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Modulators of internal Ca^{2+} stores and the spontaneous electrical and contractile activity of the guinea-pig renal pelvis

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1 The role of internal Ca^{2+} stores in the generation of the rhythmic electrical and contractile activity in the guinea-pig proximal renal pelvis was examined using intracellular microelectrode and muscle tension recording techniques.

2 Ryanodine (30 μM) transiently increased contraction amplitude, while caffeine (0.5–3 mM) reduced contraction amplitude and frequency. Contractility was also reduced by 2-aminoethoxy-diphenylborate (2-APB 60 μM), xestospongin C (1 μM), U73122 (5 μM) and neomycin (4 mM), blockers of IP_3-dependent release from Ca^{2+} stores.

3 60 mM K^+ saline-evoked contractions were reduced by caffeine (1 mM), U73122 (5 μM) and neomycin (4 mM), but little affected by ryanodine or 2-APB (60 μM).

4 Spontaneous action potentials consisting of an initial spike followed by a long plateau were recorded (frequency 8.6 ± 1.0 min^{-1}) in small urothelium-denuded strips of proximal renal pelvis.

5 Action potential discharge was blocked in 75 and 35% of cells by 2-APB (60 μM) and caffeine (1 mM), respectively. In the remaining cells, only a truncation of the plateau phase was observed.

6 Cyclopiazonic acid (CPA 10 μM for 10–180 min), blocker of CaATPase, transiently increased contraction frequency and amplitude. Action potential durations were increased 3.6 fold. Contraction amplitude and frequency slowly declined during a prolonged (>60 min) CPA exposure.

7 We conclude that the action potential in caffeine-sensitive cells and the shoulder component of caffeine-insensitive action potential arise from the entry of Ca^{2+} through Ca^{2+} channels. The inhibitory actions of modulators of internal Ca^{2+} release were partially explained by a blockade of Ca^{2+} entry.

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Abbreviations: ANOVA, analysis of variance; 2-APB, 2-aminoethoxy-diphenylborate; $[Ca^{2+}]_i$, cytosolic concentration of free Ca^{2+}; CIRC, Ca^{2+} induced release of Ca^{2+}; CPA. cyclopiazonic acid; DMSO, dimethyl sulphoxide; Hist, histamine; ICC, interstitial cells of Cajal; MI, motility index (contraction amplitude *frequency); V s^{-1}, maximal rate of rise

Introduction

Electrical and contractile autorhythmicity in many smooth muscle organs arises from the cyclic changes in the internal concentration of Ca^{2+} ($[Ca^{2+}]_i$). For example, in the urethra and lymphatic vessels, spontaneous release of Ca^{2+} from internal stores activates transient dihydropyridine-insensitive membrane depolarizations (Van Helden, 1993; Sergeant *et al*., 2001; Hashitani *et al*., 1996). The rising phase of these depolarizations promotes the opening of 'L-type' Ca^{2+} channels, which contribute to the duration of the plateau phase of the recorded 'slow wave'. In the stomach of mouse (Dickens *et al*., 2001) and guinea-pig (Dickens *et al*., 1999; Van Helden *et al*., 2000) and the urethra of the guinea-pig (Hashitani *et al*., 1996) and rabbit (Sergeant *et al*., 2000), slow wave activity is triggered by the pacemaker activity of interstitial cells (interstitial cells of Cajal (ICC) in the intestine). Intestinal slow waves (Liu *et al*., 1995; Suzuki & Hirst, 1999; Van Helden *et al*., 2000) and interstitial pacemaker potentials (Ward *et al*., 2000; Sergeant *et al*., 2001) are rapidly inhibited upon depletion of ryanodine-sensitive internal Ca^{2+} stores, upon blockade of the sacroplasmic reticulum CaATPase or upon blockade of IP_3 formation and binding. Altogether, these data suggest that the cyclic Ca^{2+} release from internal stores forms an essential part of the mechanisms underlying autorhythmicity in smooth muscle.

Although it has been well established that pyeloureteral and ureteric peristalsis is myogenic in origin, both the cells responsible and mechanisms utilized in generating the pacemaker trigger remain controversial. 'Atypical' smooth muscle cells form an outer coat of sparsely-arranged cells which does not extend distal of the pelviureteral junction (Gosling & Dixon, 1974). The decreasing presence of these histologically- and morphologically-distinct 'atypical' smooth muscle cells along the renal pelvis of the guinea-pig correlates well with the decreasing gradient of autorhythmicity observed

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in the renal pelvis, and the absence of autorhythmicity in the ureter. Indeed, relatively-short spindle shaped cells of the pelvi-calyceal junction and proximal renal pelvis of the rat and guinea-pig have been demonstrated to display spontaneous depolarizing transients which could act as the pacemaker trigger (Klemm *et al*., 1999; Lang *et al*., 2001). However, in the guinea-pig there is an additional population of interstitial cells in the proximal renal pelvis which also display spontaneous electrical activity. These interstitial cells are located in the lamina propria and contain many of the distinguishing features of ICC in the intestine, as well as form an intercommunicating network with neighbouring interstitial cells and the smooth muscle cells. Renal interstitial cells are only observed occasionally in the rat renal pelvis (Lang *et al*., 2001). As the rat renal pelvis also displays a spontaneous contractile frequency some 3 fold greater than the guinea-pig, it appears that atypical smooth muscles cells in the rat are directly driving the typical smooth muscle cells. Thus, it has been suggested that renal interstitial cells in the guinea-pig play an integrative role rather than a pacemaker role in the upper urinary tract, summing the pacemaker potentials originating in the atypical smooth muscle until action potential discharge which then triggers electrical discharge in the typical smooth muscle cells (Klemm *et al*., 1999; Lang *et al*., 2001).

In this report, we have examined the actions of modulators of Ca^{2+} release from internal stores on the spontaneous electrical and contractile activity of the guinea-pig proximal renal pelvis. In particular, we have applied (i) low concentrations of caffeine (0.5–3 mM) and ryanodine; (ii) phosphatidylinositol-specific phospholipase C inhibitors, neomycin and U73122, which prevent the formation of IP_3 (Yang *et al*., 2000); (iii) 2-aminoethoxy-diphenylborate (2-APB) and xestospongin C, which inhibit IP_3-dependent Ca^{2+} release without preventing IP_3 binding (Gafni *et al*., 1997; Ward *et al*., 2000), and (iv) the CaATPase inhibitor, cyclopiazonic acid (CPA).

Methods

Tension recordings

The upper urinary tract was dissected free from the surrounding parenchyma and kidney and opened up with a longitudinal cut extending the length of the renal pelvis enabling the whole preparation to be pinned laid out flat. Two circumferential strips (2×5 mm^2) were cut from the most proximal portion of the renal pelvis. Threads were then tied around one end of each strip. The other end of each strip was pinned into an organ bath (1 ml) and perfused with physiological saline at 3 ml min^{-1} (at 35°C). The threads were attached to isometric force transducers, which were connected to a MacLab/4s, analogue-to-digital converter, driven by a Macintosh LC. A tension of approximately 1 mN was placed on each strip and then left to equilibrate for 30–60 min, after which time strips generated contractions that were regular in both amplitude and frequency. Contraction amplitudes, frequencies and motility indexes (MI, amplitude × frequency) were calculated as previously described (Teele & Lang, 1998; Davidson & Lang, 2000). Preparations were exposed to either histamine (Hist 10 μM for 4–5 min) or 20–60 mM K^+ saline (for 2–5 min) as test agents to examine the effects of our treatments on the excitability of the proximal renal pelvis.

