

Calcium oscillations in interstitial cells of the rabbit urethra

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Measurements were made (using fast confocal microscopy) of intracellular Ca^{2+} levels in fluo-4 loaded interstitial cells isolated from the rabbit urethra. These cells exhibited regular Ca^{2+} oscillations which were associated with spontaneous transient inward currents recorded under voltage clamp. Interference with D-myoinositol 1,4,5-trisphosphate (IP_3) induced Ca^{2+} release using $100 \mu\text{M}$ 2-aminoethoxydiphenyl borate, and the phospholipase C (PLC) inhibitors 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate and U73122 decreased the amplitude of spontaneous oscillations but did not abolish them. However, oscillations were abolished when ryanodine receptors were blocked with tetracaine or ryanodine. Oscillations ceased in the absence of external Ca^{2+} , and frequency was directly proportional to the external Ca^{2+} concentration. Frequency of Ca^{2+} oscillation was reduced by SKF-96365, but not by nifedipine. Lanthanum and cadmium completely blocked oscillations. These results suggest that Ca^{2+} oscillations in isolated rabbit urethral interstitial cells are initiated by Ca^{2+} release from ryanodine-sensitive intracellular stores, that oscillation frequency is very sensitive to the external Ca^{2+} concentration and that conversion of the primary oscillation to a propagated Ca^{2+} wave depends upon IP_3 -induced Ca^{2+} release.

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The rabbit urethra contains a subpopulation of cells which are distinct both functionally and morphologically from the bulk smooth muscle cells (Sergeant *et al.* 2000). These 'interstitial cells' are noncontractile but are spontaneously active showing regular depolarizations STDs (spontaneous transient depolarizations) under current clamp conditions. The currents or STICs (spontaneous transient inward currents), which underlie the STDs, are believed to result from activation of Ca^{2+} -dependent Cl^- channels by Ca^{2+} released from intracellular stores (Sergeant *et al.* 2001a,b). In a recent study we demonstrated that interstitial cells fired large STICs and two types of STOCs (spontaneous transient outward currents), 'fast' (>100 ms in duration) and 'slow' (>1 s in duration). The latter were coupled to STICs, suggesting that they shared the same mechanism, while the former occurred independently at faster rates. All of these currents were abolished by cyclopiazonic acid, caffeine or ryanodine, suggesting that they were activated by Ca^{2+} release. When D-myoinositol 1,4,5-trisphosphate (IP_3)-sensitive stores were blocked with 2-aminoethoxydiphenyl borate (2-APB), the STICs and slow STOCs were abolished, but the fast STOCs remained. In all of the above experiments it was inferred that spontaneous intracellular Ca^{2+} oscillations were responsible for the observed changes in membrane potential or currents, so we found it curious that 2-APB

appeared to suppress the Ca^{2+} release necessary for the STICs but not that required for the STOCs. Using purely electrophysiological techniques we were unable to resolve this apparent contradiction, so the purpose of the present investigation was to measure changes in intracellular Ca^{2+} to shed further light on the nature of spontaneous activity in urethral interstitial cells.

Methods

Bladder and urethra were removed from both male and female rabbits immediately after they had been killed by lethal injection of pentobarbitone. The most proximal 1 cm of the urethra was removed and placed in Krebs solution, and from this, 0.5 cm strips were dissected and cut into 1 mm^3 pieces and stored in Hanks' Ca^{2+} -free solution for 30 min before being incubated in an enzyme medium containing (per 5 ml of Hanks' Ca^{2+} -free solution) 15 mg collagenase (Sigma, type 1A), 1 mg protease (Sigma, type XXIV), 10 mg BSA (Sigma), and 10 mg trypsin inhibitor (Sigma) for 5 min at 37°C . They were then placed in Hanks' Ca^{2+} -free solution and stirred for another 5–10 min to release single cells. These were of two types: (1) smooth muscle cells which had the characteristic spindle shape and accounted for about 95% of the total, and (2) interstitial cells which could be readily

distinguished from the former by their highly branched appearance under bright field illumination (Sergeant *et al.* 2000). In the main these latter cells were chosen for study, but in some experiments smooth muscle cells were also studied.

Solutions and drugs

The solutions used were of the following composition (mM): (1) Hanks' Ca²⁺-free solution (for cell dispersal), 141 Na⁺, 5.8 K⁺, 130.3 Cl⁻, 15.5 HCO₃⁻, 0.34 HPO₄²⁻, 0.44 H₂PO₄⁻, 10 dextrose, 2.9 sucrose; 10 Hepes (Melford), pH adjusted to 7.4 with NaOH; (2) bath solution, 130 Na⁺, 5.8 K⁺, 135 Cl⁻, 4.16 HCO₃⁻, 0.34 HPO₃²⁻, 0.44 H₂PO₄⁻, 1.8 Ca²⁺, 0.9 Mg²⁺, 0.4 SO₄²⁻, 10 dextrose, 2.9 sucrose, 10 Hepes, pH adjusted to 7.4 with NaOH; (3) Cs⁺ pipette solution, 133 Cs⁺, 1 Mg²⁺, 135 Cl⁻, 0.5 EGTA (Sigma), 10 Hepes, pH adjusted to 7.2 with CsOH. For low-Ca²⁺ bath solution, CaCl₂ was iso-osmotically substituted with MgCl₂. It could be argued that the relatively high values of Mg²⁺ that would be found in the nominally Ca²⁺-free solutions could block voltage-dependent channels (Fukushima & Hagiwara, 1985) but this would not affect the interpretation of our results. When Ca²⁺ levels were raised above normal, no osmotic adjustment was made since the change in osmolality would be trivial.

The following drugs were used: caffeine, 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC), ryanodine, tetracaine (all Sigma), SKF-96365, U73122 (Calbiochem) 2-APB (Acros) and nifedipine (Bayer). Data are presented as means ± s.e.m., and statistical differences were compared using Student's paired *t* test, taking *P* < 0.05 as significant.

Ca²⁺ imaging

Cells were incubated for 15–30 min in 2–10 μM fluo-4/AM (Molecular Probes) in Hanks' solution containing 100 μM Ca²⁺ at 37°C. They were then allowed to settle in glass-bottomed Petri dishes and placed on the stage of either a Nikon TE3000 or a Nikon TE2000 microscope. After the cells had been allowed to stick down for 30 min, they were perfused at 37°C in normal Hanks' solution and imaged using ×40, ×60 or ×100 oil immersion lenses with either an iXon 887 EMCCD camera (Andor Technology, Belfast; 512 × 512 pixels, pixel size 16 × 16 μM) coupled to a Nipkow spinning disk confocal head (CSU22, Yokogawa, Japan) or a MegaXR10 GenIII + ICCD (Stanford Photonics, USA; 1280 × 1024 pixels, pixel size 7 × 7 μM) attached to a Nipkow spinning disk confocal head (CSU10, Visitech UK). A krypton–argon laser (Melles Griot Ltd, UK) at 488 nm was used to excite the Fluo 4, and the emitted light was detected at wavelengths >510 nm. Images were usually acquired at 5 or 15 frames s⁻¹ and

analysed using ImageJ. Image grabbing and data sampling were performed on either a 2.4 Ghz Intel Xeon Dell PC using Andor software or a Power Macintosh using QED software (version 1.6). *Post-hoc* analysis and figure preparation were carried out using either NIH Image 1.62 or ImageJ (National Institutes of Health, MD, USA). Where indicated, the fluorescence intensity was normalized by dividing the Ca²⁺ signal in the region of interest by the average fluorescence intensity found in the resting cell (*F*/*F*₀). During experiments the dish containing cells was perfused with bath solution. In addition, the cell under study was continuously superfused by means of a close delivery system consisting of a pipette (tip diameter 200 μm) placed approximately 300 μm away. This could be changed, with a dead space time of around 10 s, to a solution containing a drug. All experiments were carried out at 37°C.

