

# Role of mitochondria in modulation of spontaneous $\text{Ca}^{2+}$ waves in freshly dispersed interstitial cells of Cajal from the rabbit urethra

Gerard P. Sergeant, Eamonn Bradley, Keith D. Thornbury, Noel G. McHale and Mark A. Hollywood

*The Smooth Muscle Research Centre, Dundalk Institute of Technology, Dundalk, Co. Louth, Ireland*

Interstitial cells of Cajal (ICC) isolated from the rabbit urethra exhibit pacemaker activity that results from spontaneous  $\text{Ca}^{2+}$  waves. The purpose of this study was to investigate if this activity was influenced by  $\text{Ca}^{2+}$  uptake into mitochondria. Spontaneous  $\text{Ca}^{2+}$  waves were recorded using a Nipkow spinning disk confocal microscope and spontaneous transient inward currents (STICs) were recorded using the whole-cell patch clamp technique. Disruption of the mitochondrial membrane potential with the electron transport chain inhibitors rotenone ( $10\ \mu\text{M}$ ) and antimycin A ( $5\ \mu\text{M}$ ) abolished  $\text{Ca}^{2+}$  waves and increased basal  $\text{Ca}^{2+}$  levels. Similar results were achieved when mitochondria membrane potential was collapsed using the protonophores FCCP ( $0.2\ \mu\text{M}$ ) and CCCP ( $1\ \mu\text{M}$ ). Spontaneous  $\text{Ca}^{2+}$  waves were not inhibited by the ATP synthase inhibitor oligomycin ( $1\ \mu\text{M}$ ), suggesting that these effects were not attributable to an effect on ATP levels. STICs recorded under voltage clamp at  $-60\ \text{mV}$  were also inhibited by CCCP and antimycin A. Dialysis of cells with the mitochondrial uniporter inhibitor RU360 ( $10\ \mu\text{M}$ ) also inhibited STICs. Stimulation of  $\text{Ca}^{2+}$  uptake into mitochondria using the plant flavonoid kaempferol ( $10\ \mu\text{M}$ ) induced a series of propagating  $\text{Ca}^{2+}$  waves. The kaempferol-induced activity was inhibited by application of caffeine ( $10\ \text{mM}$ ) or removal of extracellular  $\text{Ca}^{2+}$ , but was not significantly affected by the  $\text{IP}_3$  receptor blocker 2-APB ( $100\ \mu\text{M}$ ). These data suggest that spontaneous  $\text{Ca}^{2+}$  waves in urethral ICC are regulated by buffering of cytoplasmic  $\text{Ca}^{2+}$  by mitochondria.

(Received 4 July 2008; accepted after revision 4 August 2008; first published online 14 August 2008)

**Corresponding author** G. P. Sergeant: The Smooth Muscle Research Centre, Dundalk Institute of Technology, Dundalk, Co. Louth, Ireland. Email: gerard.sergeant@dkit.ie

Interstitial cells of Cajal have now been reported in various smooth muscle organs located throughout the body including the gastro-intestinal (GI) tract (Sanders *et al.* 2006) and the upper and lower urinary tract (Klemm *et al.* 1999; Sergeant *et al.* 2000; McCloskey & Gurney, 2002). In the GI tract they are well recognized as specialized pacemaker cells which are responsible for the generation and co-ordination of electrical slow waves that regulate the phasic contractile activity of the gut (Sanders, 1996; Hirst & Ward, 2003; Sanders *et al.* 2006). ICC in the urethra are also thought to act as putative pacemakers, which regulate spontaneous myogenic tone in a frequency-dependent manner (Sergeant *et al.* 2000). The frequency of pacemaker activity in urethra ICC is regulated by both excitatory and inhibitory neurotransmitters, thus application of nitric oxide (NO) agonists decreased the frequency of the activity (Sergeant *et al.* 2006a), whereas noradrenaline increased the frequency (Sergeant *et al.* 2002). Pacemaker activity in isolated ICC from the rabbit urethra is characterized by spontaneous transient inward currents

(STICs) recorded under voltage clamp and spontaneous transient depolarizations (STDs) under current clamp (Sergeant *et al.* 2000). Simultaneous patch clamp and  $\text{Ca}^{2+}$  imaging experiments revealed that spontaneous electrical activity of these cells is associated with global  $\text{Ca}^{2+}$  waves (Johnston *et al.* 2005; Sergeant *et al.* 2006a,b). The cellular mechanisms responsible for the regulation and generation of  $\text{Ca}^{2+}$  waves in these cells has been partly elucidated. For example, it was shown that inhibition of ryanodine receptors (RyRs) abolished the activity, whereas inhibition of inositol trisphosphate ( $\text{IP}_3$ ) production or  $\text{IP}_3$  receptors ( $\text{IP}_3\text{Rs}$ ) reduced the propagation of  $\text{Ca}^{2+}$  waves, unmasking multiple, uncoupled  $\text{Ca}^{2+}$  release events.  $\text{Ca}^{2+}$  waves were also found to be dependent on  $\text{Ca}^{2+}$  influx, thus removal of extracellular  $\text{Ca}^{2+}$  was found to inhibit  $\text{Ca}^{2+}$  waves by a mechanism that did not involve depletion of  $\text{Ca}^{2+}$  from stores (Johnston *et al.* 2005; Bradley *et al.* 2005).

It has now been shown in a range of cell types that the temporal and spatial profile of  $\text{Ca}^{2+}$  oscillations

are regulated by the  $\text{Ca}^{2+}$  handling properties of mitochondria, independently from an action involving ATP (Graier *et al.* 2007). For example, it has been shown that  $\text{Ca}^{2+}$  uptake into mitochondria is involved in the regulation of frequency and duration of  $\text{Ca}^{2+}$  sparks (Pacher *et al.* 2002) and can influence the propagation of  $\text{Ca}^{2+}$  waves (Jouaville *et al.* 1995; Boitier *et al.* 1999; Tinel *et al.* 1999). There is also evidence that  $\text{Ca}^{2+}$  handling by mitochondria is a key regulator of pacemaker activity in ICC in the GI tract (Ward *et al.* 2000). The aim of the present study was to investigate if spontaneous  $\text{Ca}^{2+}$  waves in ICC isolated from the rabbit urethra are also regulated by the  $\text{Ca}^{2+}$  handling properties of mitochondria.

## Methods

### Cell isolation

All procedures were carried out in accordance with current EU legislation and with the approval of Dundalk Institute of Technology Animal Use and Care Committee. Male and female New Zealand white rabbits (16–20 weeks old) were humanely killed with a lethal injection of pentobarbitone (i.v.). The most proximal 1.5 cm of the urethra was removed and placed in Krebs solution and individual ICC were isolated enzymatically as previously described (Bradley *et al.* 2006).

### Calcium imaging

Cells were placed in Hanks solution and allowed to settle in glass-bottomed Petri dishes until they had stuck down. They were then incubated in  $0.4 \mu\text{M}$  fluo-4 AM (Molecular Probes) in Hanks solution containing  $100 \mu\text{M}$   $\text{Ca}^{2+}$  for 6–8 min in the dark at room temperature. Cells were imaged using an iXon 887 EMCCD camera (Andor Technology, Belfast;  $512 \text{ pixels} \times 512 \text{ pixels}$ , pixel size  $16 \mu\text{m} \times 16 \mu\text{m}$ ) coupled to a Nipkow spinning disk confocal head (CSU22, Yokogawa, Japan). A krypton–argon laser (Melles Griot UK) at 488 nm was used to excite the fluo-4, and the emitted light was detected at wavelengths  $> 510 \text{ nm}$ . Experiments were performed using a  $\times 60$  objective (Olympus) resulting in images of pixel size  $0.266 \mu\text{m} \times 0.266 \mu\text{m}$ . Images were acquired at five frames per second. Background fluorescence from the camera, obtained using a null frame, was subtracted from each frame to obtain 'F'.  $F_0$  was determined as the minimum fluorescence measured between oscillations under control conditions. To obtain *post hoc* line-scan images for display in figures, a 1 pixel thick line was drawn centrally through the entire length of the cell and the 'reslice' command in Image J was invoked. A spatial calibration bar representing  $40 \mu\text{m}$  is shown in yellow at the right hand side of each image. Plots of  $F/F_0$  were obtained from the *post hoc* line-scan by drawing a rectangle

around the entire area of the line-scan image and plotting the intensity profile in Image J.

Summary data are presented as the mean  $\pm$  S.E.M., and statistical differences in wave frequency and, where relevant, amplitude were compared using Student's paired *t* test, taking the  $P < 0.05$  level as significant. 'Basal  $\text{Ca}^{2+}$ ' during oscillatory activity was defined as the diastolic  $\text{Ca}^{2+}$  levels (in  $F/F_0$  units) in the control period just prior to an experimental intervention. In the presence of a drug, basal  $\text{Ca}^{2+}$  was also measured as the  $\text{Ca}^{2+}$  level between oscillations; however, when oscillations were abolished, basal  $\text{Ca}^{2+}$  levels were determined as the mean  $\text{Ca}^{2+}$  level for the last 30 s during drug application.  $\Delta F/F_0$  refers to the measurement of the change in  $\text{Ca}^{2+}$  levels from basal to peak. Changes in basal  $\text{Ca}^{2+}$  were compared using the Wilcoxin signed rank test for paired data. Throughout, '*n*' refers to the number of cells in each experimental series. In each case, *n* was obtained from a minimum of two animals.