Electrophysiological recordings

The renal pelvis was dissected free of the kidney, opened along its longitudinal axis and loosely pinned out in a dissecting dish with the urothelial layer uppermost. The urothelial layer was then removed by rubbing. Small portions (2×2 mm^2) were then dissected free and firmly pinned, urothelial side uppermost, into the recording chamber, taking great care to establish the orientation of the preparation. The bath was then mounted on an inverted microscope and perfused with the physiological saline (see below) at 2 ml min^{-1} (at 35°C).

Electrophysiological recordings were made using standard intracellular microelectrode techniques and one or two glass microelectrodes with resistances of >100 MΩ when filled with 0.5 M KCl. Changes in membrane potential were recorded with a high impedance pre-amplifier (Axoclamp-2 Axon Instruments Inc. Foster City, California, U.S.A.), low pass filtered at 1 kHz and stored digitally on a personal computer for later analysis. To examine the propagation of spontaneous action potentials, preparations were impaled with two microelectrodes 50–200 μm apart placed either in an axial or circumferencial orientation. Various parameters of the spontaneous action potentials were measured: (i) the frequency of action potential discharge; (ii) the maximum rate of rise of the initial spike; (iii) the peak membrane potential between spontaneous electrical events, and (iv) the duration of the action potential measured from the time the initial spike was half maximal. In each experiment, the parameters of 3–6 action potentials were averaged. A number of similar experiments (n) were then averaged as indicated. One way ANOVAs or paired or unpaired Student's t-test were used for tests for significance, $P<0.05$ was considered to be statistically significant (Klemm *et al*., 1999).

Measurements of internal Ca^{2+} concentrations

Preparations of urothelial-denuded proximal renal pelvis (2×2 mm^2) were pinned out on the bottom of a recording chamber similar to that used for the microelectrode recordings. After 30 min incubation with warmed (35°C) physiological saline and establishing that spontaneous electrical events were present, preparations were loaded with fluorescent dye, fura-PE3 by incubation in a low Ca^{2+} (1 mM) physiological saline containing fura-PE3 AM (10 μM) for 1 h at room temperature. After loading, preparations were perfused with dye-free physiological saline (at 2 ml min^{-1}) for 30 min. Preparations, loaded with fura-PE3, were illuminated with ultraviolet light, wave lengths 340 and 380 nm, alternating at a frequencies >40 Hz. The ratio of the emission fluorescence (F340/F380) in a desired region of the preparation was measured through a barrier filter of 510 nm (sampling time 100 ms), using a micro photoluminescence measurement system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). Changes in this ratio were used as an indication of changes in the internal concentration of Ca^{2+} ($[Ca^{2+}]_i$).

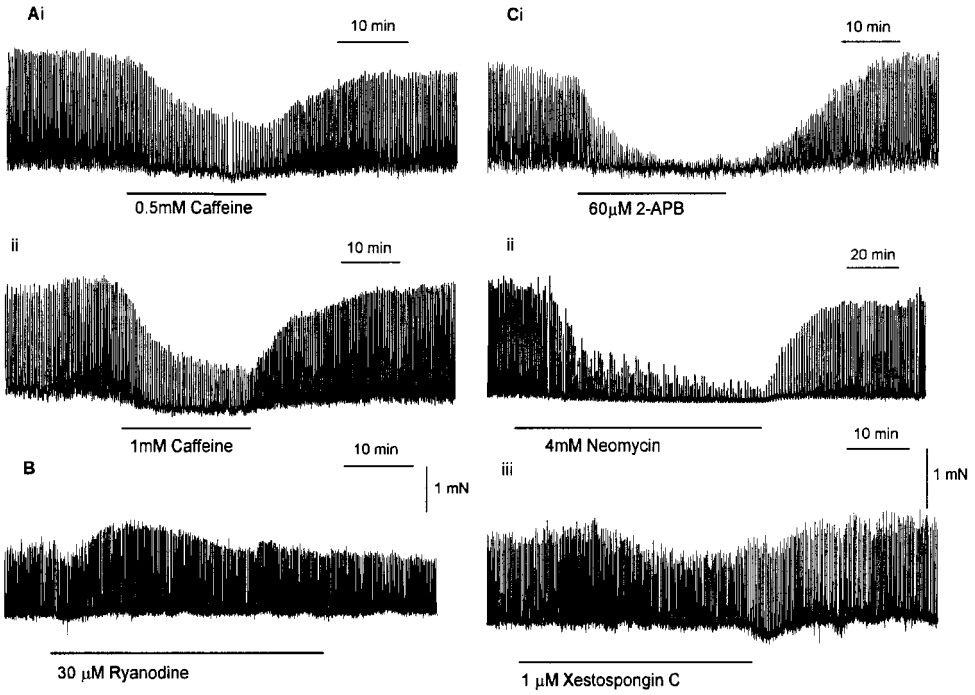

Figure 1 Effects of modulators of Ca^{2+} mobilization from ryanodine-sensitive and IP_3-dependent stores on the spontaneous contractile activity of the guinea-pig renal pelvis. Spontaneous contractions of the proximal renal pelvis in the absence and presence of caffeine (0.5 and 1 mM, Ai,ii), ryanodine (30 μM, B), 2-APB (60 μM, Ci), neomycin (4 mM, Cii) or xestospongin C (1 μM, Ciii).

Physiological saline

The physiological saline had the following composition (in mM): NaCl 122, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 15.5, and glucose 11.5, bubbled with a 95% O_2 : 5% CO_2 gas mixture to establish a pH of 7.3–7.4.

Drugs used included: caffeine, cyclopiazonic acid (CPA), ryanodine, U73122 (Sigma, St Louis Missouri, U.S.A.), fura-PE3 AM, xestospongin C, neomycin and 2-aminoethoxydiphenylborate (2-APB) (Calbiochem, San Diego, California, U.S.A.). Caffeine and neomycin were directly dissolved in the physiological saline. 2-APB was dissolved in 100% ethanol while ryanodine was dissolved in distilled water. CPA, U73122, xestospongin C and fura-PE3 AM were dissolved in dimethyl sulphoxide (DMSO). Stock solutions were generally added at least 1 : 1000 dilution. 0.1% ethanol or DMSO had no effect on the contractility of the renal pelvis.

Results

Spontaneous contractions in the renal pelvis

Spontaneous contractions regular in both amplitude and frequency were recorded in muscle strips of proximal renal pelvis. In one set of 11 control experiments, the averaged amplitude, frequency and calculated motility index (MI) were 1.95 ± 0.3 mN, 5.87 ± 0.5 min^{-1} and 10.93 ± 1.83 mN min^{-1}, respectively. All three parameters declined slowly with time, such that they were reduced to 60–70% of control after 4 h. As previously reported (Teele & Lang, 1998), the tonic release of sensory neuropeptides helps to maintain but not initiate these spontaneous contractions. The mean amplitude, frequency and MI, measured 60 min after a pretreatment of capsaicin (10 μM for 15 min), were decreased to $73 \pm 4\%$ ($P<0.05$ paired t-test, $n=12$), $94 \pm 4\%$ ($P>0.05$) and $69 \pm 5\%$ ($P<0.05$) of their respective control values (Davidson & Lang, 2000).

