Inward current oscillation underlying tonic contraction caused via ET\textsubscript{A} receptors in pig detrusor smooth muscle

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\textsuperscript{1}Department of Pharmacology, University of Oxford, Oxford OX1 3QZ, United Kingdom; \textsuperscript{2}Department of Cell Physiology, Nagoya University Graduate School of Medicine, Nagoya 466-8550; and \textsuperscript{3}Special Care Unit of Dentistry, Faculty of Dentistry, Kyushu University, Fukuoka 812-0012, Japan

Submitted 3 October 2002; accepted in final form 28 August 2003

Kajioka, Shunichi, Shinsuke Nakayama, Rachel McCoy, Gordon McMurray, Kihachiro Abe, and Alison F Brading. Inward current oscillation underlying tonic contraction caused via ET\textsubscript{A} receptors in pig detrusor smooth muscle. Am J Physiol Renal Physiol 286: F77–F85, 2004; 10.1152/ajprenal.00355.2002.—Endothelin-1 (ET-1) is a powerful vasoconstricting peptide. Recent studies showed synthesis of ET-1 and the presence of ET receptors in urinary bladder smooth muscle cells. In the present study, we investigated the possible role of ET-1 in detrusor contraction and its underlying mechanisms in terms of electrical activity. ET-1 caused dose-dependent tonic contraction of bladder smooth muscle strips. Whole cell patch-clamp experiments revealed that ET-1 induced a single transient inward current in the majority of detrusor cells and that additional inward current oscillations were induced in one-third of the cells. The inward current oscillation and tonic contraction shared several characteristic features: 1) both activities lasted for a considerable time after ET-1 washout and 2) only prior application of ET\textsubscript{A} receptor antagonists, not ET\textsubscript{B} receptor antagonists, significantly suppressed ET-1-induced contractions and the oscillating inward currents. It was concluded that the inward current oscillation underlies ET-1-induced tonic contraction. Experiments with ion substitution and channel blockers suggested that periodic activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels caused the oscillating inward currents.

endothelin receptors; urinary bladder

THE POWERFUL VASOCONSTRICTOR actions of endothelin (ET) are well established; however, ET is also known to induce contraction in many nonvascular smooth muscle cells, including the urinary bladder (23, 32). Recently, Tajeda et al. (34) demonstrated the synthesis of ET-1 by epithelial, fibroblast, and muscle cells within the urinary bladder, suggesting a possible role for ET as an autocrine hormone in the regulation of bladder wall structure and smooth muscle tone, and potentially even for paracrine effect in regulating or facilitating the urinary bladder (23, 32). Recently, Tajeda et al. (34) showed that ET A receptors mediate the mechanical response via ETA receptors and that Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels are probably responsible for the oscillating current itself. Also, Ca\textsuperscript{2+} influx through nifedipine-insensitive cation channels seems to play an important role in pacemaking.

METHODS

Preparation of isolated detrusor cells. Porcine urinary bladders were obtained from a local abattoir, and the tissue was transported to the laboratory in cold (4°C) physiological saline solution. The isolated detrusor cells were prepared as described previously (17). After the urethelium was removed, bladder smooth muscle samples were dissected into small pieces (~1–2-mm cube) with the aid of a dissecting microscope. Samples were then incubated in a nominally Ca\textsuperscript{2+}-free solution containing 0.1% BSA. Finally, after being washed with Ca\textsuperscript{2+}-free solution, the digested pieces were placed in a test tube and single cells were yielded through gentle agitation. The single cells obtained were kept in a low-Ca\textsuperscript{2+} (0.5 mM) solution with 0.5% BSA and stored at 5°C. All experiments were performed within 3 h after cell isolation.

