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Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder

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1 To investigate the cellular mechanisms underlying spontaneous excitation of smooth muscle of the guinea-pig urinary bladder, isometric tension was measured in muscle bundles while recording the membrane potential from a cell in the bundle with a microeletrode. Changes in the intracellular calcium concentration ($[Ca^{2+}]_i$; calcium transients) were recorded in strips loaded with the fluorescent dye, fura-PE3.

2 In 40% of preparations, individual action potentials and contractions, which were abolished by nifedipine (1 μM), were generated. In the remaining preparations, bursting action potentials and contractions were generated. Contractions were again abolished by nifedipine (1 μM), while higher concentrations of nifedipine (10–30 μM) were required to prevent the electrical activity.

3 Carbachol (0.1 μM) increased the frequency of action potentials and corresponding contractions. Apamin (0.1 μM) potentiated bursting activity and enhanced phasic contraction. Charybdotoxin (CTX, 50 nM) induced prolonged action potentials that generated enlarged contractions. In contrast, levcromakalim (0.1 μM) reduced the frequency of action potentials, action potential bursts and the size of the contractions.

4 Forskolin (0.1 μM), 8-bromoguanosin 3′, 5′ cyclic monophosphate (8Br-cGMP, 0.1 mM) and Y-26763 (10 μM) suppressed contractions without reducing the amplitude of either action potentials or Ca transients.

5 This paper confirms that action potentials and associated calcium transients are fundamental mechanisms in generating spontaneous contractions in smooth muscles of the guinea-pig bladder. However, in parallel with the excitation–contraction coupling, the sensitivity of the contractile proteins for Ca^{2+} may play an important role in regulating spontaneous excitation and can be modulated by cyclic nucleotides and Rho kinase.
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Keywords: Urinary bladder; action potential; cyclic nucleotide; Ca sensitivity

Abbreviations: AHP, after-hyperpolarization; 2-APB, 2-aminoethoxydiphenylborate; BK channel, large conductance Ca^{2+}-activated K^+ channel; 8Br-cGMP, 8-bromoguanosin 3′,5′ cyclic monophosphate; $[Ca^{2+}]_i$, intracellular concentration of free calcium ions; CICR, calcium-induced calcium release; CPA, cyclopiazonic acid; CTX, charybdotoxin; DMSO, dimethyl sulphoxide; dV/dt_L, leading slope; dV/dt_T, trailing slope; $InsP_3$, inositol1,4,5-trisphosphate; MLCP, myosin light-chain phosphatase; SK channel, small conductance Ca^{2+}-activated K^+ channel

Introduction

Detrusor smooth muscle strips taken from the bladder of all species, including human, develop spontaneous phasic contractions at frequencies that are species dependent (Sibley, 1984; Herrera *et al*., 2000). Under physiological conditions, these contractions do not contribute to a rise in the intravesical pressure, and thus the excitation of parasympathetic nerves is required to initiate synchronized contractions of the whole bladder to void urine. Consistently, it has been shown that the electrical coupling between detrusor smooth muscle cells is relatively low (Bramich & Brading, 1996; Hashitani *et al*., 2001), and that spontaneous contractions occur locally and do not readily spread throughout the tissue (Hashitani *et al*., 2000). However, this situation could be changed in some pathological conditions such as 'the overactive bladder', and myogenic contractions may cause an involuntary rise in the intravesical pressure, which results in urinary incontinence (Brading & Turner, 1994). Therefore, understanding the regulation of spontaneous excitation in detrusor smooth muscles may provide a key for developing successful pharmacological treatment of the overactive bladder.

A double sucrose gap study in the guinea-pig bladder demonstrated that increasing action potential frequency induced by current injections facilitated phasic contractions, suggesting a correlation between electrical and mechanical activity in detrusor smooth muscles (Mostwin, 1986).

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Spontaneous contractions are blocked by dihydropyridine L-type calcium channel blockers, for example, nifedipine and nisoldipine, indicating that they result from the opening of L-type calcium channels and the Ca^{2+} entry through these channels (Mostwin, 1986; Herrera *et al*., 2000; Buckner *et al*., 2002). Spontaneous action potentials and corresponding calcium transients were also blocked by nifedipine, but not by either CPA or ryanodine, indicating a critical role of calcium entry through L-type calcium channels in the initiation of spontaneous excitation in detrusor smooth muscles (Hashitani *et al*., 2001; Hashitani & Brading, 2003a).

Regardless of the apparent correlation between electrical and mechanical activity in isolated smooth muscle bundles, significant differences between the frequency of spontaneous action potentials and contractions in larger strip preparations have caused concern (Sibley, 1984; Herrera *et al*., 2000; Hashitani *et al*., 2001). Furthermore, a recent study on detrusor smooth muscles of the guinea-pig bladder (Imai *et al*., 2002) showed that 2-APB, a blocker for inositol 1,4,5-trisphosphate ($InsP_3$) receptors (Maruyama *et al*., 1997; Ascher-Landsberg *et al*., 1999), caused dissociation between membrane electrical and mechanical activity. Therefore, although spontaneous action potentials and associated increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) seem to play a predominant role in the regulation of spontaneous excitations in detrusor smooth muscles, there may be other mechanisms that modulate the correlation between electrical and mechanical activity.

Besides the excitation–contraction coupling process, accumulating evidence reveals the physiological importance of 'Ca^{2+}-independent' regulation of smooth muscle tone by alteration of the sensitivity of contractile proteins for Ca^{2+} (Ca sensitivity; Somlyo & Somlyo, 1994; Peitzer, 2001). The activity of myosin light-chain phosphatase (MLCP) is increased through a cyclic nucleotide-dependent protein kinase, resulting in a decrease in the Ca sensitivity of contractile proteins (Nishimura & Van Breemen, 1989; Sauzeau *et al*., 2000). Conversely, the activity of MLCP is inhibited by Rho-kinase and protein kinase C, leading to an increase in Ca sensitivity (Parsons *et al*., 1996; Fu *et al*., 1998). Recently, increased Ca sensitivity in response to muscarinic stimulation was reported in the bladder smooth muscle (Wibberley *et al*., 2003), whereas factors that reduce the Ca sensitivity in the bladder have not yet been identified.

In detrusor smooth muscles, cyclic AMP (cAMP) has been considered to play a major role in initiating relaxation (Morita *et al*., 1992; Truss *et al*., 1996), while the physiological significance of the cGMP pathway has not been established (Persson *et al*., 2000). It has been reported that isoproterenol, a β-agonist, reduced the frequency of spontaneous action potentials and associated calcium transients in detrusor smooth muscle of the guinea-pig (Nakahira *et al*., 2001). At the higher concentrations, isoproterenol hyperpolarized the membrane to prevent the generation of action potentials and corresponding Ca transients. However, the possible role of either cAMP or cGMP in the modulation of Ca sensitivity in detrusor smooth muscle remains to be established.

In this paper, first, changes in the membrane potential were recorded simultaneously with tension changes in individual detrusor smooth muscle bundles, using intracellular microelectrodes and sensitive isometric force transducers. In order to investigate the correlation of electrical and mechanical activity, the effects of several pharmacological agents known to modulate electrical activity in detrusor were examined. Second, to investigate the involvement of Ca sensitivity in the regulation of spontaneous excitation, the effects activators of cyclic nucleotides and a Rho kinase inhibitor on the correlation between calcium and contractile responses were examined by measuring changes in $[Ca^{2+}]_i$ and tension simultaneously.