In control saline, both histamine (Hist 10 μM for 4–5 min, $n=37$) and high K^+ saline (20–60 mM) caused a transient, positive inotropic and chronotropic effect, significantly increasing contraction amplitude and frequency above their control values. For example, Hist (10 μM for 5 min) and 20 mM K^+ saline (for 2 min) increased the MI to $183 \pm 30\%$ ($P<0.05$, $n=37$) and $275 \pm 34\%$ ($P<0.05$, $n=10$) of their respective control values. This increase in contractility in high K^+ saline and Hist was also associated with a transient increase in the basal tension, the peak of which was about 0.2 mN (0.2 ± 0.05, and 0.17 ± 0.02, respectively; both $P<0.05$) above the control basal tension. Invariably, the peak increase in frequency occurred on the rising phase of the increase in contraction amplitude, before the peak increase in either the basal tension or contraction amplitude. 60 mM K^+ saline (for 5 min) also had transient positive inotropic and chronotropic effects on the spontaneous contractions of the renal pelvis. However these tension oscillations ceased within 2–3 min as the muscle tension rose to a maintained tension plateau 0.9 ± 0.4 mN above the base line ($P<0.05$) (Figure 2). The effects of Hist and high K^+ saline exposure slowly decayed over 10–20 min after washout.

Effects of caffeine and ryanodine

Low concentrations of caffeine (<3 mM) have been recently reported to block slow waves in the gastrointestinal tract (Dickens *et al*., 1999) in a manner not involving a modulation

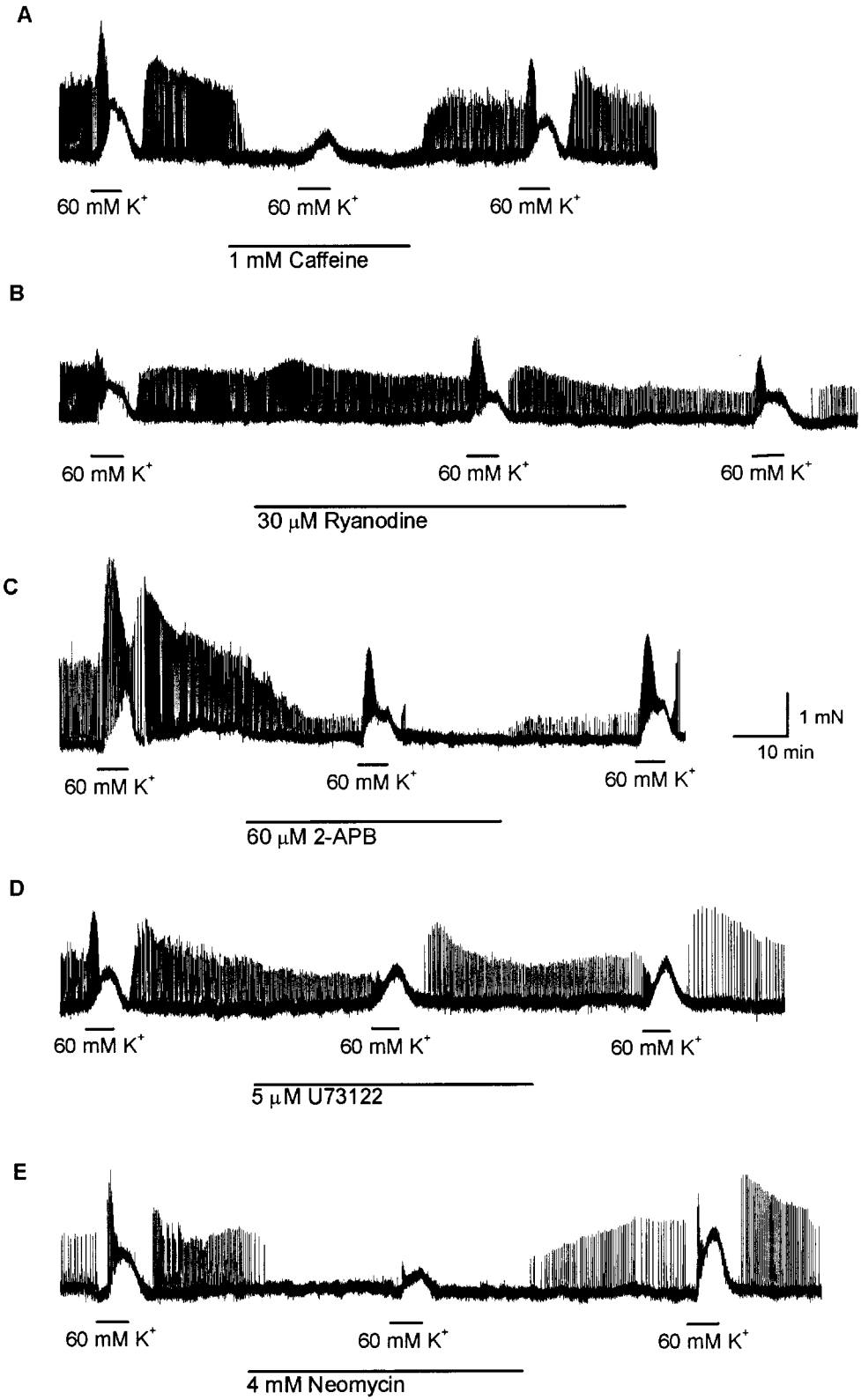

Figure 2 Comparison of the effects of modulators of ryanodine-sensitive or IP_3-dependent Ca^{2+} mobilization on the positive inotropic and chronotropic effects of high K^+ saline (60 mM). Contractions to K^+ saline (60 mM for 5 min) were evoked in the absence and presence of caffeine (1 mM, A), ryanodine (30 μM, B), 2-APB (60 μM, C), U73122 (5 μM, D) or neomycin (4 mM, E). Calibration bars apply to all traces.

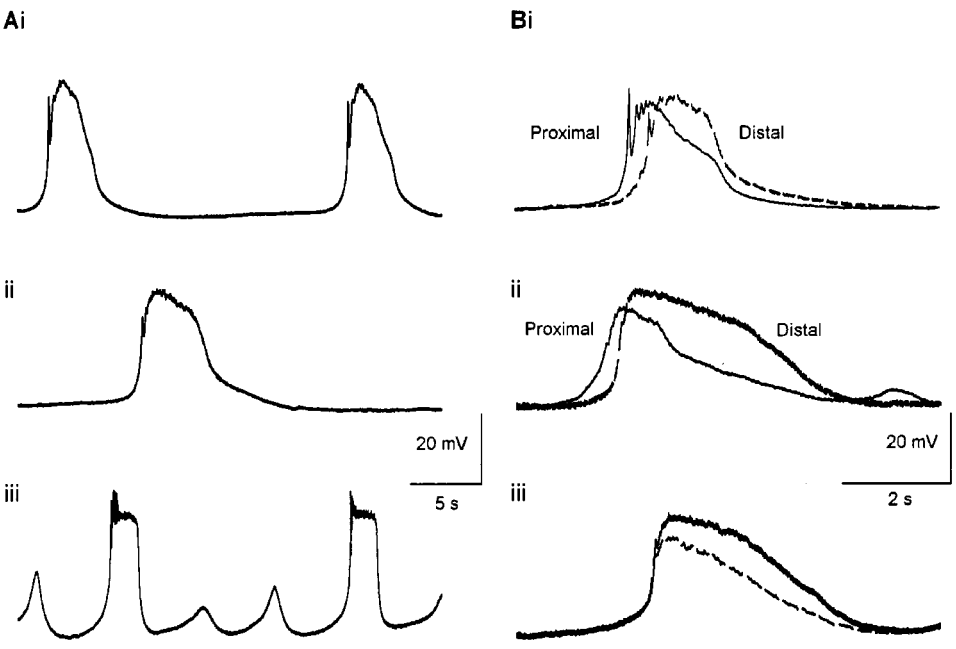

Figure 3 Typical examples of electrical discharge recorded in the urothelium-denuded proximal renal pelvis with a single (A) or a pair (B) of intracellular microelectrodes. Most commonly recorded action potentials were 'intermediate' in appearance (Ai,ii, Bi) with quiescent diastolic potentials, however 'ureteric-like' or 'driven' action potentials were also recorded (Aiii, Bii,iii). Both intermediate and driven action potentials displayed sub-threshold potential transients (Aiii, Bii). (B) Recordings from pairs of intracellular microelectrodes (100–200 μm apart) revealed that electrical responses propagated in both the axial (Bi,ii) and transverse (Biii) direction. Recordings could also occur simultaneously, suggesting that the electrodes were placed in the same smooth muscle bundle (Biii). Peak negative membrane potential between electrical events was −48 (Ai), −43 (Aii), −50 (Aiii), −43 (Bi), −40 (Bii) and −48 (Biii) mV, respectively. Time and voltage calibration bars apply to all traces.

