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Electrophysiological effects of endothelin-1 and their relationship to contraction in rat renal arterial smooth muscle

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1 The electophysiological effects of endothelin-1 (ET-1) and their relationship to contraction remain unclear in the renal circulation. Using endothelium-denuded arteries from the main branch of the renal artery proximal to the kidney of the rat, we have examined its effects on tension and conducted parallel patch-clamp measurements using freshly isolated smooth muscle cells from this tissue.

2 Pharmacological experiments revealed that ET-1 produced constriction of renal arteries dependent on the influx of extracellular Ca^{2+}, mediated solely through ET_A receptor stimulation.

3 Current-clamp experiments revealed that renal arterial myocytes had a resting membrane potential of $\sim$32 mV, with the majority of cells exhibiting spontaneous transient hyperpolarizations (STHPs). Application of ET-1 produced depolarization and in those cells exhibiting STHPs, either caused their inhibition or made them occur regularly.

4 Under voltage-clamp conditions cells were observed to exhibit spontaneous transient outward currents (STOCs) inhibited by iberiotoxin. Application of voltage-ramps revealed an outward current activated at ~ -30 mV, sensitive to both 4-AP and TEA. Taken together these results suggest that renal arterial myocytes possess both delayed rectifying K^+ (K_V) and Ca^{2+}-activated K^+ (BK_{Ca}) channels.

5 Under voltage-clamp, ET-1 attenuated the outward current and reduced the magnitude and incidence of STOCs: effects mediated solely as a consequence of ET_A receptor stimulation.

6 Thus, in conclusion, activation of ET_A receptors by ET-1 causes inhibition of K_V and BK_{Ca} channel activity, which could promote and/or maintain membrane depolarization. This effect is likely to favour L-type Ca^{2+} channel activity providing an influx pathway for extracellular Ca^{2+} essential for contraction.

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Abbreviations: 4-AP, 4-aminopyridine; BK_{Ca} channel, large conductance Ca^{2+}-activated K^+ channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ET-1, endothelin-1; IbTX, iberiotoxin; IK_{Ca}, Ca^{2+}-activated K^+ current; IK_V, delayed-rectifying K^+ current; I_{out}, outward current; K_V channel, delayed-rectifying K^+ channel; PSS, physiological salt solution; SR, sarcoplasmic reticulum; STHP(s), spontaneous transient hyperpolarization(s); STOC(s), spontaneous transient outward current(s); TEA, tetraethylammonium; VSM, vascular smooth muscle

Introduction

Endothelin-1 (ET-1) is a 21 amino acid, vasoconstrictor peptide released from the vascular endothelium (Yanagisawa *et al*., 1988). It exerts its vascular effects through two mammalian receptor subtypes: ET_A and ET_B. ET_A receptors (Arai *et al*., 1990) are found on vascular smooth muscle (VSM), stimulation of these receptors induces constriction. ET_B receptors can be sub-divided into ET_{B1} and ET_{B2} receptors: ET_{B1} receptors are found on the endothelial layer and are involved in vasodilation (Warner *et al*., 1989); ET_{B2} receptors are located on VSM and mediate vasoconstriction (White *et al*., 1993). ET-1 generally induces VSM depolarization associated with a biphasic elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell contraction (see review: Haynes & Webb, 1993). Initially there is a rapid rise in $[Ca^{2+}]_i$, due to Ca^{2+} release from the sarcoplasmic reticulum (SR), followed by a fall to plateau level, which is thought to be maintained by the persistent influx of extracellular Ca^{2+} through membrane ion channels (Yanagisawa *et al*., 1988; Goto *et al*., 1989). The contribution of intracellular and extracellular Ca^{2+} sources to the ET-1-induced vasoconstrictor response varies between vessel type. Collective evidence has revealed that following activation of ET receptors a number of intracellular processes are activated, some of which involve the modulation of ion channel activity, with differential effects in different arteries (Hu *et al*., 1991; Chen & Wagoner, 1991; Miyoshi *et al*., 1992; Salter & Kozlowski, 1998).

The renal vasculature is known to play a major role in the long-term control of blood pressure, however the molecular mechanisms for controlling renal blood flow and pressure are poorly understood (see review: Navar *et al*., 1996). The renal circulation is acutely sensitive to the vasoconstrictor effects of ET-1 (Maddedu *et al*., 1990; Braun *et al*., 1998). In the rat kidney ET-1 produces sustained vasoconstriction, a dramatic decrease in renal blood flow and an increase in renal vascular resistance (Loutzenhiser *et al*., 1990; Murakawa *et al*., 1990). Indeed, ET-1 has been implicated in the regulation of the renal vasculature under physiological and pathological conditions (Simonson & Dunn, 1993). Studies in anaesthetized rats have revealed the importance of both ET_A and ET_B receptors (Cristol *et al*., 1993; Pollock & Opgenorth, 1994; Wellings *et al*., 1994) in constriction of the renal microvasculature. Recent

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research in isolated, hydronephrotic kidneys has provided evidence that ET_A receptors are important for constriction of preglomerular vessels, whilst ET_B receptors are important for mediating constriction of both preglomerular vessels and efferent arterioles (Endlich *et al*., 1996). In isolated, main branches of the renal artery, *in vitro* studies have demonstrated the presence of ET_A receptors and absence of ET_B receptors (Clark & Pierre, 1995; Devadason & Henry, 1997).

In vivo studies and the isolated, perfused kidney technique have provided a wealth of information about responses of the renal microvasculature to ET-1. However, interpretation of *in vivo* data is difficult due to the influence of multiple interactions with other mediators involved in regulation of renal haemodynamics. Furthermore, the precise mechanism of ET-1-induced renal vasoconstriction following receptor stimulation remains uncertain: L-type Ca^{2+} channels have been implicated in this process, although their role remains unclear. *In vivo*, nifedipine partially reverses the renal effects of ET-1 (Madeddu *et al*., 1990), while others have reported no antagonism (Cao & Banks, 1990). In isolated perfused kidneys, the effects of ET-1 are fully reversed by nifedipine (Loutzenhiser *et al*., 1990) and partially reversed by isradipine (Takenaka *et al*., 1993). In spite of such functional data, the electrophysiological events underlying ET-1-induced constriction throughout the renal circulation remain unknown. Moreover, there is little data describing the electrophysiological properties of VSM cells from the renal circulation. We have, therefore, undertaken an investigation into the effects of ET-1 on tension in main renal artery and correlated these findings with results of patch-clamp experiments carried out on VSM cells isolated from this tissue. The results of these studies are presented below.

