mV, $n=7$, $P<0.05$). Interestingly, apamin (0.1 μM) abolished the ET-1-induced hyperpolarisation and significantly depolarized the membrane by about 3 mV (Figure 5). The ET-1-induced hyperpolarisation was insensitive to TEA (1 mM; $n=3$), glibenclamide (10 μM; $n=3$), 4-AP (1 mM; $n=3$) and Ba^{2+} (0.3 mM; $n=3$).

Effects of apamin on ET-1-induced mechanical responses

To investigate the possible relationship between hyperpolarisation and biphasic mechanical responses, the effects of apamin (0.1 μM) on ET-1-induced mechanical responses were studied in a separate series of experiments. Apamin elevated the resting tone from 3.5 ± 0.2 to 5.4 ± 0.4 mN ($n=5$, $P<0.05$) and increased the amplitude of the spontaneous oscillatory contractions from 4.2 ± 0.3 to 7.6 ± 1.3 mN ($n=5$, $P<0.05$). In the presence of apamin, the initial relaxation was not observed ($n=5$) and the amplitude of the sustained contraction, measured from the original resting level of tone, was enhanced to about twice that of the control (Figure 6).

Role of extracellular Ca^{2+} in the ET-1-induced hyperpolarisation

ET-1 was applied in Ca^{2+}-free Krebs solution to elucidate the mechanisms involved in the activation of apamin-sensitive Ca^{2+}-activated K^+ channels. The smooth muscle membrane was depolarized in Ca^{2+}-free Krebs solution (control, -44.6 ± 1.1 mV; Ca^{2+}-free, -39.8 ± 1.5 mV, $n=5$, $P<0.05$). In Ca^{2+}-free Krebs solution, ET-1 (10 nM) caused a small hyperpolarisation, and following the re-introduction of Krebs solution containing 1.8 mM Ca^{2+}, the ET-1-induced hyperpolarisation recovered to values comparable to those elicited under control conditions (Figure 7A). These data suggest that apamin-sensitive K^+ channels are activated mainly by Ca^{2+} entry from the extracellular fluid, and partly by the release of Ca^{2+} from intracellular stores. Since the hyperpolarisation was resistant to nifedipine, the candidate pathway for Ca^{2+} influx was a Ca^{2+} channel other than the L-type. SK&F

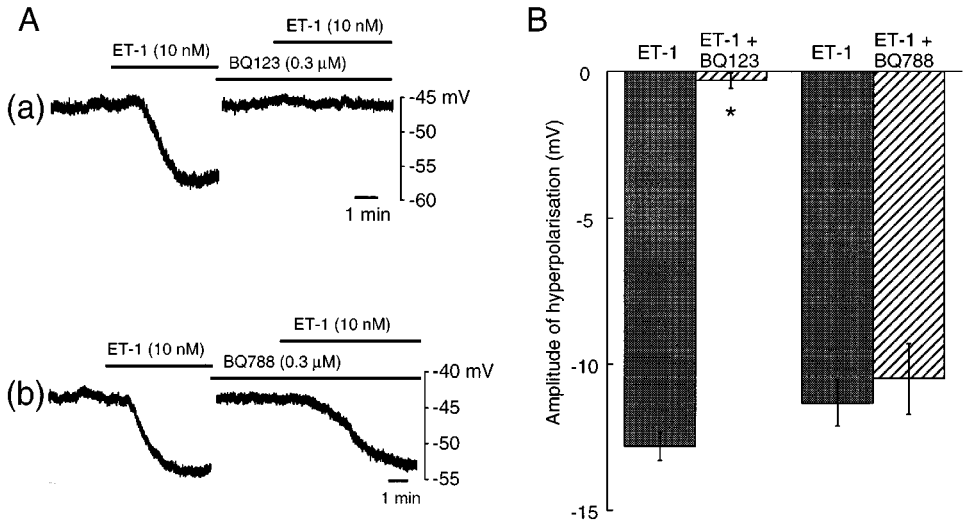

Figure 4 Effects of BQ123 and BQ788 on ET-1-induced hyperpolarisation. (A) Representative traces of the effects of (a) BQ123 (0.3 μM) and (b) BQ788 (0.3 μM) on ET-1-induced hyperpolarisation. BQ123 (a) or BQ788 (b) was applied for 10 min before ET-1 (10 nM). Nifedipine (1 μM) was present throughout the recording. The resting membrane potentials were -42 mV (a) and -43 mV (b) respectively. (B) Amplitude of the ET-1-induced hyperpolarisation in the absence and presence of BQ123 (0.3 μM) or BQ788 (0.3 μM). Values are mean$\pm$s.e.mean ($n=4$). *$P<0.05$ *vs* ET-1.