Patch clamping

In some experiments, cells were simultaneously imaged and voltage clamped. Recordings were made using the amphotericin B perforated patch method as previously described (Sergeant *et al.* 2000). Voltage clamp commands were delivered with an Axopatch 1D patch-clamp amplifier (Axon Instruments), and currents were recorded by means of a 12-bit AD/DA converter (Labmaster, Scientific Solutions) interfaced to an Intel computer running pCLAMP software (Axon Instruments). Recordings were synchronized by placing a light-emitting diode within the light path and giving a light pulse at the beginning of the voltage-clamp sweep, thus enabling the two records to be lined up afterwards.

Results

In any given dispersal, about 5% of the cells showed (under bright field illumination) the highly branched appearance typical of interstitial cells (Sergeant *et al.* 2000). Of these, more than 60% showed regular spontaneous increases in fluorescence intensity when viewed with the confocal microscope. In contrast the smooth muscle cells, which again were readily identified under bright field illumination by their smooth spindle-shape, rarely exhibited spontaneous Ca²⁺ oscillations. Figure 1 illustrates this point. Each of the 10 frames shows a highly branched cell on the right of the frame (a typical interstitial cell) and a darker spindle-shaped cell (smooth muscle cell) on the left, scarcely visible under control conditions. Regular spontaneous increases in fluorescence intensity were apparent in the perinuclear region of the branched cell, while the smooth muscle cell remained dark. However, when 10 mM caffeine was added to the perfusate, both cells lit up simultaneously. When the caffeine-induced transient was complete, the smooth muscle cell again became dark

and remained so for the rest of the experiment, while the interstitial cell, after a brief pause, resumed its oscillatory behaviour. This experiment also illustrated the different contractile properties of both cells. Thus, while the smooth muscle cell contracted vigorously in response to caffeine addition, the interstitial cell did not contract either in response to the spontaneous increases in intracellular Ca^{2+} or in response to the caffeine-induced Ca^{2+} transient.

The oscillations described above were spontaneous (i.e. they occurred in the absence of external agonist) but we have demonstrated previously (Sergeant *et al.* 2001a,b, 2002) that agonists such as noradrenaline that are known to raise intracellular IP_3 levels have the effect of increasing the frequency of spontaneous depolarizations or spontaneous inward currents. It was therefore of interest to examine the effects of three substances that are known to inhibit IP_3 -induced Ca^{2+} release.

Effect of 2-APB

This drug is known (among its other actions) to inhibit Ca^{2+} release from IP_3 R-modulated channels (Bootman *et al.* 2002; Peppiatt *et al.* 2003), and we have demonstrated previously (Sergeant *et al.* 2001a) that a dose of $100 \mu\text{M}$ completely abolished STICs. In the experiment shown in Fig. 2A, the upper record shows Ca^{2+} oscillations imaged in the perinuclear region of the cell, while the lower record shows the STICS resulting from the intracellular increases in Ca^{2+} . Addition of $100 \mu\text{M}$ 2-APB had the effect of almost abolishing the STICS, but the underlying Ca^{2+} oscillations, while reduced in amplitude, retained their normal rhythm and returned to control amplitude after washout of the drug. In 11 such experiments (Fig. 2B), the frequency of Ca^{2+} oscillation was 4.8 ± 0.49 (s.e.m.) per minute before, and 4.1 ± 0.39 during drug perfusion (not significant, paired *t* test). Oscillation amplitude decreased

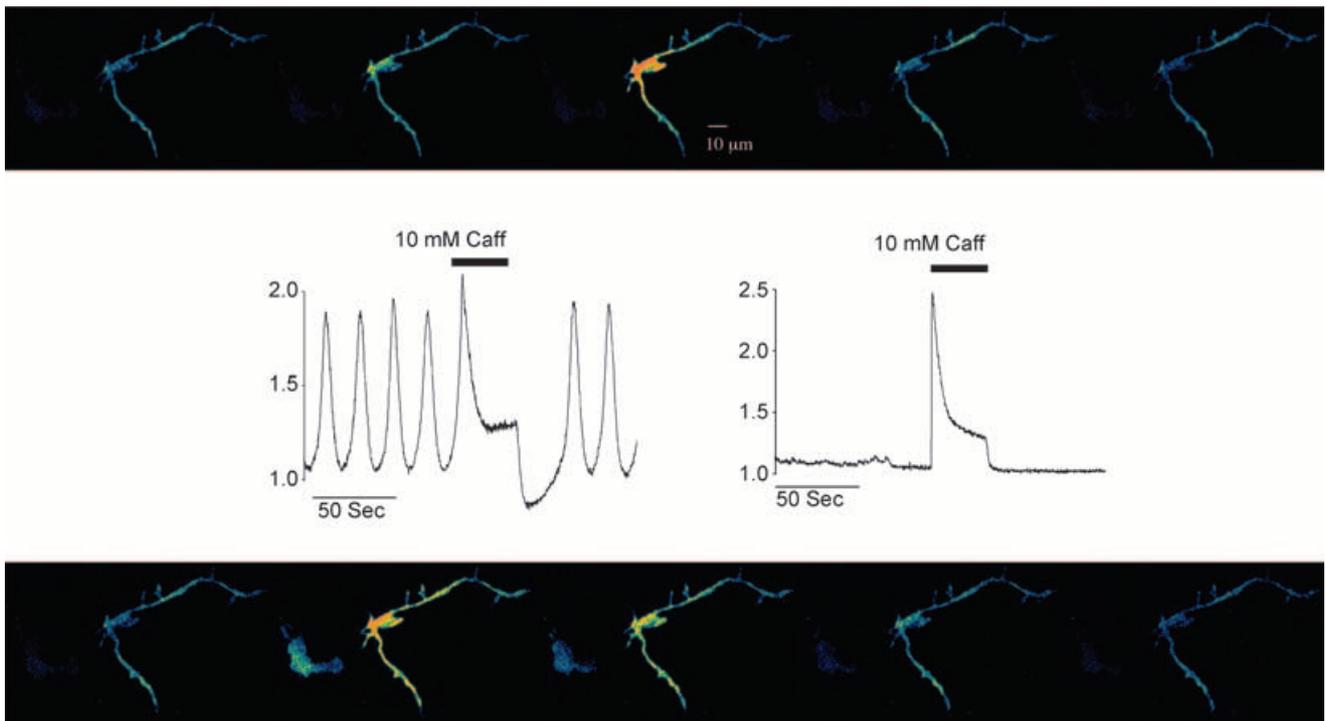


Figure 1. Ca^{2+} changes in interstitial and smooth muscle cells

Sequence of 10 frames before (top panel, taken at 4 s intervals) and during addition of 10 mM caffeine (bottom panel, at 3 s intervals). The highly branched cell on the right of each frame is a typical interstitial cell, while the darker spindle-shaped cell on the left, scarcely visible under control conditions, is a smooth muscle cell. Regular spontaneous increases in fluorescent intensity were apparent in the perinuclear region of the branched cell, while the smooth muscle cell remained dark. However, when 10 mM caffeine was added both cells lit up simultaneously (frame taken at 64 s). When the caffeine-induced transient was complete, the smooth muscle cell again became dark, and remained so for the rest of the experiment, while the interstitial cell, after a brief pause, resumed its oscillatory behaviour. This experiment also illustrated the different contractile properties of both cells. Thus, while the smooth muscle cell contracted vigorously in response to caffeine addition, the interstitial cell did not contract either in response to the spontaneous increases in intracellular Ca^{2+} or in response to the caffeine-induced Ca^{2+} transient. Scale bar, $10 \mu\text{m}$.

from 1.3 ± 0.32 ($\Delta F/F_0$) before, to 0.47 ± 0.07 during drug perfusion ($P < 0.05$, paired t test). The effect of 2-APB on STICs was more dramatic than its effects on Ca^{2+} oscillations. In seven such experiments (Fig. 2C), average STIC amplitude was reduced from -427 ± 88 to -14 ± 9 pA during drug perfusion.