Methods

Tissue isolation, tension measurement and analysis

Male Wistar rats (250–350 g) were sacrificed with an overdose of pentobarbitone (600 mg kg^{-1} body weight, i.p.). The kidneys were excised, together with the abdominal aorta and the renal arteries were dissected free of surrounding connective tissue and adventitia. Rings (internal diameter 200–500 μm; length 1–2 mm) of main renal arterial branches, proximal to the kidney, were obtained. Following dissection, two arteries were immediately mounted in a Mulvany & Halpern type Myograph (Mulvany & Halpern, 1977; JP Trading, Aarhus, Denmark). The arterial endothelium was denuded by rubbing the inner surface with thin surgical thread. Effective removal of the endothelium was assessed by the inability of 100 μM acetylcholine to relax constrictions induced by 100 μM phenylephrine. The bath (volume 10 ml) was maintained at 37±1°C and continuously aerated with 100% O_2. The effects of ET-1 (prepared as a 50 μM stock in H_2O) were examined initially in physiological salt solution (PSS) containing (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.2; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH. For Ca^{2+}-free experiments, $CaCl_2$ was replaced with equimolar $MgCl_2$ and 1 mM EGTA was added to the solution (Ca^{2+}-free PSS). Renal arterial rings were subjected to an initial tension of 100 mmHg (13.3 kPa). Isometric muscle tension was sampled and analysed by a Macintosh computer (Power Book G3) *via* a Myo-Interface (Model 500A, JP Trading, Aarhus, Denmark) and a MacLab interface (AD Instruments Pty Ltd, Castle Hill, Australia).

All data were sampled at a rate of 2 Hz and analysed using the MacLab 3.4.6 software (AD Instruments Pty Ltd, Castle Hill, Australia). Original tension, T, was normalized using the following equation:

$$T_N = (T - T_0)/(L \cdot D)$$

where T_N is the normalized force, T_0 and D are initial tension and diameter which were calculated at 100 mmHg and L is tissue wall length.

Prior to experiments being carried out, arteries were constricted by KCl (70 mM) several times to verify that the tissue was viable and to allow for tissue equilibration. All drugs were applied directly to the bath solution. Concentration response curves in the presence of antagonist were obtained from different renal arterial preparations than those used to construct control responses. The results are presented as the mean±s.e.mean. Statistical significance was evaluated using Student's *t*-test as required. P values <0.05 were considered significant.

Cell isolation, electrophysiological recordings and data analysis

In order to isolate single arterial smooth muscle cells, the small branches of the main renal artery (internal diameter approximately 200–700 μm) were subjected to an enzymatic dispersion procedure (Salter & Kozlowski, 1996), modified to include an incubation with collagenase (type VIII; 1.75 mg ml^{-1}), protease (type I; 0.1 mg ml^{-1}) and elastase (type IIA; 0.5 mg ml^{-1}) for 6 min at 37°C. Cells were stored at 4°C prior to patch-clamp experiments and remained viable for up to 10 h.

Renal arterial myocytes were subjected to perforated-patch experiments (Horn & Marty, 1988) in both current- and voltage-clamp modes of the patch-clamp configuration (Hamill *et al*., 1981). Patch pipettes were pulled from borosilicate glass capillaries, with an external diameter of 1.2 μm and internal diameter of 0.49 μm (Clark Electromedical, Pangbourne, U.K.), using a vertical puller (Narishige Ltd., Tokyo, Japan) and had resistances of 2–6 MΩ when filled with intracellular solution (in mM: KCl, 125; $MgCl_2$, 4; HEPES, 10; EGTA, 0.02; pH adjusted to 7.3 with KOH) containing 240 μg ml^{-1} amphotericin B (prepared as a 20 mg ml^{-1} stock in 100% DMSO). All experiments were performed at ambient temperature (about 25°C) and the cells were perfused with a physiological bath solution containing (in mM): NaCl, 150; KCl, 5.4; $CaCl_2$, 1.2; $MgCl_2$, 1.2; HEPES, 5; glucose, 10; pH adjusted to 7.4 with NaOH. Drugs were added at the appropriate concentrations to the bath solution as required.

An Axopatch 200A amplifier (Axon Instruments, Foster City, U.S.A.) was used to measure ionic currents. To initiate voltage-activated outward currents, cells were voltage-clamped at −50 mV and the voltage stepped to −100 mV for 100 ms before application of ramp pulses from −100 mV to +50 mV (dV dt^{-1}=1 V s^{-1}) and back to −100 mV (dV dt^{-1}=0.5 V s^{-1}) every 5 s. This protocol also allowed the background current at a holding potential of −50 mV to be continually recorded. However, to record uninterrupted background current, cells were voltage-clamped at −60, −40, −20 or 0 mV in the absence of any other voltage protocols. Voltage-activated currents were also elicited using voltage-steps: cells were voltage-clamped at −50 mV and the membrane potential was then clamped periodically at −100 mV for 1 s and stepped in increments of 10 mV up to a maximum of +30 mV. Series resistance and capacity compensation facilities were utilized where necessary. Data

were filtered at 2 kHz and digitized at 5 kHz (voltage-steps) or 3.3 kHz (voltage-ramps) using a Digidata 1200 interface (Axon Instruments), either on- or off-line following recording on a modified DAT recorder (Sony DTC-1000ES). pClamp6 software (Axon Instruments Inc.) was used for data acquisition and analysis. Data are presented as mean ± s.e.mean. Statistical significance was assessed using a Student's *t*-test. *P* values < 0.05 were considered significant.

Drugs

Acetylcholine, amphotericin B, 4-aminopyridine (4-AP), collagenase (type VIII), EGTA, elastase (type IIA) ET-1, iberiotoxin (IbTX), nicardipine, papain (papaya latex), phenylephrine, protease (type I) and tetraethylammonium (TEA) were purchased from Sigma (Poole, U.K.). BQ-123 and BQ-788 were purchased from Alexis (Nottingham, U.K.).