Methods

General

The procedures described have been approved by the animal experimentation ethics committee at the University of Oxford and Nagoya City University Medical School. Guinea-pigs of either sex weighing 250–400 g were killed by a blow to the head followed by cervical dislocation. The urinary bladder was removed and its ventral wall was opened longitudinally from the top of the dome to the bladder neck. The mucosal layer, connective tissues and several smooth muscle layers were then removed leaving underlying single smooth muscle bundles attached to the serosal layer. A serosal sheet, which contained one or a few single bundles of smooth muscle, 2–3 mm long and 0.2–0.7 mm wide was then prepared as described previously (Hashitani *et al*., 2001). An approximately 0.5–1 mm length from one end of the preparation was pinned out on a Sylgard plate (silicone elastomer, Dow Corning Corporation, Midland, MI, U.S.A.) at the bottom of the recording chamber (volume, approximately 1 ml) which was mounted on a stage of an inverted microscope, and a thread was tied around the other end. The preparations were superfused with warmed (35°C) Krebs solution at a constant flow rate ($2\,ml\,min^{-1}$).

Isometric tension recordings

The thread from the muscle strips was attached to an isometric force transducer that was connected to a bridge amplifier (ADInstruments Ltd, Grove House, Hastings, U.K.). Isometric tension changes were digitized using either PowerLab/4SP (ADInstruments Ltd, Grove House, Hastings, U.K.) or Digidata 1200 interface (Axon Instruments, Inc., Foster City, CA, U.S.A.) and stored on a personal computer for later analysis. A tension of approximately 1 mN was applied to preparations that were then left to equilibrate for 60–90 min until spontaneous phasic contractions, which were stable in both amplitude and frequency, were generated.

Microelectrode recordings

Individual bladder smooth muscle cells in the muscle bundles were impaled with glass capillary microelectrodes, filled with 0.5 M KCl (tip resistance, 120–250 MΩ). Membrane potential changes were recorded using a high input impedance amplifier (Axoclamp-2A, Axon Instruments, Inc., Foster City, CA, U.S.A.), and displayed on a cathode-ray oscilloscope (Data SYS 740, Gould Nicolet Technologies, Ilford, Essex, U.K. or SS-9622, Iwatsu, Tokyo, Japan). After low-pass filtering (cutoff frequency, 1 kHz), membrane potential changes were digitized using either PowerLab/4SP (ADInstruments Ltd, Grove House, Hastings, U.K.) or Digidata 1200 interface

(Axon Instruments, Inc., Foster City, CA, U.S.A.) and stored on a personal computer for later analysis.

Intracellular calcium measurements

For measurements of changes in $[Ca^{2+}]_i$, bladder preparations were pinned out on the bottom of a recording chamber which was similar to that used for electrical and mechanical recordings. After 30 min incubation with warmed (35°C) physiological saline, spontaneous action potentials and corresponding contractions of the tissues were detected, and then the preparations were loaded with fluorescent dye, fura-PE3, by incubation in low Ca^{2+} physiological saline (Ca^{2+}, 1 mM) containing 10 μM fura-PE3 AM for 1 h at room temperature. After loading, preparations were superfused with dye-free, warmed (35°C) physiological saline at a constant flow (about 2 ml min^{-1}) for 30 min. Preparations, loaded with fura-PE3, were illuminated with two periods of ultraviolet light, wavelengths 340 and 380 nm, alternating at a frequency of higher than 40 Hz. The ratio of the emission fluorescence ($R_{340/380}$) in a desired size of rectangular window was measured through a barrier filter of 510 nm (sampling interval less than 200 ms), using a microphotoluminescence measurement system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan).

Solutions

The composition of physiological saline was (in mM): NaCl, 120; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 15.5; NaH_2PO_4, 1.2 and glucose, 11.5. The solution was bubbled with 95% O_2 and 5% CO_2, and pH of the solution was maintained at 7.2–7.3.

Drugs used were 2-aminoethoxydiphenylborate (2-APB), fura-PE3 AM and Y-27632 (from Calbiochem-Novabiochem Ltd, San Diego, CA, U.S.A.), apamin, 8-bromo cyclic GMP (8Br-cGMP), charybdotoxin (CTX), carbachol (CCh), cyclopiazonic acid (CPA), forskolin and nifedipine (from Sigma, St Louis, MO, U.S.A.). Levcromakalim was a generous gift from GlaxoSmithKline (Harlow, U.K.). Apamin, 8Br-cGMP, CCh, CTX and Y-27632 were dissolved in distilled water. Nifedipine was dissolved in 100% ethanol, 2-APB, CPA, forskolin and levcromakalim were dissolved in dimethyl sulphoxide (DMSO). The final concentration of these solvents in the physiological saline did not exceed 1 : 1000.

Calculations and statistics

Measured values were expressed as mean ± s.d.. Statistical significance was tested using paired *t*-test, and probabilities of less than 5% different from the control were considered significant. When drug effects were studied, the numbers of preparations refer to all the successful experiments carried out for each investigation.

The following parameters of action potentials were measured: peak amplitude, measured as the value from the resting membrane potential to the action potential peak (which was defined as an average of 0.1 ms on either side of the maximum point); leading dV/dt (dV/dt_L), measured as the slope between 20 and 80% of the peak amplitude of the events on the raising phase; half-width, measured as the time between 50% peak amplitude on the rising and falling phases; trailing dV/dt (dV/dt_T), measured as the slope between 20 and 80% of the peak amplitude of the events on the falling phase. The amplitude of after-hyperpolarizations (AHPs) was measured as the value from the resting membrane potential to the peak of the AHP.

For isometric tension changes, the following parameters were measured: peak amplitude, measured as the value from the basal tension level to the peak of phasic contractions (which was defined as an average of 2 ms on either side of the maximum point); half-width, measured as the time between 50% peak amplitude on the rising and falling phases; frequency which was defined as an average of 5 min recordings.

For calcium measurements, the following parameters were measured: peak amplitude, measured as the value from the basal calcium level to the peak of calcium transients (which was defined as an average of 2 ms on either side of the maximum point); half-width, measured as the time between 50% peak amplitude on the rising and falling phases; frequency, which was defined as an average of 5 min recordings.

Results

Correlation between spontaneous electrical and mechanical activity in detrusor smooth muscles

In all preparations examined, bladder smooth muscles exhibited spontaneous action potentials and corresponding phasic contractions.

In 16 out of 41 preparations, spontaneous action potentials were generated individually (Figure 1Aa), and corresponding contractions were invariably recorded (Figure 1Ab). Their frequency ranged between 8 and 27 min^{-1} (mean 14.5 ± 6.2 min^{-1}). The resting membrane potential determined at the most negative potential between each action potential ranged between −53 and −42 mV (mean −46.3 ± 2.9 mV). Each action potential was followed by an AHP, and invariably preceded phasic contraction (Figure 1Ac, full line). Action potentials had peak amplitudes ranging between 53 and 72.6 mV (mean 60.7 ± 6.3 mV), leading dV/dt (dV/dt_L) ranging between 1.2 and 5.4 mV ms^{-1} (mean 2.8 ± 1.3 mV ms^{-1}), half-widths ranging between 4.3 and 8.2 ms (mean 5.8 ± 1.2 ms) and trailing dV/dt (dV/dt_T) ranging between −22.9 and −11.9 mV ms^{-1} (mean −16.2 ± 3.4 mV ms^{-1}). Amplitudes of AHPs ranged between 7 and 14 mV (mean 11.4 ± 2.2 mV).

Corresponding contractions had peak amplitudes ranging between 0.4 and 1.1 mN (mean 0.79 ± 0.23 mN) and half-widths ranging between 0.38 and 1.37 s (mean 0.92 ± 0.25 s; Figure 1Ac, dotted line).

Both spontaneous action potentials and contractions were abolished by nifedipine (1 μM, $n = 5$), indicating the critical role of calcium entry through L-type Ca channels in the initiation of spontaneous excitations.