### Whole-cell patch clamp recordings

Currents were recorded using the ruptured and perforated patch configurations of the whole-cell patch clamp technique (Rae *et al.* 1991). In the latter experiments the membrane was perforated using the antibiotic amphotericin B ( $600 \mu\text{g ml}^{-1}$ ). Pipettes were pulled from borosilicate glass capillary tubing ( $1.5 \text{ mm}$  outer diameter,  $1.17 \text{ mm}$  inner diameter; Clark Medical Instruments) to a tip of diameter approximately  $1\text{--}1.5 \mu\text{m}$  and resistance of  $2\text{--}4 \text{ M}\Omega$ . Voltage clamp commands were delivered via an Axopatch 1D patch clamp amplifier (Axon Instruments) connected to a Digidata 1322A AD/DA converter (Axon Instruments) interfaced to a computer running pCLAMP software (Axon Instruments).

### Solutions and drugs

The solutions used were of the following composition (mM): Hanks:  $130 \text{ Na}^+$ ,  $5.8 \text{ K}^+$ ,  $135 \text{ Cl}^-$ ,  $4.16 \text{ HCO}_3^-$ ,  $0.34 \text{ HPO}_3^{2-}$ ,  $0.44 \text{ H}_2\text{PO}_4^-$ ,  $1.8 \text{ Ca}^{2+}$ ,  $0.9 \text{ Mg}^{2+}$ ,  $0.4 \text{ SO}_4^{2-}$ ,  $10$  dextrose,  $2.9$  sucrose,  $10$  Hepes, pH adjusted to  $7.4$  with NaOH;  $\text{Ca}^{2+}$ -free Hanks: as Hanks but with  $\text{Mg}^{2+}$  ( $1.8 \text{ mM}$ ) substituted for  $\text{Ca}^{2+}$  and addition of EGTA ( $5 \text{ mM}$ ). Perforated patch solution: CsCl ( $133$ ),  $\text{MgCl}_2$  ( $1.0$ ), EGTA ( $0.5$ ), Hepes ( $10$ ), pH adjusted to  $7.2$  with CsOH. Whole-cell patch solution: CsCl ( $133$ ),  $\text{MgCl}_2$  ( $1.0$ ), Hepes ( $10$ ),  $\text{Na}_2\text{ATP}$  ( $1$ ),  $\text{NaGTP}$  ( $0.1$ ),  $\text{Na}_2$ -phosphocreatine ( $2.5$ ) pH adjusted to  $7.2$  with CsOH.

Drugs used were: CCCP (Sigma), FCCP (Ascent), rotenone (Sigma), oligomycin (Sigma) antimycin A (Sigma), kaempferol (Sigma), 2-APB (Acros), RU-360 (Calbiochem). During experiments, the dish containing the cells was superfused with Hanks solution. In addition, the cell under study was continuously superfused with

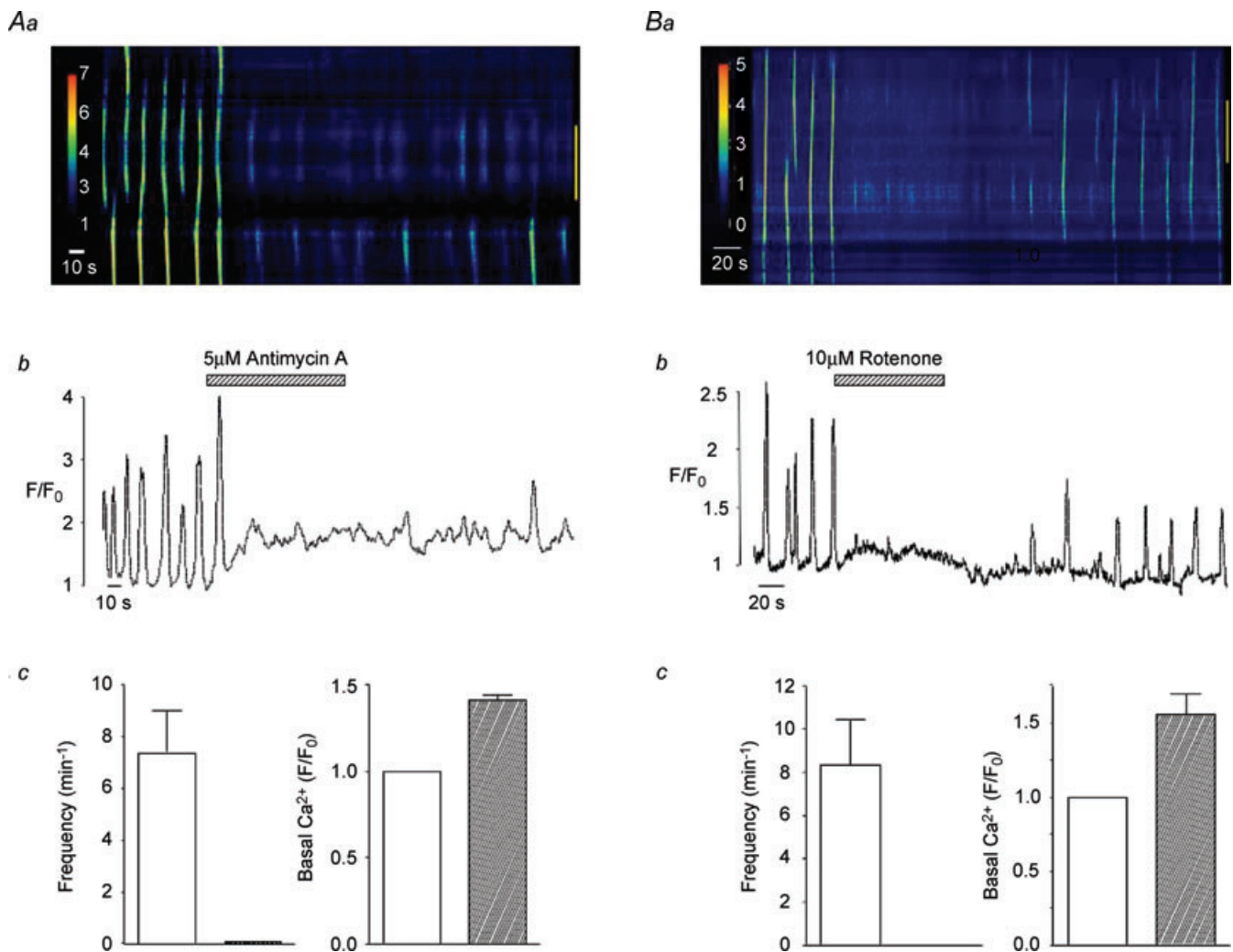
Hanks solution by means of a close delivery system consisting of a pipette (tip diameter  $200\ \mu\text{m}$ ) placed approximately  $300\ \mu\text{m}$  away. This could be switched, with a dead space time of around 5 s, to a solution containing a drug. All experiments were carried out at  $35\text{--}37^\circ\text{C}$ .

## Results

Individual ICC isolated from the rabbit urethra displayed regularly occurring  $\text{Ca}^{2+}$  waves as previously described by Johnston *et al.* (2005) and Sergeant *et al.* (2006a,b). Examples of  $\text{Ca}^{2+}$  waves are presented in the 'post hoc' line-scans shown in Figs 1–9. The mean frequency and amplitude of these events measured under resting conditions in 32 cells were  $6.2 \pm 0.69\ \text{min}^{-1}$  and  $\Delta F/F_0 = 1.71 \pm 0.13$ , respectively.

## Effect of ETC inhibitors on spontaneous $\text{Ca}^{2+}$ waves

Mitochondrial  $\text{Ca}^{2+}$  uptake is driven by the large negative potential ( $\sim -180\ \text{mV}$ ) across the inner mitochondrial membrane created by the extrusion of protons along the electron transport chain (ETC; Mitchell, 1961). In order to investigate the relationship between  $\text{Ca}^{2+}$  waves and mitochondrial  $\text{Ca}^{2+}$  uptake, we examined the effects of the ETC (complex III) inhibitor antimycin A ( $5\ \mu\text{M}$ ) and the complex I inhibitor rotenone ( $10\ \mu\text{M}$ ). These agents have been shown to dissipate the negative mitochondrial membrane potential and thus reduce the ability of mitochondria to take up  $\text{Ca}^{2+}$  (Tinel *et al.* 1999). Figure 1Aa is a *post hoc* line-scan image showing the effect of antimycin A on spontaneous  $\text{Ca}^{2+}$  waves in an isolated ICC and Fig. 1Ab is an intensity profile plot of this