Results

General characterization of ET-1-induced contractile response in rat renal artery

In order to exclude any modulatory influences which endothelial ET_B receptor activation may have on the VSM response to ET-1, renal arterial rings were denuded of their endothelium. ET-1 (30 nM) evoked a large constriction which gradually increased to maximum peak tension (Figure 1a) of 7.8 ± 0.8 mN mm^{-2} with an average half-time to maximum ($T_{1/2max}$) of 72 ± 8 s ($n = 29$). Upon reaching a maximum the ET-1-induced constriction steadily relaxed in the continued presence of ET-1, with a time to half-decay from maximum ($T_{1/2decay}$) of 698 ± 260 s ($n = 29$). Cumulative application of ET-1 from 0.1 to 100 nM constricted rat renal arterial rings in a concentration-dependent manner (Figure 1b). A best fit concentration-response curve constructed from nine vessels revealed an EC_{50} of ~21 nM for ET-1 (Figure 1c). These experiments revealed that 30 nM ET-1 induced a sub-maximal response. This concentration was therefore used routinely in subsequent tension experiments. In order to determine which ET receptors were involved in the contractile response described above the effects of BQ-123 ($n = 4$), an ET_A receptor antagonist (Ihara *et al.*, 1995), were examined. At a concentration of 0.3 μM, BQ-123 reduced the response of ET-1 (not shown) while 3 μM abolished ET-1 induced constriction over the concentration range used to achieve its maximum effect under control conditions (Figure 1d). Application of 3 μM BQ-788, an ET_B antagonist (Ishikawa *et al.*, 1994), had no significant effect on the concentration response curve produced by ET-1, while sarafotoxin S6c, a selective ET_B receptor agonist (Williams *et al.*, 1991), failed to evoke any constriction of renal arterial rings between 0.1 and 300 pM. These results suggest that in arterial rings isolated from the rat main renal artery ET-1-induced constriction is due solely to ET_A receptor stimulation.

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

Pre-treatment of renal artery rings with Ca^{2+}-free PSS reduced resting tone and subsequent application of ET-1 (30 nM) evoked a maximum increase in tension equal to ~12% of the maximum tension produced by ET-1 under control conditions (Figure 2a). Renal arterial rings bathed in PSS were constricted by ET-1 until a peak response was attained. Subsequent exposure to Ca^{2+}-free PSS produced a dramatic relaxation, as seen in Figure 2b. In order to investigate whether Ca^{2+} influx through voltage-gated, L-type Ca^{2+} channels was involved in ET-1-induced constriction the effect of nicardipine (1 μM), a selective blocker of these channels, was studied. Pre-treatment

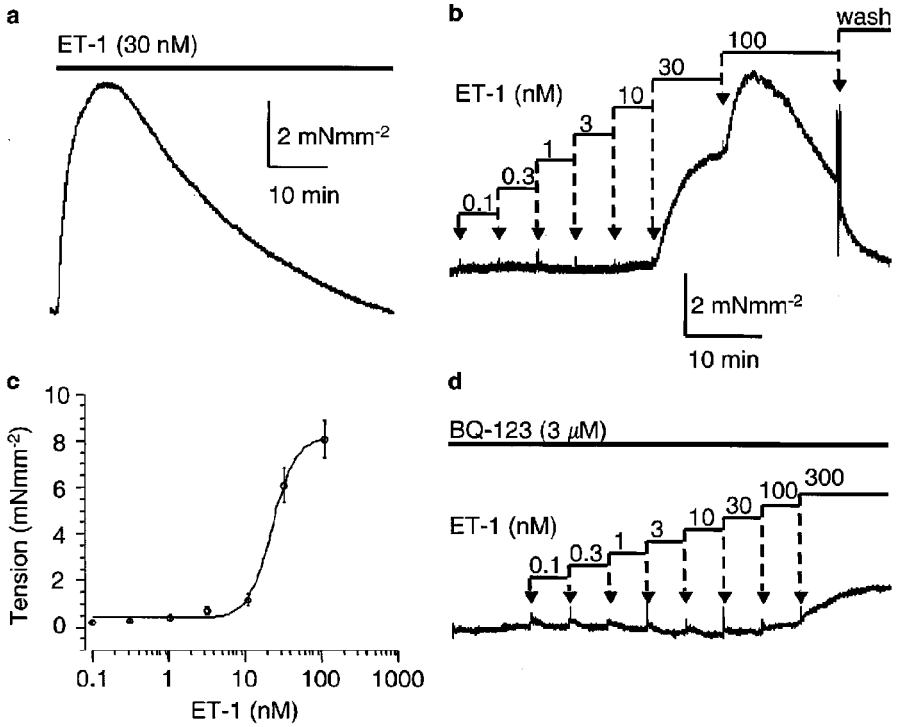

Figure 1 Contractile effects of ET-1 on endothelium-denuded renal arterial rings. (a) Typical trace illustrating constriction produced by ET-1 (30 nM). (b) Example recording of the constriction produced by cumulative application of ET-1 from 0.1 to 100 nM. (c) Mean concentration-response curve for ET-1-induced constriction ($n = 9$). (d) BQ-123 (3 μM) clearly inhibits the ET-1-induced constriction. Note, the scale bar between b and d is applicable to both.

of vessels with nicardipine produced a small relaxation of the resting tone. Subsequent application of ET-1 produced a significantly attenuated constriction ($n=6$) as seen in Figure 3a. The constriction was ~32% of that produced by ET-1 under control conditions. Figure 3b illustrates the relaxing effect of nicardipine on constriction produced by ET-1, where it significantly reduced $T_{1/2decay}$. Importantly, the magnitude of the responses in the presence of Ca^{2+}-free PSS (2.5 ± 0.2 mN mm^{-2}; $n=11$) or nicardipine (1.1 ± 0.2 mN mm^{-2}; $n=6$) were also significantly different ($P<0.05$). Taken