Membrane current recording. Whole cell membrane currents were recorded using a standard patch-clamp technique, as described previously (17, 33). A patch-clamp amplifier (EPC-7, List, Germany) was operated through a Macintosh computer equipped with an AD/DA converter (ITC-16, INSTRUTECH). The resistance of the patch pipette was 3–4 MΩ, when a Cs\textsuperscript{+}-rich pipette solution was used. After rupture of the cell membrane, the series resistance was normally less than 10 MΩ. Pig smooth muscle cells used in this investigation had a mean membrane capacitance of 62 ± 12 pF (n = 25). The capacitive surge was electrically compensated, and a cut-off frequency of 10 kHz was applied to reduce noise. Unless otherwise it is intriguing to explore the mechanisms underlying ET-1-induced detrusor contractions. Furthermore, bladder ET receptor expression and activities are known to change under pathological conditions, such as urethral obstruction (20–22), diabetes (5, 26, 31), and ageing (35). Therefore, it is possible that ET receptors and/or their downstream intracellular mechanisms may become a target for pharmacological intervention in the treatment of urinary tract disorders and incontinence.

To address the receptor subtype and ion channels responsible for ET-1-induced urinary bladder contraction, and because the pig provides a good model for human micturition (17), we carried out whole cell patch-clamp experiments in isolated pig detrusor. We found that inward current oscillation is induced via ET\textsubscript{A} receptors and that Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels are probably responsible for the oscillating current itself. Also, Ca\textsuperscript{2+} influx through nifedipine-insensitive cation channels seems to play an important role in pacemaking.

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increasing the ET-1 concentration up to 30 nM. Tonic contractions, and this concentration of ET-1 was nor-
cation of 10 nM ET-1 produced comparable or rather larger
an index compared with normal detrusor contraction.) Appli-
cation with the magnitude of CCh-induced contraction provides
tonic contraction in pig detrusor muscle strips. The normaliza-
tion of the ET-1-induced contraction was estimated to be 106 nM using a commercial software
EQCAL system (ChemCAD, Obernai, France). With the “normal”
extracellular and pipette solutions, the equilibrium potential for Cl-
ions calculated is −0.5 mV (at 20°C).

Solutions and chemicals. The “normal” extracellular solution had the following composition (in mM): 140 NaCl, 6 KCl, 2.4 CaCl₂, 12 glucose, and 5 HEPES; pH was adjusted to 7.4 with Tris base. Isosmotic substitution was used for all changes in the ionic composition of the extracellular medium, including solutions used for cell isolation. In the Ca²⁺-free, Mg²⁺-free solution used for cell isolation, the osmotic pressure was also adjusted by modifying the NaCl concentration. The composition of the “normal” pipette solution was (in mM) 144 CsCl, 2 MgCl₂, 0.025 CaCl₂, 0.05 EGTA, 2 ATP, and 10 HEPES/Tris (pH 7.2). The free Ca²⁺ concentration in the pipette solution was estimated to be 106 nM using a commercial software
EQCAL system (ChemCAD, Obernai, France). With the “normal” extracellular and pipette solutions, the equilibrium potential for Cl⁻ ions calculated is −0.5 mV (at 20°C).

The following chemicals, drugs, and enzymes were used in the present study: ATP (disodium salt), N-methyl-D-glucamine (NMDG), nifedipine, niflumic acid, disodium 4,4’-disothiocyanatostilbene-2,2’-disulphonic acid (DIDS), ryanodine, caffeine, EGTA (free acid), trypsin inhibitor (type 1-S), collagenase (type H), papain, and carbamyl choline chloride (carbachol, CCh) from Sigma (St. Louis, MO); and ET-1, BQ-123, BQ-788, and sarafotoxin S6c from Calbiochem (San Diego, CA).

Tension development. Fine strips (1.5–2 mm in length, 0.5–1 mm in diameter) were prepared as previously described (4, 10), transferred to a small chamber (0.2 ml in volume), and attached to a Dynamometer UFI transducer (Harvard Apparatus, Canterbury, UK) for isometric tension measurement at 35–37°C. The strips were strained by applying a 1-g (i.e., 10 mN) weight and allowed to equilibrate for 1 h before the start of the experiments. Drugs were administrated in the desired concentration in the superfusing solution (1.4 ml/min). The data were stored on DAT tapes with a digital audio tape deck through MacLab/8E system (ADInstrument, Chalgrove, UK) coupled with a Macintosh computer.

