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Pacemaker activity in urethral interstitial cells is not dependent on capacitative calcium entry

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Bradley, Eamonn, Mark A. Hollywood, Noel G. McHale, Keith D. Thornbury, and Gerard P. Sergeant. Pacemaker activity in urethral interstitial cells is not dependent on capacitative calcium entry. *Am J Physiol Cell Physiol* 289: C625–C632, 2005; doi:10.1152/ajpcell.00090.2005.—The aim of the present study was to investigate the properties and role of capacitative Ca^{2+} entry (CCE) in interstitial cells (IC) isolated from the rabbit urethra. Ca^{2+} entry in IC was larger in cells with depleted intracellular Ca^{2+} stores compared with controls, consistent with influx via a CCE pathway. The nonselective Ca^{2+} entry blockers Gd^{3+} (10 μM), La^{3+} (10 μM), and Ni^{2+} (100 μM) reduced CCE by 67% ($n = 14$), 65% ($n = 11$), and 55% ($n = 9$), respectively. These agents did not inhibit Ca^{2+} entry when stores were not depleted. Conversely, CCE in IC was resistant to SKF-96365 (10 μM), wortmannin (10 μM), and nifedipine (1 μM). Spontaneous transient inward currents were recorded from IC voltage-clamped at −60 mV. These events were not significantly affected by Gd^{3+} (10 μM) or La^{3+} (10 μM) and were only slightly decreased in amplitude by 100 μM Ni^{2+}. The results from this study demonstrate that freshly dispersed IC from the rabbit urethra possess a CCE pathway. However, influx via this pathway does not appear to contribute to spontaneous activity in these cells.

smooth muscle; patch clamp; spontaneous transient inward currents

URETHRAL INTERSTITIAL CELLS (IC) were recently proposed as specialized pacemaker cells that drive surrounding smooth muscle cells in the wall of the urethra in a fashion similar to interstitial cells of Cajal (ICC) in the gastrointestinal tract (25). ICC are known to act as pacemakers in the myenteric regions of the gut, responsible for the generation of electrical slow waves and therefore gastrointestinal motility (23). Although pacemaker activity in ICC involves release of Ca^{2+} from D-*myo*-inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (30, 33), it now appears that Ca^{2+} influx is also involved. Torihashi et al. (32) demonstrated that spontaneous Ca^{2+} oscillations in ICC of the murine small intestine are sustained by store-operated Ca^{2+} influx via a pathway that may involve canonical transient receptor potential (TRPC)4 channels. This pathway is in line with the model of "capacitative Ca^{2+} entry" (CCE) as originally described by Putney (21).

IC of the urethra are spontaneously active. When voltage-clamped at −60 mV, they develop spontaneous transient inward currents (STICs) due to activation of Ca^{2+}-activated Cl^- channels (25). It was shown recently that these events are mediated by regularly occurring global Ca^{2+} oscillations that arise through periodic release of Ca^{2+} from intracellular stores (12). A feature of these events is that they are acutely sensitive to changes in the external Ca^{2+} concentration and cease immediately on application of Ca^{2+}-free medium, suggesting that they are also dependent on Ca^{2+} influx. However, it appears that influx via L-type Ca^{2+} channels is not involved, as spontaneous Ca^{2+} oscillations in isolated IC were unaffected by application of nifedipine (12). The nature of the influx pathway involved therefore remains elusive; however, it is possible that a store-operated Ca^{2+} influx pathway may contribute to this process, as occurs in ICC in the murine small intestine (32). The aim of the present study was to investigate whether CCE is important for sustaining pacemaker activity in isolated urethral IC.

METHODS

Male and female New Zealand White rabbits were humanely killed by lethal injection of pentobarbitone in accordance with current UK home office guidelines.

