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

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Interstitial cells of Cajal (ICC) isolated from the rabbit urethra exhibit pacemaker activity that results from spontaneous Ca^{2+} waves. The purpose of this study was to investigate if this activity was influenced by Ca^{2+} uptake into mitochondria. Spontaneous 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 μM) and antimycin A (5 μM) abolished Ca^{2+} waves and increased basal Ca^{2+} levels. Similar results were achieved when mitochondria membrane potential was collapsed using the protonophores FCCP (0.2 μM) and CCCP (1 μM). Spontaneous Ca^{2+} waves were not inhibited by the ATP synthase inhibitor oligomycin (1 μM), suggesting that these effects were not attributable to an effect on ATP levels. STICs recorded under voltage clamp at −60 mV were also inhibited by CCCP and antimycin A. Dialysis of cells with the mitochondrial uniporter inhibitor RU360 (10 μM) also inhibited STICS. Stimulation of Ca^{2+} uptake into mitochondria using the plant flavonoid kaempferol (10 μM) induced a series of propagating Ca^{2+} waves. The kaempferol-induced activity was inhibited by application of caffeine (10 mM) or removal of extracellular Ca^{2+}, but was not significantly affected by the IP_3 receptor blocker 2-APB (100 μM). These data suggest that spontaneous Ca^{2+} waves in urethral ICC are regulated by buffering of cytoplasmic Ca^{2+} by mitochondria.

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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.* 2006*a*), 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 Ca^{2+} imaging experiments revealed that spontaneous electrical activity of these cells is associated with global Ca^{2+} waves (Johnston *et al.* 2005; Sergeant *et al.* 2006*a*,*b*). The cellular mechanisms responsible for the regulation and generation of 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 (IP_3) production or IP_3 receptors (IP_3Rs) reduced the propagation of Ca^{2+} waves, unmasking multiple, uncoupled Ca^{2+} release events. Ca^{2+} waves were also found to be dependent on Ca^{2+} influx, thus removal of extracellular Ca^{2+} was found to inhibit Ca^{2+} waves by a mechanism that did not involve depletion of 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 Ca^{2+} oscillations

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are regulated by the Ca^{2+} handling properties of mitochondria, independently from an action involving ATP (Graier *et al.* 2007). For example, it has been shown that Ca^{2+} uptake into mitochondria is involved in the regulation of frequency and duration of Ca^{2+} sparks (Pacher *et al.* 2002) and can influence the propagation of Ca^{2+} waves (Jouaville *et al.* 1995; Boitier *et al.* 1999; Tinel *et al.* 1999). There is also evidence that 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 Ca^{2+} waves in ICC isolated from the rabbit urethra are also regulated by the 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 μM fluo-4 AM (Molecular Probes) in Hanks solution containing 100 μM 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 pixels × 512 pixels, pixel size 16 μm × 16 μm) 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 nm. Experiments were performed using a ×60 objective (Olympus) resulting in images of pixel size 0.266 μm × 0.266 μm. 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 μm 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 ± 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 Ca^{2+}' during oscillatory activity was defined as the diastolic 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 Ca^{2+} was also measured as the Ca^{2+} level between oscillations; however, when oscillations were abolished, basal Ca^{2+} levels were determined as the mean Ca^{2+} level for the last 30 s during drug application. $\Delta F/F_0$ refers to the measurement of the change in Ca^{2+} levels from basal to peak. Changes in basal 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 μg ml^{-1}). Pipettes were pulled from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.17 mm inner diameter; Clark Medical Instruments) to a tip of diameter approximately 1–1.5 μm and resistance of 2–4 MΩ. 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 Na^+, 5.8 K^+, 135 Cl^-, 4.16 HCO_3^-, 0.34 HPO_3^{2-}, 0.44 H_2PO^{4-}, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4 SO_4^{2-}, 10 dextrose, 2.9 sucrose, 10 Hepes, pH adjusted to 7.4 with NaOH; Ca^{2+}-free Hanks: as Hanks but with Mg^{2+} (1.8 mM) substituted for Ca^{2+} and addition of EGTA (5 mM). Perforated patch solution: CsCl (133), $MgCl_2$ (1.0), EGTA (0.5), Hepes (10), pH adjusted to 7.2 with CsOH. Whole-cell patch solution: CsCl (133), $MgCl_2$ (1.0), Hepes (10), Na_2ATP (1), NaGTP (0.1), 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 μm) placed approximately 300 μm 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 35–37°C.

Results

Individual ICC isolated from the rabbit urethra displayed regularly occurring Ca^{2+} waves as previously described by Johnston *et al.* (2005) and Sergeant *et al.* (2006*a*,*b*). Examples of 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 Ca^{2+} waves

Mitochondrial Ca^{2+} uptake is driven by the large negative potential (~−180 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 Ca^{2+} waves and mitochondrial Ca^{2+} uptake, we examined the effects of the ETC (complex III) inhibitor antimycin A (5 μM) and the complex I inhibitor rotenone (10 μM). These agents have been shown to dissipate the negative mitochondrial membrane potential and thus reduce the ability of mitochondria to take up Ca^{2+} (Tinel *et al.* 1999). Figure 1*Aa* is a *post hoc* line-scan image showing the effect of antimycin A on spontaneous Ca^{2+} waves in an isolated ICC and Fig. 1*Ab* is an intensity profile plot of this