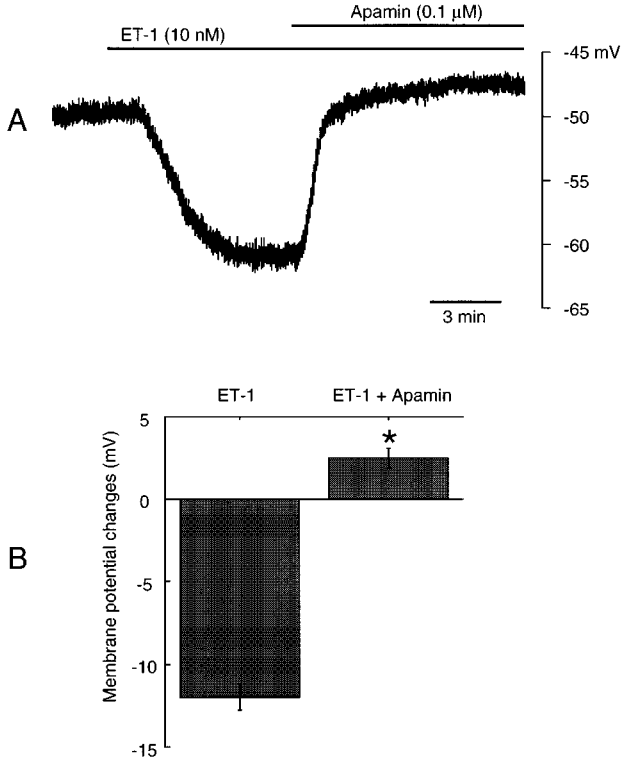

Figure 5 Effects of apamin on the ET-1-induced hyperpolarisation. (A) Representative trace of ET-1-induced hyperpolarisation and the effect of apamin on the hyperpolarisation. Apamin (0.1 μM) was applied in the presence of ET-1 (10 nM). Nifedipine (1 μM) was present throughout the recording. The initial resting membrane potential was -50 mV. (B) Each column indicates the maximum change from the resting membrane potential in the presence of ET-1 (10 nM) or ET-1 (10 nM) plus apamin (0.1 μM). Values are mean$\pm$ s.e.mean ($n=6$). *$P<0.05$ *vs* ET-1.

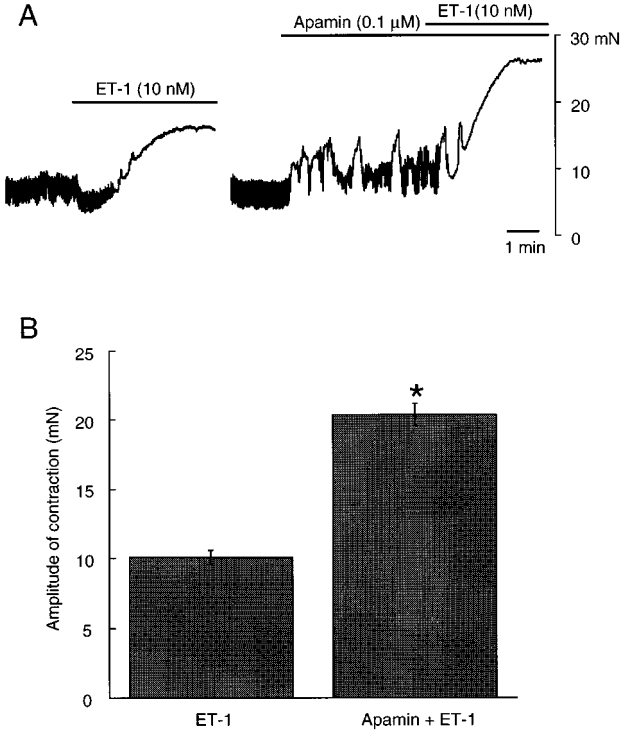

Figure 6 Effect of apamin on ET-1-induced biphasic mechanical responses of LOS. (A) Representative traces of ET-1-induced mechanical responses in the absence and presence of apamin (0.1 μM). Apamin was applied 10 min before ET-1. The resting tone was 4.6 mN. (B) ET-1-induced a sustained contraction in the absence and presence of apamin. Each column indicates the amplitude of contraction from the resting tone. Values are mean$\pm$s.e.mean ($n=5$). *$P<0.05$ *vs* ET-1.