In the remaining 25 preparations, bursts of action potentials (Figure 1Ba) and corresponding contractions were generated (Figure 1Bb), and their frequency ranged between 1 and 5.8 min^{-1} (4.2 ± 2.2 min^{-1}).

Each burst consisted of 2–21 action potentials (8.9 ± 5.9 action potentials) and lasted for 1.5–13 s (6.2 ± 2.9 s; Figure 1Bc, full line). The resting membrane potential determined at the most negative potential between bursts ranged between

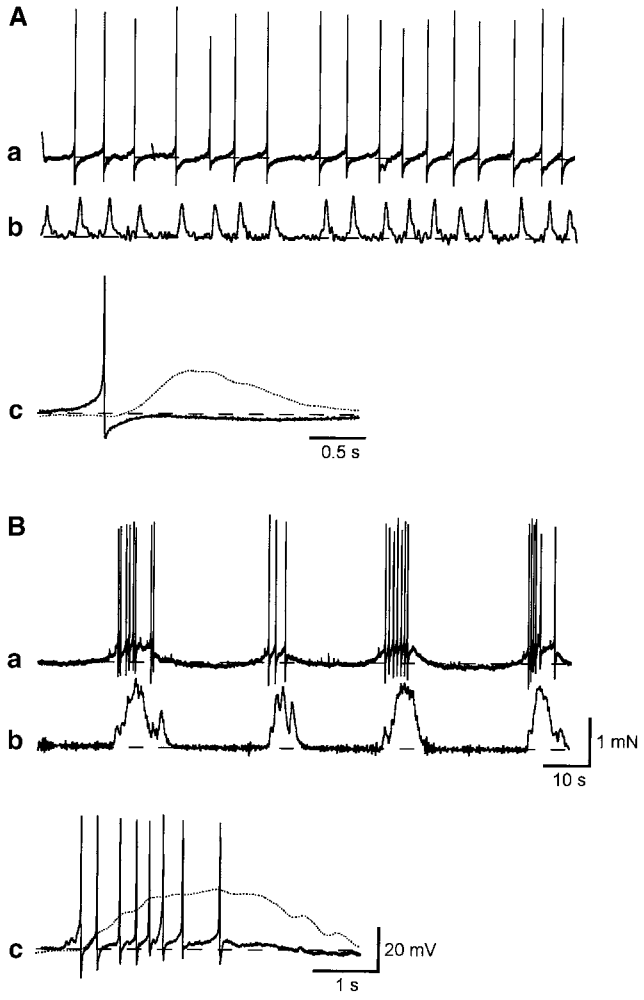


Figure 1 Correlation between spontaneous electrical and mechanical activity in detrusor smooth muscles of the guinea-pig bladder. A single bladder smooth muscle bundle exhibited individual action potentials (Aa) and corresponding phasic contractions (Ab). Each action potential was followed by a phasic contraction after a delay of some 0.2 s (Ac). In another preparation, bursts of action potentials (Ba) and corresponding phasic contractions were generated (Bb). Each burst was composed of some 5–20 action potentials, and was followed by a phasic contraction (Bc). Resting membrane potentials were −44 mV in (A) and −46 mV in (B).

−57 and −42 mV (mean −46.8±3.8 mV). Since action potentials tended to become smaller and to have a slower time course during each burst, action potential parameters were measured on the first action potential in each burst. Action potentials had peak amplitudes ranging between 48 and 73.5 mV (mean 58.5±6.3 mV), leading dV/dt (dV/dt_L) ranging between 1 and 5.8 mV ms^{-1} (mean 3.0±1.2 mV ms^{-1}), half-widths ranging between 4.5 and 9.2 ms (mean 5.9±1.0 ms) and trailing dV/dt (dV/dt_T) ranging between −22.9 and −10.7 mV ms^{-1} (mean −15.2±2.7 mV ms^{-1}). Amplitudes of AHPs ranged between 7 and 16 mV (mean 11.6±2.5 mV). These values were similar to those of action potentials that were generated individually.

Corresponding contractions had peak amplitudes ranging between 0.5 and 3.5 mN (mean 1.2±0.35 mN) and half-widths ranging between 1.3 and 5.2 s (mean 3.1±1.1 s; Figure 1Bc, dotted line).

Nifedipine (1 μM) again abolished spontaneous phasic contractions but failed to prevent the generation of action potentials ($n=8$). Nifedipine (1 μM) converted the bursts of action potentials into transients depolarizations that occurred individually, and higher concentrations (10 μM, $n=5$ or 30 μM, $n=3$) were required to block the 'nifedipine-resistant' depolarizations. Since the depolarizations did not contribute to the generation of spontaneous contractions, we did not investigate further the nature of this activity in the present study.

Effects of pharmacological agents which modulate action potentials on spontaneous contractions

Since ACh released from parasympathetic nerves is considered a principal excitatory transmitter in bladder smooth muscles, the effects of CCh, a muscarinic agonist, on electrical and mechanical activity were examined.

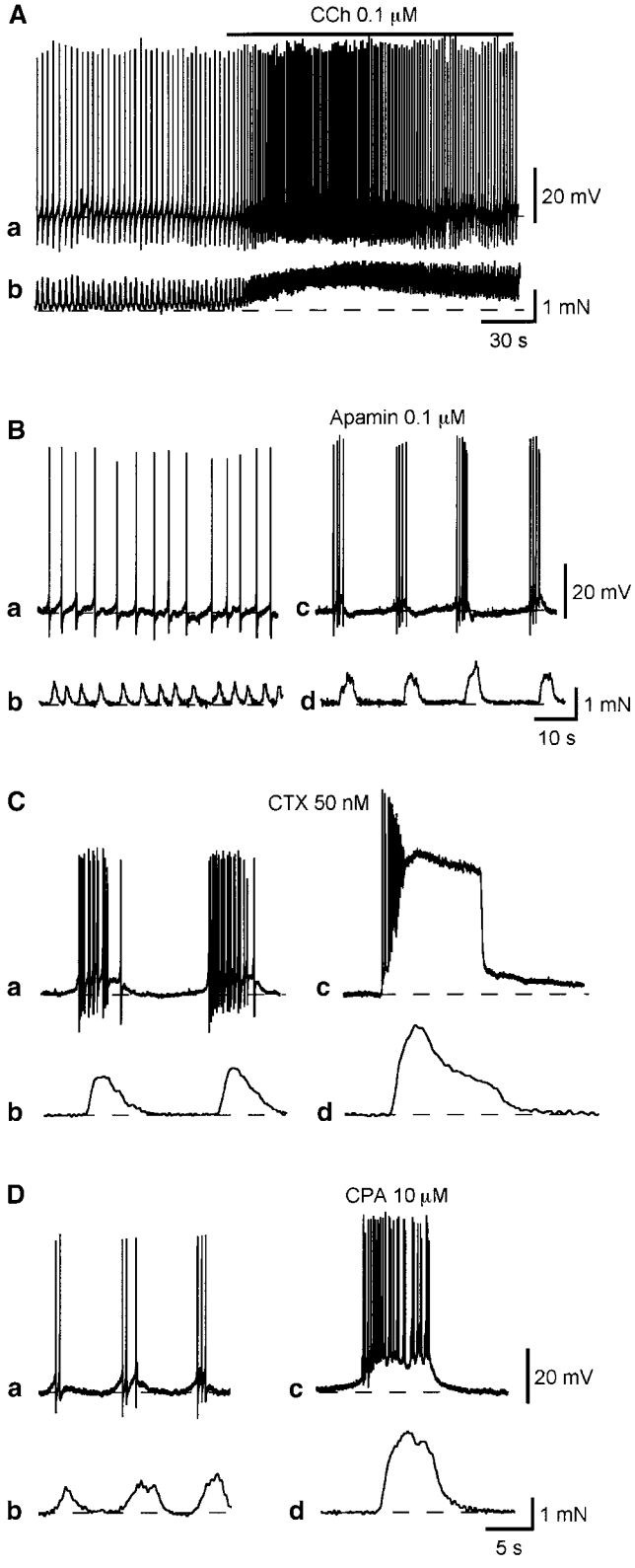
In tissues showing individual action potentials, application of CCh (0.1 μM) increased the frequency of action potentials without changing the membrane potential (Figure 2Aa). After the initial increase, the frequency declined but remained higher than that of control conditions (13±2.4 min^{-1} in control, 44.8±15 min^{-1} in CCh, $n=4$). CCh also increased the frequency of corresponding contractions to cause a sustained rise in the basal tension level (Figure 2Ab). In four other preparations, which exhibited bursting action potentials, CCh (0.1 μM) initially increased the frequency of bursts until action potentials became continuously generated. After 2–4 min, bursts of action potentials reappeared. Each bursts consisted of an increased number of action potentials, and the amplitude and the half-width of the corresponding contractions was increased (0.94±0.24 mN and 3.1±0.74 s in control, 1.8±0.55 mN and 5.7±1.9 s in CCh, $n=4$).