**Figure 1. Effect of electron transport chain inhibitors on spontaneous  $\text{Ca}^{2+}$  waves in urethra ICC**

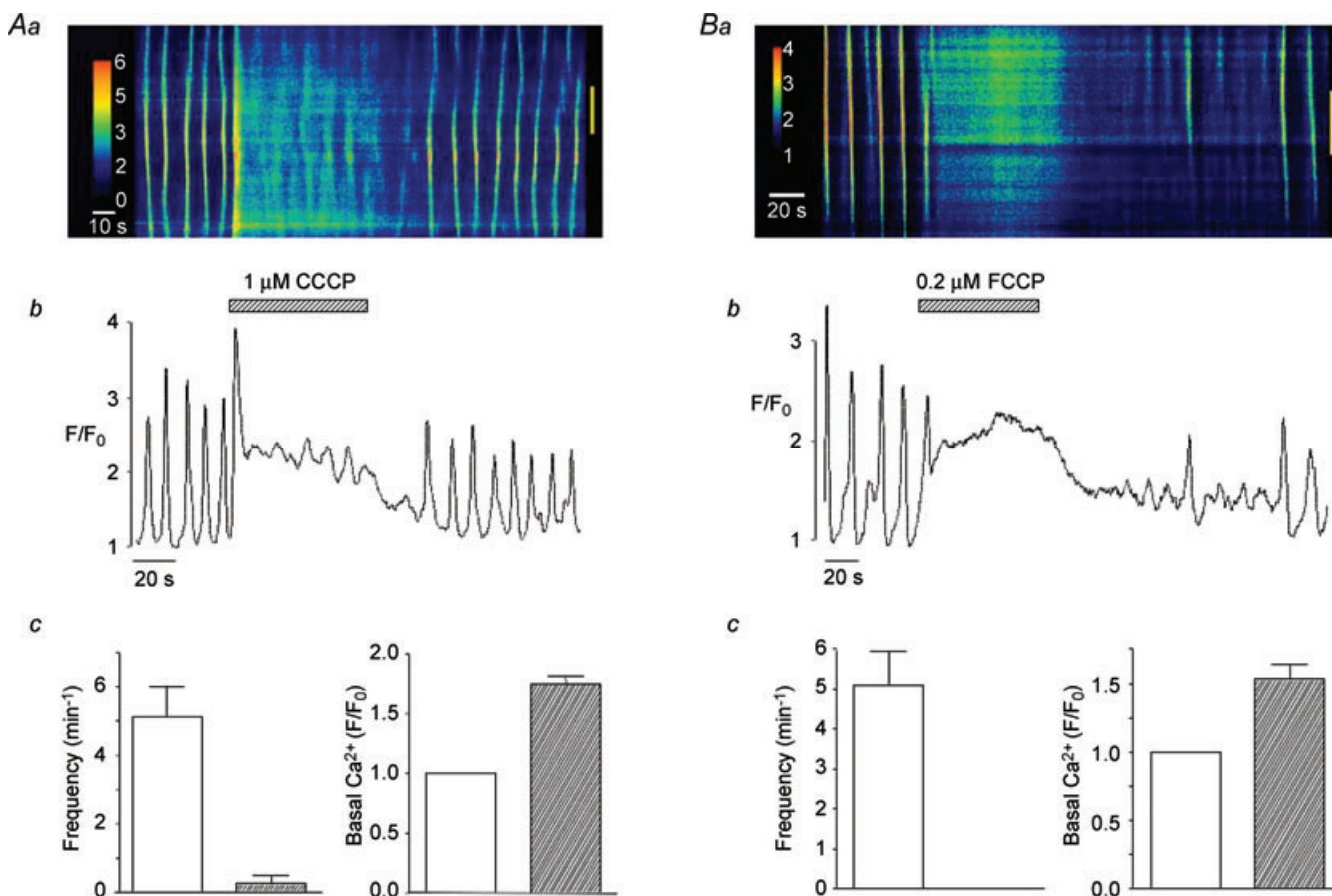
Aa shows a pseudo-line-scan image of spontaneous  $\text{Ca}^{2+}$  waves from an isolated ICC in the absence and presence of antimycin A ( $5\ \mu\text{M}$ ). Ab shows an intensity profile plot of this activity measured over the entire image. Summary graphs of the effects of antimycin A on mean frequency ( $\text{min}^{-1}$ ) of  $\text{Ca}^{2+}$  waves and on basal  $\text{Ca}^{2+}$  levels ( $F/F_0$ ) are shown in Ac. Ba–c show that rotenone produces similar effects to antimycin A.

experiment obtained by placing a region of interest around the entire line-scan. This cell exhibited spontaneous  $\text{Ca}^{2+}$  waves at a frequency of around  $5 \text{ min}^{-1}$ . Application of  $5 \mu\text{M}$  antimycin A reversibly inhibited  $\text{Ca}^{2+}$  waves and this effect was associated with a rise in basal  $\text{Ca}^{2+}$ . This experiment was performed in a total of five cells where, in each case, the inhibition was complete, while basal  $\text{Ca}^{2+}$  significantly increased from  $F/F_0 = 1$  to  $1.4 \pm 0.033$  ( $P < 0.05$ , Fig. 1Ac). Similar effects were obtained using rotenone, as illustrated in Fig. 1B. In eight cells, application of rotenone ( $10 \mu\text{M}$ ) completely inhibited  $\text{Ca}^{2+}$  waves and this was also accompanied by an increase in basal  $\text{Ca}^{2+}$  from  $F/F_0 = 1$  to  $1.56 \pm 0.14$  ( $P < 0.05$ , Fig. 1Bc).

### Effect of protonophores on spontaneous $\text{Ca}^{2+}$ waves

Reduction of mitochondrial  $\text{Ca}^{2+}$  uptake can also be induced by the protonophores FCCP and CCCP, which are also known to collapse the mitochondrial membrane

potential (Farkas *et al.* 1989). In eight cells, CCCP ( $1 \mu\text{M}$ ) raised basal  $\text{Ca}^{2+}$  to a mean of  $1.75 \pm 0.9 F/F_0$  ( $P < 0.05$ ) and reduced the frequency of  $\text{Ca}^{2+}$  waves from  $5.13 \pm 0.9$  to  $0.25 \pm 0.25 \text{ min}^{-1}$  ( $P < 0.05$ , Fig. 2Ac). Similar results were achieved using FCCP ( $0.2 \mu\text{M}$ , Fig. 2B), thus  $\text{Ca}^{2+}$  waves were abolished in each cell tested ( $n = 6$ ) and basal  $\text{Ca}^{2+}$  was increased from  $F/F_0 = 1$  to  $1.53 \pm 0.11$  ( $P < 0.05$ , Fig. 2Bc). These findings support the idea that the  $\text{Ca}^{2+}$  waves depend on the ability of mitochondria to take up  $\text{Ca}^{2+}$ . It is unlikely that the effects of the ETC inhibitors and the protonophores were due to depletion of ATP as oligomycin, an ATP synthase inhibitor, failed to inhibit spontaneous  $\text{Ca}^{2+}$  waves (Fig. 3A and B). In five cells, the mean frequency and amplitude of  $\text{Ca}^{2+}$  waves under control conditions was  $4.6 \pm 1.3 \text{ min}^{-1}$  and  $1.93 \pm 0.28 \Delta F/F_0$ , respectively, compared to  $4.5 \pm 0.92 \text{ min}^{-1}$  and  $1.65 \pm 0.18 \Delta F/F_0$  in the presence of oligomycin ( $1 \mu\text{M}$ ,  $P > 0.05$ , Fig. 3C).



**Figure 2.** Effect of protonophores on spontaneous  $\text{Ca}^{2+}$  waves in urethra ICC

A line-scan image showing the effect of CCCP ( $1 \mu\text{M}$ ) on spontaneous  $\text{Ca}^{2+}$  waves is shown in Aa. Ab shows an intensity profile plot of this record. Summary bar charts plotting the mean frequency ( $\text{min}^{-1}$ ) of  $\text{Ca}^{2+}$  waves and basal  $\text{Ca}^{2+}$  levels ( $F/F_0$ ) in the absence and presence of CCCP are shown in Ac. Ba–c shows that FCCP produces similar effects to CCCP.

### Effect of mitochondrial inhibitors on caffeine-evoked $\text{Ca}^{2+}$ transients

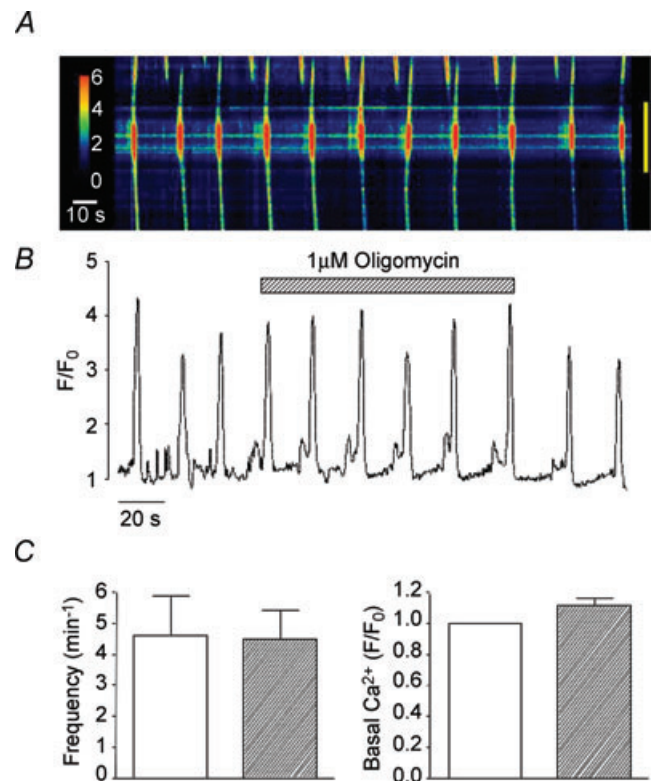
It could be argued that the inhibition of  $\text{Ca}^{2+}$  waves produced by the mitochondrial uncouplers and inhibitors was due to depletion of the intracellular  $\text{Ca}^{2+}$  stores, therefore experiments were performed to test if caffeine-evoked  $\text{Ca}^{2+}$  transients were affected by application of these drugs. Figure 4A and B shows the effect of CCCP on caffeine-evoked  $\text{Ca}^{2+}$  transients. When caffeine (10 mM) was applied under control conditions a maximal  $\text{Ca}^{2+}$  transient was induced, followed by a period of inhibition of the spontaneous oscillations. This effect was reproducible when caffeine was re-applied after a 70 s interval. The cell was then exposed to CCCP (1  $\mu\text{M}$ ) which inhibited the spontaneous activity, as explained above. When caffeine was re-applied during this inhibitory period, a  $\text{Ca}^{2+}$  transient of similar amplitude to the control was evoked, suggesting that the stores had not been depleted by CCCP. Similar results were observed using FCCP, rotenone and antimycin A. These data are summarized in Fig. 4C. It is clear that amplitude of the caffeine-evoked  $\text{Ca}^{2+}$  transients were not affected by the mitochondrial inhibitors ( $P > 0.05$ ).