Cell dispersal. Strips of urethral smooth muscle (5 mm in width) were dissected, cut into 1-mm^3 pieces, and stored in Ca^{2+}-free Hanks' solution (see *Solutions*) at 4°C for 30 min before cell dispersal. Tissue pieces were incubated in dispersal medium containing (per 5 ml of Ca^{2+}-free Hanks' solution) 15 mg of collagenase (Sigma type 1A), 1 mg of protease (Sigma type XXIV), 10 mg of bovine serum albumin (Sigma), and 10 mg of trypsin inhibitor (Sigma) for 10–15 min at 37°C. Tissue was then transferred to Ca^{2+}-free Hanks' solution and stirred for a further 15–30 min to release single smooth muscle cells and IC. These cells were plated in petri dishes containing 100 μM Ca^{2+} Hanks' solution and stored at 4°C for use within 8 h.

Perforated-patch recordings from single cells. Currents were recorded with the perforated patch-configuration of the whole cell patch-clamp technique as described previously (25–27). This circumvented the problem of current rundown encountered when using the conventional whole cell configuration. The cell membrane was perforated with the antibiotic amphotericin B (600 μg/ml). Patch pipettes were initially frontfilled by dipping into pipette solution and then backfilled with the amphotericin B-containing solution. Pipettes were pulled from borosilicate glass capillary tubing (1.5-mm outer diameter, 1.17-mm inner diameter; Clark Medical Instruments) to a tip with a diameter of ∼1–1.5 μm and resistance of 2–4 MΩ.

Voltage clamp commands were delivered via an Axopatch 1D patch-clamp amplifier (Axon Instruments), and membrane currents were recorded by a 12-bit analog digital/digital analog converter (Axodata 1200 or Labmaster, Scientific Solutions) interfaced to an Intel computer running pCLAMP software. During experiments, the dish containing the cells was continuously perfused with Hanks' solution at 36 ± 1°C. Additionally, the cell under study was continuously superfused by means of a custom-built close delivery system with a pipette of ∼200-μm tip diameter placed ∼300 μm from the cell. The Hanks' solution in the close delivery system could be switched to a drug-containing solution with a "dead space" time of <5 s. In all experiments *n* refers to the number of cells studied, and

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each experimental set usually contained samples from a minimum of four animals. Summary data are presented as means ± SE, and statistical comparisons were made on raw data with Student's paired *t*-test, taking $P < 0.05$ levels as significant.

Ca^{2+} measurements with fura-2 microfluorimetry. Ca^{2+} measurements were made from IC incubated in fura-2 AM (5 μM) for 15 min at 37°C in Ca^{2+}-free Hanks' solution. Cells were placed in a glass-bottomed dish and then mounted on the stage of an inverted microscope. The Ca^{2+} microfluorimetry system consisted of a dual monochromator passing 340 nm/380 nm light (5-nm bandwidth), a light chopper (PTI DeltaScan), and an inverted microscope with an oil immersion objective (×40, numerical aperture 1.3). The emission side of the microscope comprised an adjustable rectangular window, a filter (510 nm), and a photon-counting photomultiplier tube in the light path. Fluorescence equipment was controlled by PTI Felix software, which also performed storage and analysis of the acquired data. Before experimentation, cells were superfused with normal Hanks' solution for 10 min. Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured as the change in ratio of fluorescence at the 340- and 380-nm wavelengths. CCE was plotted as the total amplitude of the Ca^{2+} transient produced on introduction of 1.8 mM Ca^{2+} solution after incubation in Ca^{2+}-free medium containing cyclopiazonic acid (CPA; 20 μM).

Solutions. The compositions of the solutions used were as follows (in mM): Ca^{2+}-free Hanks' solution (for cell dispersal): 125 NaCl, 5.36 KCl, 10 glucose, 2.9 sucrose, 15.5 $NaHCO_3$, 0.44 KH_2PO_4, 0.33 Na_2HPO_4, and 10 HEPES, pH adjusted to 7.4 with NaOH; Hanks' solution: 125 NaCl, 5.36 KCl, 10 glucose, 2.9 sucrose, 4.17 $NaHCO_3$, 0.44 KH_2PO_4, 0.33 Na_2HPO_4, 0.4 $MgSO_4$, 0.5 $MgCl_2$, 1.8 $CaCl_2$, and 10 HEPES, pH adjusted to 7.4 with NaOH; Ca^{2+}-free Hanks' solution (superfusate for CCE measurement): 125 NaCl, 5.36 KCl, 10 glucose, 2.9 sucrose, 4.17 $NaHCO_3$, 0.44 KH_2PO_4, 0.33 Na_2HPO_4, 0.4 $MgSO_4$, 2.3 $MgCl_2$, 10 glucose, 2.9 sucrose, 5.0 EGTA, and 10 HEPES, pH adjusted to 7.4 with NaOH; Cs^+ perforated-patch solution: 133 CsCl, 1.0 $MgCl_2$, 0.5 EGTA, and 10 HEPES, pH adjusted to 7.2 with CsOH.