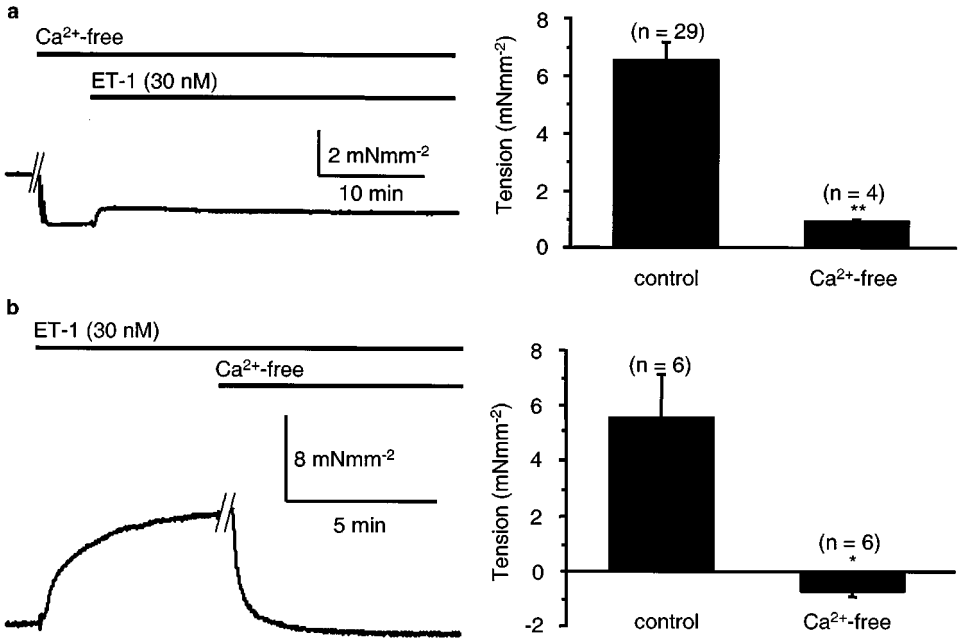

Figure 2 Contribution of extracellular Ca^{2+} to the constriction evoked by ET-1. (a) Representative trace of tension recorded when ET-1 (30 nM) was applied to arterial ring bathed in Ca^{2+}-free PSS. Summarized data reveals the attenuated constriction evoked by ET-1 in Ca^{2+}-free PSS. (b) ET-1-induced constriction is dramatically relaxed by substitution of bath solution from normal PSS to Ca^{2+}-free PSS. Summarized data indicates that ET-1-induced tension is significantly reduced in Ca^{2+}-free PSS. Perfusion artefacts were removed from the areas between the pairs of diagonal bars in each trace. Data are mean$\pm$s.e. mean. $*P<0.05$; $**P<0.01$; number of vessels is shown in parenthesis.

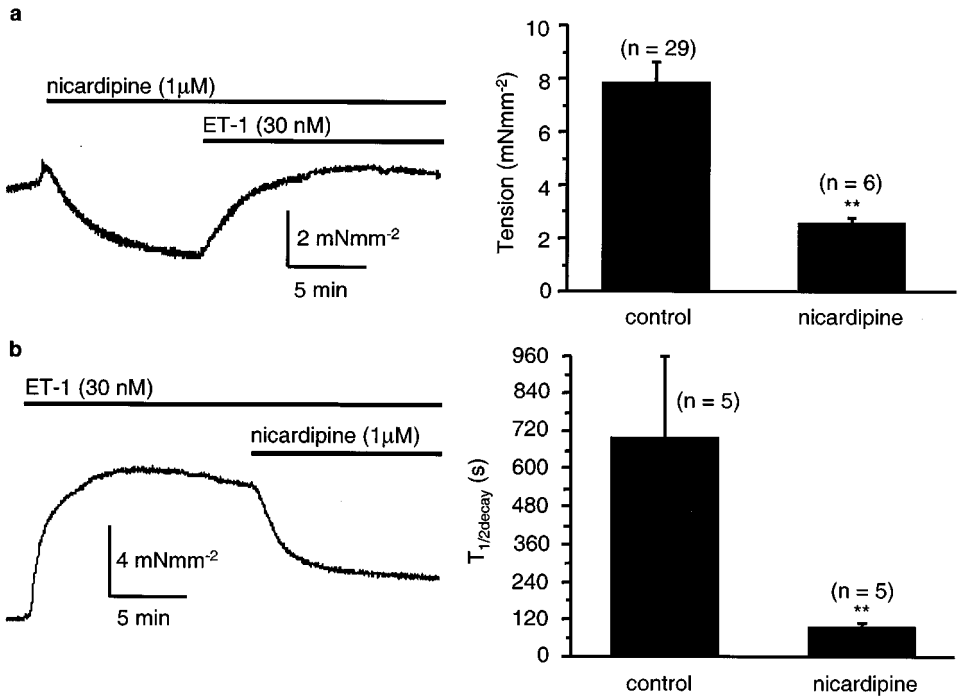

Figure 3 Effect of L-type Ca^{2+} channel antagonism on ET-1-induced constriction. (a) Pre-incubation with nicardipine (1 μM) for 10 min results in an attenuated ET-1-induced constriction. Summary of data shows significant inhibition of ET-1-induced constriction by nicardipine. (b) Application of nicardipine (1 μM) to the bath, after the peak response to ET-1 had been reached, accelerated the rate of relaxation of the response to ET-1. This can be seen as a reduction in the value for $T_{1/2decay}$ produced by nicardipine relative to control. Data are mean$\pm$s.e. mean. $**P<0.01$; number of vessels is shown in parenthesis.

together these data provide evidence that, in rat renal artery, ET-1-induced constriction is largely dependent on extracellular Ca^{2+}, with L-type Ca^{2+} channels constituting an important influx pathway, with the possibility of an additional Ca^{2+} influx pathway. In view of these data, it was important to determine whether ET-1 would induce electrophysiological effects that favour Ca^{2+} entry (for example depolarization). Thus, we undertook a characterization of the electrophysiological properties of renal arterial myocytes and studied the effects of ET-1 on them.

Characterization of renal arterial myocyte electrophysiology

Individual acutely dissociated renal arterial myocytes were maintained in the perforated-patch configuration, under a physiological cation gradient. The resting potentials of all cells used for data recording were measured in current-clamp mode at the start of each experiment. Membrane potential data was then allocated into 10 mV bins and plotted as a histogram (Figure 4a). The resting potential ranged from −57 to −10 mV and a single Gaussian distribution was fitted with a mean of -32.3 ± 10.0 mV ($n=74$). In cells where the membrane potential was examined in detail ($n=15$), visual analysis of current-clamp recordings revealed the existence of two electrophysiolgical profiles (Figure 4b). In 4 out of 15 cells, the membrane potential was quiescent. In the remaining 11 out of 15 cells, which perhaps represented a second population, the membrane potential was erratic with frequent, irregular hyperpolarizations. These spontaneous transient hyperpolarizations (STHPs) were superimposed on, what was regarded as the resting membrane potential and mirrored the activity seen during voltage-clamp experiments (in which current was recorded at holding potentials of −60, −40, −20 or 0 mV; Figure 4c). Indeed, at positive voltages, spontaneous transient outward currents (STOCs) were observed in 16 out of 26 cells. Extracellular application of IbTX (10–20 nM), a selective inhibitor of BK_{Ca} channels (Galvez *et al*., 1990), markedly reduced the incidence and magnitude of the STOCs at a potential of 0 mV ($n=3$) as shown in Figure 4c. STOCs and their corresponding STHPs are therefore likely to be the result of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activity, induced by spontaneous release of Ca^{2+} from the SR.