### Effect of mitochondrial inhibitors on spontaneous transient inward currents

Previous studies have shown that under voltage clamp conditions, spontaneous  $\text{Ca}^{2+}$  waves in urethral ICC are associated with the occurrence of STICs (Johnston *et al.* 2005). Therefore, in order to verify that STICs were regulated by mitochondrial  $\text{Ca}^{2+}$  handling we examined the effect of CCCP, antimycin A and the mitochondrial uniporter inhibitor RU360 on isolated urethral ICC voltage clamped at  $-60$  mV. Figure 5A shows the effect of CCCP (1  $\mu\text{M}$ ) on STICs recorded using the perforated patch configuration of the whole-cell patch clamp technique. In each cell tested, STICs were abolished by application of CCCP ( $P < 0.05$ ). A representative recording of this effect is shown in Fig. 5Aa. Summary data plotting the mean frequency and amplitude of STICs measured from seven cells in the absence and presence of the drug are shown in Fig. 5Ab. Figure 5B shows that similar results were achieved using antimycin A (5  $\mu\text{M}$ ). These recordings were made using the ruptured patch configuration of the whole-cell patch clamp technique. In 4 out of 5 cells, antimycin A abolished STICs, resulting in a mean amplitude and frequency of  $-212 \pm 131$  pA and  $14 \pm 4$   $\text{min}^{-1}$  under control conditions *versus*  $-6.6 \pm 6.6$  pA and  $0.6 \pm 0.6$   $\text{min}^{-1}$  in the presence of the drug ( $P < 0.05$ ,  $n = 5$ ).

We next examined if STICs were inhibited by application of the mitochondrial uniporter inhibitor

RU360 (10  $\mu\text{M}$ ). The mitochondrial uniporter is a  $\text{Ca}^{2+}$ -selective channel that spans the inner mitochondrial membrane and is responsible for  $\text{Ca}^{2+}$  uptake into the mitochondria (Kirichok *et al.* 2004). These recordings were made under ruptured patch conditions as noted above and RU360 was dialysed into the cell via the pipette solution. RU360 inhibited STICs within 3 min following rupture of the membrane. In 4 out of 7 cells RU360 completely abolished the activity within this time period. This resulted in an overall mean frequency and amplitude of STICs (measured between 2 and 3 min from the beginning of the recording) of  $0.86 \pm 0.46$   $\text{min}^{-1}$  and  $-65.3 \pm 54$  pA, respectively. This compared with values of  $9.6 \pm 3.03$   $\text{min}^{-1}$  and  $-358 \pm 124$  pA in cells recorded under similar conditions without RU360, in the same time period ( $n = 9$ ,  $P < 0.05$  unpaired *t* test). These data are plotted in the summary bar charts in Fig. 5Cb and a representative example is shown in Fig. 5Ca.



**Figure 3.** Effect of oligomycin on spontaneous  $\text{Ca}^{2+}$  waves in urethra ICC

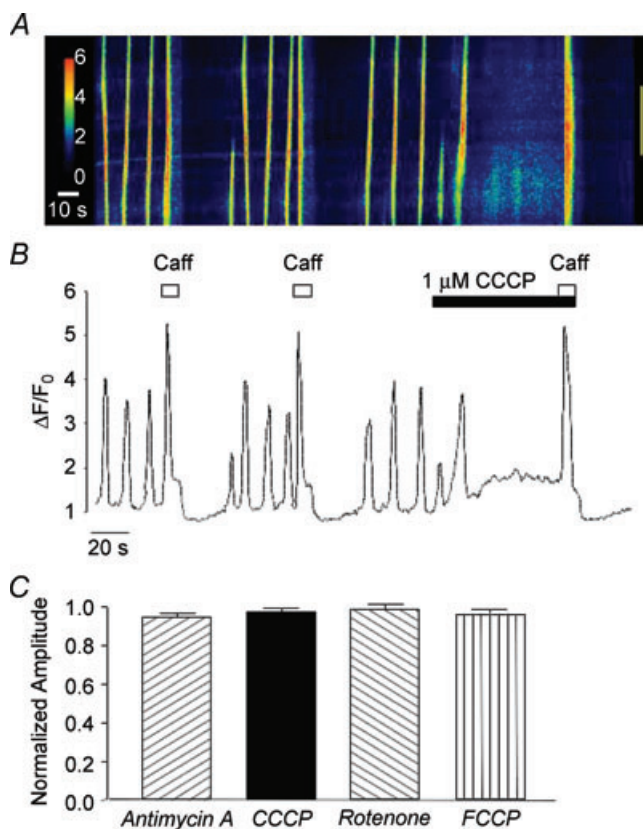
A representative pseudo-line-scan image and corresponding intensity profile plot demonstrating that oligomycin did not inhibit spontaneous  $\text{Ca}^{2+}$  waves is shown in A and B. Summary data, plotting the effects of oligomycin on the mean frequency of  $\text{Ca}^{2+}$  waves and basal  $\text{Ca}^{2+}$  levels, are shown in C.

### Effect of activation of the mitochondrial uniporter on spontaneous Ca<sup>2+</sup> waves

In order to investigate the effect of activation of the mitochondrial uniporter in more detail, we examined the effect of the uniporter opener kaempferol on spontaneous Ca<sup>2+</sup> waves. Montero *et al.* 2004 reported that kaempferol, a naturally occurring plant flavonoid, was a potent activator of the uniporter and could dramatically increase mitochondrial Ca<sup>2+</sup> uptake in HeLa cells. Vay *et al.* (2007) went on to show that stimulation of the uniporter with kaempferol (10  $\mu\text{M}$ ) in HeLa cells and in human fibroblasts induced a burst of repetitive Ca<sup>2+</sup> oscillations of diminishing amplitude which then ceased. Figure 6 shows that application of 10  $\mu\text{M}$  kaempferol to a spontaneously active ICC induced a burst of Ca<sup>2+</sup> oscillations, comprised of an initial Ca<sup>2+</sup> transient followed by a series of Ca<sup>2+</sup> oscillations with a progressively smaller amplitude. This activity ceased on washout before returning to control levels. In 20 cells, the mean amplitude of the initial Ca<sup>2+</sup>

transient induced by kaempferol was  $2.8 \pm 0.32 \Delta F/F_0$ . The overall frequency of the kaempferol-induced Ca<sup>2+</sup> oscillations (measured by dividing the total amount of oscillations during exposure to kaempferol by the duration of the exposure time) was  $9.95 \pm 0.96 \text{ min}^{-1}$ . The propagation velocity of Ca<sup>2+</sup> waves was also significantly enhanced by kaempferol ( $31.4 \pm 2.5 \mu\text{m s}^{-1}$  under control conditions compared to  $55.9 \pm 4 \mu\text{m s}^{-1}$  in the presence of the drug). These values were obtained by analysis of 41 Ca<sup>2+</sup> waves before and 37 during addition of kaempferol in the same 15 cells. Basal Ca<sup>2+</sup> levels did not change significantly during the presence of kaempferol ( $F/F_0 = 1$  under control conditions and  $0.97 \pm 0.04$  during its presence).

In order to verify that these effects were dependent on the Ca<sup>2+</sup> handling properties of mitochondria, we tested if they were affected by pre-treatment with FCCP. Figure 7A and B shows that kaempferol-induced Ca<sup>2+</sup> oscillations were greatly attenuated in the presence of FCCP (0.2  $\mu\text{M}$ ). Figure 7A also shows that addition of FCCP following the initial application of kaempferol caused a sharp rise in Ca<sup>2+</sup>. This effect was variable from cell to cell; however, in each instance it was associated with a marked reduction in the amplitude of the kaempferol response. The summary bar chart illustrated in Fig. 7C shows that in six cells the mean amplitude of the initial Ca<sup>2+</sup> transient evoked by kaempferol significantly decreased from  $2.8 \pm 0.4 \Delta F/F_0$  under control conditions to  $1.2 \pm 0.4 \Delta F/F_0$  in the presence of FCCP ( $P < 0.05$ ).