Drugs. The following drugs were used: amphotericin B (Sigma); lanthanum chloride (Hopkin and Williams); 2-aminoethoxydiphenyl borate (2-APB, ACROS); gadolinium chloride, nickel chloride, and wortmannin (WT; Sigma); SKF-96365 and CPA (Calbiochem); and nifedipine (Bayer). All drugs were made up in the appropriate stock solution before being diluted to their final concentrations in Hanks' solution.

RESULTS

CCE in urethral IC. Global Ca^{2+} measurements were made from interstitial cells loaded with fura-2 (5 μM) as detailed

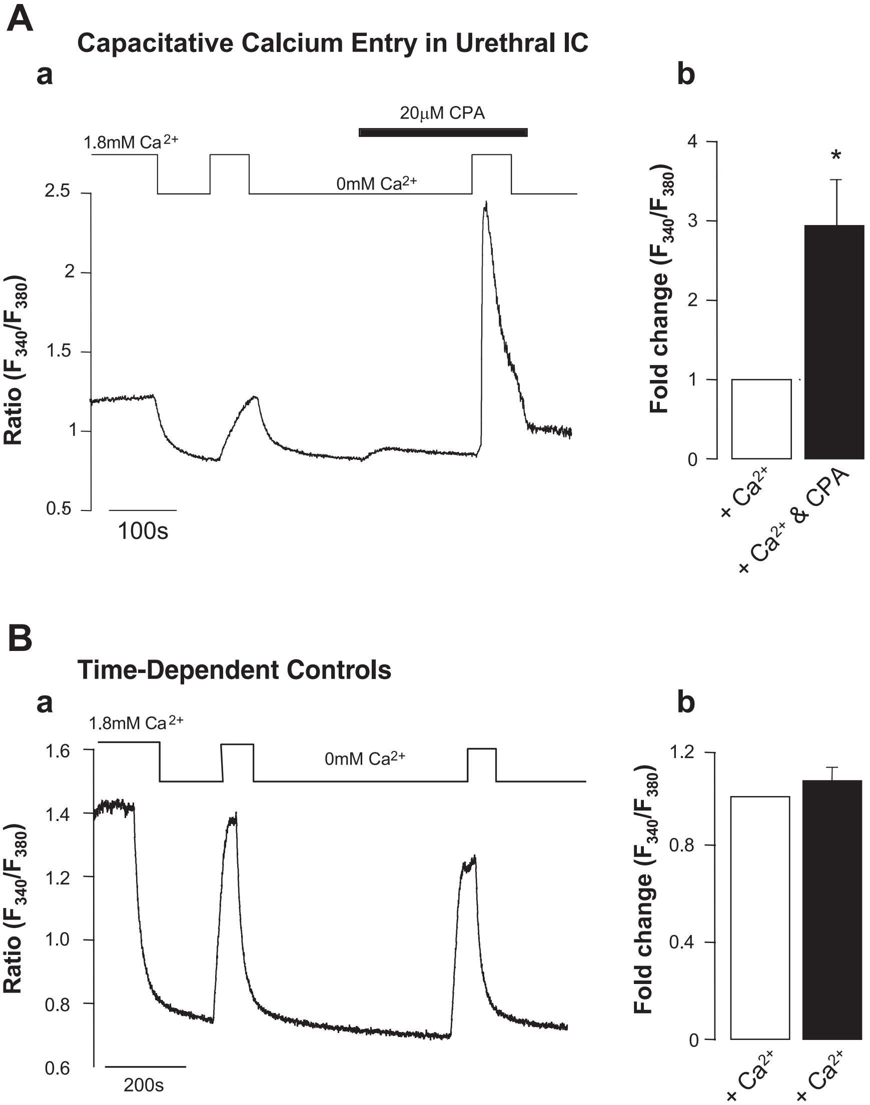

Fig. 1. Capacitative Ca^{2+} entry (CCE) in urethral interstitial cells (IC). *A*: representative example of CCE in a urethral interstitial cell (IC) (*a*) and summary bar chart showing an ~3-fold increase in the amplitude of the Ca^{2+} transient evoked by introduction of 1.8 mM Ca^{2+} Hanks' solution when stores are depleted compared with control conditions (*b*). CPA, cyclopiazonic acid; F_{340}/F_{380}, ratio of fluorescence at 340- and 380-nm wavelengths. $^*P < 0.05$, statistical significance. *B*: time-dependent controls. *a*: Representative example of Ca^{2+} entry evoked in the absence of CPA at the same time points as in *A*. *b*: Summary bar chart of a total of 9 similar experiments showing that the overshoot in Ca^{2+} shown in *A* is not a function of time.

above. The protocols used to evoke CCE and "non-CCE" are illustrated in the insets of Fig. 1, *Aa* and *Ba*, respectively. Under control conditions cells were bathed in normal Hanks' solution containing 1.8 mM Ca^{2+}. Removal of Ca^{2+} from the bathing solution caused a decrease in $[Ca^{2+}]_i$. However, when 1.8 mM Ca^{2+} was returned to the medium, $[Ca^{2+}]_i$ was restored to control levels. To evoke CCE, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor CPA (20 μM) was included in the solution to deplete Ca^{2+} stores. In