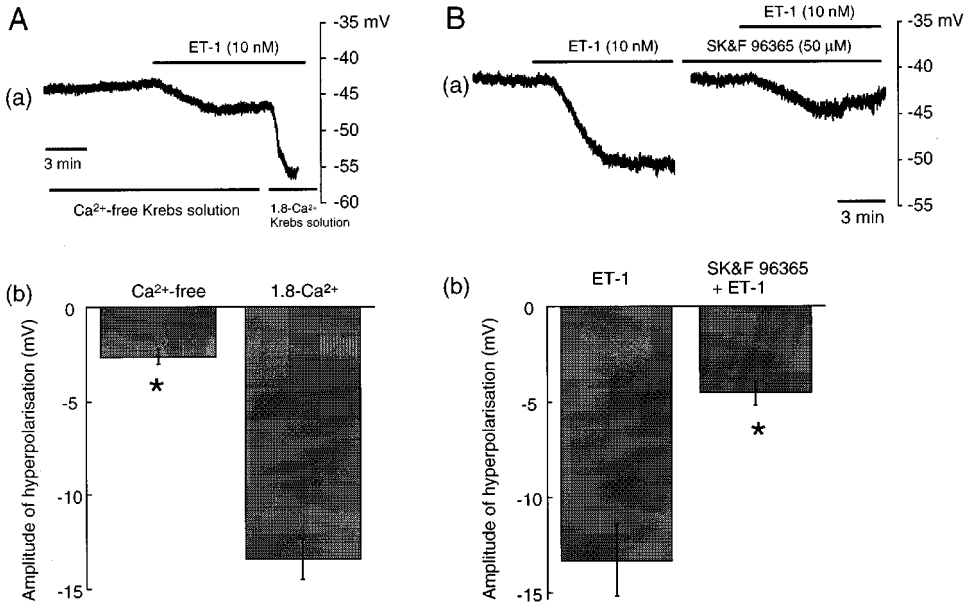

Figure 7 Role of extracellular Ca^{2+} in the ET-1-induced hyperpolarisation. (A(a)) Representative trace showing inhibition of the ET-1-induced hyperpolarisation in Ca^{2+}-free Krebs solution and its recovery following reperfusion with Krebs solution containing 1.8 mM Ca^{2+}. Nifedipine (1 μM) was present throughout the recording. The resting membrane potential was -44 mV. (b) Amplitude of the ET-1-induced hyperpolarisations in Ca^{2+}-free or 1.8 mM Ca^{2+} Krebs solution (1.8-Ca^{2+}). Values are mean$\pm$s.e.mean ($n=5$). *$P<0.05$ *vs* 1.8-Ca^{2+}. (B(a)) Representative trace of the effect of SK&F 96365 (50 μM) on ET-1-induced hyperpolarisation. SK&F 96365 (50 μM) was applied for 10 min before ET-1 (10 nM). Nifedipine (1 μM) was present throughout the recording. The resting membrane potential was -41 mV. (b) Amplitude of the ET-1-induced hyperpolarisation in the absence or presence of SK&F 96365. Values are mean$\pm$s.e.mean ($n=6$). *$P<0.05$ *vs* ET-1.

96365 (50 μM), an inhibitor of receptor-mediated Ca^{2+} entry (RMCE, Merritt *et al*., 1990) significantly attenuated the ET-1-induced hyperpolarisation (Figure 7Ba,b). This result suggests that RMCE is involved in the genesis of the ET-1-induced hyperpolarisation.

Role of intracellular Ca^{2+} in the ET-1-induced hyperpolarisation

To investigate the possible involvement of intracellular Ca^{2+} stores in the ET-1-induced hyperpolarisation, thapsigargin, an inhibitor of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (Treiman *et al*., 1998), was tested on the SK&F 96365-insensitive component of the ET-1-induced hyperpolarisation. Pretreatment with thapsigargin (1 μM) for more than 30 min significantly reduced the residual component of the ET-1-induced hyperpolarisation (Figure 8).