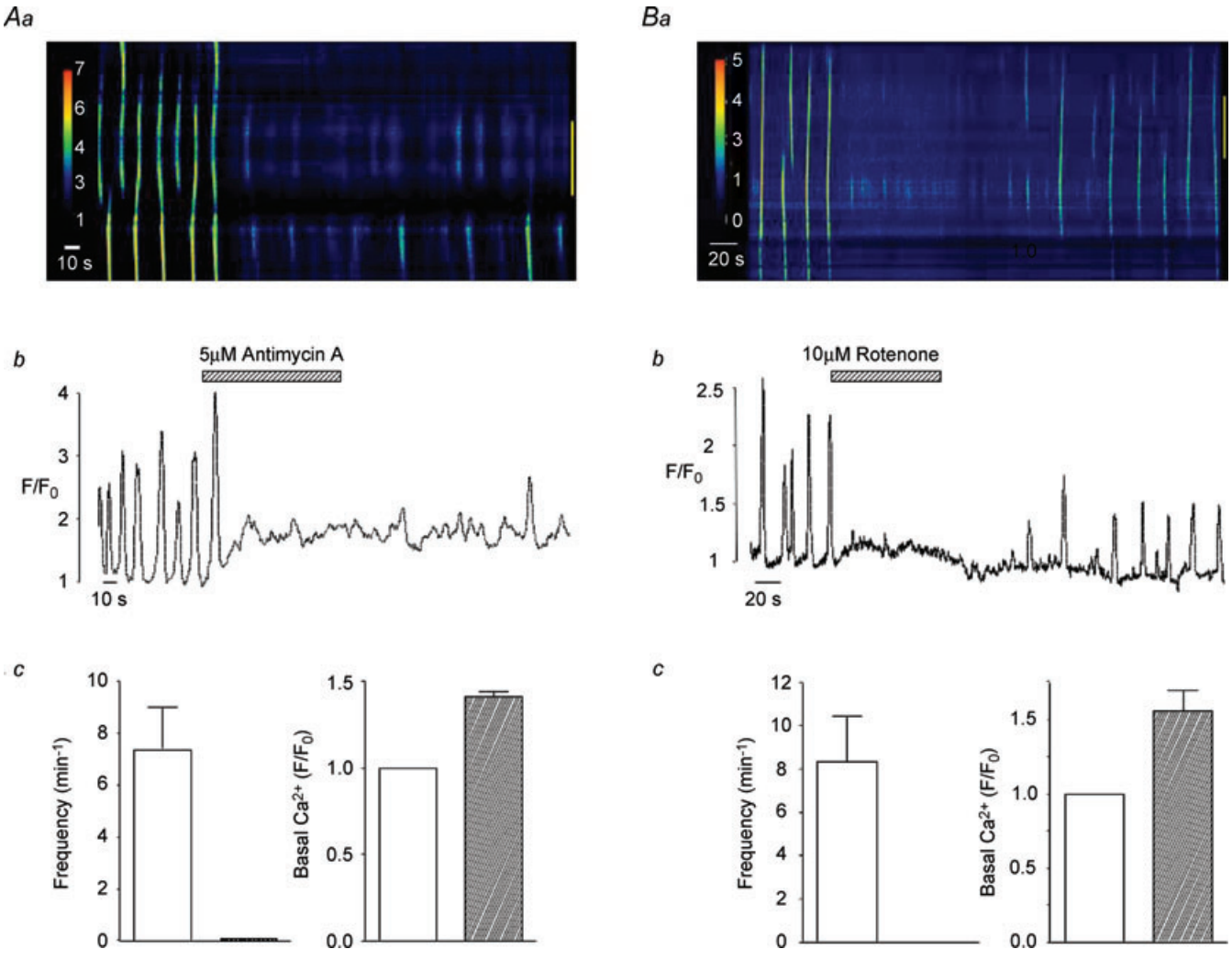

Figure 1. Effect of electron transport chain inhibitors on spontaneous Ca^{2+} waves in urethra ICC
Aa shows a pseudo-line-scan image of spontaneous Ca^{2+} waves from an isolated ICC in the absence and presence of antimycin A (5 μM). *Ab* shows an intensity profile plot of this activity measured over the entire image. Summary graphs of the effects of antimycin A on mean frequency (min^{-1}) of Ca^{2+} waves and on basal 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 Ca^{2+} waves at a frequency of around 5 min^{-1}. Application of 5 μM antimycin A reversibly inhibited Ca^{2+} waves and this effect was associated with a rise in basal Ca^{2+}. This experiment was performed in a total of five cells where, in each case, the inhibition was complete, while basal Ca^{2+} significantly increased from $F/F_0 = 1$ to 1.4 ± 0.033 ($P < 0.05$, Fig. 1*Ac*). Similar effects were obtained using rotenone, as illustrated in Fig. 1*B*. In eight cells, application of rotenone (10 μM) completely inhibited Ca^{2+} waves and this was also accompanied by an increase in basal Ca^{2+} from $F/F_0 = 1$ to 1.56 ± 0.14 ($P < 0.05$, Fig. 1*Bc*).