of ryanodine-sensitive Ca^{2+} release channels (Van Helden *et al*., 2000). In the proximal renal pelvis, the application of caffeine (0.5 and 1 mM) produced a concentration-dependent reduction in the amplitude of the spontaneous contractions recorded (Figure 1Ai,ii). For example, caffeine (1 mM for 20 min) rapidly reduced the amplitude and frequency of the spontaneous contractions to 25 ± 0.3 mN and $6.2\pm.7$ min^{-1}; both $P<0.05$, $n=16$). This resulted in a significant reduction in the calculated MI to $48\pm10\%$ of control ($P<0.05$). Caffeine (3 mM for 20 min) ($n=3$) or bathing preparations in a Ca^{2+}-free saline (for 30–60 min) ($n=3$) caused a complete blockade of all contractile activity.

In contrast, ryanodine (30 μM for 30 to 60 min) evoked a transient significant increase in contraction amplitude in the proximal renal pelvis that peaked after 5–10 min and decayed over the next 20 min (Figure 1B). After 5 min exposure to ryanodine contraction amplitudes were $118\pm6\%$ of their control values ($P<0.05$, $n=6$). This increase in contraction amplitude was not accompanied by any significant increase in the contraction frequency or the calculated MI ($107\pm5\%$ and $164\pm36\%$ of control, respectively). After a 30 min exposure to ryanodine (30 μM), contraction amplitude, frequency and the calculated MI returned to $89\pm8\%$, $102\pm10\%$ and $117\pm33\%$ of their respective control values (all $P>0.05$).

Effects of 2-APB and xestospongin C

We examined the effects of selective inhibitors of the IP_3-dependent Ca^{2+} release channels which do not inhibit IP_3 binding (Gafni *et al*., 1997) on the spontaneous contractility of the renal pelvis. Application of 2-aminoethoxy-diphenylborate (2-APB) (60 μM for 30–60 min) (Figure 1Ci) caused a rapid reduction in both the contraction amplitude and frequency to $37\pm7\%$ and $55\pm9\%$ of their respective control values (both $P<0.05$, $n=11$). In contrast, xestospongin C (1 μM for 60–120 min) ($n=4$) significantly reduced the frequency (to $98\pm0.6\%$ of control) ($P<0.05$) of the spontaneous contractions of the renal pelvis, but had no significant effect on either the amplitude ($98\pm4\%$ of control) or the calculated MI ($98\pm5\%$ of control) (both $P>0.05$, (Figure 1Ciii).

Effects of neomycin and U73122

We have further examined the involvement of IP_3-dependent Ca^{2+} release in the generation of the spontaneous contractile activity in the proximal renal pelvis using neomycin and U73122, blockers of the formation of IP_3. Neomycin (4 mM for 60 min) caused a slowly-developing reduction in both the amplitude and frequency of the contractions in the proximal renal pelvis (Figure 1Cii). After 60 min exposure to neomycin, the averaged amplitude, frequency and MI were decreased to $13.8\pm0.5\%$, $29.9\pm8.1\%$ and $7.2\pm2.5\%$ of their respective control values (all $P<0.05$ $n=12$). It should be noted that five of these 12 preparations were completely inhibited after 60 min exposure to 4 mM neomycin. U73122 (5 μM) ($n=3$) was less effective at reducing contractions of the renal pelvis (Figure 2D). After 30 min exposure to U73122, contraction amplitudes, frequency and the calculated MI were $80\pm13\%$, $85\pm15\%$ and $67\pm17\%$ of their respective control values (all $P>0.05$).

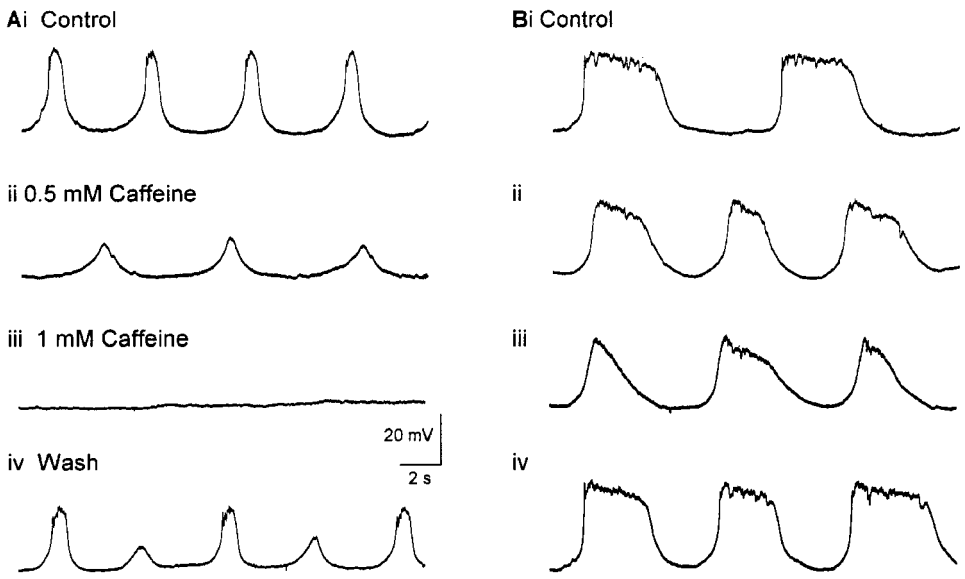

Figure 4 Effects of caffeine on the spontaneous electrical activity recorded in the guinea-pig renal pelvis. Application of caffeine (0.5 mM) produced a substantial block of the spontaneous action potentials (Ai,ii) in 35% of cells (6 of 17 cells) and only a reduction in both the maximum rate of rise and the plateau phase in the remaining 65% of cells (11 of 17) (Bi,ii). Raising the caffeine concentration (1 mM) abolished electrical discharge in the caffeine-sensitive cells (Aiii) and further decreased the plateau of the caffeine-insensitive cells (Biii). The effects of caffeine were readily reversible after 5 min wash out (Aiv, Biv). Membrane potentials in control saline were −42 (A) and −40 (B) mV, respectively. Calibration bars apply to all traces.

Effects of CPA

Exposure of preparations to cyclopiazonic acid (CPA 10 μM for 1–3 hours), a blocker of the CaATPase and therefore the refilling of internal Ca^{2+} stores, led to a transient increase in the contraction frequency associated with an increase in contraction amplitude (Figure 8A) (Santicioli & Maggi, 1997). After a 60 min exposure to CPA the percentage increase in the contraction amplitude was $280 \pm 21\%$ of control, while the frequency of contraction decreased to $49 \pm 4\%$ ($n=12$) of control. This resulted in an average increase in the calculated MI to $135 \pm 14\%$ of control. This increase in contraction amplitude was long lasting, the frequency of contraction also gradually decreased during this maintained exposure to CPA. Even after 3 h exposure to CPA (10 μM), spontaneous contractions of raised amplitudes but decreased frequency were still observed (Figure 8A). Moreover the positive inotropic effects of histamine (10 μM for 5 min) were still partially evident after this period of exposure to CPA ($n=3$) (data not shown).