some experiments caffeine (10 mM) was also applied for 10 s immediately before addition of CPA to fully discharge stores. Subsequent addition of 1.8 mM Ca^{2+} under these conditions resulted in an "overshoot" in $[Ca^{2+}]_i$ above control levels, similar to CCE in other cell types (9, 10, 34). An example of this effect is shown in Fig. 1*Aa*. These data were typical of 16 experiments, demonstrating that Ca^{2+} entry was significantly increased by approximately threefold when stores were depleted compared with control levels (Fig. 1*Ab*; $n = 16$, $P < 0.05$). To test whether these effects were due to the time lag involved in the second application of 1.8 mM Ca^{2+} and not the presence of CPA, a series of time-dependent control experiments were performed. The protocol for these experiments was the same as that described above (Fig. 1*A*), with the exception that CPA and caffeine were omitted from the solutions. Results from these experiments are illustrated in Fig. 1*Ba*. Summary data for nine similar experiments are plotted in Fig. 1*Ba* and show that this protocol did not result in an overshoot in $[Ca^{2+}]_i$ ($P > 0.05$).

Pharmacological characterization of CCE in urethral IC. To assess the functional role of CCE in urethral IC it was necessary to obtain a pharmacological profile of this entry pathway. Figures 2 and 3 show the effect of a range of putative CCE inhibitors on CCE in urethral IC. In these experiments CCE was evoked as described in Fig. 1*A* before, during, and after washout of the various blockers. Figure 2 shows the effect of Ni^{2+} (100 μM), La^{3+} (10 μM), and Gd^{3+} (10 μM). Each of these agents significantly and reversibly inhibited CCE in urethral IC (Fig. 2; $P < 0.05$). Application of Ni^{2+} (100 μM) reduced CCE by 55 ± 5% ($n = 9$), whereas Gd^{3+} and La^{3+} reduced CCE by 67 ± 5% ($n = 14$) and 63 ± 5% ($n = 11$), respectively.