Discussion

The effects of ET-1 on membrane potential and mechanical responses of the circular smooth muscle cells of the guinea-pig LOS have been investigated. The results showed contrasting aspects between membrane potential changes and mechanical responses. For example, ET-1 induced an initial transient relaxation followed by a sustained contraction, actions which have been observed in other gastrointestinal smooth muscles (Lin & Lee, 1990; Miasiro & Paiva, 1990; Allcock *et al*., 1995; Irie *et al*., 1995; Chakder & Rattan, 1999). In contrast, and quite unexpectedly, ET-1 produced a sustained hyperpolarisation of the smooth muscle cells with a similar time course to the ET-1-induced sustained contraction.

Spontaneous oscillatory contractions

Under resting control conditions, the circular smooth muscle cells of the guinea-pig LOS exhibited spontaneous oscillatory contractions that were abolished by nifedipine, indicating that L-type Ca^{2+} channels are necessary to produce the oscillatory contractions. In separate experiments, some but not all LOS preparations exhibited spontaneous, irregular fluctuations in membrane potential that were abolished by nifedipine. These results suggest that spontaneous contractions of LOS are triggered by L-type Ca^{2+}-channel activated oscillatory depolarizations of the membrane. However, electrical rhythms occurred at a higher frequency than mechanical oscillations. Moreover, not all preparations exhibited spontaneous electrical oscillations in the membrane potential. The dissociation between spontaneous electrical and mechanical responses has also been confirmed in some preparations. The balance of evidence suggests that spontaneous electrical events at the membrane are not causally related to the mechanical properties of LOS. In the present study, ET-1 hyperpolarized the smooth muscle membrane and abolished the oscillatory contractions and conversely, apamin depolarized the membrane and potentiated the amplitude of the oscillatory contractions. Interestingly, the frequency of the oscillations was decreased (data not shown). These observations suggest that L-type Ca^{2+} channels are functionally deactivated by hyperpolarisation and activated by depolarization (Nelson *et al*., 1990), providing a powerful mechanism to modulate smooth muscle tone.

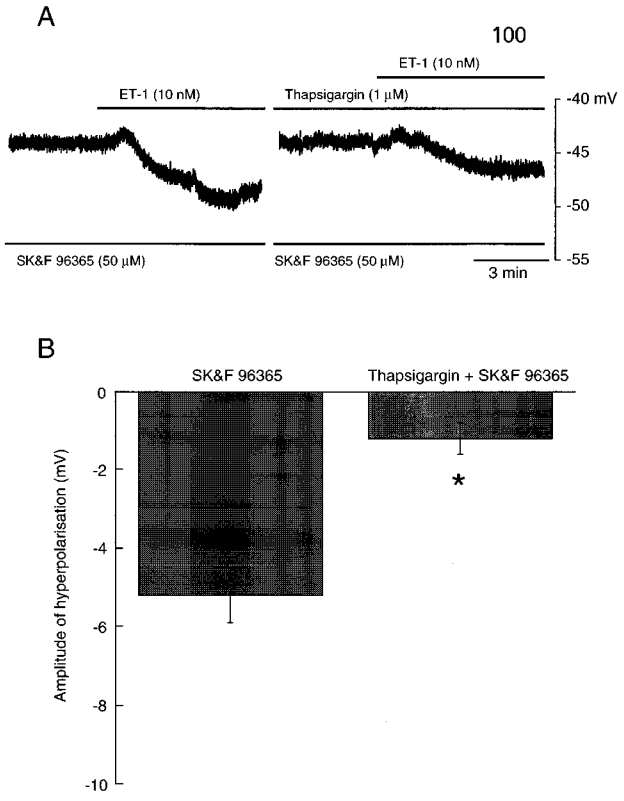

Figure 8 Role of intracellular Ca^{2+} stores in the ET-1-induced hyperpolarisation (A) Representative traces of the ET-1-induced hyperpolarisation after pretreatment of either SK&F 96365 (50 μM) or a combination of thapsigargin (1 μM) and SK&F 96365 (50 μM). Thapsigargin was applied for 30 min before ET-1. Nifedipine (1 μM) was present throughout the recording. The resting membrane potential was -43 mV. (B) Amplitude of the ET-1-induced hyperpolarisation after pretreatment with either SK&F 96365 (50 μM) or a combination of thapsigargin (1 μM) and SK&F 96365 (50 μM). Values are mean$\pm$s.e.mean ($n=4$). *$P<0.05$ *vs* SK&F 96365.