The differential effects of 2-APB on Ca^{2+} oscillations and STICs can be explained, perhaps, by the experiment shown in Fig. 3. The upper panel of Fig. 3 shows a series of images of the initiation and spread of a Ca^{2+} wave. Under control conditions this arose at the top of the cell (region of interest 1 (ROI 1)) and propagated without decrement to the other end of the cell. Occasionally the wave originated at ROI9 and propagated in the opposite direction but generally the global event was well co-ordinated throughout the cell. However in the presence of 2-APB, the pattern was quite different. Oscillations continued in all parts of the cell, but these were now propagated only to one or two of the neighbouring regions of interest, with the result that activity in the cell as a whole was now very poorly coordinated. Upon washout of 2-APB the well-coordinated global events were quickly restored. These experiments would suggest that the role of IP_3 in these cells was to amplify the Ca^{2+} signal and ensure propagation of the wave. Such a global event appears to be necessary for the production of STICs but not

apparently for the generation of STOCs (Sergeant *et al.* 2001a).

Effect of PLC inhibitors

Another approach to assessing the role of IP_3 in the genesis and modulation of Ca^{2+} oscillations in the interstitial cells was to inhibit the enzyme necessary for its production. We used two different compounds for this purpose, NCDC (Cremaschi & Sterin-Borda, 1989; Gotoh *et al.* 1993) and U73122 (Smith *et al.* 1990). Figure 4A shows the effect of $100 \mu\text{M}$ NCDC on frequency and amplitude of Ca^{2+} oscillations. Before drug addition, oscillations were occurring at a frequency of about 3 min^{-1} . After drug addition, the amplitude of oscillations was much reduced, but they continued at the slightly slower frequency of 2 min^{-1} . In 11 such experiments (Fig. 4B), frequency was reduced from a mean of 3.86 ± 0.52 before drug addition to 2.3 ± 0.55 oscillations min^{-1} during drug addition ($P < 0.05$). Normalized change in amplitude ($\Delta F/F_0$) was reduced from a control value of 1.58 ± 0.13 to 0.41 ± 0.14 in the presence of drug ($P < 0.05$). Basal normalized amplitude (F/F_0) was little affected. Similar results were obtained with U73122. In six experiments, mean frequency of oscillation was 3.9 ± 0.9 in control conditions and this decreased to 3.4 ± 1.2 oscillations min^{-1} during drug

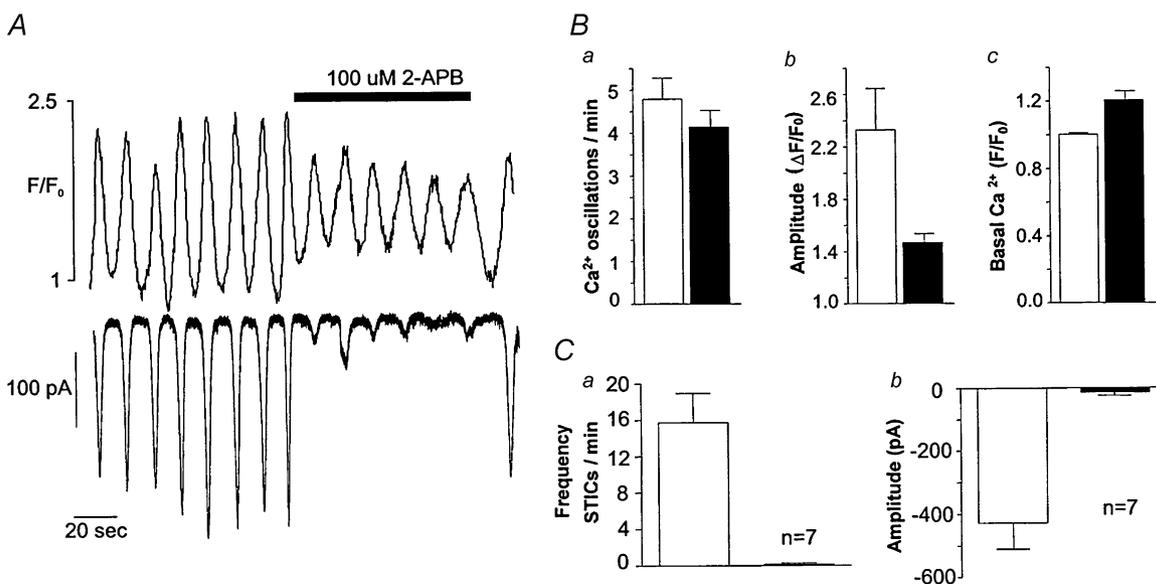
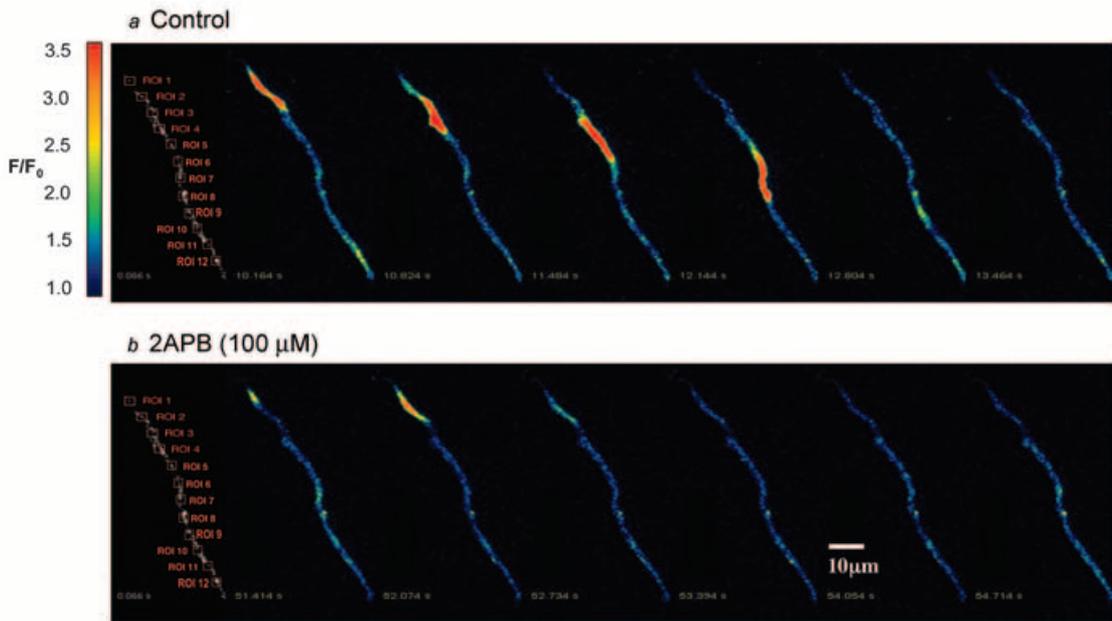


Figure 2. The effect of 2-APB on Ca^{2+} oscillations and STICs

A, the upper record shows Ca^{2+} oscillations imaged in the perinuclear region of the cell, while the lower record shows the spontaneous transient inward currents (STICs) resulting from the intracellular increases in Ca^{2+} . Addition of $100 \mu\text{M}$ 2-aminoethoxydiphenyl borate (2-APB) had the effect of almost abolishing the STICs, but the underlying Ca^{2+} oscillations, while reduced in amplitude, retained their normal rhythm and returned to control amplitude after washout of the drug. The summarized results (B and C) show essentially the same pattern as the original record in A.