In a previous study (Hashitani & Brading, 2003a), we examined how the modulation of Ca^{2+}-activated K^+ channels altered the electrical activity of the tissue. Large conductance Ca^{2+}-activated K^+ (BK) channels contribute to the repolarization of action potentials and AHPs, while, the inhibition of small conductance Ca^{2+}-activated K^+ (SK) channels with apamin converted the electrical activity from individual action potentials into bursts without changing the action potential shape.

In the present study, we extended the observations to include simultaneous recordings of contractile activity. In four preparations, which generated individual action potentials, apamin (0.1 μM) converted individual action potentials into bursts without changing either the action potential shape or AHPs (Figure 2Bc). It increased the amplitude and half-width of phasic contractions (0.71±0.13 mN and 0.78±0.16 s in control, 2.5±0.6 mN and 3.3±0.67 s in apamin, $n=4$), and reduced their frequency (16±4.3 min^{-1} in control, 2.5±0.6 min^{-1} in apamin, Figure 2Bd). In three preparations, which generated bursts of action potentials, apamin increased the number of action potentials in each burst. It again increased the amplitude and half-width of corresponding contractions (1.4±0.43 mN and 3.5±1.1 s in control, 2.3±0.5 mN and 5.9±2.2 s in apamin, $n=3$) and reduced their frequency (3.8±2.9 min^{-1} in control, 2.1±1.2 min^{-1} in apamin).

In four preparations, which generated bursts of action potentials, CTX (50 nM) initiated prolonged action potentials (Figure 2Cc). The plateau phase of these depolarizations

ranged between 0 and 5 mV and lasted for 5–15 s. CTX increased the amplitude and half-width of corresponding contractions (2.5 ± 0.8 mN and 2.2 ± 0.45 s in control, 5.6 ± 0.9 mN and 9.9 ± 4.6 s in CTX, $n = 4$) and reduced their frequency ($3.2 \pm 1.6\,min^{-1}$ in control, $2.3 \pm 0.9\,min^{-1}$ in CTX, Figure 2Cd).



In four preparations, which generated bursts of action potentials, CPA increased the amplitude and duration of individual action potentials and suppressed AHPs as shown previously (see Hashitani & Brading, 2003a). In addition, CPA clustered action potentials so that prolonged bursts of action potentials, which lasted for 5–10 s, were generated (Figure 2Dc). CPA increased the amplitude and the half-width of phasic contractions (1.6 ± 0.5 mN and 2.9 ± 0.58 s in control, 4.5 ± 1.2 mN and 6.2 ± 0.6 s in CPA, $n = 4$) and reduced their frequency ($3.7 \pm 2.9\,min^{-1}$ in control, $1.3 \pm 0.5\,min^{-1}$ in CPA, Figure 2Dd).

Effects of levcromakalim on spontaneous electrical and mechanical activity

To determine the effects of the inhibition of electrical activity on mechanical activity in bladder smooth muscles, levcromakalim, an ATP-sensitive K^+ channel opener, was used. Levcromakalim (0.1 μM) reduced the frequency of either individual action potentials or bursts of action potentials without changing the membrane potential (Figure 3Ba). It also reduced the frequency of the corresponding phasic contractions, but did not change their amplitude ($14 \pm 6.1\,min^{-1}$ in control, $4.2 \pm 1.5\,min^{-1}$ in levcromakalim, $P < 0.05$, $n = 6$, Figure 3Bb). These results are consistent with those of the previous study (Petkov *et al*., 2001). A higher concentration of levcromakalim (1 μM) hyperpolarized the membrane by about 10 mV and prevented the generation of action potentials and phasic contractions ($n = 3$, Figure 3Ca, b).

Modulation of the correlation between electrical and mechanical activity by cyclic nucleotides

To investigate the possible role of cyclic nucleotides on the modulation of excitation–contraction coupling in the bladder smooth muscle, the effects of forskolin, an activator for adenylate cyclase, and 8Br-cGMP, a membrane permeable analogue of cGMP, on electrical and mechanical activity, were examined.

Forskolin (0.1 μM) reduced action potential frequency without changing the membrane potential ($20.2 \pm 5.8\,min^{-1}$ in control, $6.8 \pm 3.1\,min^{-1}$ in forskolin, $n = 5$, Figure 4Ba). It also reduced the amplitude and frequency of phasic contractions (Figure 4Bb). A higher concentration of forskolin (1 μM) hyperpolarized the membrane by about 5 mV and prevented the generation of spontaneous action potentials ($n = 4$, Figure 4Ca). It also abolished corresponding contractions (Figure 4Cb).

Figure 2 Effects of CCh, apamin, CTX and CPA on the correlation of electrical and mechanical activity. In a preparation, which generated individual action potentials, CCh (0.1 μM) increased the frequency of action potentials (Aa) and contractions to cause tonic contraction (Ab). Apamin (0.1 μM) converted individual action potentials into bursts (Ba) and increased the amplitude and duration of phasic contractions (Bb). In a different preparation, which generated bursts of action potentials, CTX (50 nM) suppressed AHPs, induced action potentials that had a plateau phase (Ca) and dramatically increased the amplitude and duration of corresponding contractions (Cb). In another preparation, which generated bursts of action potentials, CPA (10 μM) suppressed AHPs, clustered the action potentials (Da), and increased the amplitude and duration of corresponding contractions (Db). Resting membrane potentials were −46 mV in (A), −44 mV in (B), −44 mV in (C) and −47 mV in (D).