To ascertain whether any of the agents tested above were having any additional non-specific actions we have also examined their action on the excitatory contractile responses evoked during a 5 min exposure to 60 mM K^+ saline. In Figure 2, it can be seen that caffeine (1 mM) ($n=7$) (Figure 2A), U73122 (5 μM) ($n=4$) (Figure 2D) and neomycin (4 mM) ($n=5$) (Figure 2E) all reduced the initial excitatory response to 60 mM K^+ saline. Moreover, caffeine (1 mM) and neomycin both significantly reduced the 60 mM K^+-induced rise in basal tone to $30 \pm 14\%$ and $39 \pm 22\%$ of their respective control values (both $P<0.05$). In contrast, the high K^+ contractions evoked in the presence of ryanodine (30 μM) ($n=5$) (Figure 2B) or 2-APB (60 μM) ($n=6$) (Figure 2C) were little affected. The rise in basal tension during the plateau was $91 \pm 23\%$ ($n=5$) and $66 \pm 18\%$ ($n=6$), respec-

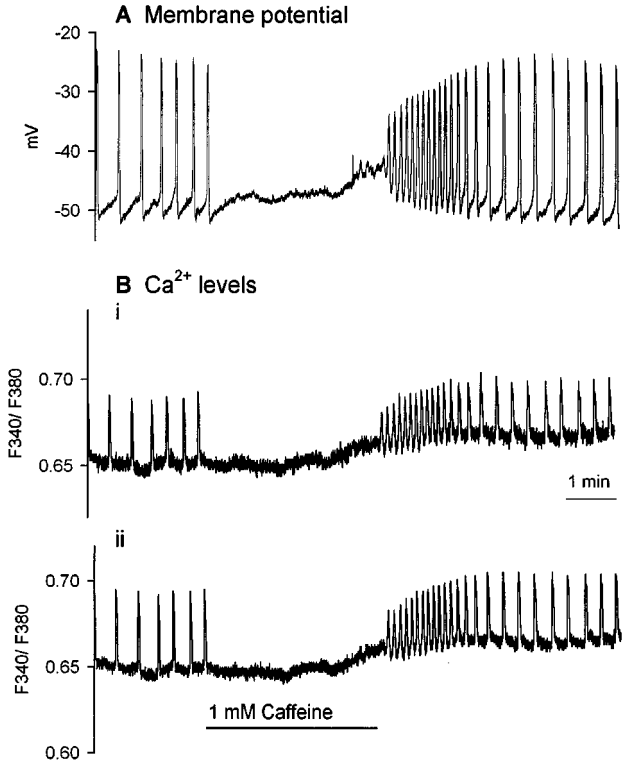

Figure 5 Caffeine-induced blockade of electrical activity in the renal pelvis was associated with cessation of transient increases of $[Ca^{2+}]_i$. Membrane potential recordings (A) were made simultaneously with relative changes in $[Ca^{2+}]_i$ in two areas (Bi,ii) of the renal pelvis adjacent to the intracellular microelectrode. Spontaneous action potential and increases in $[Ca^{2+}]_i$ were recorded synchronously. $[Ca^{2+}]_i$ transients consisted of a rapid rising phase and a slower decay phase. The application of caffeine (1 mM for min) induced a cessation of both action potential discharge and the transient increase in $[Ca^{2+}]_i$. Time calibration bar applies to all traces.

tively, not significantly different from their control rises in tension during an exposure to 60 mM K^+ saline (both $P>0.5$).

Spontaneous electrical activity in the renal pelvis

Electrical recordings were made from the urothelial side of small preparations of proximal renal pelvis, previously denuded of the urothelium. Almost all preparations displayed spontaneous contractile and electrical activity within 30 min of being bathed with warmed physiological saline. Most frequently, the spontaneous electrical activity consisted of a rapidly-rising single spike with a maximum rate of rise ($dV\ dt^{-1}$) of 0.76 ± 0.08 V s^{-1} (range 0.34–1.99) ($n=22$), which was followed by a long plateau 35.9 ± 1.4 mV (range 30.6–46.1 mV) positive of the membrane potential ($n=12$). The peak negative membrane potential between spontaneous action potentials was -43.4 ± 0.8 mV (range -28 to -50 mV) ($n=37$). Some cells did not have such a distinct initial spike and their plateau phases were generally longer in duration (Figure 3Aii,Bii). Electrical responses occurred at a mean frequency of 8.6 ± 1.0 min^{-1} (range 4.4–16.1) ($n=20$), and had a duration, as measured at the half-maximal amplitude, of 2.6 ± 0.5 s (range 0.8–5.9 s).

Recordings could be divided into cells that displayed spontaneous electrical events separated by a quiescent stable membrane potential (Figure 3Ai), and cells that displayed additional spontaneous potential transients, which appeared not to reach threshold for the triggering of the electrical events described above (Figure 3Aiii). The rapid oscillations on the plateau phase of 'driven cells' recorded in larger portions of urothelium-intact renal pelvis (Klemm *et al*., 1999) were only occasionally recorded in these urothelium-denuded preparations (Figure 3Aiii).

In four experiments, preparations were impaled with two microelectrodes. After the recording of spontaneous events was established with one electrode, a second electrode was placed within 100–200 μm of the first, either in the axial or transverse direction. Recordings made from cells which also displayed spontaneous electrical activity. Simultaneous recordings were then superimposed for comparison. On three occasions, there was a distinct delay (100–200 ms) between the firing of the proximal cell and the distal cell when the second electrode was placed 100 μM in the axial direction (Figure 3Bi–ii). On two other occasions, the spontaneous action potentials were recorded simultaneously in both cells, independent of whether the second electrode was placed (100 μm apart) either in the circular (Figure 3Biii) or the axial (data not shown) orientation, suggesting that the recordings were made in the same smooth muscle bundle. Recordings in the same bundle could be verified by examining the relative electrical coupling in the distal and circumferencial direction by passing a hyperpolarizing current (1–2.5 nA) into the electrode impaling the proximal cell and recording an electronic potential (1–5 mV) in the second electrode (data not shown).

Effects of caffeine

The effects of caffeine were examined using a single microelectrode. Cells displaying spontaneous action potential discharge could be divided into two groups by their relative sensitivity to caffeine (0.5 and 1 mM) exposure. In 35% of cells (6 of 17), caffeine (0.5 and 1 mM for 3–5 min) caused a concentration dependent reduction and finally abolition of action potential discharge (Figure 4Ai–iii). Blockade by 1 mM caffeine was preceded by a time-dependent reduction in the plateau phase, followed by an increasing failure to discharge the active responses, and finally, a cessation of all spontaneous activity (Figures 4Aiii and 5). The remaining 65% of cells (11 of 17) showed a relative insensitivity to caffeine. In these cells, caffeine (0.5 and 1 mM) caused a concentration dependent reduction in the plateau phase associated with a loss of the right 'shoulder' such that the responses became more triangular in appearance (Figure 4Bii,iii). In the presence of caffeine (1 mM for 5 min), the frequency (11.3 ± 2.4 min^{-1}) of action potential discharge in these caffeine-insensitive cells was not significantly different from control (8.9 ± 1.6 min^{-1}; $P>0.05$, $n=11$). However, the maximum rate of rise of these active responses in 1 mM