Statistics. Numerical data are expressed as means ± SD. The averaged amplitude of the inward current oscillation was calculated from the peaks of four to six currents when the amplitude of the oscillations had become stable. When differences between means were evaluated by ANOVA, a P value of < 0.05 was taken as a statistically significant difference.

RESULTS

ET-1-induced pig detrusor contraction. Application of ET-1 induced tonic contraction in pig detrusor strips. The ET-1-induced contraction reached a plateau level in 10 min and slowly returned to the baseline level after washout of ET-1. As shown in Fig. 1A, 1 μM CCh was applied to obtain a control contraction, and subsequently ET-1 was cumulatively applied to examine the dose dependency of the contractile response. In Fig. 1B, the magnitude of the ET-1-induced contraction was expressed relative to that induced by 1 μM CCh. (In both human and pig urinary bladder, it is known that cholinergic innervation plays a dominant role in voiding urine. One micromole of CCh normally produces ~80% of the maximal tonic contraction in pig detrusor muscle strips. The normalization with the magnitude of CCh-induced contraction provides an index compared with normal detrusor contraction.) Application of 10 nM ET-1 produced comparable or rather larger tonic contractions, and this concentration of ET-1 was normally used. The magnitude of the contraction increased by increasing the ET-1 concentration up to 30 nM.

Previous reports showed that two subtypes are recognized in smooth muscle, which are ET₁ and ET₂ (2, 14, 16). The effects of BQ-123 and BQ-788 were examined to determine which ET receptor subtype was responsible for the contractile response. As shown in Fig. 2A, in the presence of 1 μM BQ-123, a selective antagonist for ET₁ receptors, 10 nM ET-1 failed to induce a contraction. After washout of BQ-123 for 60 min, subsequent application of 10 nM ET-1 caused a tonic contraction [0.93 ± 0.02 (n = 6) vs. 1 μM CCh-induced contraction]. It is interesting to note that after the contraction was induced by ET-1, additional application of BQ-123 had little effect on the sustained contraction (Fig. 2B). On the other hand, BQ-788, an ET₂ receptor antagonist, affected neither resting tone nor ET-1-induced contraction even when preceding ET-1 application (Fig. 2C). Furthermore, we examined a potent ET₂ agonist, sarafotoxin S6c. Application of 10 nM sarafotoxin S6c induced a transient contraction of much smaller amplitude (0.14 ± 0.06 of 1 μM CCh-induced contraction, n = 6).

Niflumic acid is known to block Ca²⁺-activated Cl⁻ (ClCa) channels (12). As described later, the patch-clamp experiments

Fig. 1. Contraction induced by endothelin-1 (ET-1) in the pig detrusor strips. Aa: tonic contraction was induced by application of 10 nM ET-1 for ~15 min. Note that contraction of significant magnitude persisted for some time after washout of ET-1. Ab: tension recording shows a typical contractile response to cumulative application of ET-1 (0.1–10 nM). One micromolar carbachol (CCh) was applied to obtain a control tonic contraction. B: dose-response relationship of ET-1-induced contraction (cumulative application). The amplitude of the ET-1-induced contraction is expressed relative to that induced by 1 μM CCh. Each column stands for the means ± SD (n = 18).
in the present study suggested an important role of Cl\textsubscript{Ca} channels in the excitation-contraction (E-C) coupling of pig detrusor contraction via ET\textsubscript{A} receptors. We therefore examined the effect of niflumic acid on ET-1-induced contraction. As shown in Fig. 3A, after 10 nM ET-1-induced contraction had reached a plateau, niflumic acid was cumulatively applied. During application of 300 \mu M niflumic acid, ET-1-induced contraction was decreased to 0.16 ± 0.03 of the control (n = 5; Fig. 3B).