Effect of protonophores on spontaneous Ca^{2+} waves

Reduction of mitochondrial 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 μM) raised basal Ca^{2+} to a mean of 1.75 ± 0.9 F/F_0 ($P < 0.05$) and reduced the frequency of Ca^{2+} waves from 5.13 ± 0.9 to 0.25 ± 0.25 min^{-1} ($P < 0.05$, Fig. 2*Ac*). Similar results were achieved using FCCP (0.2 μM, Fig. 2*B*), thus Ca^{2+} waves were abolished in each cell tested ($n = 6$) and basal Ca^{2+} was increased from $F/F_0 = 1$ to 1.53 ± 0.11 ($P < 0.05$, Fig. 2*Bc*). These findings support the idea that the Ca^{2+} waves depend on the ability of mitochondria to take up 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 Ca^{2+} waves (Fig. 3*A* and *B*). In five cells, the mean frequency and amplitude of Ca^{2+} waves under control conditions was 4.6 ± 1.3 min^{-1} and 1.93 ± 0.28 $\Delta F/F_0$, respectively, compared to 4.5 ± 0.92 min^{-1} and 1.65 ± 0.18 $\Delta F/F_0$ in the presence of oligomycin (1 μM, $P > 0.05$, Fig. 3*C*).

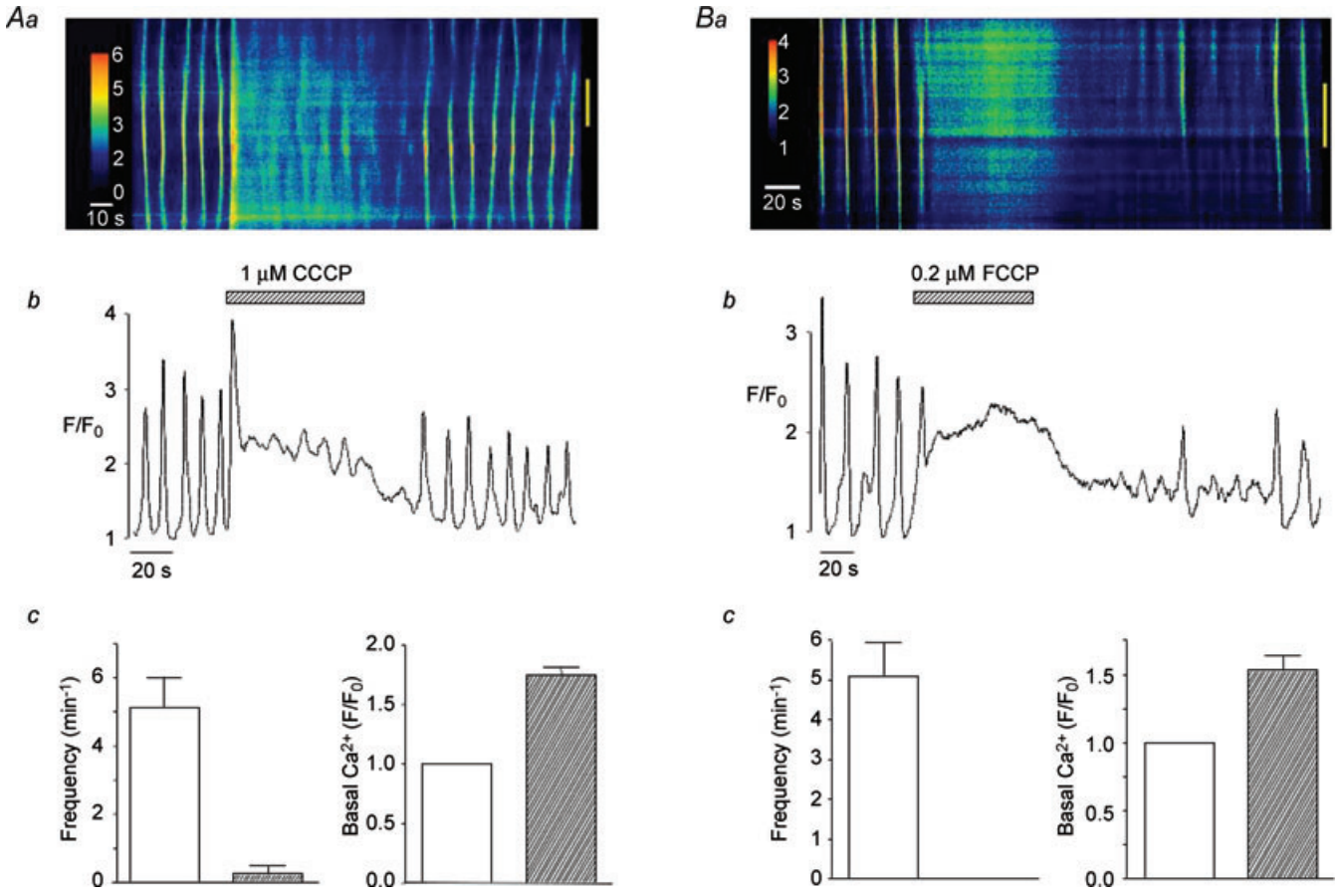

Figure 2. Effect of protonophores on spontaneous Ca^{2+} waves in urethra ICC
A line-scan image showing the effect of CCCP (1 μM) on spontaneous Ca^{2+} waves is shown in *Aa*. *Ab* shows an intensity profile plot of this record. Summary bar charts plotting the mean frequency (min^{-1}) of Ca^{2+} waves and basal 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 Ca^{2+} transients

It could be argued that the inhibition of Ca^{2+} waves produced by the mitochondrial uncouplers and inhibitors was due to depletion of the intracellular Ca^{2+} stores, therefore experiments were performed to test if caffeine-evoked Ca^{2+} transients were affected by application of these drugs. Figure 4*A* and *B* shows the effect of CCCP on caffeine-evoked Ca^{2+} transients. When caffeine (10 mM) was applied under control conditions a maximal 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 μM) which inhibited the spontaneous activity, as explained above. When caffeine was re-applied during this inhibitory period, a 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. 4*C*. It is clear that amplitude of the caffeine-evoked 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 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 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 5*A* shows the effect of CCCP (1 μM) 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. 5*Aa*. 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. 5*Ab*. Figure 5*B* shows that similar results were achieved using antimycin A (5 μM). 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 ± 131 pA and 14 ± 4 min^{-1} under control conditions *versus* -6.6 ± 6.6 pA and 0.6 ± 0.6 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 μM). The mitochondrial uniporter is a Ca^{2+}-selective channel that spans the inner mitochondrial membrane and is responsible for 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 ± 0.46 min^{-1} and -65.3 ± 54 pA, respectively. This compared with values of 9.6 ± 3.03 min^{-1} and -358 ± 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. 5*Cb* and a representative example is shown in Fig. 5*Ca*.