In current-clamp experiments, where the membrane potential was measured continually, addition of ET-1 (20 nM) to the bath solution produced sustained membrane depolarization in cells which did not show STHPs. In cells exhibiting STHPs, ET-1 induced one of two effects. In three out of six cells, a reduction in the magnitude and frequency (from 0.62 ± 0.26 to 0.07 ± 0.02 Hz over 3 min; $P<0.05$) of SHTPs occurred, resulting in a more stable membrane potential (Figure 5a). In the remaining three cells, ET-1 altered the STHP profile, approximately 1 min after application, inducing hyperpolarizations which occurred rhythmically (Figure 5b). These rhythmic, ET-1-induced hyperpolarizations were of a longer duration and more erratic than the control STHPs (see insets of Figure 5b) and gradually decreased in magnitude, ceasing after 10 min. Membrane potentials measured before and after exposure to ET-1 (between any observed STHPs), during the course of both voltage and current clamp experiments, are summarized in Figure 5c.

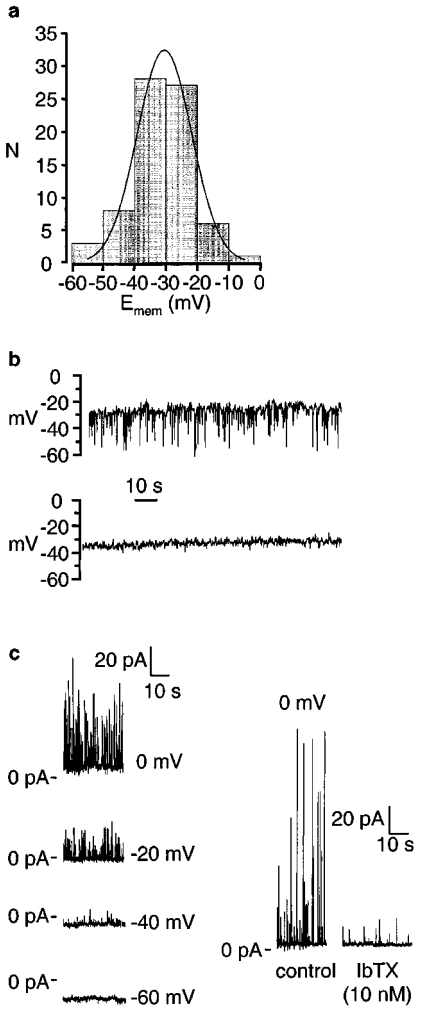

Figure 4 (a) Resting potentials from individual renal arterial myocytes, at the start of each experiment used for data recording, were allocated to 10 mV bins and the number of cells in each bin is expressed as a column. A single Gaussian distribution was fitted; *N* is the number of cells and E_{mem} is membrane potential. (b) Membrane potential recordings made in current-clamp using the perforated-patch, whole-cell method. The upper panel is an example of a cell with a mean resting potential of −30 mV which exhibits STHPs, seen as downward deflections superimposed on the resting potential. The lower panel is a recording from a cell with a stable resting potential at −35 mV. (c) Current records obtained during perforated-patch recording from a cell voltage-clamped at potentials of −60, −40, −20 and 0 mV (left panel). At depolarized holding potentials, STOCs are observed with increasing frequency and magnitude. At 0 mV, IbTX (10 nM) inhibits STOCs (right panel).

Characterization of the outward current in renal arterial myocytes

In order to determine the electrophysiological events underlying the membrane depolarization of renal arterial myocytes by ET-1, a characterization of its effects under voltage-clamp conditions was carried out. For these experiments cells were voltage-clamped at a holding potential of −50 mV and subjected to voltage-ramps or voltage-steps as described in the Methods. In voltage-ramp experiments (which allow whole-cell currents across a full voltage range to be examined during a relatively short time-course) a voltage-activated, outward current (I_{out}) was activated at a membrane potential of ~−30 mV in response to each ramp. Two distinct membrane current profiles were observed (Figure 6a). In 45 out of 78 cells, I_{out} was erratic and varied in magnitude with each ramp pulse, whilst in the remaining 33 cells the current was uniform (Figure 6a). Closer inspection of the data revealed that the erratic nature of I_{out} reflected the presence of STOCs, seen as bursts of current superimposed on I_{out}, at voltages

positive to −20 mV (Figure 6b). The erratic current profile resembled that seen under similar recording conditions in arterial myoytes isolated from rat basilar artery which was attributed to BK_{Ca} channel activity (Salter & Kozlowski, 1998). In response to voltage-steps, I_{out} increased in magnitude at depolarized potentials and showed little time-dependent inactivation during the course of the 1 s step. However in 13 out of 26 cells, I_{out} in response to voltage-steps exhibited STOCs superimposed on the steady-state current evoked at potentials positive to −10 mV (see Figure 6c).

The outward current profile evoked by the ramp and step pulses is characteristic of a K^+ current. In order to confirm this, the effects of K^+ channel blockers were studied. Typical results of an experiment with TEA, an inhibitor of BK_{Ca} channels at a concentration of 1 mM (Nelson *et al*., 1990) are illustrated in Figure 7a. Addition of TEA to the extracellular solution bathing the myocytes inhibited the erratic nature, and reduced the magnitude of I_{out}. To quantify this inhibitory effect, the mean peak I_{out} across the whole voltage-range of 3–6 successive depolarizing ramps was determined before and after the application of TEA in cells exhibiting an erratic current profile. TEA produced 21.3±5.4% inhibition of the mean peak I_{out} ($n=5$; $P<0.05$). The sensitivity of I_{out} to 4-AP, a blocker of delayed-rectifying K^+ (K_V) channels (Robertson & Nelson, 1994), was also examined (Figure 7b). Extracellular application of 4-AP (1 mM; pH adjusted to 7.4 with HCl) produced 52.6±5.4% inhibition of peak I_{out} ($n=6$; $P<0.01$). These results demonstrate that I_{out} possesses at least two components: a delayed-rectifying K^+ current (IK_V) component and an additional Ca^{2+}-activated current (IK_{Ca}) component (observed in at least 50% of cells).