**Effects of ET-1 and ET antagonists on whole cell membrane current.** With the use of the conventional whole cell patch-clamp technique, the effects of ET-1 were examined in isolated pig detrusor cells. The outward K\textsuperscript{+} currents were suppressed with Cs\textsuperscript{+} in the pipette, and intracellular Ca\textsuperscript{2+} was only weakly buffered (50 \mu M EGTA and 25 \mu M CaCl\textsubscript{2} resulting in a free Ca\textsuperscript{2+} concentration of 106 nM). A holding potential of −60 mV was normally applied. In the majority of the cells, application of ET-1 (10 nM) induced a single transient inward current (334 ± 47 pA, n = 80) as shown in Fig. 4A. However, in 39% (n = 51) of cells responding to ET-1, the initial transient inward current was followed by oscillating inward currents. The initial single inward current was observed even in Ca\textsuperscript{2+}-free solutions: the amplitude was 0.81 ± 0.04 of that seen in a normal extracellular solution (n = 4). On the other hand, the inward current oscillation was completely abolished by removal of extracellular Ca\textsuperscript{2+} (Fig. 4B). It may be noteworthy that ET-1 did not significantly affect the resting current level, whether a single transient inward current or inward current oscillation was induced.

In Fig. 5, the effects of ET agonists and antagonists were examined on detrusor cells showing a single transient inward current upon ET-1 application. Ten nanomolar ET-1 failed to induce any membrane current in the presence of 1 \mu M BQ-123. However, after washout of BQ-123 for 30 min, reapplication of 10 nM ET-1 elicited a single transient inward current in the same cell (Fig. 5A). On the other hand, 1 \mu M BQ-788 had no effect on ET-1-induced transient inward current (Fig. 5B). The mean amplitude of the inward current induced by 10 nM ET-1 in the presence of 1 \mu M BQ-788 was 280.8 ± 79.8 pA (n = 9) [not significantly different (P < 0.05) from the control]. Furthermore, S6c (10 nM) failed to induce any inward
Receptor subtypes were also investigated in detrusor cells, showing inward current oscillation upon ET-1 application. Figure 6A shows that a 1-min application of 10 nM ET-1 caused an inward current oscillation that lasts for a while after removal of ET-1. After washout for 30 min, application of BQ-123 (1 μM) itself had little effect on the membrane current; however, additional application of ET-1 (10 nM) failed to induce inward current oscillation. Furthermore, the fact that this cell remained capable of producing an inward current oscillation upon subsequent application of 10 μM CCh (in the presence of BQ-123) indicated that the underlying intracellular mechanisms were still preserved during this long patch-clamp measurement. It should also be noted that BQ-123 had no effect on the inward current oscillation induced by a preceding ET-1 application (Fig. 6B). The relative amplitude and frequency of the inward current oscillation induced by ET-1 (10 nM) were 0.96 ± 0.01 and 1.07 ± 0.15 (vs. control), when 1 μM BQ-123 was additionally applied (n = 4). This feature of BQ-123 inhibition (requirement of preapplication) was in good agreement with its action on the ET-1-induced contraction (Fig. 2, A and B). On the other hand, BQ-788 (1 μM) had no significant effect on either resting membrane current or ET-1-induced oscillating inward currents (amplitude: 164 ± 77 pA, frequency: 0.035 ± 0.014 Hz, n = 5; Fig. 6C). Furthermore, sarafotoxin S6c (10 nM) did not induce an inward current oscillation in detrusor cells that show oscillatory responses to ET-1 (n = 5; Fig. 6D). Taken together, it is suggested that whether transient or persistent, electrical activities seen during and after ET-1 applications are caused via ETα receptors.

**General features of ET-1-induced inward current oscillation.** From the essential similarities on the BQ-123 inhibitory effects and persistence after washout of ET-1, inward current oscillation was considered to be the underlying mechanism in the sustained contraction induced by ET application. We thus examined the properties of inward current oscillation in terms of the ET concentration and membrane potential.