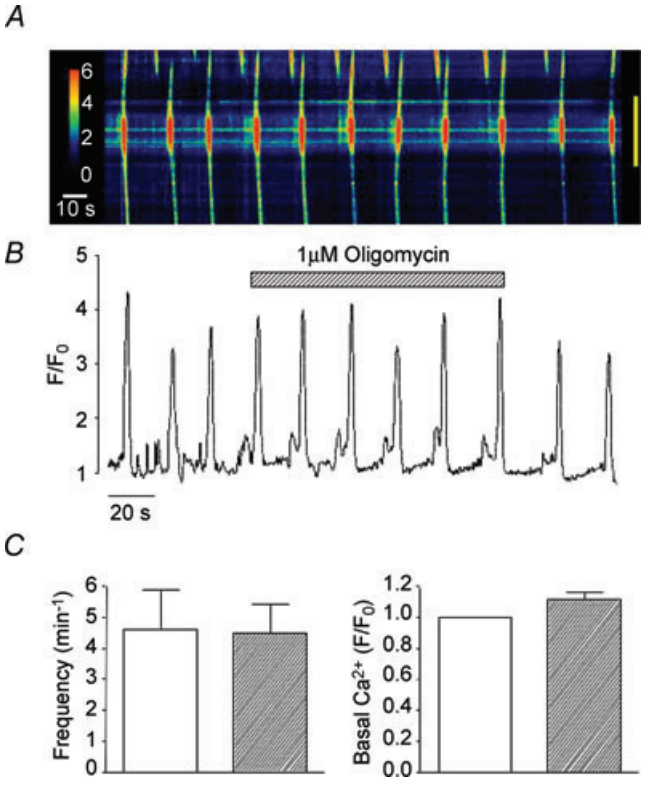

Figure 3. Effect of oligomycin on spontaneous Ca^{2+} waves in urethra ICC
A representative pseudo-line-scan image and corresponding intensity profile plot demonstrating that oligomycin did not inhibit spontaneous Ca^{2+} waves is shown in *A* and *B*. Summary data, plotting the effects of oligomycin on the mean frequency of Ca^{2+} waves and basal Ca^{2+} levels, are shown in *C*.

Effect of activation of the mitochondrial uniporter on spontaneous Ca^{2+} 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^{2+} 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^{2+} uptake in HeLa cells. Vay *et al.* (2007) went on to show that stimulation of the uniporter with kaempferol (10 μM) in HeLa cells and in human fibroblasts induced a burst of repetitive Ca^{2+} oscillations of diminishing amplitude which then ceased. Figure 6 shows that application of 10 μM kaempferol to a spontaneously active ICC induced a burst of Ca^{2+} oscillations, comprised of an initial Ca^{2+} transient followed by a series of Ca^{2+} 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^{2+} transient induced by kaempferol was $2.8 \pm 0.32\ \Delta F/F_0$. The overall frequency of the kaempferol-induced Ca^{2+} 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^{2+} 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^{2+} waves before and 37 during addition of kaempferol in the same 15 cells. Basal Ca^{2+} levels did not change significantly during the presence of kaempferol ($F/F_0 = 1$ under control conditions and 0.97 ± 0.04 during its presence).

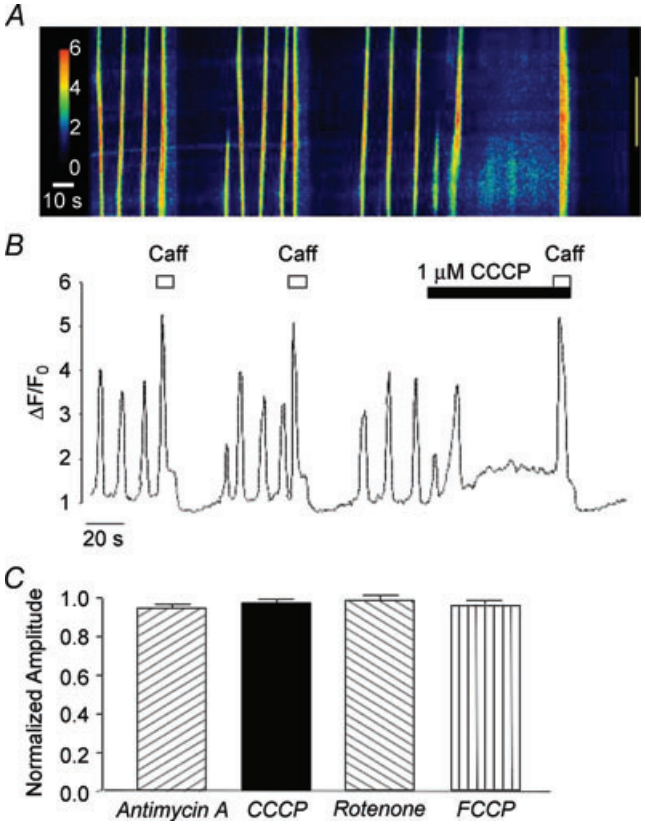

Figure 4. Effect of mitochondrial inhibitors on caffeine-evoked Ca^{2+} transients
A and *B* are a representative pseudo-line-scan image and intensity profile plot showing that caffeine-induced Ca^{2+} transients are not inhibited by application of CCCP. The bar chart shown in *C* plots the mean peak amplitude of the caffeine-evoked Ca^{2+} 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.