**Figure 4.** Effect of mitochondrial inhibitors on caffeine-evoked Ca<sup>2+</sup> transients

A and B are a representative pseudo-line-scan image and intensity profile plot showing that caffeine-induced Ca<sup>2+</sup> transients are not inhibited by application of CCCP. The bar chart shown in C plots the mean peak amplitude of the caffeine-evoked Ca<sup>2+</sup> transient in the presence of antimycin A, CCCP, rotenone and FCCP as a function of that evoked under control conditions before addition of each drug.

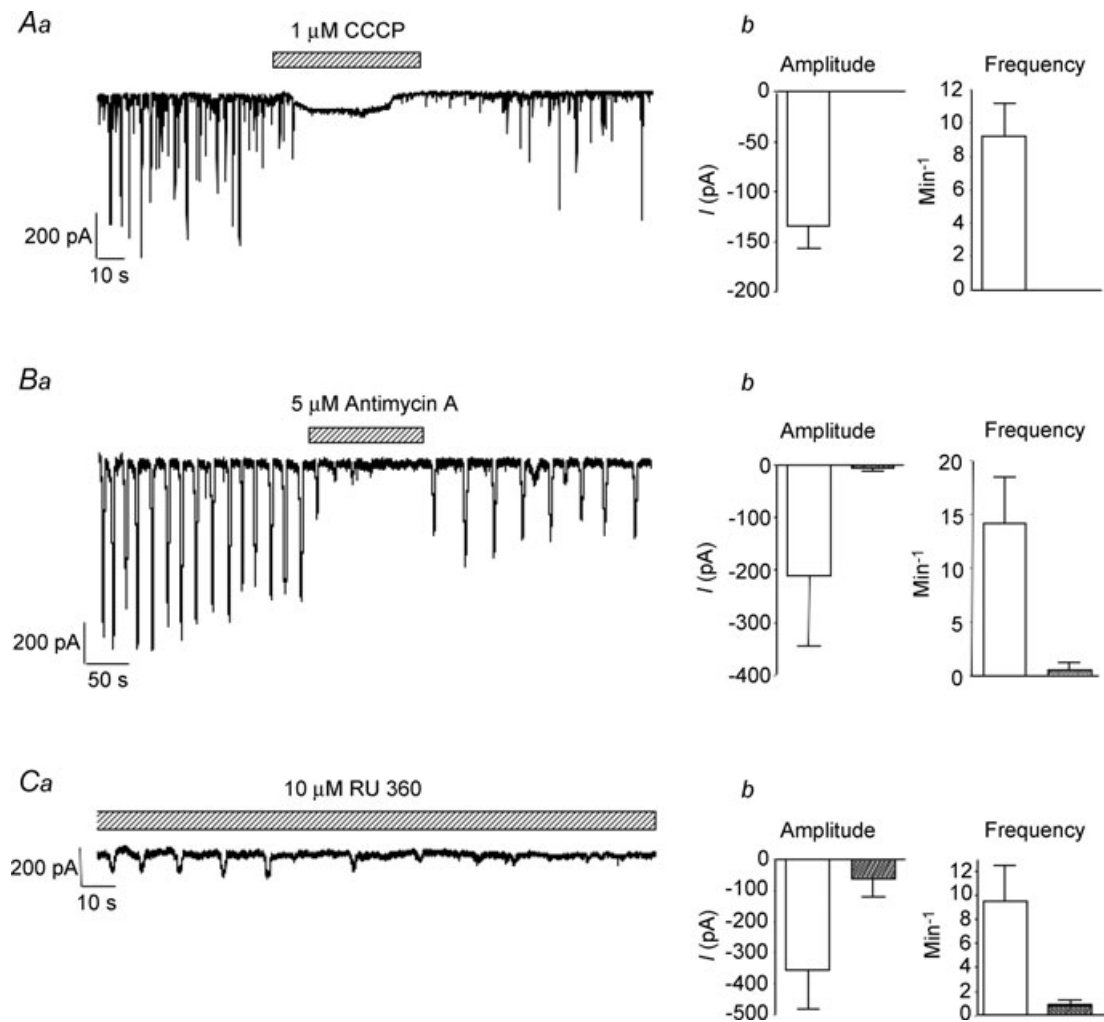
### Role of Ca<sup>2+</sup> stores in kaempferol-induced Ca<sup>2+</sup> oscillations

In order to investigate if kaempferol-induced Ca<sup>2+</sup> oscillations depended on Ca<sup>2+</sup> release from intracellular stores, we examined the effect of kaempferol before and during exposure of cells to caffeine (10 mM), used to deplete intracellular Ca<sup>2+</sup> stores. A representative example of such an experiment is shown in Fig. 8Aa and b. In the absence of drugs, application of kaempferol induced a burst of Ca<sup>2+</sup> oscillations, as described above. Cells were then treated with 10 mM caffeine. This evoked a large Ca<sup>2+</sup> transient and abolished spontaneous activity (as previously described by Johnston *et al.* 2005). Kaempferol was then re-applied in the continued presence of caffeine, but did not induce any change in Ca<sup>2+</sup> levels. The inhibitory effect of caffeine was reversible as kaempferol was able to induce a series of Ca<sup>2+</sup> oscillations upon its removal. This effect was observed in five cells, where in each case the kaempferol response was completely abolished ( $P < 0.05$ , Fig. 8Ac). These data indicate that kaempferol-induced Ca<sup>2+</sup> oscillations are dependent upon Ca<sup>2+</sup> release from stores.

The effects produced by kaempferol in the present study were notably similar to those reported by Vay *et al.* (2007). The authors of this study suggested that the kaempferol effects may result from  $\text{Ca}^{2+}$  release from  $\text{IP}_3\text{Rs}$  due to changes in their local  $\text{Ca}^{2+}$  concentration as a consequence of increased  $\text{Ca}^{2+}$  uptake into mitochondria. To investigate if the kaempferol-induced  $\text{Ca}^{2+}$  oscillations in the present study were due to  $\text{Ca}^{2+}$  release from  $\text{IP}_3\text{Rs}$ , we assessed the effects of the  $\text{IP}_3\text{R}$  blocker 2-APB. Figure 8Ba and b show that although 2-APB disrupted the pattern of spontaneous  $\text{Ca}^{2+}$  oscillations in the absence of kaempferol (as reported previously by Johnston *et al.* 2005), it had only a minor effect on the kaempferol-induced activity. In the presence of 2-APB (100  $\mu\text{M}$ ), kaempferol was still able

to induce a burst of repetitive  $\text{Ca}^{2+}$  oscillations, though the amplitude of the initial spike was slightly reduced. However, this effect was not significant and in three cells the mean amplitude of the kaempferol-induced  $\text{Ca}^{2+}$  transient was  $4.5 \pm 0.85 \Delta F/F_0$  under control conditions and  $3.9 \pm 0.77 \Delta F/F_0$  in the presence of 2-APB ( $P > 0.05$ , Fig. 8Bc).

Previous studies from our laboratory demonstrated that pacemaker activity in urethral ICC was abolished by removal of extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o$ ) (Johnston *et al.* 2005; Sergeant *et al.* 2006b); therefore we next investigated if kaempferol was able to elicit any  $\text{Ca}^{2+}$  oscillations in the absence of  $[\text{Ca}^{2+}]_o$ . A typical example of such an experiment is shown in Fig. 9A and B. Under control



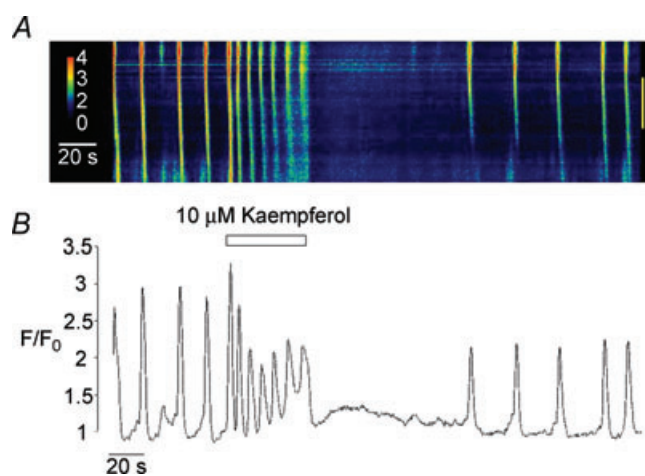
**Figure 5. Effect of mitochondrial inhibitors on STICs**

Aa shows the effect of CCCP (1  $\mu\text{M}$ ) on STICs in a cell held at  $-60$  mV. Ab, summary of the effect of CCCP on STICs from 7 cells. Ba shows the effect of antimycin A (5  $\mu\text{M}$ ) on STICs in a cell held at  $-60$  mV. Bb, summary of the effect of antimycin A on STICs from 5 cells. Ca shows the effect of dialysis of RU360 (10  $\mu\text{M}$ ) on STICs in a cell held at  $-60$  mV. The summary data shown in Cb plot the mean amplitude and frequency of STICs recorded from 7 cells dialysed with RU360 (filled bars) and 9 cells without the drug (open bars).

conditions, the cell produced regularly occurring  $\text{Ca}^{2+}$  waves and kaempferol induced a burst of repetitive  $\text{Ca}^{2+}$  oscillations, as described above. However, when  $\text{Ca}^{2+}$  was removed from the bath, the spontaneous oscillations ceased and application of kaempferol was without effect. This effect was partially reversible upon washout. The summary bar chart shown in Fig. 9C shows that the mean amplitude of the kaempferol-induced oscillations was reduced from  $3.58 \pm 0.66 \Delta F/F_0$  under control conditions to  $0.83 \pm 0.33 \Delta F/F_0$  in the absence of  $[\text{Ca}^{2+}]_o$  ( $n = 5$ ,  $P < 0.05$ ). These data demonstrate that the stimulatory effects of kaempferol on  $\text{Ca}^{2+}$  waves require  $[\text{Ca}^{2+}]_o$  and suggest kaempferol did not cause a direct release of  $\text{Ca}^{2+}$  from stores.