The effects of cumulative application of ET-1 on oscillating inward currents were examined at 1, 10, and 100 nM. The frequency of the inward current oscillation was comparable between 1 and 10 nM ET-1 (0.040 ± 0.007 Hz at 1 nM and...
A 10nM ET-1 100pA 2min
B 10nM ET-1 200pA 2min
C 10nM ET-1 200pA 2min
D 10nM ET-1 200pA 2min

Fig. 6. Effects of ET receptor antagonists on ET-1-induced inward current oscillation and of an ET<sub>B</sub> agonist. A: after observation of a control response (inward current oscillation) to 10 nM ET-1, ET-1 was washed out for ~30 min. Subsequently, 10 nM ET-1 was again applied in the presence of BQ-123 (1 μM), an ET<sub>A</sub> receptor antagonist. This treatment significantly suppressed inward current oscillation. Then, 10 μM CCh was applied in the presence of BQ-123. B: addition of 10 nM ET-1 after ET-1 (10 nM) had no effect on the inward current oscillation. C: pretreatment with BQ-788 (1 μM), an ET<sub>B</sub> receptor antagonist, had no effect on ET-1 (10 nM)-induced oscillation. D: sarafotoxin S5c (10 nM), an ET<sub>B</sub> receptor agonist, failed to induce inward current oscillation.

0.045 ± 0.004 Hz at 10 nM), whereas it was significantly increased by increasing the ET-1 concentration to 100 nM (P < 0.05, 0.070 ± 0.007 Hz). On the other hand, no statistically significant change was observed in the mean amplitude of the oscillating inward current among the three ET-1 concentrations: 196 ± 31 pA at 1 nM; 301 ± 110 pA at 10 nM; and 305 ± 47 pA at 100 nM (n = 4–7).

Figure 7 shows the voltage dependency of an inward current oscillation induced by 10 nM ET-1. As shown in Fig. 7, the frequency of the inward current oscillation was clearly increased at more negative membrane potentials. In contrast, the amplitude was normally maximal at ~60 mV. In the detrusor cell used in Fig. 7A, ramp pulses (from -120 to +40 mV for 1,000 ms) were also applied in the absence of ET-1 as a control (○) and during an oscillating inward current in the presence of ET-1 (●). Figure 7Ab shows the instantaneous current-voltage (I-V) relationship of the resting membrane current and oscillating current. The subtraction of the I-V relationship ○ from ● yielded an ohmic I-V relationship over the range between +40 and -120 mV with the reversal potential (E<sub>rev</sub>) of -0.82 ± 0.93 mV (n = 6).

Effects of ionic substitution and channel blockers. To elucidate the ionic nature of the ET-1-induced oscillating inward currents, effects of ionic substitution and channel blockers were examined. As shown in Fig. 8A, 100 nM nifedipine, which would cause complete suppression of voltage-sensitive (L-type) Ca<sup>2+</sup> currents in detrusor smooth muscle (27), had little effect on inward current oscillation (226 ± 92 pA, 0.036 ± 0.016 Hz, n = 6). This result suggests that although Ca<sup>2+</sup> influx from the extracellular space is essential for the maintenance of inward current oscillation, voltage-sensitive Ca<sup>2+</sup> channels are not required.

Because the E<sub>rev</sub> was close to 0 mV, the ion channel candidates for the ET-1-induced oscillating inward currents under the conditions used are nonselective cation channels and Cl<sup>-</sup> channels. In the experiment shown in Fig. 8B, the extracellular Na<sup>+</sup> was replaced with a large cation, NMDG, which does not permeate through nonselective cation channels (e.g., Ref. 38). Application of 10 nM ET-1 still induced inward current oscillation, and the subsequent removal of extracellular Ca<sup>2+</sup> completely abolished it. On the other hand, 100 μM niflumic acid, a blocker for ClCa channels (12), significantly and reversibly suppressed ET-1-evoked inward current oscillation in normal solution (Fig. 8C). Furthermore, DIDS (500 μM), another ClCa channel blocker, also abolished it (Fig. 8D). These results suggest that Cl<sup>-</sup> is the charge carrier for the oscillating inward current.

It has been reported that ET-1 activates L-type voltage-sensitive Ca<sup>2+</sup> channels in guinea pig portal vein (15). In detrusor smooth muscle, the same L-type voltage-sensitive Ca<sup>2+</sup> channels are well known to play an important role in E-C coupling. Thus we examined effects of ET-1 on voltage-sensitive Ca<sup>2+</sup> channel current. Application of ET-1 up to 100 nM, however, had little effect on the I-V relationship (peak current amplitude vs. step voltage) of voltage-sensitive Ca<sup>2+</sup> channel current (2.5 mM extracellular Ca<sup>2+</sup> were used as a charge carrier). The intracellular Ca<sup>2+</sup> was buffered with 4 mM EGTA (n = 4, data not shown).