Initial relaxation

NO is a likely candidate as the mediator of ET-1-induced relaxation in the vascular smooth muscle (De Nucci *et al*., 1988; Higashi *et al*., 1997) and the opossum internal anal sphincter (Chakder & Rattan, 1999). However, the initial relaxation was insensitive to TTX, guanethidine or L-NOARG, suggesting the relaxation is not due to inhibitory neurotransmitters such as NO, NA or ATP released from nerves, nor NO release from non-neural tissues. Apamin abolished the initial relaxation suggesting that the activation of apamin-sensitive K^+ channel is a major mechanism involved in the production of the initial relaxation. These findings are consistent with previous reports on the effects of ET-1 in guinea-pig ileum (Lin & Lee, 1992) and rat duodenum (Irie *et al*., 1995).

Hyperpolarisation

The sustained hyperpolarisation induced by ET-1 was insensitive to TTX, guanethidine, suramin and L-NOARG, indicating that NA, ATP and NO, which are candidates for the neurotransmitter(s) causing inhibitory junction potentials in this tissue (Imaeda *et al*., 1998), are not involved in this hyperpolarisation. BQ123, an ET_A receptor antagonist abolished, but BQ788, an ET_B receptor antagonist did not reduce the ET-1-induced hyperpolarisation. In addition, sarafotoxin S6c, a selective ET_B receptor agonist did not change the membrane potentials. These observations indicate that ET-1 acts mainly at ET_A receptors in this tissue, and that ET-1 acts, not by triggering transmitter release from nerves but rather, by direct action at ET_A receptors located on the circular smooth muscle cells.

The ET-1-induced hyperpolarisation was reversibly abolished by apamin, an inhibitor of SK_{Ca} channel, showing that the hyperpolarisation was produced by the activation of apamin-sensitive K^+ channels. The activation of K_{ATP} channels (Hasunuma *et al*., 1990; Eddahibi *et al*., 1993), BK_{Ca} channels (Hill *et al*., 1997; Betts & Kozlowski, 2000) or delayed rectifying K^+ (K_V) channels (Betts & Kozlowski, 2000) by ET-1 has been reported in vascular smooth muscle. However, because of the ineffectiveness of TEA, glibenclamide, 4-AP or Ba^{2+}, it is probable that BK_{Ca}, K_{ATP}, K_V and inward rectifying K^+ channels are not involved to any significant extent in the generation of the ET-1-induced hyperpolarisation in the guinea-pig LOS.

ET-1 is known to elevate intracellular free Ca^{2+} concentration by release from SR and/or influx (Rubanyi & Polokoff, 1994). In the present study, removal of extracellular Ca^{2+} reduced most of the ET-1-induced hyperpolarisation, and the residual component was sensitive to thapsigargin. These observations suggest that apamin-sensitive K^+ channels were activated mainly by Ca^{2+} entry from the extracellular fluid but also partially by Ca^{2+} release from the SR. The pathways of Ca^{2+} influx due to ET-1 in smooth muscle cells are under discussion. L-type Ca^{2+} channels (Goto *et al*., 1989; Inoue *et al*., 1990) are one of the candidates. However, it is unlikely that L-type Ca^{2+} channels are responsible for the ET-1-induced hyperpolarisation in this tissue, because nifedipine did not attenuate it. SK&F 96365 was originally reported as an inhibitor of RMCE (Merritt *et al*., 1990). RMCE acts through various mechanisms, namely: (1) receptor-channel complex; (2) receptors coupled to channel *via* a G protein; (3) second messenger operated channel; (4) store-regulated entry (Rink, 1990). Receptor mediated non-selective cation channels have been identified in various kinds of smooth muscle cells. For example, non-selective cation channels (NSCCs) were activated by ATP in rabbit ear arterial smooth muscle cells (Benham & Tsien, 1987) or by muscarinic receptor activation in guinea-pig jejunal (Pacaud & Bolton, 1991) and canine gastric smooth muscle cells (Sims, 1992). ET-1 also activates NSCCs in cultured vascular smooth muscle cells (Simpson *et al*., 1990; Chen & Wagoner, 1991; Iwamuro *et al*., 1998) and also in freshly dispersed smooth muscle cells of rat aorta (Minowa *et al*., 1997) and rabbit aorta (Enoki *et al*., 1995). Store-operated Ca^{2+} channels (SOCCs) may be involved in the ET-1-induced Ca^{2+} influx. Depletion of the SR acts as the stimulus for the activation of SOCCs (Berridge, 1995; Gibson *et al*., 1998). Since the ET_A receptor is a G protein-coupled receptor, its stimulation results in increased formation of IP_3 and hence, mobilization of Ca^{2+} from the SR (Rubanyi & Polokoff, 1994). Thus, it is probable that SOCC is activated following stimulation of the ET_A receptor (Iwamuro *et al*., 1999). Recently, SK&F 96365 was shown to have suppressive effects on NSCC and SOCC