Effects of ET-1 on the outward current

To investigate the mechanism of ET-1-induced membrane depolarization, ET-1 was applied to cells under voltage-clamp.

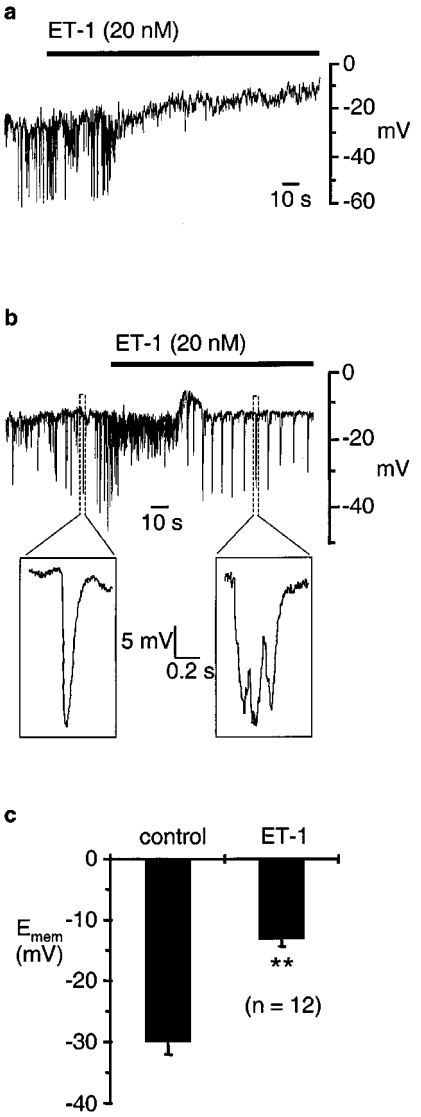

Figure 5 Effects of ET-1 on membrane potential recording in current-clamp. (a) Membrane potential recording from a cell showing STHPs superimposed on a resting potential of ~−28 mV. ET-1 induces a gradual depolarization and the STHPs cease. (b) Membrane potential recording from another cell exhibiting STHPs. Treatment with ET-1 results in rhythmic STHPs (illustrated on a faster time base in insets) which gradually decrease in magnitude. (c) Membrane potential (E_{mem}), measured at the start of each experiment and following exposure to ET-1 are summarized. Data are mean± s.e.mean. $^{**}P<0.01$; number of cells is shown in parenthesis.

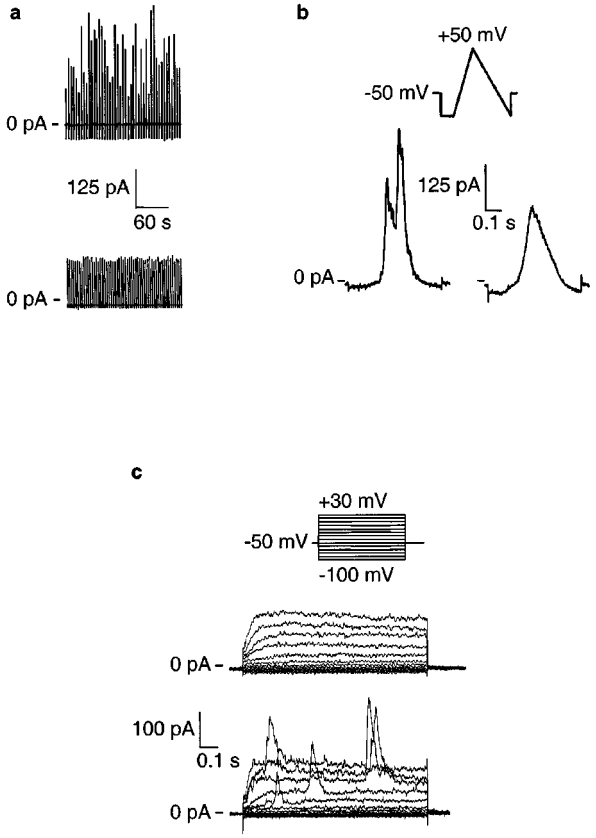

Figure 6 Current records obtained during perforated-patch recording under voltage-clamp conditions. (a) Typical current records obtained in response to ramp pulses recorded continuously in the absence of any drug additions. Vertical deflections on these traces (and all subsequent records of this nature) represent currents evoked in response to ramp pulses from −100 to +50 mV and back again. The upper trace is an example of a cell in which ramp pulses evoked currents of an erratic nature and varying magnitude. The lower trace is an example from a cell exhibiting stable I_{out} in response to each voltage-ramp. (b) An example of the voltage-ramp pulse (see Methods) and two typical control currents evoked in response to a single ramp pulse: erratic (left) and stable (right). (c) Whole-cell currents evoked by voltage-step pulses (upper panel; see Methods). The middle panel is an example of a trace in which voltage-steps evoked a stable I_{out}, which increases in magnitude at depolarized potentials, and exhibits little time-dependent inactivation. The lower panel is a representative trace from a cell in which STOCs, believed to reflect BK_{Ca} activity, are superimposed on the underlying, stable I_{out} induced at the more depolarized potentials.

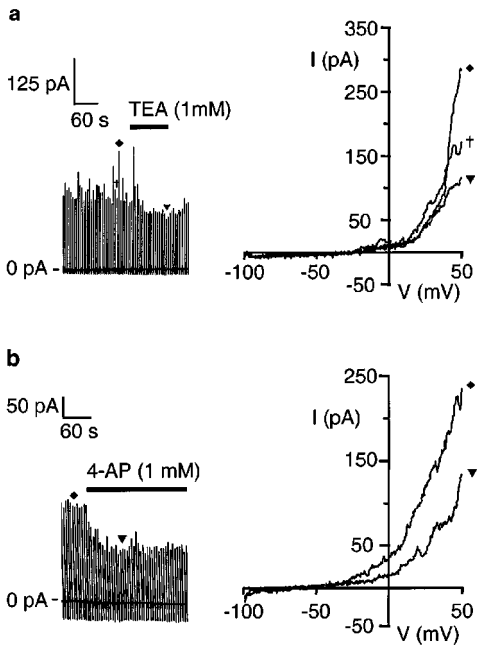

Figure 7 Effects of K^+ channel blockers on whole-cell currents recorded from renal arterial myocytes. (a) Inhibitory effect of TEA (1 mM) on I_{out} evoked in response to ramp pulses. Current evoked by the depolarizing ramp from −100 to +50 mV, at the points indicated on the current record shown was plotted as current (I) against voltage (V). Under control conditions (diamond, during a STOC containing ramp, and cross) and after exposure to TEA for 1 min (triangle). (b) Application of 4-AP (1 mM) causes a marked inhibition of I_{out} seen in response to voltage-ramps. Current-voltage (I/V) relationships from the depolarizing ramp obtained under control conditions (diamond) and 1 min after the application of 4-AP (triangle), indicated on the current record shown.