Involvement of intracellular Ca<sup>2+</sup> stores. Possible involvement of Ca<sup>2+</sup> release from the intracellular Ca<sup>2+</sup> stores in the oscillating inward current was examined. In Fig. 9A, 10 mM caffeine was applied to a pig detrusor cell showing oscillating inward currents in the presence of 10 nM ET-1. A transient inward current was evoked just after application of caffeine, and oscillating inward currents ceased during the exposure. Ryanodine (30 μM) also abolished the oscillating inward currents induced by ET-1 (Fig. 9B). The results agree well with the hypothesis that periodic Ca<sup>2+</sup> release underlies oscillating inward currents.

We further examined whether G proteins are involved in ET-1-induced inward currents. As shown in Fig. 9C, when 1 mM GDPβS was contained in the patch pipette, ET-1 was unable to induce any inward current (even a transient one). Applications of caffeine (10 mM) or ATP (100 μM), however, readily induced transient inward currents, as seen in
detrusor cells dialyzed with “normal” intracellular solution \( (n = 5). \) (The inward currents induced by the former and latter drugs were presumably through \( \text{Cl}_{\text{Ca}} \) channels and nonselective cation channels.) Furthermore, step pulse depolarizations readily evoked voltage-sensitive inward currents (data not shown).

**DISCUSSION**

Pig detrusor is often used as a good model for investigation of mechanisms underlying micturition. In particular, muscarinic receptors play a major role in voiding urine in both humans and pigs, whereas purinergic innervation is significant in other animals such as rats and rabbits \((7, 13)\).

Applications of ET-1 caused detrusor contraction from the concentration of 0.1 nM, and 10 nM ET-1 induced a tonic contraction comparable or rather larger than that induced by 1 \( \mu \)M CCh, a typical muscarinic agonist. From the high sensitivity of detrusor contraction to ET-1, plus the fact that ET-1 is synthesized in urinary bladder tissues, it is tempting to speculate that ET-1 may act as a powerful modulator of bladder function.

ET-1-induced contractions and oscillating inward currents share a number of features. First, the ET-1-induced contraction was persistent, and the tension only slowly decreased during washout of ET-1. This persistent contraction is also observed in other smooth muscles \((19, 37)\). Similarly, as shown in Fig. 6A, inward current oscillations in isolated detrusor cells last for some while after washout of ET-1. The similar persistence seen in contraction and electrical activity may be explained by the following two hypotheses: 1) a very high affinity of ET-1 to its receptor in detrusor cells and/or a very low dissociation rate and 2) ET-1-induced persistent intracellular signal(s) that can

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**Fig. 7.** Voltage dependency of ET-1-induced inward current oscillation. **Aa:** holding potential was displaced from −60 to −20 mV or −100 mV in the presence of 10 nM ET-1. Ramp pulses (from −120 to +40 mV, 1 s) were applied before (○) and during application of ET-1 (●). **Ab:** instantaneous current-voltage \((I-V)\) relationships obtained by the ramp pulses used in Aa. **B:** summary of the changes in amplitude and frequency at the membrane potential of −100, −60, and −20 mV. Each column stands for the means \( \pm \) SD \((n = 5)\). *Statistically significant differences of \( P < 0.05\). The amplitude of oscillating inward current was expressed relative to that at −60 mV.
promote mechanisms underlying inward current oscillation and tonic contraction.

Another characteristic feature shared in contraction and electrical activity is the effects of antagonists. Prior application of BQ-123, an ETA receptor antagonist, completely suppressed both contraction and inward current oscillation induced by ET-1, whereas BQ-788, an ETB receptor antagonist, had no effect on either (Figs. 2 and 6). Furthermore, sarafotoxin S6c, an ETB receptor agonist, produced only a very small contraction and no inward current (Figs. 2 and 6). These results suggest that inward current oscillation induced via ETA receptors is the primary electrical activity underlying ET-1-induced contraction. When application of BQ-123 was preceded by ET-1, neither ET-1-induced contraction nor inward current oscillation was suppressed. This order-dependent inhibitory effect of BQ-123 could be explained by the hypotheses described in the previous paragraph.