In order to verify that these effects were dependent on the Ca^{2+} handling properties of mitochondria, we tested if they were affected by pre-treatment with FCCP. Figure 7*A* and *B* shows that kaempferol-induced Ca^{2+} oscillations were greatly attenuated in the presence of FCCP (0.2 μM). Figure 7*A* also shows that addition of FCCP following the initial application of kaempferol caused a sharp rise in Ca^{2+}. 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. 7*C* shows that in six cells the mean amplitude of the initial Ca^{2+} 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 ($P < 0.05$).

Role of Ca^{2+} stores in kaempferol-induced Ca^{2+} oscillations

In order to investigate if kaempferol-induced Ca^{2+} oscillations depended on Ca^{2+} 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^{2+} stores. A representative example of such an experiment is shown in Fig. 8*Aa* and *b*. In the absence of drugs, application of kaempferol induced a burst of Ca^{2+} oscillations, as described above. Cells were then treated with 10 mM caffeine. This evoked a large Ca^{2+} 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^{2+} levels. The inhibitory effect of caffeine was reversible as kaempferol was able to induce a series of Ca^{2+} 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. 8*Ac*). These data indicate that kaempferol-induced Ca^{2+} oscillations are dependent upon Ca^{2+} 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 Ca^{2+} release from IP_3Rs due to changes in their local Ca^{2+} concentration as a consequence of increased Ca^{2+} uptake into mitochondria. To investigate if the kaempferol-induced Ca^{2+} oscillations in the present study were due to Ca^{2+} release from IP_3Rs, we assessed the effects of the IP_3R blocker 2-APB. Figure 8*Ba* and *b* show that although 2-APB disrupted the pattern of spontaneous 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 μM), kaempferol was still able to induce a burst of repetitive 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 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. 8*Bc*).