A



B



Figure 3. Effect of 2-APB on wave propagation

Series of images of the initiation and spread of a Ca^{2+} wave under control conditions (A, upper panel) The wave arose at the top of the cell (region of interest (ROI) 1) and propagated without decrement to the other end of the cell. Occasionally the wave originated at ROI 9 and propagated in the opposite direction, but generally the global event was well coordinated throughout the cell (as is apparent from the plotted regions of interest in B). In the presence of 2-APB (A, lower panel) the pattern was quite different. Oscillations continued in all parts of the cell, but these were now propagated to only one or two of the neighbouring ROIs, with the result that activity in the cell as a whole was now very poorly coordinated. Upon washout of 2-APB, the well-coordinated global events were quickly restored. Scale bar, 10 μm .

addition ($P < 0.05$). Normalized amplitude was reduced from a control value of 1.3 ± 0.18 to 0.78 ± 0.17 in the presence of $1 \mu\text{M}$ U73122.

Effect of ryanodine receptor (RyR) blockade

Ryanodine. Figure 5 shows the effect on Ca^{2+} oscillations of $30 \mu\text{M}$ ryanodine. Within 80 s of drug addition, oscillations had ceased completely and this was accompanied by an increase in basal Ca^{2+} concentration within the cell. The effects of ryanodine were irreversible even on prolonged washout. In 12 such experiments, this concentration of ryanodine invariably abolished spontaneous oscillations. The effect on basal Ca^{2+} level was also a consistent one with normalized level increasing from 1.0 ± 0.007 under control conditions to 1.30 ± 0.065 in the presence of $30 \mu\text{M}$ ryanodine.

Tetracaine. Tetracaine in concentrations greater than $50 \mu\text{M}$ is known to inhibit Ca^{2+} release from ryanodine-sensitive stores in skeletal (Csernoch *et al.* 1999) cardiac (Lukyanenko *et al.* 1996; Overend *et al.*

1997, 1998) and smooth muscle (Hyvelin *et al.* 2000; Cheranov & Jaggar, 2002). Figure 6 shows the effect on Ca^{2+} oscillations of $100 \mu\text{M}$ tetracaine. Within 10 s of drug addition, oscillations had ceased completely, and within 10 s of washout they had returned at control frequency and greater amplitude. In 12 such experiments, mean frequency was reduced from a control value of 4.8 ± 0.67 to 0.5 ± 0.25 oscillations min^{-1} during tetracaine application, while mean amplitude was reduced from a control value of 0.85 ± 0.6 to $0.12 \pm 0.45 \Delta F/F_0$. It should be noted that during tetracaine addition, the basal signal intensity fell below the normal resting value, and this was evident on visual inspection of the movie where the cell became noticeably darker during tetracaine application.

Effect of external Ca^{2+} concentration on spontaneous oscillations

The experiment shown in Fig. 7 was designed to test the dependency of oscillation frequency on external Ca^{2+} concentration. Reduction of $[\text{Ca}^{2+}]_o$ from the

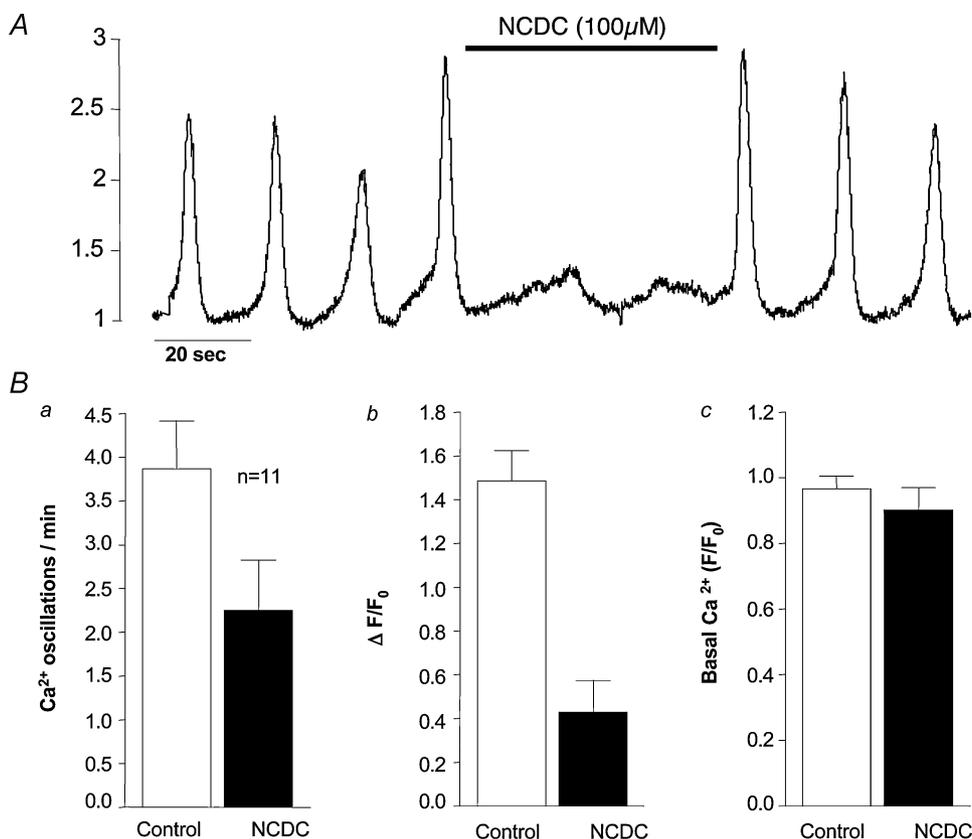


Figure 4. The effect of NCDC on Ca^{2+} oscillations

A, before drug addition oscillations were occurring at a frequency of about 3 min^{-1} . In the presence of $100 \mu\text{M}$ *N,N*-diphenylcarbamate (NCDC), the amplitude of oscillations was much reduced, but they continued but at the slightly slower frequency of 2 min^{-1} . The summarized results (B) showed a decrease in both frequency and amplitude, but there was little effect on basal Ca^{2+} levels.

normal value of 1.8 mM to 0 immediately caused cessation of oscillations and a decrease in basal cytosolic Ca^{2+} . Frequency and amplitude of oscillations were promptly restored on readmission of normal Ca^{2+} . When this solution was changed to one containing 0.9 mM Ca^{2+} , frequency of oscillation was markedly reduced. On the other hand increasing $[\text{Ca}^{2+}]_o$ to 3.6 mM had the effect of increasing frequency of oscillation. The graph in Fig. 7B shows the averaged results (numbers of experiments at each concentration are shown alongside each point) \pm s.e.m. at five different Ca^{2+} concentrations. It can be seen that there is a roughly linear relationship between oscillation frequency and external Ca^{2+} concentration. It could be argued that cessation of calcium oscillation in nominally Ca^{2+} -free solution was due to rapid depletion of the intracellular stores, but the experiment shown in Fig. 8 would suggest that this is not the case. When 10 mM caffeine was added in 1.8 mM Ca^{2+} solution, a maximal Ca^{2+} transient was induced followed by inhibition of spontaneous oscillations (eight such experiments are summarized in Fig. 8B). When nominally Ca^{2+} -free solution was introduced after normal oscillations had returned, there was the usual cessation

of oscillation. However a second introduction of 10 mM caffeine still evoked a maximal Ca^{2+} transient, suggesting that the stores had not been depleted in the Ca^{2+} -free solution. In four such experiments there was no significant difference between the transient evoked in 1.8 mM Ca^{2+} as compared with that evoked in Ca^{2+} -free solution (Fig. 8C).