## Discussion

The results of the present study show that spontaneous  $\text{Ca}^{2+}$  waves and STICs in urethral ICC are inhibited by agents which decrease mitochondrial  $\text{Ca}^{2+}$  uptake, such as FCCP, CCCP, antimycin A, rotenone and RU360. Conversely, when mitochondrial  $\text{Ca}^{2+}$  uptake was enhanced using the mitochondrial uniporter activator kaempferol, the frequency of  $\text{Ca}^{2+}$  oscillations was increased. This effect was inhibited by FCCP, consistent with an action on mitochondria. Application of the ATP synthase inhibitor, oligomycin, did not inhibit  $\text{Ca}^{2+}$  oscillations, indicating that the effects observed in this study are directly related to the  $\text{Ca}^{2+}$  handling properties of mitochondria and were not due to an indirect effect on ATP levels. Taken together, these results suggest that the occurrence of spontaneous  $\text{Ca}^{2+}$  waves in urethral ICC is

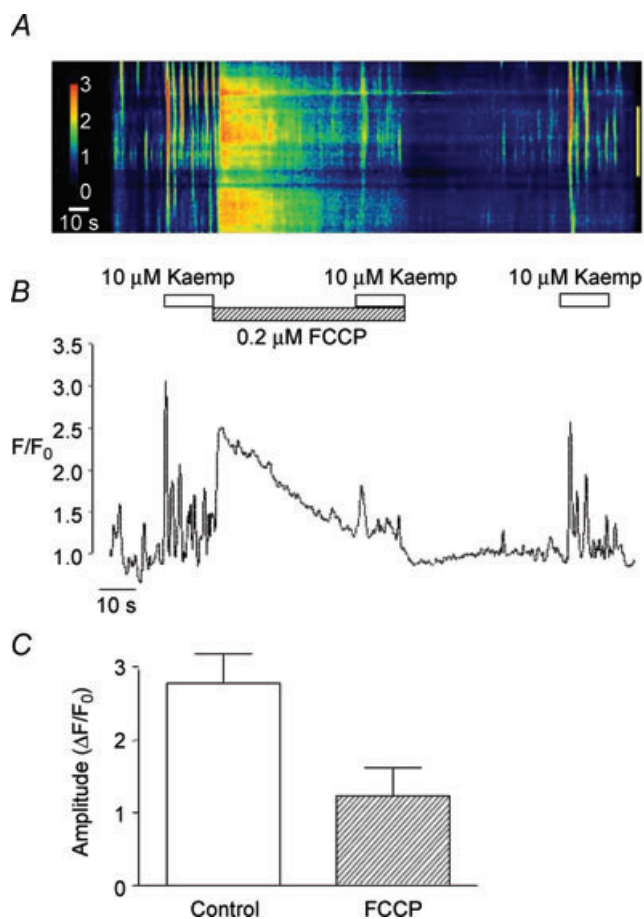


**Figure 6. Effect of the mitochondrial uniporter opener kaempferol on  $\text{Ca}^{2+}$  waves**

A is a linescan image showing the effect of kaempferol ( $10 \mu\text{M}$ ) on spontaneous  $\text{Ca}^{2+}$  waves in isolated urethra ICC. An intensity profile plot of this record is shown in B.

directly related to the ability of mitochondria to take up  $\text{Ca}^{2+}$ .

Bursts of  $\text{Ca}^{2+}$  oscillations induced by application of kaempferol in this study were inhibited when intracellular stores were depleted by application of  $10 \text{ mM}$  caffeine, but were not significantly inhibited by 2-APB, suggesting that they were more likely to be dependent on RyRs than  $\text{IP}_3\text{Rs}$ . However, spontaneous  $\text{Ca}^{2+}$  waves were significantly reduced by 2-APB, demonstrating that  $\text{IP}_3\text{Rs}$  are involved in the generation of  $\text{Ca}^{2+}$  waves under resting conditions as previously reported by Johnston *et al.* (2005). The kaempferol-induced activity was also inhibited by removal of  $[\text{Ca}^{2+}]_o$ ; therefore it seems unlikely that kaempferol released  $\text{Ca}^{2+}$  directly from stores, as under similar experimental conditions caffeine-evoked  $\text{Ca}^{2+}$  transients were unaffected by removal of  $[\text{Ca}^{2+}]_o$  (Johnston *et al.* 2005). The results of Johnston *et al.* (2005) also suggest that the effects produced by removal of  $[\text{Ca}^{2+}]_o$  in the



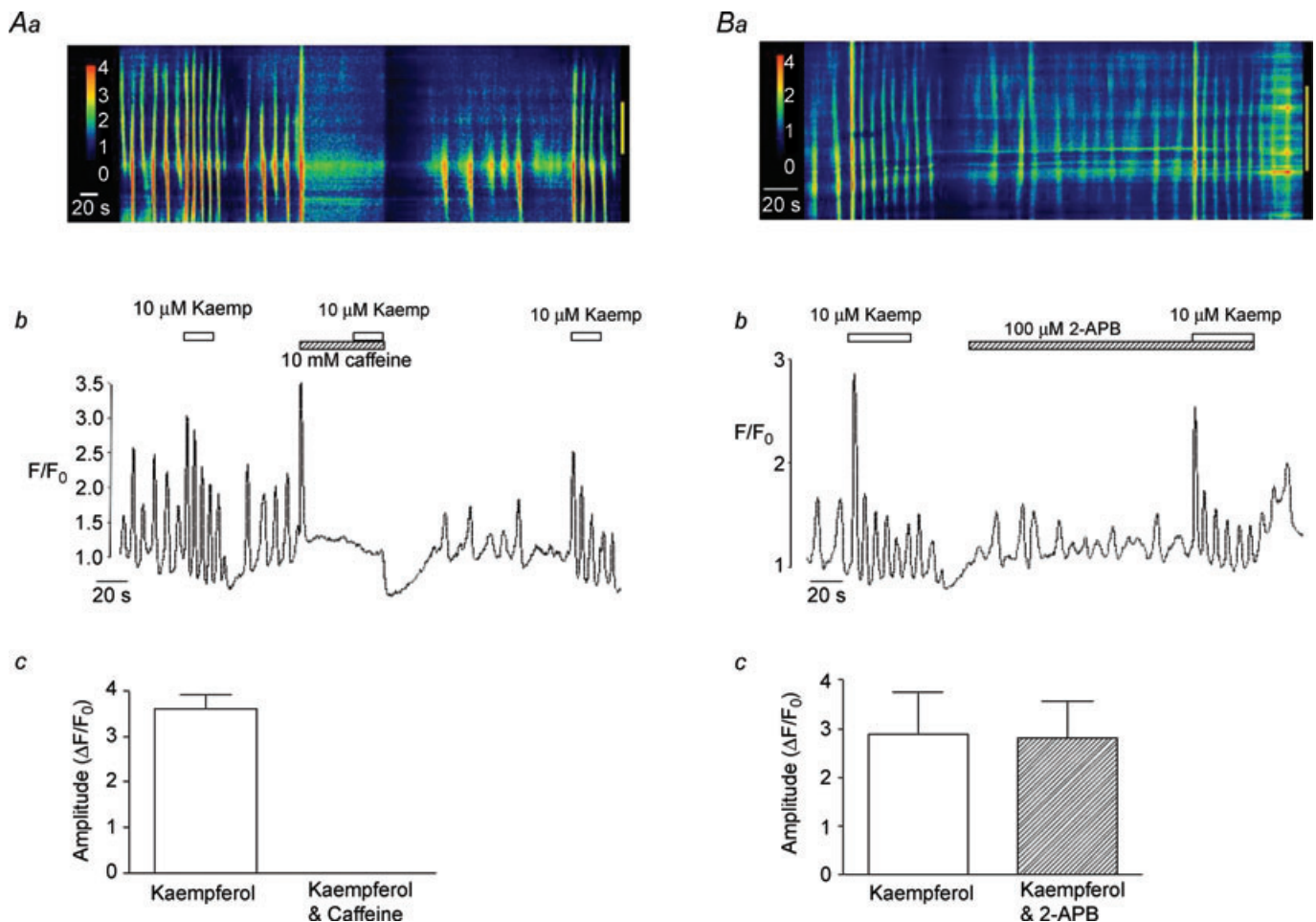
**Figure 7. Effect of FCCP on kaempferol-induced  $\text{Ca}^{2+}$  waves**

A representative line-scan image showing the effect of kaempferol ( $10 \mu\text{M}$ ) in the absence and presence of FCCP ( $0.2 \mu\text{M}$ ) is shown in A. An intensity profile plot of this record is shown in B. A summary bar chart plotting the mean amplitude ( $\Delta F/F_0$ ) of the initial kaempferol-induced  $\text{Ca}^{2+}$  transient in the absence and presence of FCCP is shown in C.

current study were not attributable to rapid depletion of stores.