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

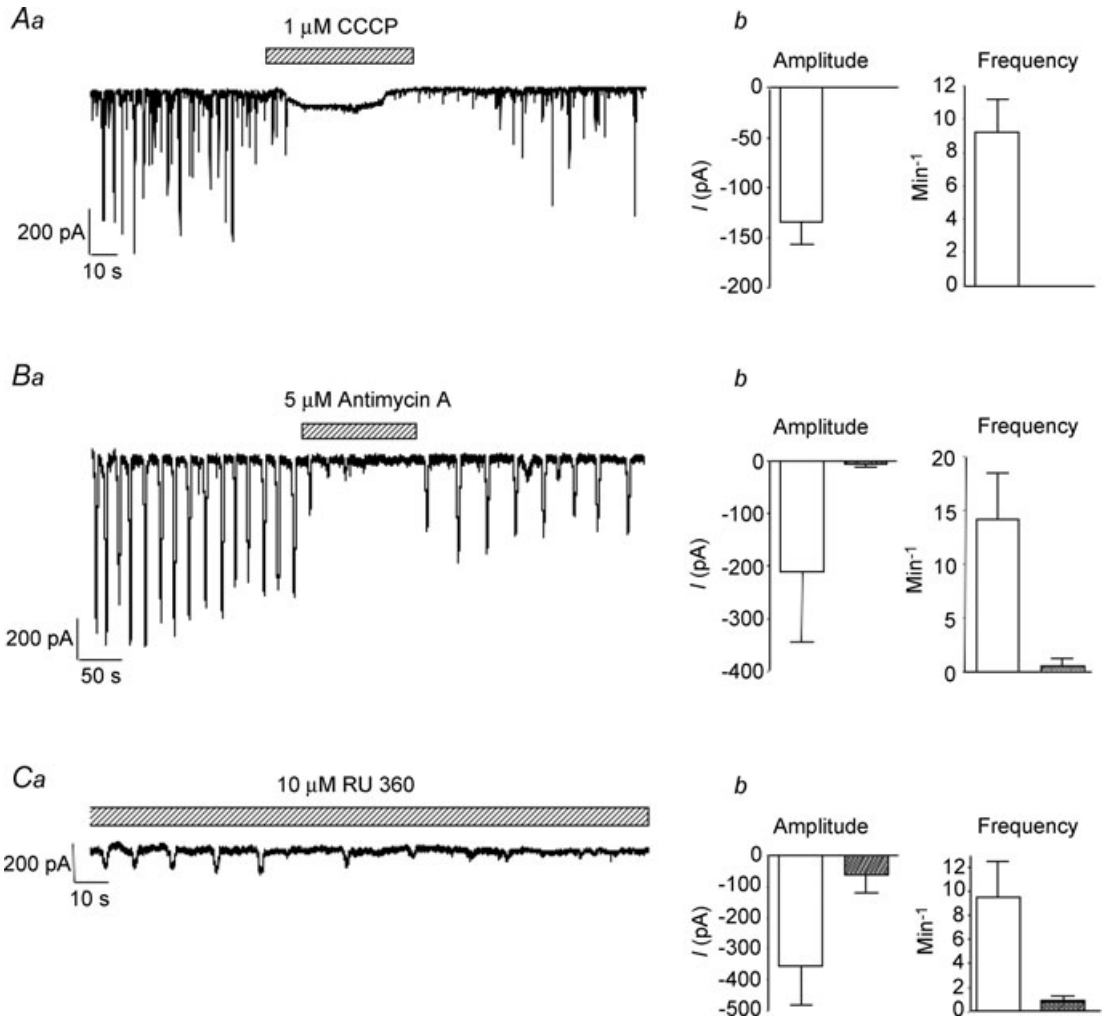

Figure 5. Effect of mitochondrial inhibitors on STICs
Aa shows the effect of CCCP (1 μM) on STICs in a cell held at −60 mV. *Ab*, summary of the effect of CCCP on STICs from 7 cells. *Ba* shows the effect of antimycin A (5 μM) on STICs in a cell held at −60 mV. *Bb*, summary of the effect of antimycin A on STICs from 5 cells. *Ca* shows the effect of dialysis of RU360 (10 μM) 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 Ca^{2+} waves and kaempferol induced a burst of repetitive Ca^{2+} oscillations, as described above. However, when 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. 9*C* 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 $[Ca^{2+}]_o$ ($n = 5$, $P < 0.05$). These data demonstrate that the stimulatory effects of kaempferol on Ca^{2+} waves require $[Ca^{2+}]_o$ and suggest kaempferol did not cause a direct release of Ca^{2+} from stores.

Discussion

The results of the present study show that spontaneous Ca^{2+} waves and STICs in urethral ICC are inhibited by agents which decrease mitochondrial Ca^{2+} uptake, such as FCCP, CCCP, antimycin A, rotenone and RU360. Conversely, when mitochondrial Ca^{2+} uptake was enhanced using the mitochondrial uniporter activator kaempferol, the frequency of 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 Ca^{2+} oscillations, indicating that the effects observed in this study are directly related to the 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 Ca^{2+} waves in urethral ICC is directly related to the ability of mitochondria to take up Ca^{2+}.

Bursts of Ca^{2+} oscillations induced by application of kaempferol in this study were inhibited when intracellular stores were depleted by application of 10 mM caffeine, but were not significantly inhibited by 2-APB, suggesting that they were more likely to be dependent on RyRs than IP_3Rs. However, spontaneous Ca^{2+} waves were significantly reduced by 2-APB, demonstrating that IP_3Rs are involved in the generation of Ca^{2+} waves under resting conditions as previously reported by Johnston *et al.* (2005). The kaempferol-induced activity was also inhibited by removal of $[Ca^{2+}]_o$; therefore it seems unlikely that kaempferol released Ca^{2+} directly from stores, as under similar experimental conditions caffeine-evoked Ca^{2+} transients were unaffected by removal of $[Ca^{2+}]_o$ (Johnston *et al.* 2005). The results of Johnston *et al.* (2005) also suggest that the effects produced by removal of $[Ca^{2+}]_o$ in the

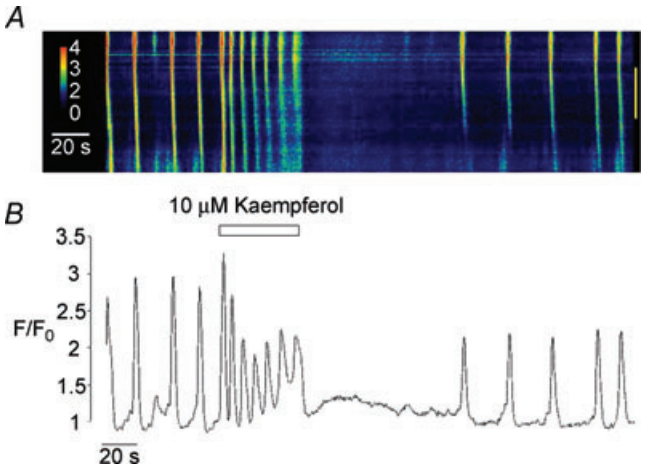

Figure 6. Effect of the mitochondrial uniporter opener kaempferol on Ca^{2+} waves
A is a linescan image showing the effect of kaempferol (10 μM) on spontaneous Ca^{2+} waves in isolated urethra ICC. An intensity profile plot of this record is shown in *B*.