ET-1 increased the frequency of inward current oscillation in a dose-dependent manner. It can be deduced that at high concentrations of ET-1, summation of oscillating inward currents results in a large tonic contraction of detrusor muscle. Gap junction channels, although less present than in other smooth muscle tissues (3), may also make an important contribution in the summation of electrical activity in the detrusor, i.e., sustained depolarization. Nifedipine, a selective blocker for L-type voltage-sensitive Ca^{2+} channels, had little effect on ET-1-induced inward current oscillation (Fig. 8A) but largely suppressed ET-1-induced tonic contraction (data not shown). We also observed these paradoxical responses to nifedipine in porcine coronary artery in which the high sensitivity to a dihydropyridine Ca^{2+} channel antagonist of the ET-1-induced contraction represents a tissue-specific rather than agonist-specific property (18). Although the tissue specificity was taken into consideration, these facts suggest that L-type Ca^{2+} channels play a central role in E-C coupling in detrusor muscle but that this Ca^{2+} pathway is not always required to maintain inward current oscillation.

In vascular smooth muscle, it has been shown that voltage-sensitive L-type Ca^{2+} channels are directly facilitated through ET-1 receptor stimulation even under voltage-clamp conditions (11, 15). However, we noted that ET-1 had no effect on voltage-sensitive Ca^{2+} channel current in detrusor cells (data not shown), supporting the conclusion that the major action of ET-1 is to produce oscillating inward currents, which depolar-
ize the cell membrane and subsequently activate voltage-sensitive Ca\(^{2+}\) channels. Further evidence was obtained using a K\(^+-\)rich solution in the patch pipette. When the cell membrane was clamped at \(-60\) or \(-40\) mV, only oscillating inward currents were observed after application of ET-1. A periodic outward component that accompanied the oscillating inward current was produced by depolarizing the membrane to \(-20\) mV (data not shown).

In the majority of detrusor cells, application of ET-1 promptly evoked a single transient inward current (Fig. 4A), but about one-third of the cells showed persistent inward current oscillation even after the removal of ET-1 (Fig. 6A). The single inward current was observed even in Ca\(^{2+}\)-free solutions, but on the other hand, the oscillatory inward currents were terminated by removal of extracellular Ca\(^{2+}\) (Fig. 4).

These results suggest that distinct mechanisms are operated in the single transient inward current and persistent inward current oscillation, although the same ET\(_\alpha\) receptors are responsible for both. Because inward current oscillation seems to underlie ET-1-induced tonic contractions, we focused on the present study on the ionic nature of this electrical activity.

The \(E_{\text{rev}}\) of the ET-1-induced oscillating inward current was very close to 0 mV (Fig. 7A). Nonselective cation channels and Cl\(^-\) channels are candidates for carrying this current under the conditions used in the present study. As shown in Fig. 8B, ET-1 still induced oscillating inward currents, even when extracellular Na\(^+\) was replaced with a large cation, NMDG, which does not permeate through nonselective cation channels (e.g., Ref. 38). On the other hand, niflumic acid, a Ca\(^{2+}\)-activated Cl\(^-\) channel blocker, significantly suppressed inward current oscillation (Fig. 8C). Taken together, these results suggest that Ca\(^{2+}\)-activated Cl\(^-\) channels are the major conductance for the oscillating inward currents induced by ET-1 and that these Cl\(^-\) channels are activated presumably by periodic increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]).