Effect of blockers of Ca^{2+} influx

The sensitivity of oscillation frequency to external Ca^{2+} concentration would suggest that Ca^{2+} influx was essential for the maintenance of normal oscillations. These cells are known to possess L-type Ca^{2+} channels, and these may be one possible influx pathway. The experiment illustrated in Fig. 9A was designed to test this possibility. Prior to the addition of 10 μM nifedipine (an L-type channel blocker), frequency of oscillation was 4 min^{-1} and this slightly increased (to about 6 min^{-1}) rather than decreased in the presence of nifedipine. In four similar experiments, mean frequency decreased slightly from a control value of 4.56 ± 1.06 to 4.1 ± 0.68 oscillations min^{-1} in the presence of nifedipine (non significant, paired *t* test).

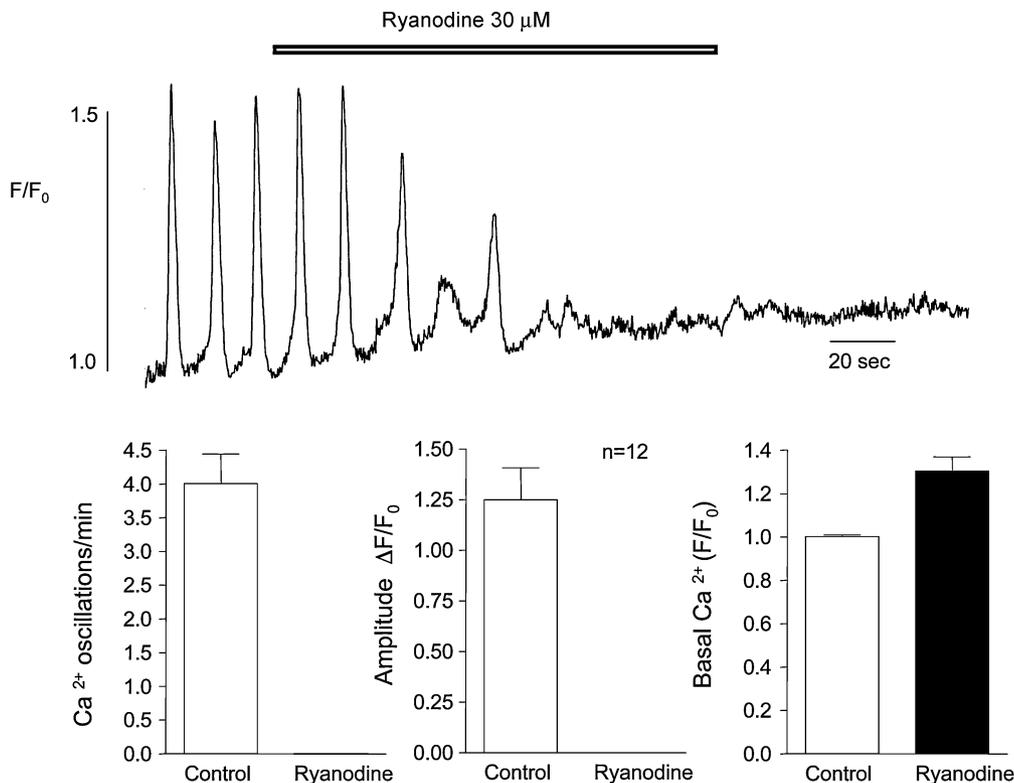


Figure 5. The effect of ryanodine

The effect of 30 mM ryanodine on Ca^{2+} oscillations is shown. Within 80 s of drug addition, oscillations had ceased completely, and this was accompanied by an increase in basal Ca^{2+} concentration within the cell. The effects of ryanodine were irreversible even on prolonged washout. In 12 such experiments this concentration of ryanodine invariably abolished spontaneous oscillations. The effect on basal Ca^{2+} level was also a consistent one with normalized level increasing from 1.0 ± 0.007 before drug addition to 1.30 ± 0.065 in the presence of 30 mM ryanodine.

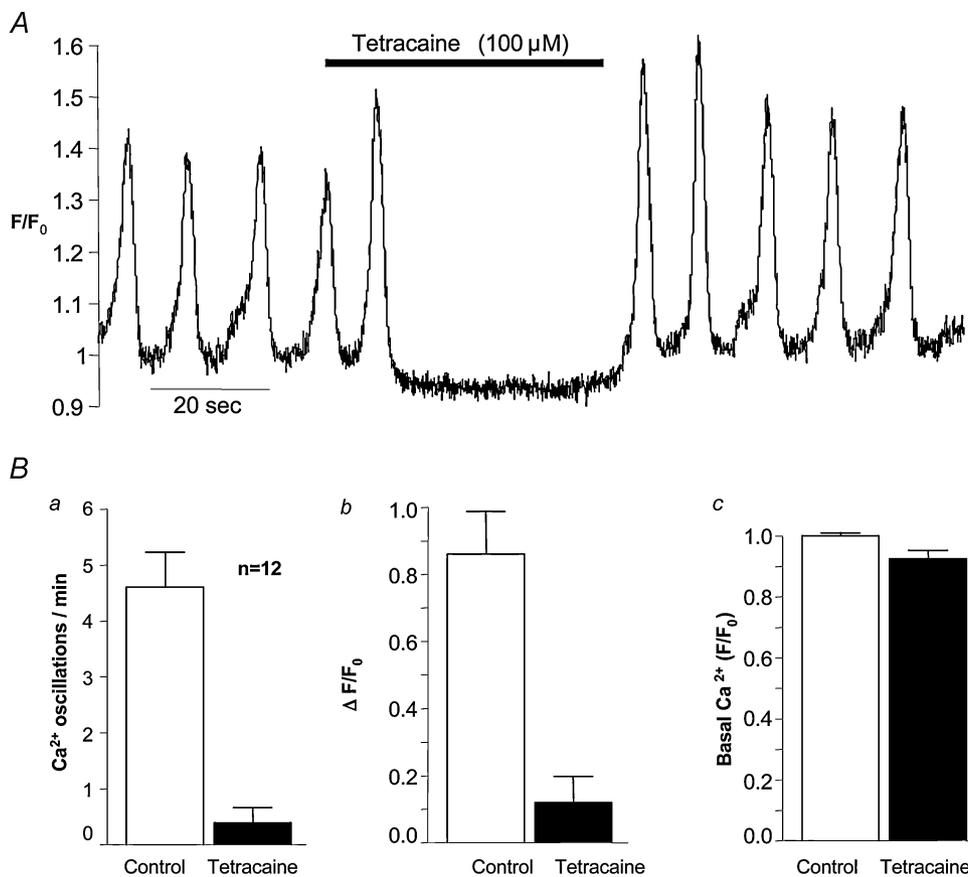


Figure 6. The effect of tetracaine

A, tetracaine at 100 μM abolished oscillations within 10 s of drug addition, and these were just as quickly restored when the drug was washed out. The summarized results (*B*) show that both frequency and amplitude of oscillations were profoundly depressed by this concentration of tetracaine.