(Gibson *et al*., 1994; Wayman *et al*., 1996; Iwamuro *et al*., 1998; 1999). In the present study, the ET-1-induced hyperpolarisation was greatly attenuated by SK&F 96365, suggesting that the Ca^{2+} influx pathway is by RMCE, including NSCC and/or SOCC.

Relationship between hyperpolarisation and sustained contraction

ET-1 showed two contrasting responses with similar time courses in guinea-pig LOS, namely a sustained hyperpolarisation and a sustained contraction. Nifedipine did not inhibit the ET-1-induced hyperpolarisation, but greatly attenuated the ET-1-induced contraction in this tissue, indicating that Ca^{2+} influx through L-type Ca^{2+} channels is essential for contraction but not for hyperpolarisation. Simultaneous recording showed that ET-1-induced contraction was increased in the repolarizing phase after ET-1 was removed, supporting the necessity of L-type Ca^{2+} channels for ET-1-induced contraction. Further studies will be needed to clarify this point. However, one possible explanation is that Ca^{2+} entry by RMCE can elevate the Ca^{2+} level just under the surface membrane of the smooth muscle cells and can activate apamin-sensitive K^+ channels in the membrane, resulting in hyperpolarisation. In arterial smooth muscle cells, local increases in intracellular Ca^{2+} can trigger Ca^{2+}-activated K^+ channel activity that produces relaxation of arterial smooth muscle (Nelson *et al*., 1995). It remains unclear whether this mechanism operates in smooth muscle of the LOS. Indeed, in porcine coronary artery, it has been suggested that ET-1 has a direct action on the L-type Ca^{2+} channel (Goto *et al*., 1989).

Apamin abolished the ET-1-induced hyperpolarisation, and in so doing provided a useful tool to clarify the relationship between the two responses. In the presence of apamin, the ET-1-induced contraction increased to about twice that of the control. This finding indicates that the ET-1-induced hyperpolarisation acts to suppress the sustained contraction, possibly by reducing the activity of L-type Ca^{2+} channels.

One remaining question is whether ET-1 is produced in the LOS under physiological conditions. In the rat gastrointestinal tract (from stomach to colon), endogenous ET-1 production has been reported to occur not only in vascular endothelial cells but also in mucosal epithelial cells (Takahashi *et al*., 1990). The LOS possesses endothelial cells in intramuscular arterioles and mucosal epithelial cells, which may be possible sites for the local production of ET-1. Further investigations are required to determine whether endogenous production of ET-1 occurs in LOS. Thus, the physiological role of ET-1 in LOS remains uncertain. It is unlikely that the action of ET-1 at ET_A receptors has a major role in maintaining high tone and/or producing oscillatory contractions under physiological conditions, since BQ123 had no effect on spontaneous tone or oscillatory contractions of guinea-pig LOS (data not shown).

In conclusion, ET-1 elicits biphasic mechanical responses, composed of an initial transient relaxation followed by a sustained contraction, but paradoxically produces a concentration-dependent, sustained membrane hyperpolarisation in smooth muscle of the guinea-pig LOS. ET-1, acting mainly through ET_A receptors, produces a hyperpolarisation by activating apamin-sensitive Ca^{2+}-activated K^+ channels. K^+ channel activity is triggered mainly by extracellular Ca^{2+}, through RMCE, and partially by Ca^{2+} release from intracellular stores. The ET-1-induced hyperpolarisation triggers an initial transient relaxation and acts to oppose the subsequent sustained contraction.

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