The results of the current study imply a functional association between the activity of RyRs and uptake of  $\text{Ca}^{2+}$  by mitochondria, and there are many examples of such interactions in the literature (Straub *et al.* 2000; Nassar & Simpson, 2000; Szalai *et al.* 2000; Shkryl & Shirokova, 2006; Kopach *et al.* 2008). It is now well established that the steady state activity of RyRs is governed by cytoplasmic  $\text{Ca}^{2+}$  concentration in a classical bell-shaped manner (Fabiato, 1985; Meissner *et al.* 1997). This represents an effective negative feedback mechanism, resulting in closure of RyRs at high  $\text{Ca}^{2+}$  concentrations (Sham *et al.* 1998; Laver & Lamb, 1998). However, as these studies were performed on lipid bilayers it is difficult to determine the precise concentration required to induce

inhibition *in situ*. The results shown in the present study suggest that  $\text{Ca}^{2+}$  waves in urethral ICC could be regulated by opening and closure of RyRs brought about by buffering of cytosolic  $\text{Ca}^{2+}$  by mitochondria. Thus, inhibition of mitochondrial  $\text{Ca}^{2+}$  uptake may lead to elevations in cytosolic  $\text{Ca}^{2+}$  which are sufficient to inhibit the opening of RyRs and prevent the development of spontaneous  $\text{Ca}^{2+}$  waves. Consistent with this idea are the recent findings of Kopach *et al.* 2008 which demonstrate that CICR at RyRs is reduced when mitochondrial  $\text{Ca}^{2+}$  uptake is decreased by FCCP. On the other hand, enhanced uptake of  $\text{Ca}^{2+}$  into mitochondria would be expected to cause a reduction in cytosolic  $\text{Ca}^{2+}$  and remove  $\text{Ca}^{2+}$ -induced inhibition of RyRs. Jouaville *et al.* (1995) demonstrated a similar mechanism involving mitochondrial regulation of  $\text{IP}_3\text{Rs}$  in *Xenopus* oocytes, whereby uptake of  $\text{Ca}^{2+}$



**Figure 8. Effect of caffeine and 2-APB on kaempferol-induced  $\text{Ca}^{2+}$  waves**

**Aa** is a line-scan image showing the effect of kaempferol before and during exposure to 10 mM caffeine. **Ba** represents the intensity profile plot of the data shown in **Aa**. A summary plot of the mean amplitude ( $\Delta F/F_0$ ) of the initial kaempferol-induced  $\text{Ca}^{2+}$  transient in the absence and presence of caffeine is shown in **Ac**. **Ba** is a line-scan image showing the effect of kaempferol before and during exposure to 2-APB (100  $\mu\text{M}$ ). **Bb** represents the intensity profile plot of the data shown in **Ba**. A summary plot of the mean amplitude ( $\Delta F/F_0$ ) of the initial kaempferol-induced  $\text{Ca}^{2+}$  transient in the absence and presence of 2-APB is shown in **Bc**.

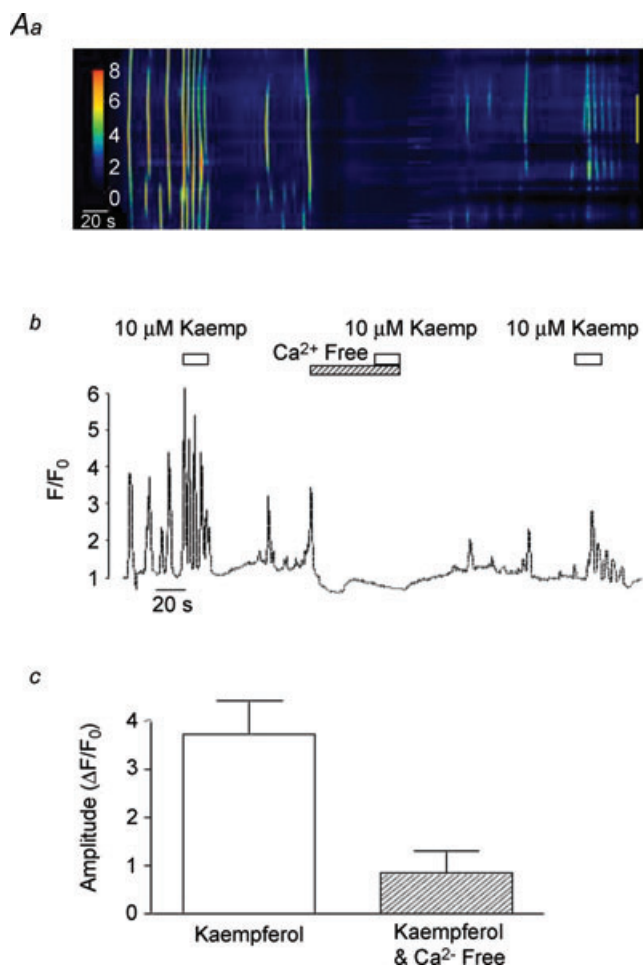
into mitochondria synchronized  $\text{Ca}^{2+}$  release from  $\text{IP}_3\text{Rs}$  resulting in an increase in the propagation velocity of  $\text{Ca}^{2+}$  waves.

The activity of RyRs is also known to be affected by the  $\text{Ca}^{2+}$  concentration in the lumen of the sarcoplasmic reticulum (Burdakov *et al.* 2005). Indeed, studies by Hoth *et al.* (1997) and Malli *et al.* 2003, 2005 demonstrated that mitochondria could affect intraluminal  $\text{Ca}^{2+}$  levels in the endoplasmic reticulum (ER) by regulating the refilling pathway. Therefore, if mitochondrial  $\text{Ca}^{2+}$  uptake enhanced the refilling of  $\text{Ca}^{2+}$  stores in urethral ICC, as noted in the studies above, then we might expect that its inhibition would decrease refilling of stores and lead to a reduction in store  $\text{Ca}^{2+}$  levels. However, it seems unlikely that this is the case, as although FCCP, CCCP, rotenone and antimycin A all inhibited spontaneous  $\text{Ca}^{2+}$  waves,

they did not affect the amplitude of caffeine-evoked  $\text{Ca}^{2+}$  transients, suggesting that the  $\text{Ca}^{2+}$  content of intracellular stores was unaffected by a reduction in uptake of  $\text{Ca}^{2+}$  into mitochondria.

Several studies have reported functional links between mitochondria and  $\text{IP}_3\text{Rs}$ ; therefore the finding that kaempferol-induced  $\text{Ca}^{2+}$  waves were not significantly affected by 2-APB was surprising in the current study. For example, Jouaville *et al.* (1995) showed that mitochondrial  $\text{Ca}^{2+}$  uptake increased the propagation speed of  $\text{Ca}^{2+}$  waves in *Xenopus* oocytes by preventing  $\text{Ca}^{2+}$ -dependent inhibition of  $\text{IP}_3\text{Rs}$ . Vay *et al.* (2007) also reported that mitochondrial regulation of  $\text{IP}_3\text{Rs}$  led to an increase in the frequency of spontaneous  $\text{Ca}^{2+}$  waves in HeLa cells and fibroblasts. Some studies have suggested that 2-APB is a poor blocker of  $\text{IP}_3\text{Rs}$ , but is an effective blocker of capacitative  $\text{Ca}^{2+}$  entry (CCE; Bootman *et al.* 2002); therefore it is possible that the results of the present study could be due to the lack of efficacy of 2-APB as an  $\text{IP}_3\text{R}$  inhibitor. However, several lines of evidence suggest that this is not the case in urethral ICC. For example, Sergeant *et al.* (2001) demonstrated that 2-APB (100  $\mu\text{M}$ ) blocked noradrenaline-induced  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  currents, but not those evoked by caffeine. This study also showed that 2-APB failed to inhibit spontaneous transient outward currents (STOCs). These data suggest that 2-APB does block  $\text{IP}_3\text{Rs}$  but does not affect release or uptake of  $\text{Ca}^{2+}$  from caffeine-sensitive stores, nor does it affect  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels or  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BK) channels in urethral ICC.

Mitochondrial  $\text{Ca}^{2+}$  handling has also been shown to be involved in the regulation of pacemaker activity in ICC of the murine small intestine (Ward *et al.* 2000). However, in these cells it is thought that the pacemaker channels are directly modulated by mitochondrial  $\text{Ca}^{2+}$  uptake. The pacemaker current in intestinal ICC is mediated by activation of non-selective cation channels (NSCC; Koh *et al.* 1998). Activation of pacemaker channels in these cells is stimulated by reductions in cytoplasmic  $\text{Ca}^{2+}$  (Koh *et al.* 2002) and it is thought that uptake of  $\text{Ca}^{2+}$  into mitochondria is an essential prerequisite for activation of pacemaker currents. It seems unlikely that a similar process could regulate STICs in urethral ICC, as in these cells STICs arise from activation of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  (ClCa) channels (Sergeant *et al.* 2000). Unlike the pacemaker NSCC channels in the gut, ClCa channels open in response to elevations of cytoplasmic  $\text{Ca}^{2+}$ , therefore mitochondrial  $\text{Ca}^{2+}$  uptake would be unlikely to stimulate these channels directly. In summary, we suggest that uptake of  $\text{Ca}^{2+}$  into mitochondria influences spontaneous  $\text{Ca}^{2+}$  waves, which underlie the pacemaker activity in urethral ICC, via a process which may involve regulation of RyRs.