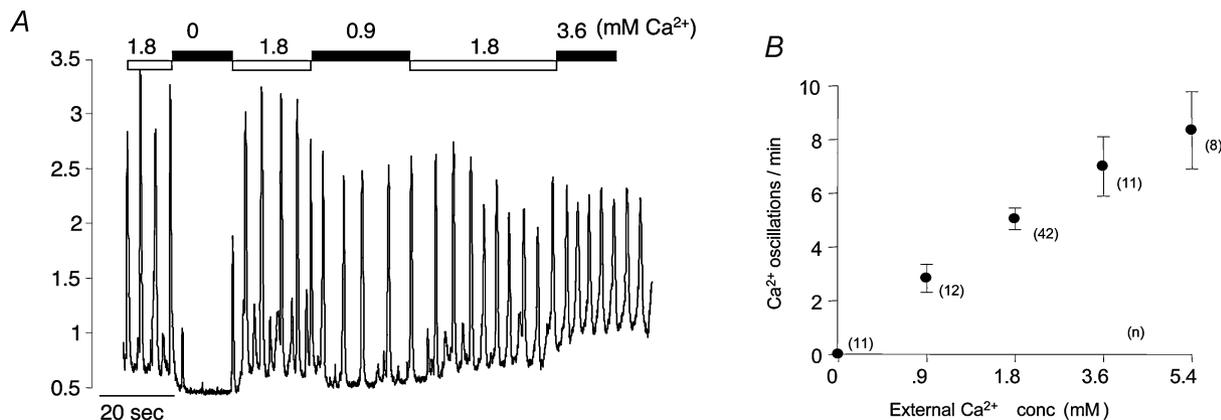


Figure 7. Effect of external Ca²⁺ concentration

Reduction of [Ca²⁺]_o from the normal value of 1.8–0 mM (nominally Ca²⁺-free, since no chelator was present) immediately caused cessation of oscillations and a decrease in basal cytosolic Ca²⁺. Frequency and amplitude of oscillations were promptly restored on readmission of normal Ca²⁺. When this solution was changed to one containing 0.9 mM Ca²⁺, frequency of oscillation was markedly reduced. On the other hand increasing [Ca²⁺]_o to 3.6 mM had the effect of increasing frequency of oscillation. *B*, the averaged results (numbers of experiments at each concentration are shown alongside each point) (±s.e.m.) at five different Ca²⁺ concentrations. It can be seen that there is a roughly linear relationship between oscillation frequency and external Ca²⁺ concentration.

These results would suggest that influx through L-type Ca^{2+} channels does not significantly modulate frequency of oscillation. In Fig. 10, the effects of three other Ca^{2+} influx blockers are illustrated. In Fig. 10Aa, $10 \mu\text{M}$ SKF-96365 (a blocker of store operated Ca^{2+} entry, Wayman *et al.* 1996) decreased frequency from a control value of 2.2 to 1.1 oscillations min^{-1} during drug addition. In four similar experiments, frequency was reduced from a control value of 3.57 ± 0.37 to 2.52 ± 0.68 oscillations min^{-1} ($P < 0.05$) in the presence of SKF-96365, suggesting that Ca^{2+} influx through store-operated Ca^{2+} channels played a role in store refilling. The nonspecific blockers lanthanum and cadmium chloride had a more dramatic blocking effect. In Fig. 10Ba and Ca, oscillations were completely inhibited when either lanthanum or cadmium were present,

Fig. 10Bb shows a summary of eight experiments where 1 mM lanthanum decreased mean oscillation frequency from 2.87 ± 0.44 to 1.57 ± 0.48 ($P < 0.05$). Figure 10Cb shows a summary of nine experiments where 1 mM cadmium decreased mean frequency from 1.09 ± 0.05 to 0.42 ± 0.17 oscillations min^{-1} ($P < 0.05$).

Discussion

The main conclusion of this study is that spontaneous electrical events in the interstitial cells of the rabbit urethra are generated by the oscillatory release of Ca^{2+} from intracellular stores as was inferred from our previous studies of these cells (Sergeant *et al.* 2000, 2001a,b). The 'prime oscillator' would appear to be the ryanodine-sensitive store rather than the IP_3 -sensitive,

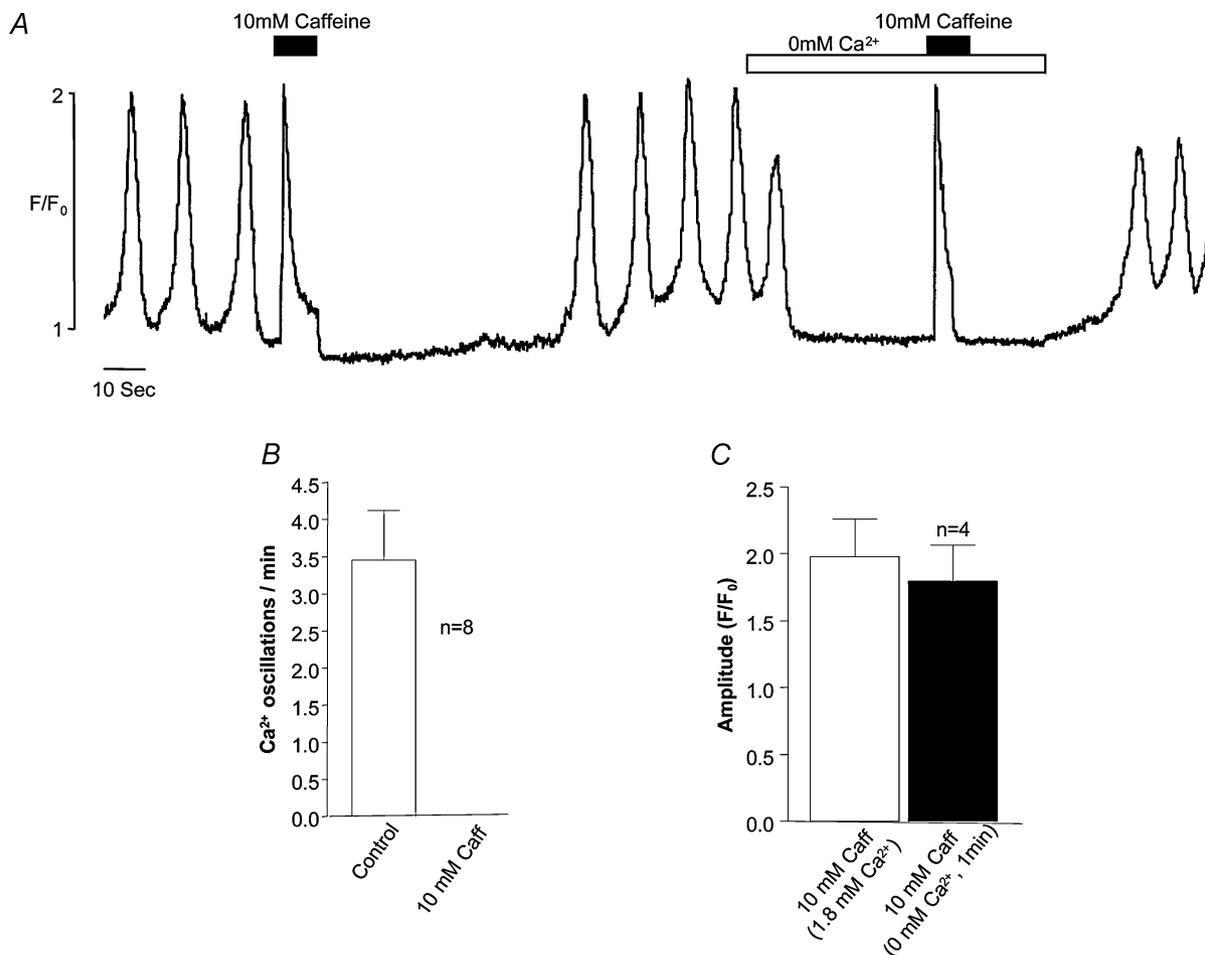


Figure 8. Removing external Ca^{2+} does not rapidly deplete stores

When 10 mM caffeine was added in 1.8 mM Ca^{2+} solution, a maximal Ca^{2+} transient was induced followed by inhibition of spontaneous oscillations (eight such experiments are summarized in B). A second introduction of 10 mM caffeine a minute after exposure to 0 mM $[\text{Ca}^{2+}]_o$ still evoked a maximal Ca^{2+} transient suggesting that the stores had not been depleted in the nominally Ca^{2+} -free solution. In four such experiments there was no significant difference between the transient evoked in 1.8 mM Ca^{2+} as compared with that evoked in nominally Ca^{2+} -free solution (C).