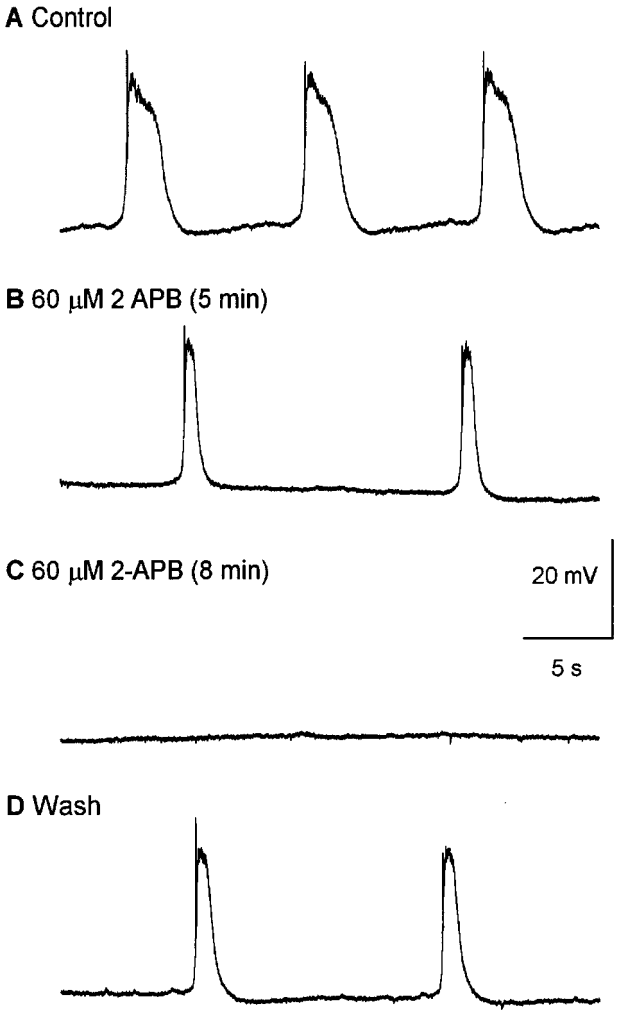

Figure 6 Effects of blocking IP_3-dependent Ca^{2+} release with 2-APB. Application of 2-APB (60 μM) caused a time-dependent reduction of the frequency and plateau component of the action potentials (A,B) recorded in the renal pelvis. In 75% of cells (six of eight cells), action potential discharge was completely abolished after 6 min exposure to 2-APB (C). The effects of 2-APB were partially reversible (D) upon washout. The membrane potential in control saline (A) was -48 mV. Calibration bars apply to all traces.

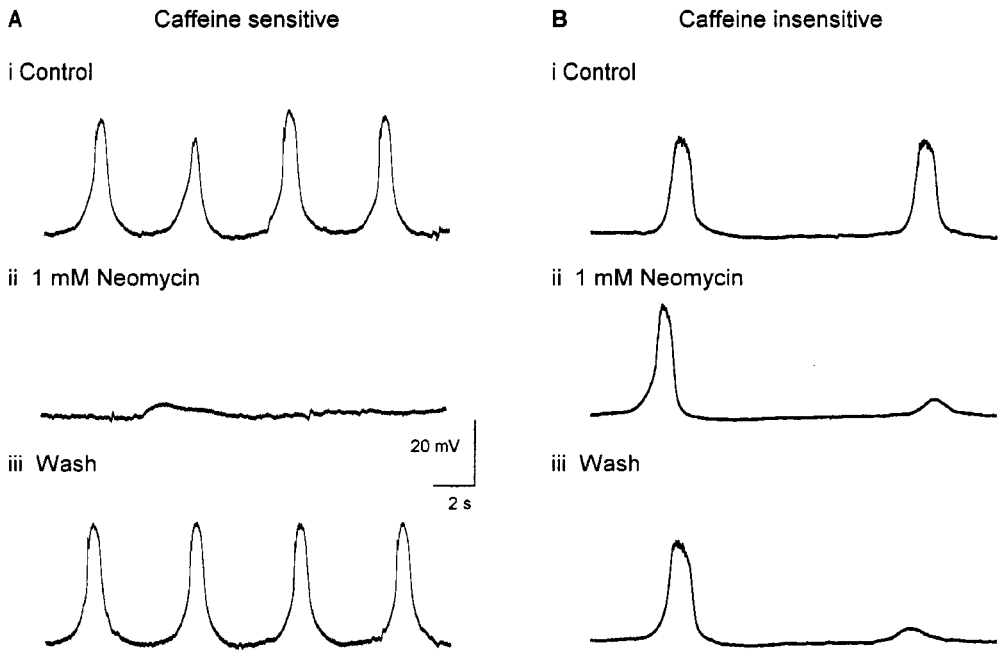

Figure 7 Effects of preventing IP_3 formation with the antibiotic, neomycin, on the electrical discharge in the proximal renal pelvis. Application of neomycin (1 mM) caused a time-dependent blockade of the action potential discharge in both caffeine-sensitive (Ai,ii) and -insensitive (Bi,ii) cells. Action potential discharge was completely restored upon wash-out (Aiii, Biii). The membrane potential in control saline was −45 (A) and −43 (B) mV, respectively. Time and voltage calibration bars apply to all traces.

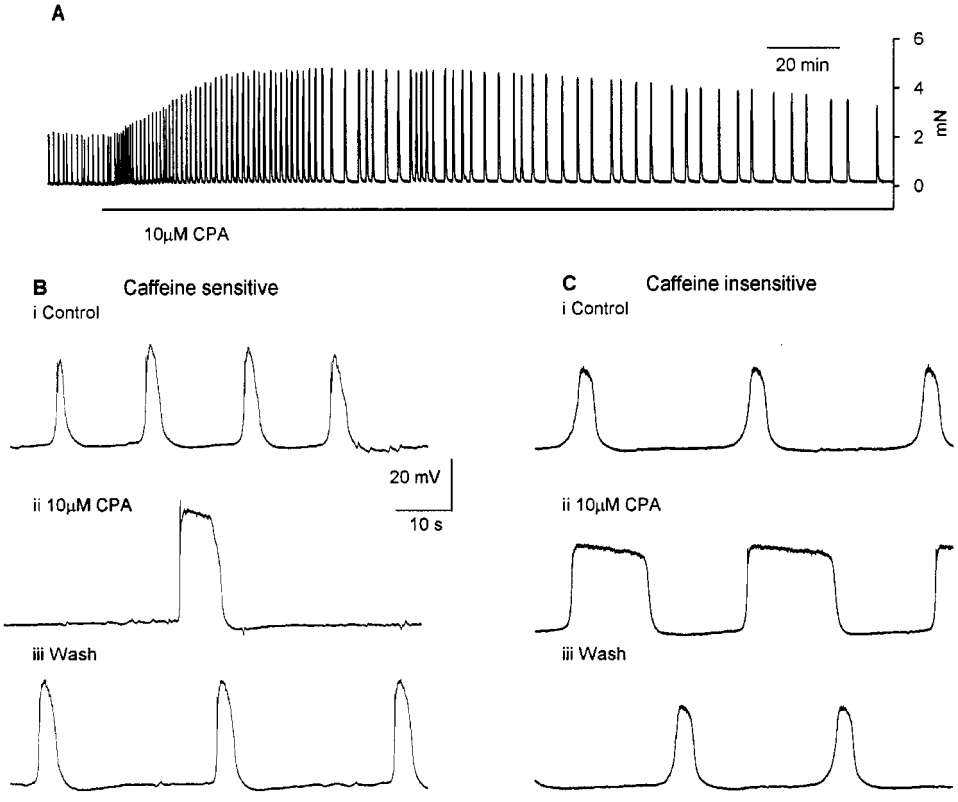

Figure 8 Effects of blocking the sarcoplasmic reticulum CaATPase with cyclopiazonic acid (CPA). (A) Contraction amplitude and frequency transiently increased and slowly declined with time in the presence of CPA (10 μM for >120 min). (B–C) Application of CPA (10 μM for 10 min) gradually increased the duration of the recorded action potential in both caffeine-sensitive (Bi,ii) and -insensitive (Ci,ii) cells. This effect of CPA was reversed upon wash-out (Biii, Ciii). The membrane potential in control saline was −48 (B) and −42 (C) mV, respectively. Time and voltage calibration bars apply to all traces.

caffeine (0.32 ± 0.026 V s^{-1}) was significantly decreased to 42% of control (0.77 ± 0.002 V s^{-1}). Washout of caffeine was associated with a transient increase in the frequency of discharge, followed by a gradual increase in the half-amplitude duration of individual responses (Figure 5A).