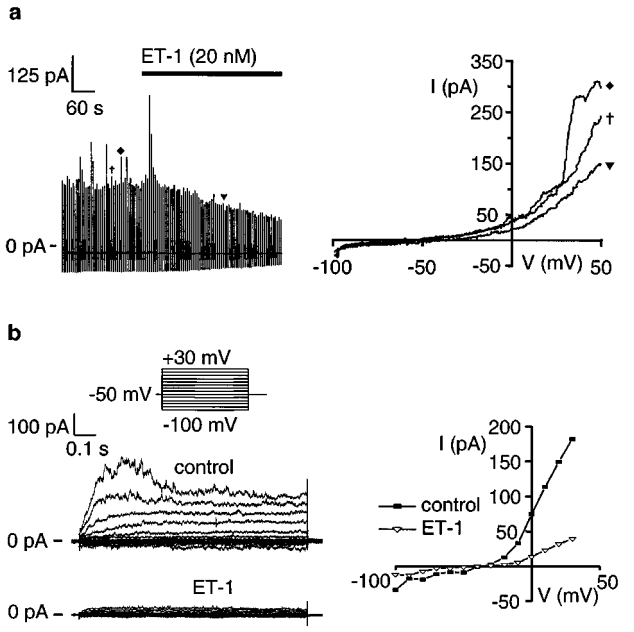

Figure 8 Effects of ET-1 on whole-cell currents. (a) Typical current record from a cell held at −50 mV and subjected to ramp pulses. ET-1 produces a slowly developing inhibition of I_{out} seen in response to each ramp. Current-voltage (I/V) relationships in response to depolarizing ramp pulses obtained at the points indicated on the current record under control conditions during a non-STOC containing current (cross), STOC-containing current (diamond) and following 3 min exposure to ET-1 (triangle). (b) The inhibitory effect of ET-1 on I_{out} was also seen during voltage-step stimulation (upper panel) of membrane currents. ET-1 produced a marked reduction of I_{out} evoked at more depolarized voltage-steps (lower panel). The mean current (I) recorded during the final 100 ms (approx.) of each voltage-step was plotted against voltage (V).

Figure 8a illustrates the effects of ET-1 on whole-cell current activated by voltage-ramps. In STOC-exhibiting cells, ET-1 reduced the incidence of the erratic, large magnitude peaks of current and induced a slowly developing, but marked inhibition of I_{out} at +50 mV of 66.9±5.3% ($n=5$; $P<0.05$). In cells which did not exhibit STOCs an inhibitory effect of 51.7±7.6% ($n=3$; $P<0.05$) was observed. Shown in Figure 8b are the effects of ET-1 on whole-cell currents evoked by voltage-steps. Following analysis of the final 100 ms (approx.) of the steady-state current in response to each voltage-step and construction of an I/V relationship (Figure 8b) it was found that ET-1 inhibited the steady state current at +30 mV by 64.1±4.2% ($n=11$; $P<0.01$). In conclusion, the electrophysiological effects of ET-1 appear to include an inhibitory effect on IK_V and IK_{Ca}.

Pharmacology of renal arterial ET-1 receptors

In order to determine whether ET_A and/or ET_B receptors were responsible for the electrophysiological effects described above, pharmacological experiments using ET receptor antagonists were performed. The electrophysiological effects of ET-1 were prevented by BQ-123 (0.3 μM; $n=5$), as shown in Figure 9a. In contrast, BQ-788 (0.3 μM; $n=3$) did not affect the response to ET-1 (Figure 9b). The results of experiments using BQ-123 and BQ-788 are quantified in Figure 9c. Membrane potential measurements made following exposure to ET-1 in the presence of BQ-123 were not significantly different from control values at the start of each experiment: −46.9±6.8 and −46.2 3±5.5 mV respectively ($n=5$). These results provide evidence that ET_A receptor stimulation by ET-1 is associated with membrane depolarization through inhibition of IK_V and IK_{Ca}. This action is likely to favour L-type Ca^{2+} channel activity allowing the Ca^{2+} influx necessary for contraction.

Discussion

In this study we have examined the consequences of ET receptor stimulation in smooth muscle of rat main renal arteries, proximal to the kidney, using myography and the patch-clamp recording technique. We have for the first time made electrophysiological recordings from myocytes acutely dissociated from these arteries and described the electrophysiological effects of ET receptor stimulation in these cells.

ET-1 induced constriction of rat, endothelium-denuded renal arterial rings in a concentration-dependent manner: an effect inhibited by BQ-123 and unaffected by BQ-788 suggesting that ET_A receptors are involved in this process (in agreement with results from other studies; Clark & Pierre, 1995; Devadason & Henry, 1997). Pre-treatment with Ca^{2+}-free PSS or subsequent exposure to Ca^{2+}-free PSS resulted in a dramatic relaxation of ET-1-induced tension. L-type Ca^{2+} channel blockade also inhibited ET-1-induced constriction. These observations indicate that ET-1 induced vasoconstriction of branches of the main renal artery, proximal to the kidney is dependent upon influx of extracellular Ca^{2+}, mainly through L-type Ca^{2+} channels. This contrasts with the lack of antagonism by Ca^{2+} channel blockers *in vivo* (Cao & Banks, 1990; Maddedu *et al.*, 1990) and supports the notion that