store since blockade of the former with tetracaine or with ryanodine (Sergeant *et al.* 2001a) stopped oscillations completely, whereas inhibition of the action or production of IP₃ decreased the amplitude or propagation of the Ca²⁺ wave but did not entirely prevent Ca²⁺ oscillations. These observations help to explain our earlier observations (Sergeant *et al.* 2001a) that STICs were almost completely blocked by 2-APB and by PLC inhibitors, whereas STOCs were not. The channels underlying STICs, low conductance Ca²⁺-activated Cl⁻ channels (Large & Wang, 1996; Piper & Large, 2003), appear not to be clustered sufficiently for small local increases in Ca²⁺ to produce measurable currents. Thus it appears that large STICs result only from global increases in Ca²⁺. The channels underlying STOCs, on the other hand, are large conductance Ca²⁺-activated K⁺ channels which can produce measurable currents in response to small local changes in Ca²⁺ concentration. Recent studies by Rossi *et al.* (2002) and Aoyama *et al.* (2004) in HEK 293 cells (and by the latter authors in isolated Interstitial cells of Cajal (ICC) from the mouse small intestine) would support the idea that spontaneous Ca²⁺ oscillations (as distinct from agonist-induced oscillations) require the presence of functional ryanodine receptors. HEK 293 cells, which expressed all three subtypes of IP₃ receptor (IP₃R) as well as RyR₁, did not exhibit spontaneous Ca²⁺ oscillations

although they were responsive to caffeine and carbachol. On the other hand those cells that expressed the RyR₃ subtype did show spontaneous Ca²⁺ oscillations. Many of the observations of Aoyama *et al.* (2004) on the properties of mouse ICC would concur with the results of our present study. For example, they also showed that nifedipine did not affect spontaneous Ca²⁺ oscillations, while tetracaine and ryanodine abolished them. However, in contrast to the results we report here, they found that 2-APB in doses as low as 1 μM could abolish spontaneous oscillations. An interesting aspect of this finding is that the effects of even this low dose of 2-APB were not reversible on washout. Some of the actions of 2-APB other than that of preventing IP₃-induced Ca²⁺ release (sarcoplasmic reticulum Ca²⁺-ATPase pump (SERCA) inhibition, blockade of store-operated Ca²⁺ channels, swelling of mitochondria, Bootman *et al.* 2002; Peppiatt *et al.* 2003) are irreversible or reversed only slowly. In the present study, we found that a much higher concentration of 2-APB (100 μM) was very rapidly reversed on washout.

We are well aware that 2-APB is not as 'clean' a drug as we would like it to be. It is known to have three actions in addition to its effect of inhibiting IP₃-mediated Ca²⁺ release (Peppiatt *et al.* 2003; Ma *et al.* 2003). These are: (1) inhibition of store-operated Ca²⁺ entry; (2) inhibition of SERCA pumps (both of these could result in store rundown

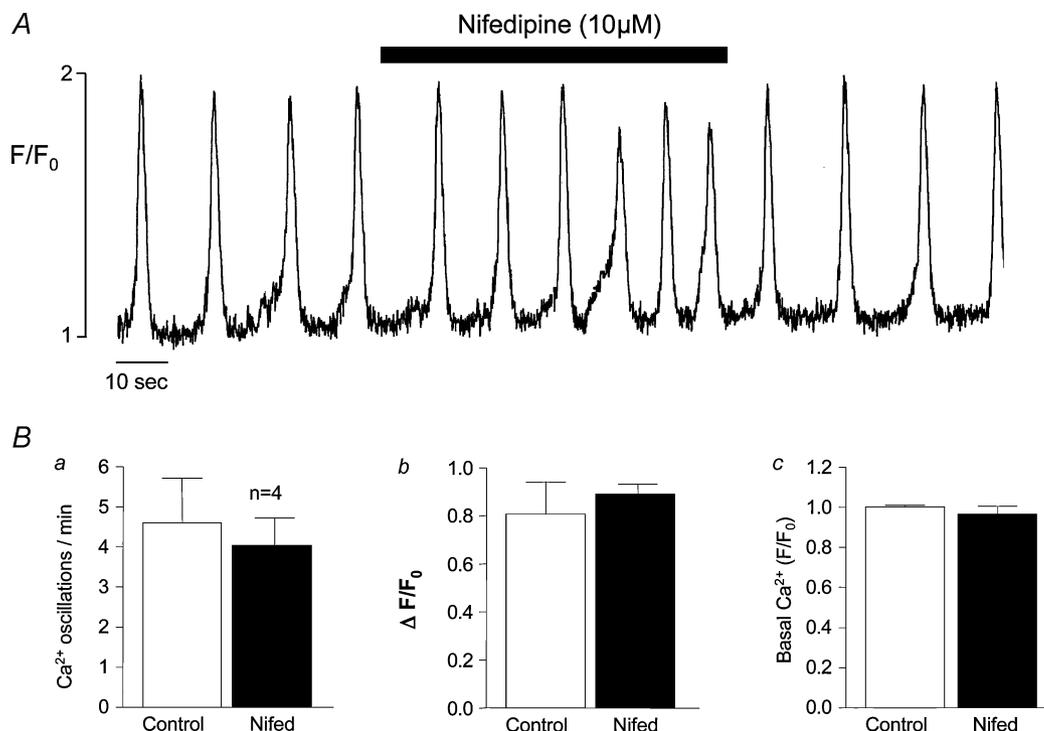


Figure 9. The effect of nifedipine

Nifedipine at 10 μM increased rather than decreased oscillation frequency while having little effect on amplitude (an effect that was consistent in four preparations as shown in the summarized data, B) suggesting that Ca²⁺ influx through L-type channels is not important for store refilling.

while the latter effect could slow the recovery of the Ca^{2+} transient); and (3) inhibition of the plasma membrane Ca^{2+} pump. However, we have several reasons for believing that these three actions are of much less significance than the inhibition of IP_3 -mediated Ca^{2+} release under the conditions of the present study. (1) We have demonstrated that caffeine-induced Ca^{2+} transients (G. P. Sergeant, K. D. Thornbury, M. A. Hollywood and N. G. McHale, unpublished data) and caffeine-induced STICs (Sergeant *et al.* 2001a) persisted in the presence of $100 \mu M$ 2-APB (suggesting that store filling and SR uptake were working normally) while noradrenaline-induced transients were abolished. (2) Direct measurement of capacitative Ca^{2+} entry showed that 2-APB decreased this by less than 20% (G. P. Sergeant, K. D. Thornbury, M. A. Hollywood and N. G. McHale, unpublished data) and that a reduction of this magnitude was not enough to affect STICs. (3) We have observed that basal Ca^{2+} does increase in the

presence of 2-APB and this is consistent with inhibition of the plasma membrane Ca^{2+} -ATPase. However this does not pose a serious challenge to our interpretation of our results. The above observations and the fact that oscillation frequency was not significantly decreased (as it would be if influx through store-operated channels were blocked) would suggest that in isolated urethral interstitial cells 2-APB is acting preferentially on IP_3 -induced Ca^{2+} release.