In six experiments, spontaneous action potential discharge was recorded simultaneously with the measurement of $[Ca^{2+}]_i$ in two areas adjacent (<100 μm) to the cell being impaled by the intracellular microelectrode. In all experiments, action potential discharge occurred synchronously with transient increases in $[Ca^{2+}]_i$ (Figure 5A,B). It was generally difficult to ascertain whether the Ca^{2+} transients preceded or followed the action potential due to the technical limitation of having to record the membrane potentials in a region electrically distant of the areas of Ca^{2+} measurement. However, when all electrical activity was blocked by caffeine (Figure 5A), the associated Ca^{2+} transients were also blocked (Figure 5Bi–ii).

Finally, there were no distinguishing features in the parameters of the action potentials in the absence of caffeine that could identify whether a cell was sensitive or insensitive to caffeine. There was no significant difference in either their membrane potentials, frequency of discharge or maximum rate of rise of the initial spike. Thus, in the following experiments all cells were first tested for their sensitive/insensitivity to caffeine (1 mM for 3–5 min).

Effects of 2-APB

We have demonstrated above (Figure 1Ci) that 2-APB reduced both the amplitude and frequency of the spontaneous contractions in the proximal renal pelvis. In the first 4–5 min during an exposure to 2-APB (60 μM for <10 min), there was a time-dependent reduction in both the frequency and the duration of the plateau of the action potentials recorded in both caffeine-sensitive ($n=3$) and caffeine-insensitive ($n=3$) cells (Figure 6A,B). In 75% (6 of 8) of cells, action potential discharge and any spontaneous potential transients were completely abolished after 6–8 min (Figure 6C). The effects of 2-APB were partially reversible, action potentials of a reduced frequency and duration returned after 10 min washout of 2-APB (Figure 6D).

There were two additional cells that were insensitive to both caffeine and 2-APB. These cells displayed spontaneous sub-threshold transients of membrane potential which were also insensitive to 2-APB and caffeine.

Effects of neomycin and CPA

The effects of neomycin (1 mM for 10–20 min) were examined in seven cells. Neomycin (1 mM) caused a time dependent reduction in the frequency of action potential discharge in both caffeine-sensitive (Figure 7A, $n=5$) and -insensitive (Figure 7B, $n=2$) cells. When these cells were pooled, the mean frequency was 2.61 ± 1.03 min^{-1} in neomycin, compared with the control frequency of 9.12 ± 2.2 min^{-1} ($P<0.05$). However, when there was an electrical discharge in the presence of neomycin, the rate of rise of the action potential was significantly reduced to 0.32 ± 0.03 V s^{-1} (0.54 ± 0.05 V s^{-1} in control saline) ($P<0.05$, $n=4$). The average amplitude of the plateau was not affected, being 34.3 ± 1.6 mV positive of the membrane potential in control, and 35.9 ± 1.6 mV in neomycin ($P>0.05$ $n=4$) (Figure 7Aii,Bii). After 10 min washout of neomycin, action potential discharge returned, both the frequency and the rate of rise of the initial spike (7.9 ± 1.6 min-1 and 0.53 ± 0.05 V s^{-1}, respectively) were restored to near control values (Figure 7Aiii,Biii). Finally, neomycin (4 mM for 10 min) reversibly abolished all spontaneous electrical activity ($n=3$) (data not shown).

We also examined the effects of CPA (10 μM for 10 min) on the spontaneous action potentials in both caffeine-sensitive ($n=3$) (Figure 8B) and -insensitive ($n=2$) (Figure 8C) cells in the urothelial-denuded proximal renal pelvis. When the pooled data was examined, CPA (after 10 min) significantly increased the half-amplitude duration to $363 \pm 74\%$ ($n=5$) of the control (control duration of 1.65 ± 0.051 s; $P<0.05$) (Figure 8Bii,Cii). However, CPA (for 5–10 min) had no significant effect on the frequency ($122.2 \pm 33.5\%$ of control), maximum rate of rise ($193.8 \pm 38.4\%$ of control) or amplitude ($107.8 \pm 7\%$ of control) of the spontaneous action potentials. These effects of CPA were readily reversed upon washout. It was generally not possible to maintain impalements to test the effects of longer exposures (>10 min) to CPA.

Discussion

When intracellular recordings are made from the serosal surface of large (5×5–15 mm^2) urothelial-intact strips of the proximal renal pelvis of guinea-pig and rat, three populations of cells have been identified, based on their characteristic electrical behaviour and their cell profile when filled with the cell marker, neurobiotin (Lang *et al*., 1995; 2001; Klemm *et al*., 1999). Small depolarizing membrane potential transients are recorded in 'atypical' smooth muscle cells of the most proximal regions of the renal pelvis, while 'driven' action potentials are recorded in 'typical' smooth muscle cells (Exintaris & Lang, 1999b). In contrast, 'intermediate' action potentials consisting of a single initial spike and a long quiescent plateau are recorded in irregularly-shaped interstitial cells, which closely resemble intestinal ICC even though they are not immuno-reactive for *c-Kit* (Klemm *et al*., 1999). Electrical activity in all three cell types is blocked in the presence of the 'L-type; Ca^{2+} channel blocker, nifedipine (1–3 μM) (Lang *et al*., 1995; Santicioli & Maggi, 1997), suggesting that these electrical events are active membrane responses, action potentials (Exintaris & Lang, 1999a), and not slow waves as in the gastrointestinal tract.

In the present experiments, much smaller (2×2 mm^2) portions of proximal renal pelvis were dissected, the urothelium was removed and the preparation was firmly pinned with the urothelial surface facing uppermost. Under these conditions, only two types of electrical events were evident: action potentials resembling 'intermediate' action potentials (Figure 3Ai,ii), and action potentials similar to 'driven' action potentials except that the initial spike and potential oscillations at the beginning of these action potentials were only seldom evident (Figure 3Aiii). However, these two types of action potential could not be clearly distinguished in terms of their frequency of discharge, resting or peak diastolic potentials, or initial spike amplitude, as was the case in the larger urothelium-intact preparations (Lang &

Zhang, 1996). This was presumably due to the shorter cable properties of the small urothelium-denuded portions of renal pelvis used and the membrane depolarization associated with the greater application of stretch when preparations were pinned in the organ bath. The short cable properties of these preparations would also mean that the electrical activity recorded with a single electrode may well reflect the conductance changes occurring in both the impaled cell and any cells to which it was electrically connected, be they other smooth muscle cells in the same or an adjacent bundle, or an interstitial cell. Although it has been difficult to unequivocally identify individual cells, we propose that the recordings made in the present experiments were mostly from 'intermediate' cells, which were distinguished by their initial spike, and from 'driven cells' which were somewhat depolarized, with truncated initial spikes and potential oscillations. The presence and communication between the two action potential types was confirmed in Figure 3Bii when two intracellular microelectrodes recorded simultaneously both types of electrical behaviour. Moreover, the 'intermediate' cell preceded the 'driven' cell by 100–200 ms, perhaps confirming our previous suggestions that intermediate interstitial cells provide the electrical excitation for driven typical smooth muscle cells (Klemm *et al*., 1999; Lang *et al*., 2001). Finally, cells firing 'only' pacemaker potentials were never recorded in the present experiments, perhaps confirming previous evidence that these cells were predominantly situated on the serosal surface of the most proximal regions of the guinea-pig renal pelvis (Gosling & Dixon, 1974).