With respect to the contractile response, niflumic acid suppressed the ET-1-induced contraction with IC\(_{50}\) between 100 and 300 \(\mu\)M (Fig. 3). It may be noteworthy that phasic contractions appeared when niflumic acid significantly reduced the amplitude of the ET-1-induced tonic contraction. This might be evidence for the summation of phasic activities (contraction and/or electrical oscillations) induced via ET\(_\alpha\) receptors. Compared with patch-clamp experiments, a slightly higher concentration was required to inhibit the contractile response to ET-1. This is presumably due to higher spontaneous activity at a higher temperature (35–37\(\degree\)C) used for tension recordings and/or due to the low permeability (efficacy) of niflumic acid in tissue level experiments. Also, it has been reported that niflumic acid activates K\(^+\) currents (25). This effect might be involved in the inhibitory action of niflumic acid on the ET-1-induced contraction. In addition, it is important to consider the contribution of K\(^+\) channels on the detrusor excitability (36), although this was not the subject of the present study.

The mechanism(s) of the periodic [Ca\(^{2+}\)], increases seems to require Ca\(^{2+}\) influx from the extracellular space (Figs. 4B and 8B) through nifedipine-insensitive Ca\(^{2+}\)-permeable channels (Fig. 8A). The fact that the frequency of the ET-1-induced inward current oscillation was increased by increasing the negativity of the membrane potential over the range \(-20\) to \(-100\) mV supports the involvement of a Ca\(^{2+}\) permeability other than voltage-sensitive Ca\(^{2+}\) channels. On the other hand, the amplitude of the currents decreased despite the increase in frequency. We hypothesize that the periodic [Ca\(^{2+}\)], increase that underlies ET-1-induced inward current oscillation is mainly due to Ca\(^{2+}\) release from the sarcoplasmic reticulum. The inhibitory effects of caffeine and ryanodine on the inward current oscillation (Fig. 9, A and B) support this hypothesis. If the release is triggered by Ca\(^{2+}\) influx through the nifedipine-insensitive Ca\(^{2+}\) permeability, the increase in frequency of the oscillating inward current at \(-100\) mV could be due to amplification of the driving force for Ca\(^{2+}\) entry, and the decrease in size might reflect the insufficiency of refilling Ca\(^{2+}\) in the intracellular Ca\(^{2+}\) stores due to shorter intervals.

The inclusion of GDP\(_\beta\)S in the patch pipette completely abolished ET-1-induced electrical activities (both single and oscillatory inward currents; Fig. 9C), implying that factors (or mechanisms) causing periodic changes in [Ca\(^{2+}\)]\(_i\) are downstream of GTP binding protein(s) activated via ET\(_\alpha\) receptors (6, 28). Further experiments are necessary to elucidate details of intracellular signals: for example, whether G protein-related signals primarily activate periodic Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores or enhance the nifedipine-insensitive Ca\(^{2+}\) permeability in the plasma membrane consequently activating it.

What is the role of ET-1 and its receptors in urinary bladder function? It seems likely that ET-1 may be released from some tissues in the bladder wall during micturition and may play some facilitatory role in enhancing the responses to acetylcholine released from the parasympathetic nerves. The sustained nature of the oscillating inward currents induced by ET-1 may contribute to the maintenance of the pressure rise, preventing the buildup of residual urine. It is further speculated that changes in the amount of released ET-1 might alter the duration of sustained activation of individual smooth muscle cells and consequently alter the threshold for activation of afferent nerves and initiation of the next micturition. Ageing (35) and some popular diseases, such as diabetes mellitus (5, 26, 31), are known to alter ET receptor expression and bladder activity.

In conclusion, we demonstrated that ET-1 induces inward current oscillation via ET\(_\alpha\) receptors in a considerable number of detrusor cells. The inward current oscillation is associated with tonic and persistent contraction in the smooth muscle. The results with ionic substitution and channel blockers suggested that the oscillating inward currents flow through Ca\(^{2+}\)-activated Cl\(^-\) channels that are periodically activated. Ca\(^{2+}\) influx through nifedipine-insensitive Ca\(^{2+}\) permeability plays an important role in pacing the oscillation.

ACKNOWLEDGMENTS

The authors are deeply grateful to Dr. Ken Kato for pertinent help and advice.

GRANTS

This work was supported by a Medical Research Council project grant of UK. S. Nakayama was supported by grants-in-aid for Scientific Research from Ministry of Education, Science and Culture of Japan.

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