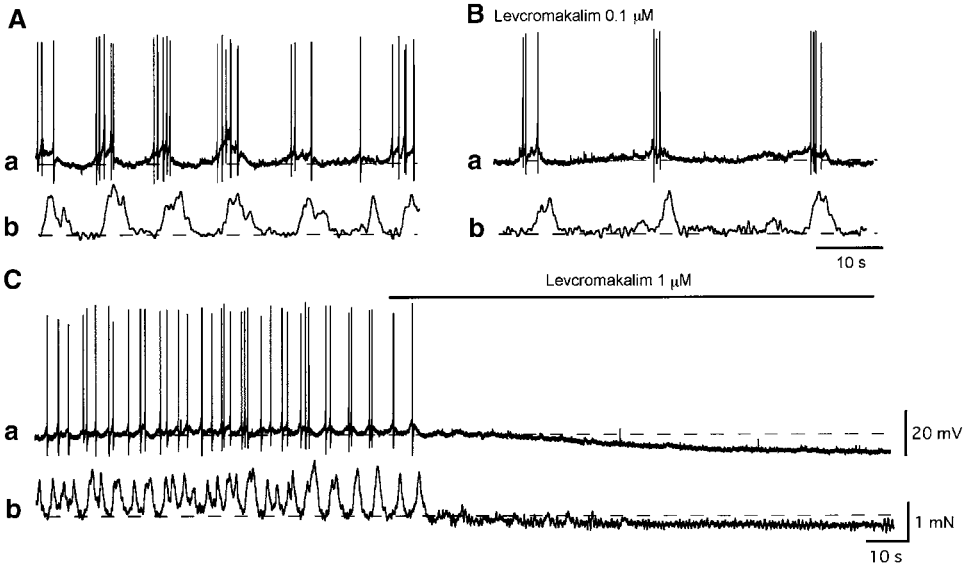


Figure 3 Effects of levcromakalim on the correlation between electrical and mechanical activity. In a bladder smooth muscle preparation in which bursts of action potentials and corresponding contractions were generated (Aa, b), levcromakalim (0.1 μM) reduced the frequency of the bursts without changing either action potential shape or resting membrane potential (Ba), and reduced the frequency of the contractions (Bb). In another preparation, which generated individual action potentials, a higher concentration of levcromakalim (1 μM) hyperpolarized the membrane and prevented the generation of spontaneous action potentials (Ca). It also abolished contractions and reduced the resting tension level (Cb).

8Br-cGMP (0.1–1 mM) reduced the frequency of spontaneous action potentials without changing the membrane potential ($15\pm3.8\,min^{-1}$ in control, $3.7\pm1\,min^{-1}$ in 0.1 mM 8Br-cGMP, $n=6$, Figure 5Ba; $16.3\pm43\,min^{-1}$ in control, $3.5\pm1.3\,min^{-1}$ in 1 mM 8Br-cGMP, $n=4$). It also reduced the frequency of corresponding contractions and strongly suppressed their amplitude (Figure 5Bb). In three preparations, which exhibited bursts of action potentials, 8Br-cGMP (0.1 mM) again strongly suppressed corresponding contractions (Figure 5Db) without inhibiting electrical activity (Figure 5Da).

Dissociation of the correlation between calcium and contractile responses by forskolin, 8Br-cGMP, 2-APB and Y-27632

Since forskolin and 8Br-cGMP were found to reduce the amplitude of spontaneous contractions without changing action potential amplitudes, in a separate series of experiments, changes in $[Ca^{2+}]_i$ and tension were simultaneously recorded to investigate the correlation between calcium and contractile responses.

Prior to carrying out the next series of experiments, the correlation between electrical, calcium and contractile responses was examined in nine preparations.

In five detrusor smooth muscle strips, which generated individual action potentials, associated calcium transients and corresponding phasic contractions were recorded (Figure 6A). In four preparations, which exhibited bursts of action potentials, each burst triggered large calcium transients and caused phasic contractions (Figure 6B). These observations clearly demonstrated the correlation between electrical, calcium and contractile responses.

In the following experiments, intracellular recordings were not routinely performed unless otherwise stated.

Forskolin (0.1 μM) reduced the frequency of calcium transients ($9.6\pm2.2\,min^{-1}$ in control, 4.1 ± 1.5 in forskolin, $P<0.05$, $n=7$) without changing their amplitude ($0.09\pm0.03R_{340/380}$ in control, $0.08\pm0.03R_{340/380}$ in forskolin, $P>0.05$, $n=7$, Figure 7Aa). It also reduced the amplitude and frequency of corresponding contractions (1.3 ± 0.7 mN in control, 0.4 ± 0.2 mN in forskolin, $P<0.05$, $n=7$, Figure 7Ab). A higher concentration of forskolin (1 μM, $n=3$) abolished calcium transients and contractions.

8Br-cGMP (0.1 mM) reduced the frequency of calcium transients ($8\pm3.1\,min^{-1}$ in control, 3.1 ± 1.9 in 8Br-cGMP, $P<0.05$, $n=7$) without changing their amplitude ($0.1\pm0.02R_{340/380}$ in control, $0.1\pm0.03R_{340/380}$ in 8Br-cGMP, $P>0.05$, $n=7$, Figure 7Ba). It reduced the amplitude and frequency of corresponding contractions (1.6 ± 0.6 mN in control, 0.4 ± 0.1 mN in 8Br-cGMP, $P<0.05$, $n=7$, Figure 7Bb).

2-APB (30 μM), an inhibitor for $InsP_3$-induced calcium release from intracellular stores, has been reported to inhibit spontaneous contractions without inhibiting action potentials (Imai *et al*., 2002). To further investigate the cellular mechanism of its inhibitory effects, effects of 2-APB on spontaneous calcium and mechanical activity were examined.

2-APB (30 μM) increased the frequency of calcium transients ($6.6\pm2.2\,min^{-1}$ in control, 8.1 ± 2.6 in 2-APB, $P>0.05$, $n=7$) without reducing their amplitude ($0.1\pm0.03R_{340/380}$ in control, $0.11\pm0.04R_{340/380}$ in 2-APB, $P>0.05$, $n=7$, Figure 7Ca). It also increased the frequency of corresponding contractions but reduced their amplitude (1.4 ± 0.5 mN in control, 0.5 ± 0.2 mN in 2-APB, $P<0.05$, $n=7$, Figure 7Cb).

One might expect that the reduction in the frequency of calcium transients itself may cause reductions in the amplitude of phasic contractions by reducing the resting calcium level. Therefore, we have examined the effects of a low concentration of levcromakalim (0.1 μM) on calcium and contractile responses.

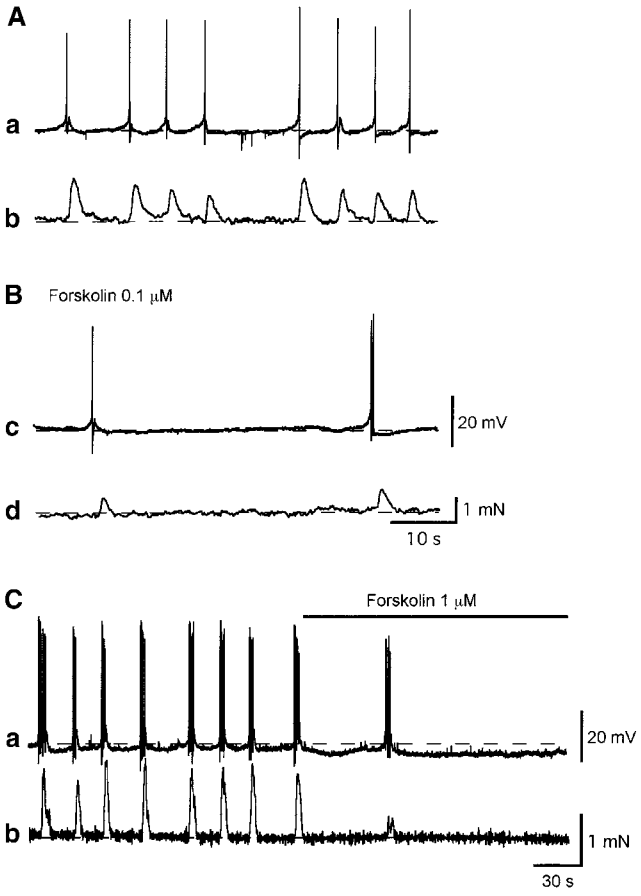


Figure 4 Effects of forskolin on the correlation between electrical and mechanical activity. In a bladder smooth muscle preparation, which exhibited individual action potentials (Aa), forskolin (0.1 μM) reduced the frequency of action potentials (Ba). It also reduced the frequency and amplitude of corresponding contractions (Bb). In another preparation, which generated bursts of action potentials, a higher concentration of forskolin (1 μM) hyperpolarized the membrane and prevented the generation of action potentials (Ca). It also abolished contractions (Cb). The resting membrane potentials were -44 mV in (A and B), and -45 mV in (C).