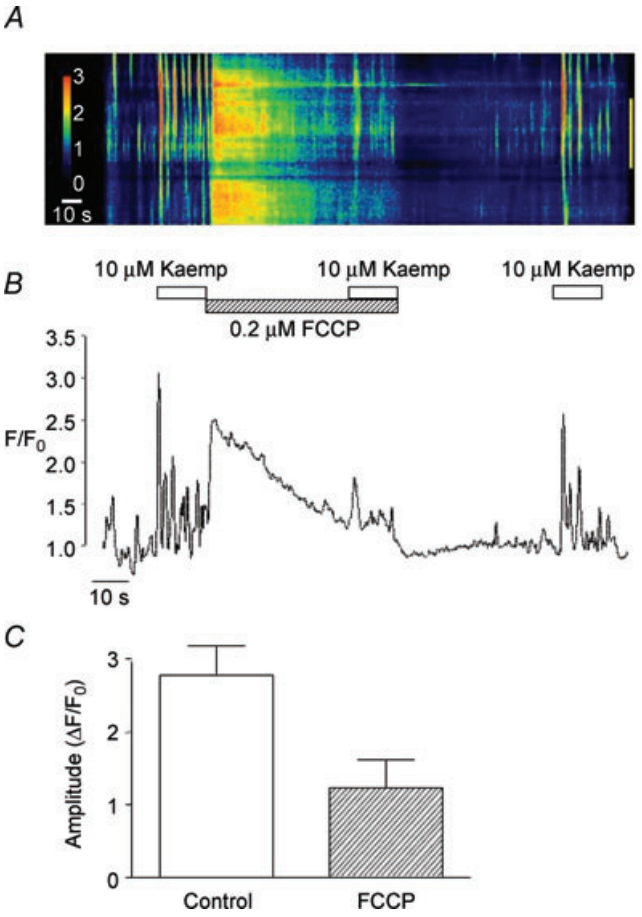

Figure 7. Effect of FCCP on kaempferol-induced Ca^{2+} waves
A representative line-scan image showing the effect of kaempferol (10 μM) in the absence and presence of FCCP (0.2 μM) is shown in *A*. An intensity profile plot of this record is shown in *B*. A summary bar chart plotting the mean amplitude ($\Delta F/F_0$) of the initial kaempferol-induced 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 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 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 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 Ca^{2+} waves in urethral ICC could be regulated by opening and closure of RyRs brought about by buffering of cytosolic Ca^{2+} by mitochondria. Thus, inhibition of mitochondrial Ca^{2+} uptake may lead to elevations in cytosolic Ca^{2+} which are sufficient to inhibit the opening of RyRs and prevent the development of spontaneous 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 Ca^{2+} uptake is decreased by FCCP. On the other hand, enhanced uptake of Ca^{2+} into mitochondria would be expected to cause a reduction in cytosolic Ca^{2+} and remove Ca^{2+}-induced inhibition of RyRs. Jouaville *et al.* (1995) demonstrated a similar mechanism involving mitochondrial regulation of IP_3Rs in *Xenopus* oocytes, whereby uptake of Ca^{2+}

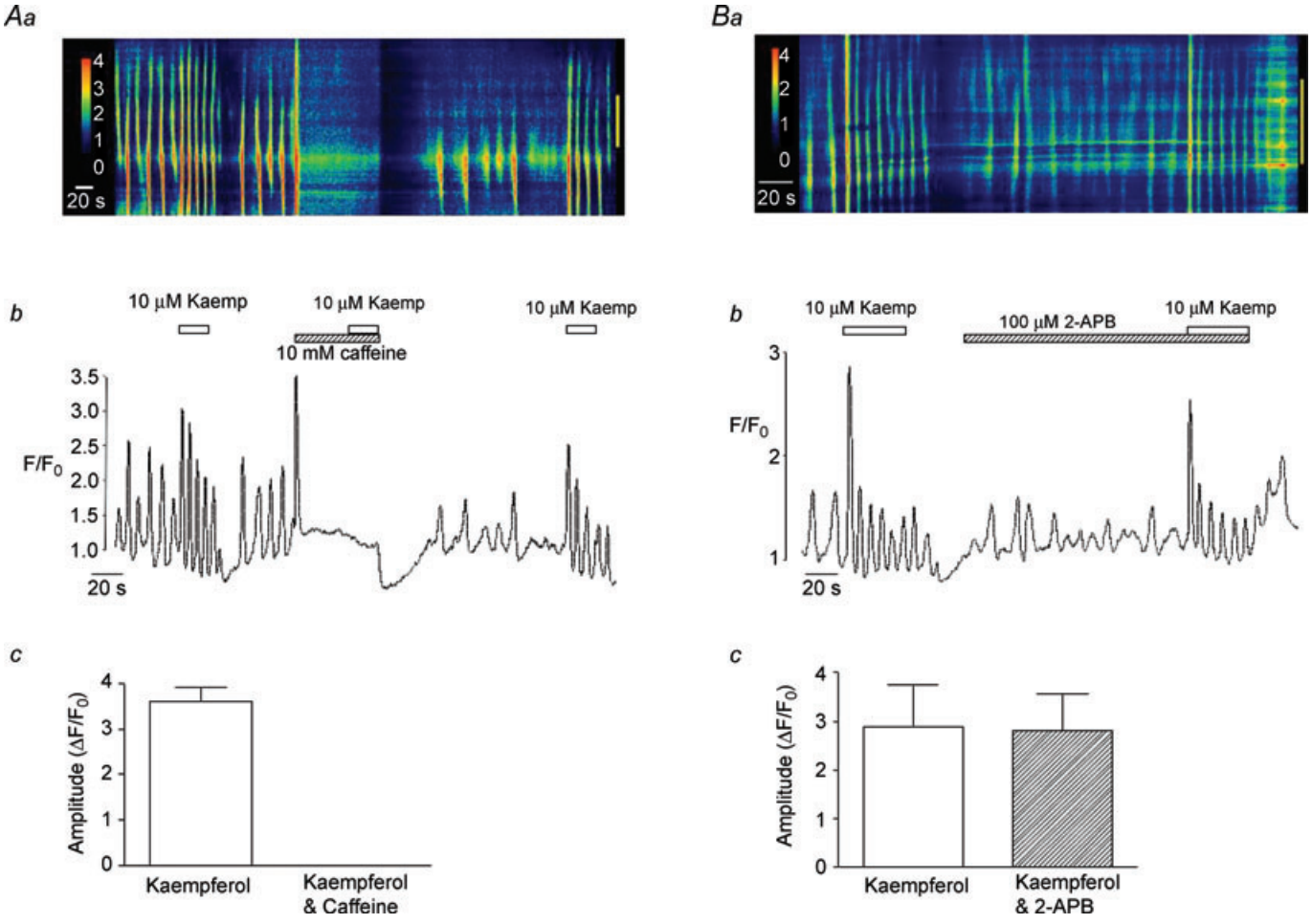

Figure 8. Effect of caffeine and 2-APB on kaempferol-induced Ca^{2+} waves
Aa is a line-scan image showing the effect of kaempferol before and during exposure to 10 mM caffeine. *Ab* represents the intensity profile plot of the data shown in *Aa*. A summary plot of the mean amplitude ($\Delta F/F_0$) of the initial kaempferol-induced 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 μM). *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 Ca^{2+} transient in the absence and presence of 2-APB is shown in *Bc*.