Although Ca^{2+} release via the IP_3R may not be the primary oscillator in urethral interstitial cells, the results of the present study bear out the importance of IP_3 -induced Ca^{2+} release in the amplification and coordination of the Ca^{2+} wave. A well-coordinated wave appears to be necessary for the STICs which underlie the normal spontaneous electrical activity in these cells. This means that both RyR and IP_3R are necessary for normal electrical activity. Several other studies have shown that calcium oscillations require both RyR and IP_3R . For example,

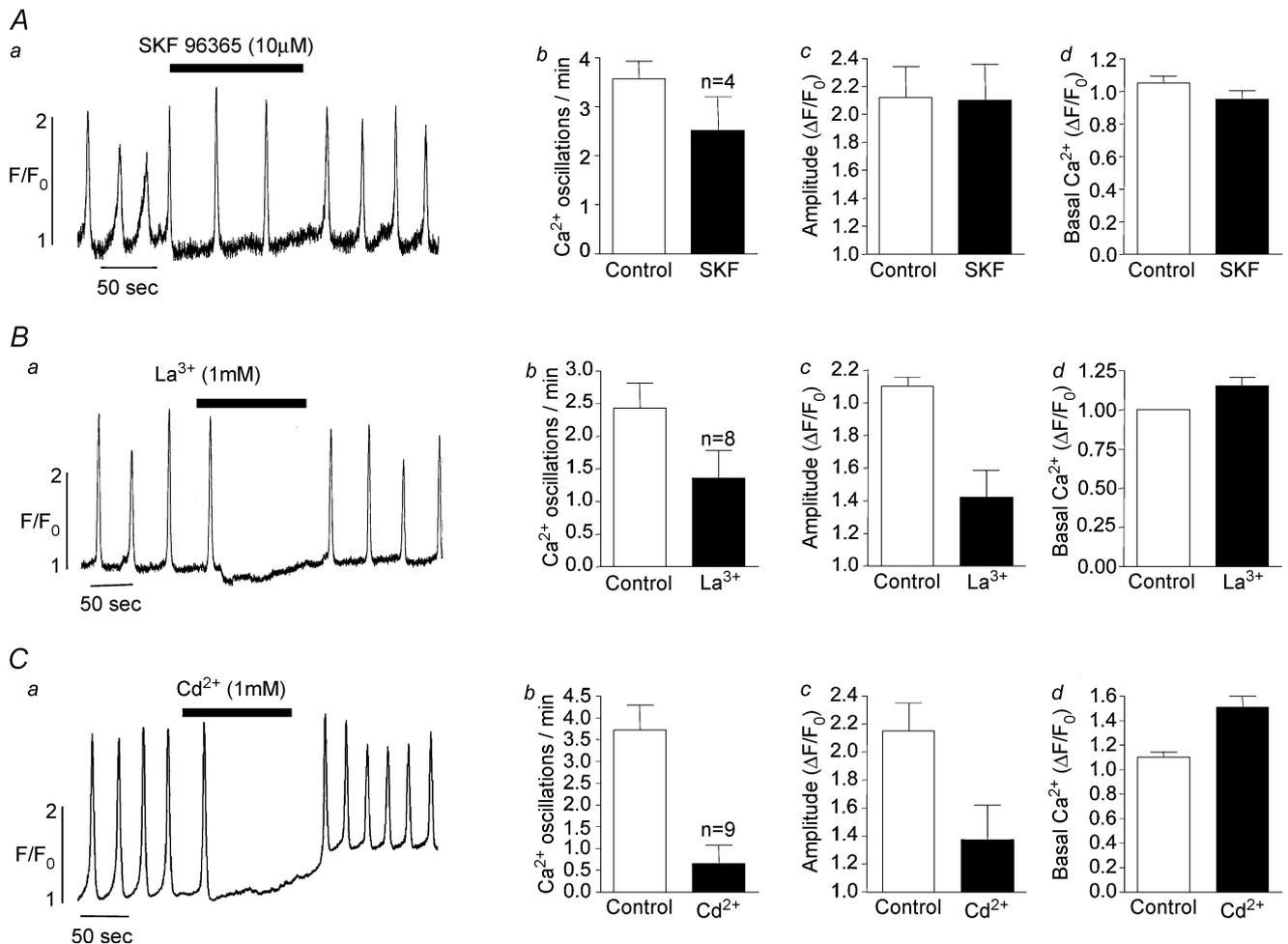


Figure 10. The effect of blockers of Ca^{2+} influx

SKF-96365 at $10 \mu M$ approximately halved the frequency of oscillation (Aa) and, although this was a greater reduction than the average of four such experiments (30% reduction, Ab), it would suggest that Ca^{2+} entry through store-operated channels does play a part in modulating oscillation frequency. The nonspecific blockers lanthanum and cadmium chloride had a more dramatic blocking effect confirming the importance of Ca^{2+} influx in the modulation of intracellular oscillations.

Koizumi *et al.* (1999) showed that elementary Ca^{2+} signals in PC12 cells appeared to arise from clusters containing both RyR and IP_3R since they had the spatial spread or kinetics of neither Ca^{2+} sparks nor Ca^{2+} puffs. This is very similar to the pattern of elementary events observed in urethral interstitial cells. We never observed events that had the spatiotemporal properties of sparks such as those seen in myocytes isolated from the portal vein (Boittin *et al.* 1998), although we have observed such events in isolated bladder myocytes under the same experimental conditions as those of the present study (unpublished observations). Bayguinov *et al.* (2000) reported that spontaneous localized Ca^{2+} -release events (insensitive to ryanodine and mediated by IP_3Rs) were generated in murine colonic myocytes in the absence of external agonists presumably due to basal activity of PLC. These were referred to as 'Ca²⁺ puffs' similar to those observed in *Xenopus* oocytes (Berridge, 1997; Boittin *et al.* 2000). Similarly Boittin *et al.* (2000) observed Ca^{2+} puffs discharged through IP_3 -gated channels in response to low concentrations of IP_3 which was photo-released from its caged precursor. On the other hand, Gordienko & Bolton (2002) did not observe discrete Ca^{2+} -release events arising from coordinated opening of IP_3R clusters in rabbit portal vein myocytes. All spontaneous Ca^{2+} -release events observed in these myocytes were completely abolished by high (50–100 μM) concentrations of ryanodine. Nevertheless these authors also confirmed the importance of IP_3 -induced Ca^{2+} release in the amplification and coordination of the Ca^{2+} wave.

It is well known that external Ca^{2+} concentration affects oscillation frequency in many cell types (Kawanishi *et al.* 1989; Bootman *et al.* 1996; Shuttleworth & Thompson, 1996; Sneyd *et al.* 2004). For example, Bootman *et al.* (1996) found that raising external Ca^{2+} from 0 to 1.3 mM increased both frequency of oscillations and intracellular [³H]inositol phosphate levels in HeLa cells. When external Ca^{2+} concentrations were increased above this level, oscillation frequency continued to rise even though the PLC response had already saturated. They took this to mean that external Ca^{2+} (at least at levels above 1.3 mM) could control the release of Ca^{2+} from intracellular stores by modulating the rate of store refilling between each Ca^{2+} spike. Since PLC activity was essentially saturated they argued that the positive feedback responsible for Ca^{2+} spiking must be generated by calcium-induced Ca^{2+} release (CICR). The obvious mechanism for such an oscillator would be cyclical filling and emptying of a ryanodine-sensitive store. Sneyd *et al.* (2004) argued that the Ca^{2+} influx pathway which was responsible for modulating frequency of oscillation did not depend on store depletion or capacitative Ca^{2+} entry, since oscillations could occur in the absence of significant store depletion (Park *et al.* 2000). This would accord with the results of the present study that blockade of

store-operated Ca^{2+} entry with SKF-96365, although it decreased frequency, did not abolish oscillations. This contrasted with the effect of nonspecific blockers of Ca^{2+} entry such as lanthanum or cadmium.

In conclusion, the results presented here confirm our previous inference (Sergeant *et al.* 2001a) that regular intracellular Ca^{2+} oscillations underlie the spontaneous electrical activity of the interstitial cells of the rabbit urethra. This activity can be modulated by substances which raise intracellular levels of IP_3 , and IP_3 -induced Ca^{2+} release appears to be necessary for the coordination of the global Ca^{2+} event underlying STICs (and thus pacemaking); however, localized Ca^{2+} oscillations (and thus STOCs) can occur in an IP_3 -independent manner.

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