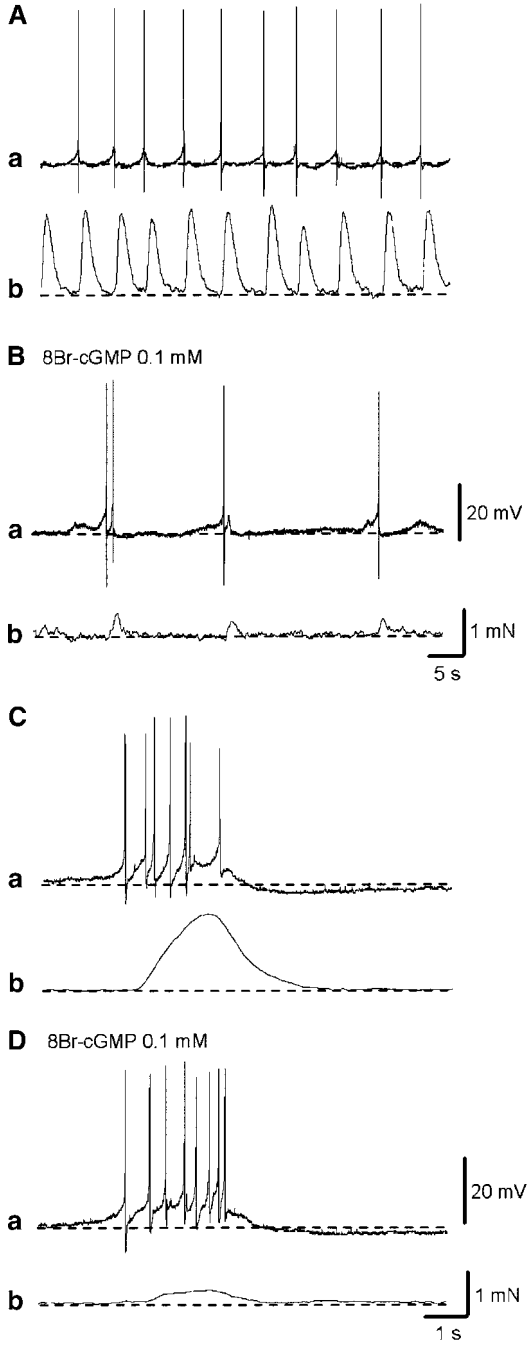


Figure 5 Effects of 8Br-cGMP on the correlation between electrical and mechanical activity. In a bladder smooth muscle preparation, which generated individual action potentials (Aa), 8Br-cGMP (0.1 mM) reduced the frequency of action potentials (Ba). It also reduced the frequency and amplitude of phasic contractions (Bb). In another preparation, which generated bursts of action potentials (Ca), 8Br-cGMP (0.1 mM) reduced the amplitude of corresponding contraction (Db) without affecting the bursts of action potentials (Da). The resting membrane potentials were -46 mV in (A and B) and -48 mV in (C and D).

Levcromakalim (0.1 μM) reduced the frequency of calcium transients ($9.8 \pm 2.9\,\text{min}^{-1}$ in control, 4.5 ± 2.6 in levcromakalim, $P > 0.05$, $n = 4$) without changing their amplitude ($0.08 \pm 0.03 R_{340/380}$ in control, $0.09 \pm 0.03 R_{340/380}$ in levcromakalim, $P > 0.05$, $n = 4$, Figure 7Da). It also reduced the frequency of spontaneous contractions, but did not change their amplitude (1.1 ± 0.5 mN in control, 0.1 ± 0.5 mN in levcromakalim, $P < 0.05$, $n = 4$, Figure 7Db).

Finally, the effects of Y-27632, a specific inhibitor of Rho-kinase (Uehata *et al*., 1997), on electrical, calcium and contractile responses were examined. In three preparations, Y-27632 (10 μM) did not inhibit either spontaneous action potentials or associated calcium transients (Figure 8Ba,b). However, Y-27632 (10 μM) reduced the amplitude of spontaneous contractions without affecting their frequency (Figure 8Bc).

Discussion

In the present study, the close correlation between electrical, calcium and mechanical activity in isolated detrusor smooth muscle bundles of the guinea-pig has been demonstrated. Increases in either the frequency or the duration of action potentials resulted in corresponding changes in $[Ca^{2+}]_i$ and mechanical activity, while the inhibition of electrical activity caused associated inhibition of the calcium transients and the

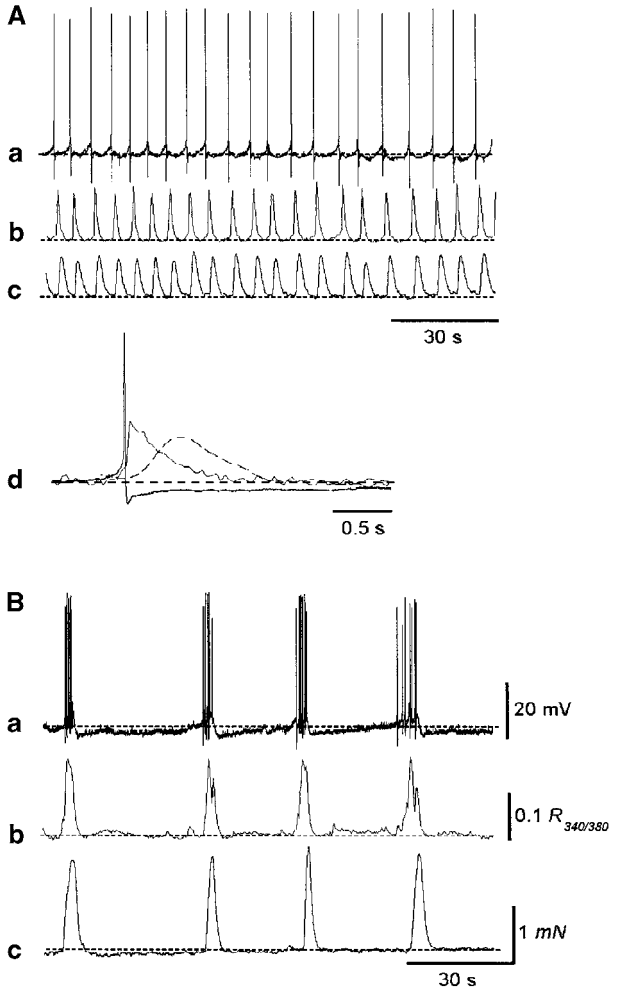


Figure 6 The correlation between electrical and mechanical activity and intracellular calcium simultaneously recorded from guinea-pig bladder smooth muscle. In a detrusor smooth muscle preparation in which spontaneous action potentials were individually generated (Aa), each action potential and associated calcium transient (Ab) produced a corresponding contraction (Ac). On a fast time scale (Ad), the action potential (full line) was associated with a calcium transient which lasted for some 0.5 s (dotted line), and was followed by a contraction which lasted for about 1 s (dashed line). In another preparation, bursts of action potentials were generated (Bb). Each burst consisted of some 20 action potentials and was associated with a summed calcium transient (Bb) which produced a phasic contraction (Bc). The resting membrane potentials were −45 mV in (A) and −47 mV in (B).