into mitochondria synchronized Ca^{2+} release from IP_3Rs resulting in an increase in the propagation velocity of Ca^{2+} waves.

The activity of RyRs is also known to be affected by the 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 Ca^{2+} levels in the endoplasmic reticulum (ER) by regulating the refilling pathway. Therefore, if mitochondrial Ca^{2+} uptake enhanced the refilling of 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 Ca^{2+} levels. However, it seems unlikely that this is the case, as although FCCP, CCCP, rotenone and antimycin A all inhibited spontaneous Ca^{2+} waves, they did not affect the amplitude of caffeine-evoked Ca^{2+} transients, suggesting that the Ca^{2+} content of intracellular stores was unaffected by a reduction in uptake of Ca^{2+} into mitochondria.

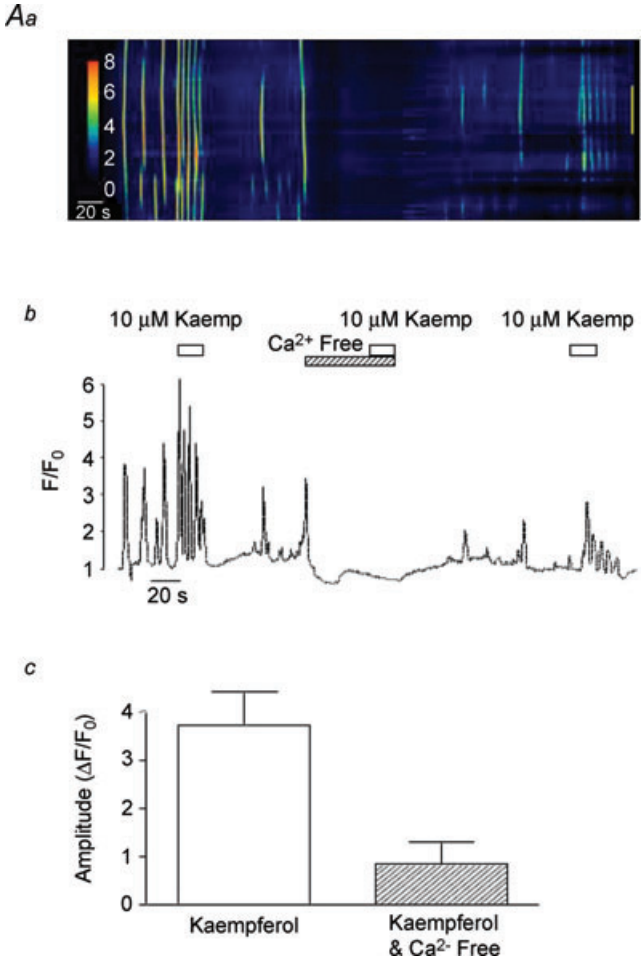

Figure 9. Effect of Ca^{2+}-free media on kaempferol-induced Ca^{2+} waves
A typical line-scan image showing the effect of kaempferol before and during exposure to Ca^{2+}-free media is shown in *A*. An intensity profile plot of this record is plotted in *B* and a summary plot of the mean amplitude ($\Delta F/F_0$) of the initial kaempferol-induced Ca^{2+} transient in the absence and presence of Ca^{2+}-free media is shown in *C*.

Several studies have reported functional links between mitochondria and IP_3Rs; therefore the finding that kaempferol-induced 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 Ca^{2+} uptake increased the propagation speed of Ca^{2+} waves in *Xenopus* oocytes by preventing Ca^{2+}-dependent inhibition of IP_3Rs. Vay *et al.* (2007) also reported that mitochondrial regulation of IP_3Rs led to an increase in the frequency of spontaneous Ca^{2+} waves in Hela cells and fibroblasts. Some studies have suggested that 2-APB is a poor blocker of IP_3Rs, but is an effective blocker of capacitative 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 IP_3R 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 μM) blocked noradrenaline-induced Ca^{2+}-activated 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 IP_3Rs but does not affect release or uptake of Ca^{2+} from caffeine-sensitive stores, nor does it affect Ca^{2+}-activated Cl^- channels or Ca^{2+}-activated K^+ (BK) channels in urethral ICC.

Mitochondrial 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 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 Ca^{2+} (Koh *et al.* 2002) and it is thought that uptake of 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 Ca^{2+}-activated Cl^- (ClCa) channels (Sergeant *et al.* 2000). Unlike the pacemaker NSCC channels in the gut, ClCa channels open in response to elevations of cytoplasmic Ca^{2+}, therefore mitochondrial Ca^{2+} uptake would be unlikely to stimulate these channels directly. In summary, we suggest that uptake of Ca^{2+} into mitochondria influences spontaneous Ca^{2+} waves, which underlie the pacemaker activity in urethral ICC, via a process which may involve regulation of RyRs.

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