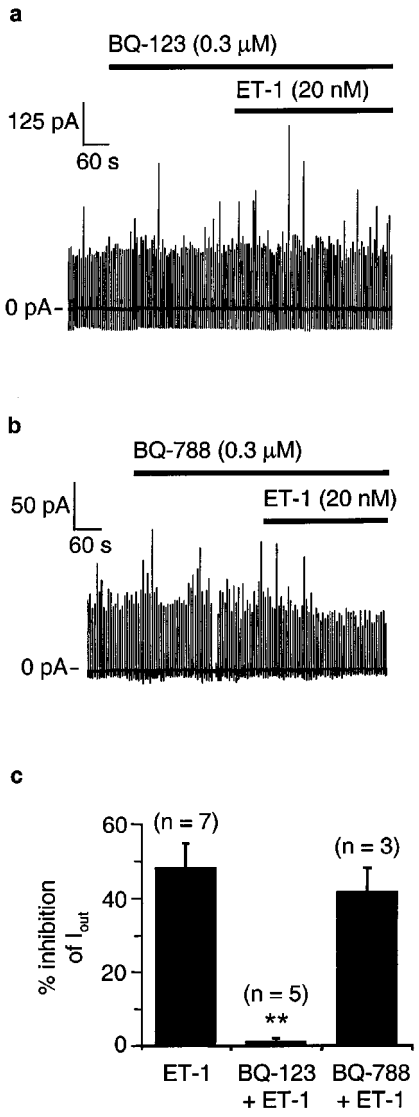

Figure 9 Effects of ET-1 on whole-cell currents recorded in the presence of ET receptor antagonists. (a) BQ-123 (0.3 μM) inhibits the electrophysiological effects of ET-1. (b) Electrophysiological effects of ET-1 are observed in the presence of BQ-788 (0.3 μM). (c) Bar graph showing mean $\pm$ s.e. mean inhibition of I_{out} by ET-1 alone and ET-1 in the presence of BQ-123 or BQ-788. $^{**}P<0.01$; number of cells is shown in parenthesis.

different regions of the renal circulation have a differential sensitivity to Ca^{2+} channel blockers and dependence on extracellular Ca^{2+} for contraction (see Loutzenhiser *et al.*, 1990; Takenaka *et al.*, 1993). Interestingly, it appears that a small residual contraction can be mediated in the absence of Ca^{2+} influx, which may be mediated through Ca^{2+} release from IP_3-sensitive stores (ET_A receptor stimulation is known to promote Ca^{2+} release from the SR: Kasuya *et al.*, 1989; Little *et al.*, 1992; Xuan *et al.*, 1994). Alternatively, these results do not exclude the possibility of a novel Ca^{2+} influx pathway involved in constriction.

From the contraction data presented above it was concluded that activation of L-type Ca^{2+} channels occurs in the presence of ET-1. This process is associated with depolarization (Nelson *et al.*, 1990) which can be produced by a decrease in K^+ channel activity (Nelson & Quayle, 1995). Consequently, a mechanism of depolarization must exist in renal arterial myocytes to maintain L-type Ca^{2+} channel activity following ET receptor stimulation (as shown in other types of vascular myocyte; van Renterghem *et al.*, 1988; Klöckner & Isenberg, 1991). Thus, prior to examining the electophysiological effects of ET-1, we undertook a characterization of the electrical properties of renal arterial myocytes. Under voltage-clamp conditions, I_{out} appears to be composed of two distinct, macroscopic K^+ currents. One component of I_{out} evoked by voltage-ramps at depolarized potentials was STOC-like, inhibited by TEA and therefore attributed to BK_{Ca} channel activity (in agreement with the IbTX sensitivity of STOCs observed at positive holding potentials). A second component of I_{out} was voltage-activated, sensitive to 4-AP and therefore believed to reflect K_V channel involvement. This K^+ channel profile indicates that subtle differences in K^+ channel expression exist in myocytes isolated from different regions of the renal circulation (Gordienko *et al.*, 1994; Gebremedhin *et al.*, 1996; Martens & Gelband, 1996).

Freshly isolated renal arterial myocytes had a mean resting potential of -32 mV close to the activation threshold for L-type Ca^{2+} channels (see Nelson *et al.*, 1990): a value which corresponds with findings using pressurized rat renal arteries (-40 mV; Loutzenhiser *et al.*, 1997). Under current-clamp, the majority of cells exhibited STHPs due to spontaneous activity of BK_{Ca} channels. These electrophysiological events, associated with sporadic release of Ca^{2+} from the SR (Benham & Bolton, 1986), have a hyperpolarizing influence and can promote inhibition of L-type Ca^{2+} channel activity, with profound effects on vascular tone (Brayden & Nelson, 1992). ET-1 either reduced STHP frequency and magnitude or induced a rhythmic STHP profile. In both cases depolarization was observed: an effect which would serve to maintain L-type Ca^{2+} channel activity.

Pharmacological studies revealed that ET_A, but not ET_B, receptor antagonism prevented the electrophysiological effects produced by ET-1 in agreement with the finding that ET_A receptors mediate contraction. In order to examine the mechanism of ET-1 induced depolarization, its effects on the ramp- and step-induced currents were studied. ET-1 produced a reduction in I_{out} and a decrease in the erratic nature of the current (attributed to inhibition of IK_V and IK_{Ca}, respectively). On the basis of these observations it appears that inhibition of either or both IK_V and IK_{Ca} represents a mechanism for depolarization. We have previously provided evidence that ET-1 induced inhibition of IK_V in aorta, pulmonary artery and vein and inhibition of IK_{Ca} in basilar artery are possible mechanisms for inducing depolarization (Salter & Kozlowski, 1996, 1998). Thus it seems likely that inhibition of K_V and BK_{Ca} channels by ET-1 may represent a generic mechanism for maintaining, and perhaps promoting, depolarization thereby allowing Ca^{2+} influx and constriction. Both the physiological role of ET-1 induced rhythmic STHPs and the mechanism underlying their generation is uncertain. However, it is tempting to speculate that they are the product of changes in the activity of BK_{Ca} channels associated with complex patterns of Ca^{2+} release from the SR. Alternatively, their presence may be indicative of two populations of smooth muscle cell which respond in subtly different ways to ET_A receptor stimulation.

In conclusion, our electrophysiological data suggest that ET-1 inhibits BK_{Ca} and K_V channel activity and produces membrane depolarization through ET_A receptor stimulation in renal myoctes from main arteries, proximal to the kidney. These effects would serve to promote Ca^{2+} influx through L-type Ca^{2+} channels thereby inducing and/or maintaining constriction.

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