**Figure 9.** Effect of  $\text{Ca}^{2+}$ -free media on kaempferol-induced  $\text{Ca}^{2+}$  waves

A typical line-scan image showing the effect of kaempferol before and during exposure to  $\text{Ca}^{2+}$ -free media is shown in A. An intensity profile plot of this record is plotted in B and a summary plot of the mean amplitude ( $\Delta F/F_0$ ) of the initial kaempferol-induced  $\text{Ca}^{2+}$  transient in the absence and presence of  $\text{Ca}^{2+}$ -free media is shown in C.

## References

- Boitier E, Rea R & Duchen MR (1999). Mitochondria exert a negative feedback on the propagation of intracellular  $\text{Ca}^{2+}$  waves in rat cortical astrocytes. *J Cell Biol* **145**, 795–808.
- Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ & Peppiatt CM (2002). 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated  $\text{Ca}^{2+}$  entry but an inconsistent inhibitor of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release (Review). *FASEB J* **16**, 1145–1150.
- Bradley E, Hollywood MA, Johnston L, Large RJ, Matsuda T, Baba A, McHale NG, Thornbury KD & Sergeant GP (2006). Contribution of reverse  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange to spontaneous activity in interstitial cells of Cajal in the rabbit urethra. *J Physiol* **574**, 651–661.
- Bradley E, Hollywood MA, McHale NG, Thornbury KD & Sergeant GP (2005). Pacemaker activity in urethral interstitial cells is not dependent on capacitative calcium entry. *Am J Physiol Cell Physiol* **289**, C625–C632.
- Burdakov D, Petersen OH & Verkhratsky A (2005). Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium* **38**, 303–310.
- Fabiato A (1985). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J Gen Physiol* **85**, 247–289.
- Farkas DL, Wei MD, Febroriello P, Carson JH & Loew LM (1989 (December)). Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys J* **56**, 1053–1069.
- Graier WF, Frieden M & Malli R (2007). Mitochondria and  $\text{Ca}^{2+}$  signaling: old guests, new functions. *Pflugers Arch* **455**, 375–396.
- Hirst GD & Ward SM (2003). Interstitial cells: involvement in rhythmicity and neural control of gut smooth muscle (Review). *J Physiol* **550**, 337–346.
- Hoth M, Fanger CM & Lewis RS (1997). Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J Cell Biol* **137**, 633–648.
- Johnston L, Sergeant GP, Hollywood MA, Thornbury KD & McHale NG (2005). Calcium oscillations in interstitial cells of the rabbit urethra. *J Physiol* **565**, 449–461.
- Jouaville LS, Ichas F, Holmuhamedov EL, Camacho P & Lechleiter JD (1995). Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. *Nature* **377**, 438–441.
- Kirichok Y, Krapivinsky G & Clapham D (2004). The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**, 360–364.
- Klemm MF, Exintaris B & Lang RJ (1999). Identification of the cells underlying pacemaker activity in the guinea-pig upper urinary tract. *J Physiol* **519**, 867–884.
- Koh SD, Jun JY, Kim TW & Sanders KM (2002). A  $\text{Ca}^{2+}$ -inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cell of Cajal. *J Physiol* **540**, 803–814.
- Koh SD, Sanders KM & Ward SM (1998). Spontaneous electrical rhythmicity in cultured interstitial cells of cajal from the murine small intestine. *J Physiol* **513**, 203–213.
- Kopach O, Kruglikov I, Pivneva T, Voitenko N & Fedirko N (2008). Functional coupling between ryanodine receptors, mitochondria and  $\text{Ca}^{2+}$  ATPases in rat submandibular acinar cells. *Cell Calcium* **43**, 469–481.
- Laver DR & Lamb GD (1998). Inactivation of  $\text{Ca}^{2+}$  release channels (ryanodine receptors RyR1 and RyR2) with rapid steps in  $[\text{Ca}^{2+}]$  and voltage. *J Biophys* **74**, 2352–2364.
- McCloskey KD & Gurney AM (2002). Kit positive cells in the guinea pig bladder. *J Urol* **168**, 832–836.
- Malli R, Frieden M, Osibow K & Graier WF (2003). Mitochondria efficiently buffer subplasmalemmal  $\text{Ca}^{2+}$  elevation during agonist stimulation. *J Biol Chem* **278**, 10807–10815.
- Malli R, Frieden M, Trenker M & Graier WF (2005). The role of mitochondria for  $\text{Ca}^{2+}$  refilling of the endoplasmic reticulum. *J Biol Chem* **280**, 12114–12122.
- Meissner G, Rios E, Tripathy A & Pasek DA (1997). Regulation of skeletal muscle  $\text{Ca}^{2+}$  release channel (ryanodine receptor) by  $\text{Ca}^{2+}$  and monovalent cations and anions. *J Biol Chem* **272**, 1628–1638.
- Mitchell P (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**, 144–148.
- Montero M, Lobatón CD, Hernández-Sanmiguel E, Santodomingo J, Vay L, Moreno A & Alvarez J (2004). Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. *Biochem J* **384**, 19–24.
- Nassar A & Simpson AW (2000). Elevation of mitochondrial calcium by ryanodine-sensitive calcium-induced calcium release. *J Biol Chem* **275**, 23661–23665.
- Pacher P, Thomas AP & Hajnóczky G (2002).  $\text{Ca}^{2+}$  marks: miniature calcium signals in single mitochondria driven by ryanodine receptors. *Proc Natl Acad Sci U S A* **99**, 2380–2385.
- Rae J, Cooper K, Gates P & Watsky M (1991). Low access resistance perforated patch recordings using amphotericin B. *J Neurosci Methods* **37**, 15–26.
- Sanders KM (1996). A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract (Review). *Gastroenterology* **111**, 492–515.
- Sanders KM, Koh SD & Ward SM (2006). Interstitial cells of cajal as pacemakers in the gastrointestinal tract (Review). *Annu Rev Physiol* **68**, 307–343.
- Sergeant GP, Hollywood MA, McCloskey KD, McHale NG & Thornbury KD (2001). Role of  $\text{IP}_3$  in modulation of spontaneous activity in pacemaker cells of rabbit urethra. *Am J Physiol Cell Physiol* **280**, C1349–C1356.
- Sergeant GP, Hollywood MA, McCloskey KD, Thornbury KD & McHale NG (2000). Specialised pacemaking cells in the rabbit urethra. *J Physiol* **526**, 359–366.
- Sergeant GP, Johnston L, McHale NG, Thornbury KD & Hollywood MA (2006a). Activation of the cGMP/PKG pathway inhibits electrical activity in rabbit urethral interstitial cells of Cajal by reducing the spatial spread of  $\text{Ca}^{2+}$  waves. *J Physiol* **574**, 167–181.
- Sergeant GP, Thornbury KD, McHale NG & Hollywood MA (2006b). Interstitial cells of Cajal in the urethra (Review). *J Cell Mol Med* **10**, 280–291.

- Sergeant GP, Thornbury KD, McHale NG & Hollywood MA (2002). Characterization of norepinephrine-evoked inward currents in interstitial cells isolated from the rabbit urethra. *Am J Physiol Cell Physiol* **283**, C885–C894.
- Sham JS, Song LS, Chen Y, Deng LH, Stern MD, Lakatta EG & Cheng H (1998). Termination of  $\text{Ca}^{2+}$  release by a local inactivation of ryanodine receptors in cardiac myocytes. *Proc Natl Acad Sci U S A* **95**, 15096–15101.
- Shkryl VM & Shirokova N (2006). Transfer and tunneling of  $\text{Ca}^{2+}$  from sarcoplasmic reticulum to mitochondria in skeletal muscle. *J Biol Chem* **281**, 1547–1554.
- Straub SV, Giovannucci DR & Yule DI (2000). Calcium wave propagation in pancreatic acinar cells: functional interaction of inositol 1,4,5-trisphosphate receptors, ryanodine receptors, and mitochondria. *J Gen Physiol* **116**, 547–560.
- Szalai G, Csordás G, Hantash BM, Thomas AP & Hajnóczky G (2000). Calcium signal transmission between ryanodine receptors and mitochondria. *J Biol Chem* **275**, 15305–15313.
- Tinel H, Cancela JM, Mogami H, Gerasimenko JV, Gerasimenko OV, Tepikin AV & Petersen OH (1999). Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic  $\text{Ca}^{2+}$  signals. *EMBO J* **18**, 4999–5008.
- Vay L, Hernández-Sanmiguel E, Santo-Domingo J, Lobatón CD, Moreno A, Montero M & Alvarez J (2007). Modulation of  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  oscillations in HeLa cells and fibroblasts by mitochondrial  $\text{Ca}^{2+}$  uniporter stimulation. *J Physiol* **580**, 39–49.
- Ward SM, Ordog T, Koh SD, Baker SA, Jun JY, Amberg G, Monaghan K & Sanders KM (2000). Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J Physiol* **525**, 355–361.

### Acknowledgements

This study was supported by grant number RO1 DK68565 from NIH, and G.P.S. is in receipt of a Research Fellowship awarded by the Health Research Board, Ireland (PD/2005/4).