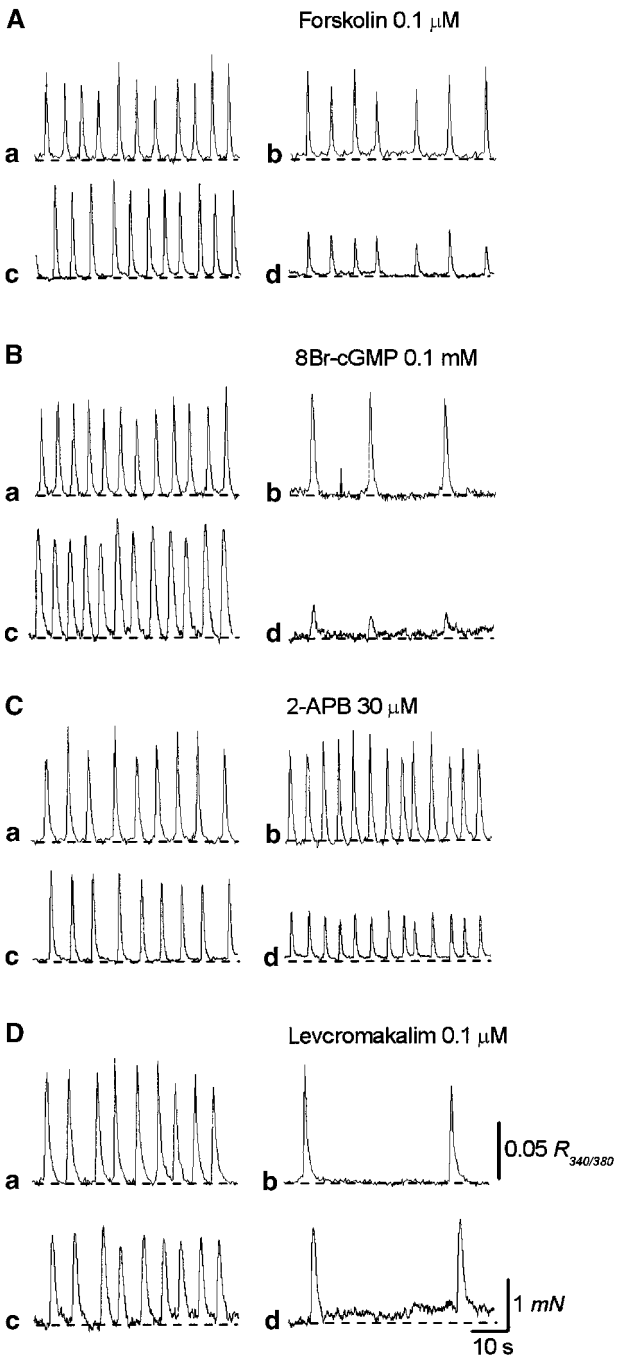


Figure 7 Effects of forskolin, 8Br-cGMP, 2-APB and levcromakalim on the correlation between calcium transients and mechanical activity. In a bladder smooth muscle preparation, which exhibited spontaneous calcium transients (Aa) and corresponding contractions (Ac), forskolin (0.1 μM) reduced the frequency of calcium transients without changing their amplitude (Ab), and reduced the amplitude of the contractions (Ad). In the same preparation, 8Br-cGMP (0.1 mM) reduced the frequency of calcium transients without changing their amplitude (Bb), and suppressed corresponding contractions (Bd). In a different preparation, 2-APB (30 μM) increased the frequency of calcium transients without changing their amplitude (Cb), and reduced the amplitude of corresponding contractions (Cd). In another preparation, levcromakalim (0.1 μM) reduced the frequency of calcium transients without changing their amplitude (Db). It also reduced the frequency of phasic contractions but did not change their amplitude (Dd).

contractile responses. In addition to this excitation–contraction coupling, alteration of the Ca sensitivity may play an important role in the regulation of spontaneous excitation in the bladder. This sensitivity could be modulated by cyclic nucleotides and Rho kinase, and allowed changes in the muscle tone without changing [Ca^{2+}].

Spontaneous contractions of detrusor smooth muscle strips from various species have been investigated in organ-bath studies using strips normally weighing several milligrams, and thus considerably larger than the single muscle bundles used in the present study. In such preparations, spontaneous contractions occur with relatively low frequencies (rabbit 7.3 min^{-1}, pig 1.1 min^{-1} and human 2.2 min^{-1}, Sibley (1984); guinea-pig 3.1 min^{-1}, Herrera *et al*. (2000); pig, 1.5 min^{-1}, Buckner *et al*.

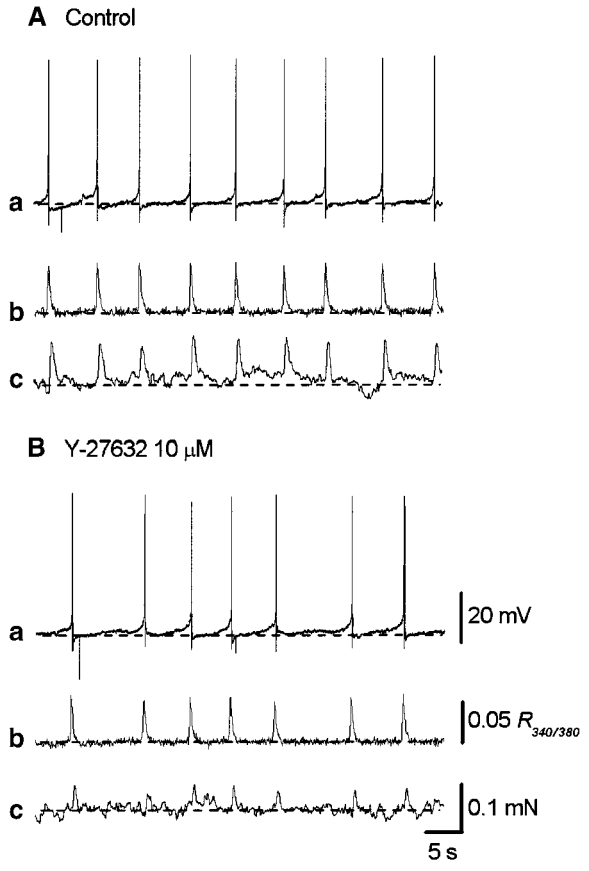


Figure 8 Effects of Y-27632 on electrical, calcium and mechanical activity. In a detrusor smooth muscle preparation, which exhibited individual action potentials (Aa), Y-27632 (10 μM) did not change either the amplitude or frequency of action potentials (Ba) or associated calcium transients (Bb) but it reduced the amplitude of the corresponding contractions (Bc). The resting membrane potential was −46 mV.

(2002)). The frequency of spontaneous action potentials recorded from a single cell in muscle bundles using intracellular recording techniques is significantly higher than that of contractions (guinea-pig 21.1 min^{-1}, Hashitani *et al.* (2001); pig 10.1 min^{-1} and human 7.9 min^{-1}, Hashitani & Brading (2003b)). Regardless of the obvious correlation between electrical and mechanical activity that we see in single detrusor muscle bundles, therefore, the fundamental role of spontaneous action potentials in the initiation of spontaneous contractions in strips and in the intact bladder remain to be established. It could be that only action potentials successfully propagated between several bundles and thus synchronizing their activity can generate recordable contractions in larger preparations, and the frequency of electrical activity recorded from cells in such preparations may thus be higher than that of the contractions. Indeed, the electrical coupling between detrusor smooth muscle cells is relatively poor (Bramich & Brading, 1996; Hashitani *et al.*, 2001), and thus in preparations with multiple smooth muscle bundles spontaneous excitation could possibly occur locally and not spread throughout the preparation. However, simultaneous recordings of electrical and mechanical activity in single detrusor smooth muscle bundles demonstrated that individual action potentials invariably initiated phasic contractions. In about 60% of preparations, bursts of action potentials were generated with a reasonably low frequency (4.2 min^{-1}), and caused corresponding contractions. It is therefore likely that in physiological conditions, burst of action potentials may be the normal pattern and the individual action potential pattern seen in some preparations may result from their stretch, since stretch has been reported to depolarize the membrane and increase the frequency of action potentials (Wellner & Isenberg, 1994). However, the resting membrane potentials of burst type of cells were not significantly different from those type of cells that generated individual action potentials, and we will need to consider other factors that may regulate the pattern of spontaneous action potentials.