Modulators of ryanodine-sensitive Ca^{2+} release

Low concentrations of caffeine (0.3–1 mM) have been previously used to markedly truncate the second component or plateau phase of slow waves in 'follower' smooth muscle cells within small preparations of the guinea-pig stomach. The shape of the 'driver' potentials recorded in the neighbouring ICC was little affected. Caffeine (2 mM) also reduced the plateau phase of driver potentials to reveal a caffeine-insensitive initial component, which fired at a greater frequency than in control saline (Dickens *et al*., 1999). These effects of caffeine were readily reversible and did not involve the modulation of ryanodine-sensitive Ca^{2+} stores (Van Helden *et al*., 2000). It has therefore been suggested that these low concentrations of caffeine inhibit IP_3-mediated Ca^{2+} release in a manner yet to be elucidated, but not involving a depletion of the internal Ca^{2+} stores. In Figure 1, we have demonstrated that caffeine (0.5 and 1 mM) reduces the amplitude and frequency of the spontaneous contractions in the guinea-pig renal pelvis in a concentration-dependent manner. This effect of caffeine is in direct contrast to the transient excitatory action of ryanodine (Figure 1B). Caffeine (1 mM) also reduced the maximum rate of rise and the right hand shoulder of the spontaneous action potentials in 65% of cells recorded in urothelium-denuded preparations of the proximal renal pelvis (Figure 4B). In the remaining 35% of cells, 1 mM caffeine blocked all electrical discharge (Figures 4A and 5). This block of caffeine was similar in time course to the inhibitory effects of nifedipine in the renal pelvis (Zhang & Lang, 1994; Lang *et al*., 1995; Santicioli & Maggi, 1997), being characterized by a gradual reduction in action potential duration followed by an increase in the number of 'failures' to discharge an action potential, and finally a complete cessation of all spontaneous electrical activity. The reduction of the plateau contraction to 60 mM K^+ saline in caffeine (1 mM) (Figure 2A) supports this notion that caffeine may well be decreasing Ca^{2+} entry through L-type Ca^{2+} channels. Thus, although our experiments are consistent in some respects with previous experiments in the stomach, we believe that it is more likely that caffeine is directly blocking Ca^{2+} channels (Zahradnik & Palade, 1993). If this is the case, the action potential in caffeine-sensitive cells and the shoulder component of the action potential of caffeine-insensitive cells are arising from a similar mechanism, i.e. from the entry of Ca^{2+} through voltage activated Ca^{2+} channels (Dickens *et al*., 1999).

Modulators of IP_3-dependent Ca^{2+} release

Exposure to 2-APB caused a rapid reduction of both the amplitude and frequency of spontaneous contractions of the guinea-pig renal pelvis. This inhibition was associated with decrease in frequency and duration, and finally a complete abolition of the action potentials recorded in both caffeine-sensitive and caffeine-insensitive cells. However, the contractions to 60 mM K^+ saline were not significantly affected by 2-APB, suggesting that Ca^{2+} entry through L-type Ca^{2+} channels was not significantly affected. Although the effects of 2-APB were superficially mimicked by U73122 and neomycin, both agents readily reduced the contractions to 60 mM K^+ saline (Figure 2), suggesting that part of their inhibitory action arises from a blockade of voltage-activated Ca^{2+} entry. The lack of an inhibitory effect in the presence of xestospongin C, when compared to 2-APB (Figure 1C), also suggests that the primary action of 2-APB does not arise from a blockade of IP_3-dependent Ca^{2+} release. Altogether, these data suggest that 2-APB must be having an additional inhibitory action. Recently, 2-APB has been demonstrated to inhibit the refilling of internal Ca^{2+} stores by blocking store-operated channels without affecting IP_3-dependent Ca^{2+} release (Gregory *et al*., 2001). If this were the case in the renal pelvis, 2-APB would cause a slow depletion of all internal stores, reducing any Ca^{2+} release evoked upon Ca^{2+} entry during the upstroke of each action potential.

Blockade of CaATPase

The relative resistance of the spontaneous contractions of the renal pelvis to CaATPase inhibitors has been previously described. Blockade of the Ca^{2+} re-uptake into internal stores with thapsigargin (Maggi *et al*., 1995a) or CPA (Figure 8A), leads to a transient increase in contraction amplitude and frequency in the guinea-pig renal pelvis, which is followed by a slow reduction (over 60 min) towards control values (Santicioli & Maggi, 1997). CPA and ryanodine also increase the amplitude and duration of electrically-evoked contractions and their accompanying $[Ca^{2+}]_i$ transients in the ureter (Maggi *et al*., 1995b; Burdyga & Wray, 1999). During short-term exposures (<10 min) to CPA, the increase in contraction amplitude in the guinea-pig renal pelvis was accompanied by a 2–4 fold increase in the duration of the spontaneous action potentials in both caffeine-sensitive and -insensitive cells (Figure 8B,C). Even after 120–180 min exposure to CPA, occasional spontaneous contractions were

still recorded, albeit infrequently (Figure 8A). We were not able to obtain continuous electrical recordings during these prolonged exposures to CPA. Thus it is not known whether pacemaker activity continues in the absence of action potential discharge, as in the presence of indomethacin (Zhang & Lang, 1994). Alternatively, these occasional contractions in the prolonged presence of CPA could well arise from random action potential discharge triggered directly in driven smooth muscle cells. This resistance of the renal pelvis to CaATPase inhibitors has led us to conclude that the release of Ca^{2+} from internal stores is not the primary pacemaker mechanism as has been suggested in smooth muscles which display nifedipine-insensitive slow waves. At this point we can only conclude that the main role of Ca^{2+} re-uptake *via* CaATPases in the renal pelvis under control conditions appears to be limiting both the action potential duration and the amount of Ca^{2+} reaching the contractile proteins (Burdyga & Wray, 1999). Thus the mechanisms underlying autorhythmicity in the renal pelvis remain to be elucidated.

In summary, the contractile and electrical activity of the proximal renal pelvis displays a marked resistance to blockade in the presence of agents which rapidly block autorhythmicity in other smooth muscles (Hashitani *et al*., 1996; Ward *et al*., 2000; Sergeant *et al*., 2001). In addition, all activity in the renal pelvis is readily blocked in nifedipine, while slow waves are usually resistant to L-type Ca^{2+} blockade. These two fundamental differences suggest that the role of Ca^{2+} stores in the mechanisms underlying pacemaking in the upper urinary tract must not be similar to those in the intestine, mesenteric lymphatic vessels or urethra. In the renal pelvis, the release from ryanodine-sensitive and IP_3-dependent Ca^{2+} stores appears to contribute little to the frequency generator of the spontaneous action potentials. However, a cyclic movement of Ca^{2+} into and out of these stores appears to be involved in regulating the rise of $[Ca^{2+}]_i$, and therefore contraction, evoked during the time course of each spontaneous action potential.

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