Other populations of cells might be involved to determine the pattern of spontaneous action potentials. Indeed, ICC-like cells have been identified in the guinea-pig bladders, and preferentially located along the muscle boundary where spontaneous excitations originated (Hashitani *et al.*, 2001, McCloskey & Gurney, 2002). Consistent with previous reports, spontaneous contractions were abolished by nifedipine (1 μM) regardless of the pattern of underlying action potentials (Herrera *et al.*, 2000; Buckner *et al.*, 2002). However, when bursting action potentials were generated, nifedipine converted the bursts into individual transient depolarizations, which required at least 10 times higher concentrations of nifedipine (10 or 30 μM) to be blocked. Although we will need to await further investigations into the nature of the depolarizations, these potentials might be originating from ICC-like cells and would be unmasked only when L-type calcium channels in the bulk of smooth muscle cells had been blocked.

The stimulation of muscarinic receptors has been suggested to increase $[Ca^{2+}]_i$ by producing $InsP_3$ to contract detrusor smooth muscles (Iacovou *et al.*, 1990; Andersson *et al.*, 1991). In the present study, CCh (0.1 μM) increased the frequency of action potentials and corresponding contractions to cause a sustained rise in the muscle tone, indicating that increased electrical activity contributes to the muscarinic contractions, as it has been suggested for the contractions mediated by neurally released ACh (Hashitani *et al.*, 2000).

A study using isolated detrusor smooth muscle cells indicated that calcium entry through L-type calcium channels activates both BK and SK channels, but calcium release through ryanodine receptors activates only BK channels (Herrera & Nelson, 2002). Consistently, intracellular recordings showed that calcium entry through L-type calcium channels activates BK channels to repolarize the action potentials, and calcium-induced calcium release (CICR) is required to generate AHPs by opening BK channels (Hashitani & Brading, 2003a). In the same study, apamin often converted individual action potentials into bursts without changing either action potentials shapes or AHPs. In the present study, apamin had more emphasized excitatory effects in preparations which generated individual action potentials than in those that exhibited bursting action potentials. Therefore, the contribution of SK channels to the regulation of spontaneous excitation in detrusor smooth muscles may not be large under physiological condition, in which bursts of action potentials seem to be a dominant pattern. Indeed, apamin was shown to reinforce contractile responses in ryanodine-treated

preparations where the 'negative-feedback' function of BK channels had been diminished (Herrera *et al*., 2000).

CTX changed bursts of action potentials into prolonged depolarizations, which had a plateau lasting for some 5–20 s, and greatly increased the amplitude and duration of corresponding contractions. In a preliminary study, we have found that a combination of CTX and apamin initiated prolonged depolarizations, which were very similar to those observed in the present study. Therefore, it is again suggested that SK channels play a less extensive role in the regulation of spontaneous electrical activity in physiological conditions than BK channels, and that BK channels are critical for regulation of spontaneous excitation of detrusor smooth muscles.

The release of calcium from intracellular stores seems to have two 'opposite' effects on the excitability of detrusor smooth muscles. The calcium entry through L-type channels has been suggested to stimulate CICR from intracellular stores to contract muscles (Ganitkevich & Isenberg, 1992; Imaizumi *et al*., 1998; Hashitani *et al*., 2001). Conversely, the CICR has been proposed to cause a 'negative-feedback' on L-type calcium channels by the opening of BK channels (Herrera *et al*., 2000, Herrera & Nelson, 2002). In the present study, CPA increased the number of action potentials in each burst to increase the amplitude and duration of the phasic contractions. Therefore, CPA may be capable of facilitating contractile responses by clustering action potentials, even though the net calcium increase associated with each action potential might be reduced.

Surprisingly, Y-27632, a specific inhibitor of Rho-kinase (Uehata *et al*., 1997), suppressed spontaneous contractions without inhibiting either action potentials or associated calcium transients, indicating that Rho-kinase in detrusor smooth muscles may be activated in unstimulated conditions. It has recently been reported that Y-27632 inhibited CCh-induced but not high potassium-induced contractions in the rat urinary bladder (Wibberley *et al*., 2003). On the other hand, activation of Rho-kinase by membrane depolarizations has been reported (Mita *et al*., 2002). Since detrusor smooth muscle exhibits spontaneous depolarizations and relatively depolarized membranes, Rho-kinase could be contributing to spontaneous excitation in this smooth muscle. Alternations of Ca-sensitivity have been reported in animals with a partial bladder outlet obstruction (Stanton *et al*., 2003), and thus further investigations of this pathway might provide clues to understand the mechanisms of bladder overactivity.

Cyclic nucleotides exert their relaxation effects on smooth muscles in a number of ways, which may or may not involve a reduction in $[Ca^{2+}]_i$ (Lincoln & Cornwell, 1993; Carvajal *et al*., 2000). In the guinea-pig bladder, the activation of β-adrenoceptors has been shown to inhibit Ca transients by reducing the frequency of spontaneous action potentials (Nakahira *et al*., 2001). In the present study, a low concentration of forskolin (0.1 μM), which only reduced the action potential frequency, suppressed spontaneous contraction without reducing the amplitude of calcium transients. The simplest explanation of this observation would be a reduction of Ca sensitivity of the contractile proteins, and this mechanism may play a critical role in detrusor smooth muscle relaxations induced by β-adrenoceptors. Similarly, the prominent effect of the cGMP-pathway in detrusor smooth muscles seems to be the reduction of Ca sensitivity. It has been reported that cGMP-dependent protein kinase inhibits Rho kinase-induced Ca sensitization (Sauzeau *et al*., 2000), and thus cGMP decreases Ca sensitivity either directly by activating MLCP or indirectly by inhibiting Rho kinase activity.

2-APB, a blocker for $InsP_3$ receptors, has been reported to increase the frequency of action potential discharges while suppressing spontaneous contractions (Imai *et al*., 2002). Consistently, 2-APB increased the frequency of Ca transients; however, it suppressed phasic contractions without reducing the amplitude of calcium transients. These results suggest that 2-APB may also be capable of reducing the Ca sensitivity in guinea-pig detrusor smooth muscles, and that calcium release through $InsP_3$-sensitive Ca stores do not contribute to either the generation or amplification of spontaneous Ca transients. In gastrointestinal smooth muscles, in which spontaneous electrical activity in the form of slow waves is generated from ICCs, calcium release from $InsP_3$ receptors is a fundamental process in their generation, and in these tissues slow waves are readily blocked by either 2-APB or xestospongin C (Ward *et al*., 2000; Fukuta *et al*., 2002). Therefore, although ICC-like cells have been identified in the guinea-pig bladder (McCloskey & Gurney, 2002), the function of these cells in the bladder may be different from that of ICCs in the gastrointestinal tract.

In conclusion, spontaneous action potentials and associated Ca transients are confirmed to be fundamental processes for spontaneous excitation of detrusor smooth muscles. In addition, the modulation of Ca sensitivity may play an important role in regulating contractile activity of detrusor smooth muscles. Both cAMP and cGMP may be capable of reducing Ca sensitivity, while the activation of Rho kinase, presumably by membrane depolarizations may cause Ca sensitization. The manipulation of Ca sensitivity could be a novel target for the pharmacological treatment of the overactive bladder.

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