The effects of various pharmacological agents known to inhibit CCE in other cell types (22) are shown in Fig. 3. SKF-96365 is a potent blocker of CCE in many tissues (7, 15, 18) and has also been shown to inhibit STICs in smooth muscle cells isolated from the sheep urethra (27). Therefore, we investigated whether CCE is inhibited by SKF-96365 in isolated IC from the rabbit urethra. Figure 3*A* demonstrates that

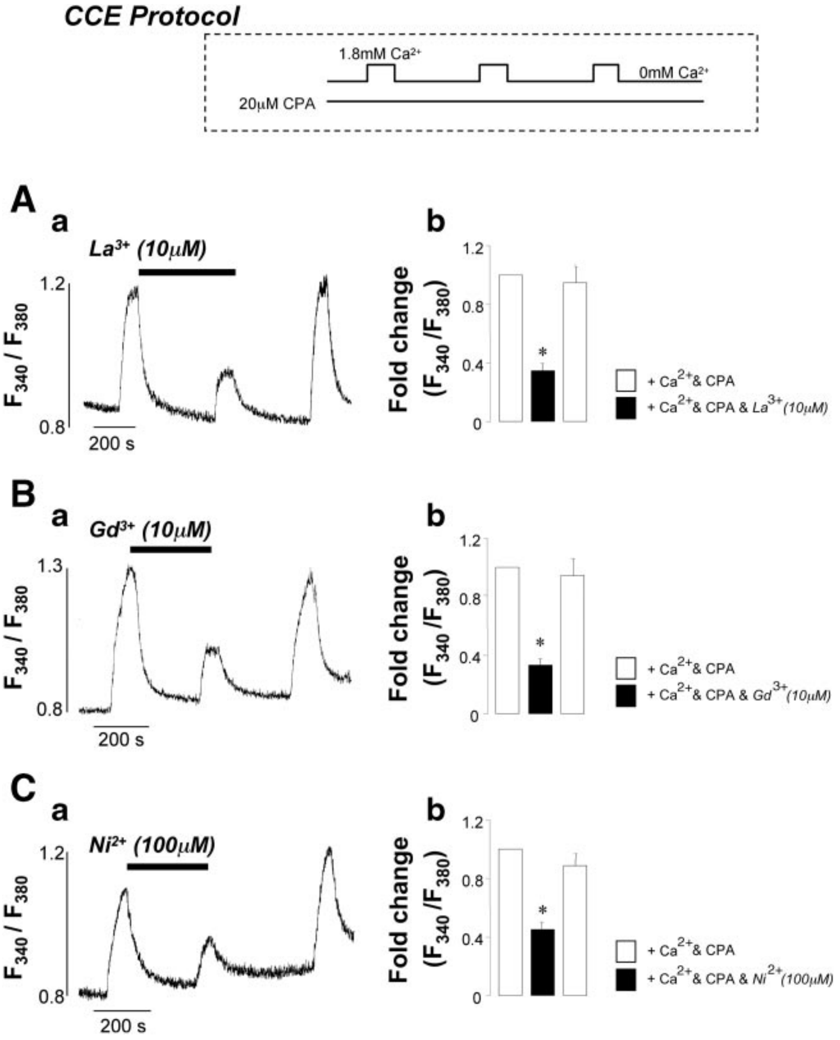

Fig. 2. CCE in IC is inhibited by La^{3+} (10 μM), Gd^{3+} (10 μM), and Ni^{2+} (100 μM). *A*: representative example of an experiment that shows that application of 10 μM La^{3+} reversibly inhibits CCE (*a*) and summary bar chart of 11 similar experiments that show that 10 μM La^{3+} reduced CCE by 65% (*b*). $^*P < 0.05$, statistical significance. *B*: representative example of an experiment that shows that application of 10 μM Gd^{3+} reversibly inhibits CCE (*a*) and summary bar chart of 14 similar experiments that show that 10 μM Gd^{3+} reduced CCE by 67% (*b*). *C*: representative example of an experiment that shows that application of 100 μM Ni^{2+} reversibly inhibits CCE (*a*) and summary bar chart of 9 similar experiments that show that 100 μM Ni^{2+} reduced CCE by 55% (*b*).

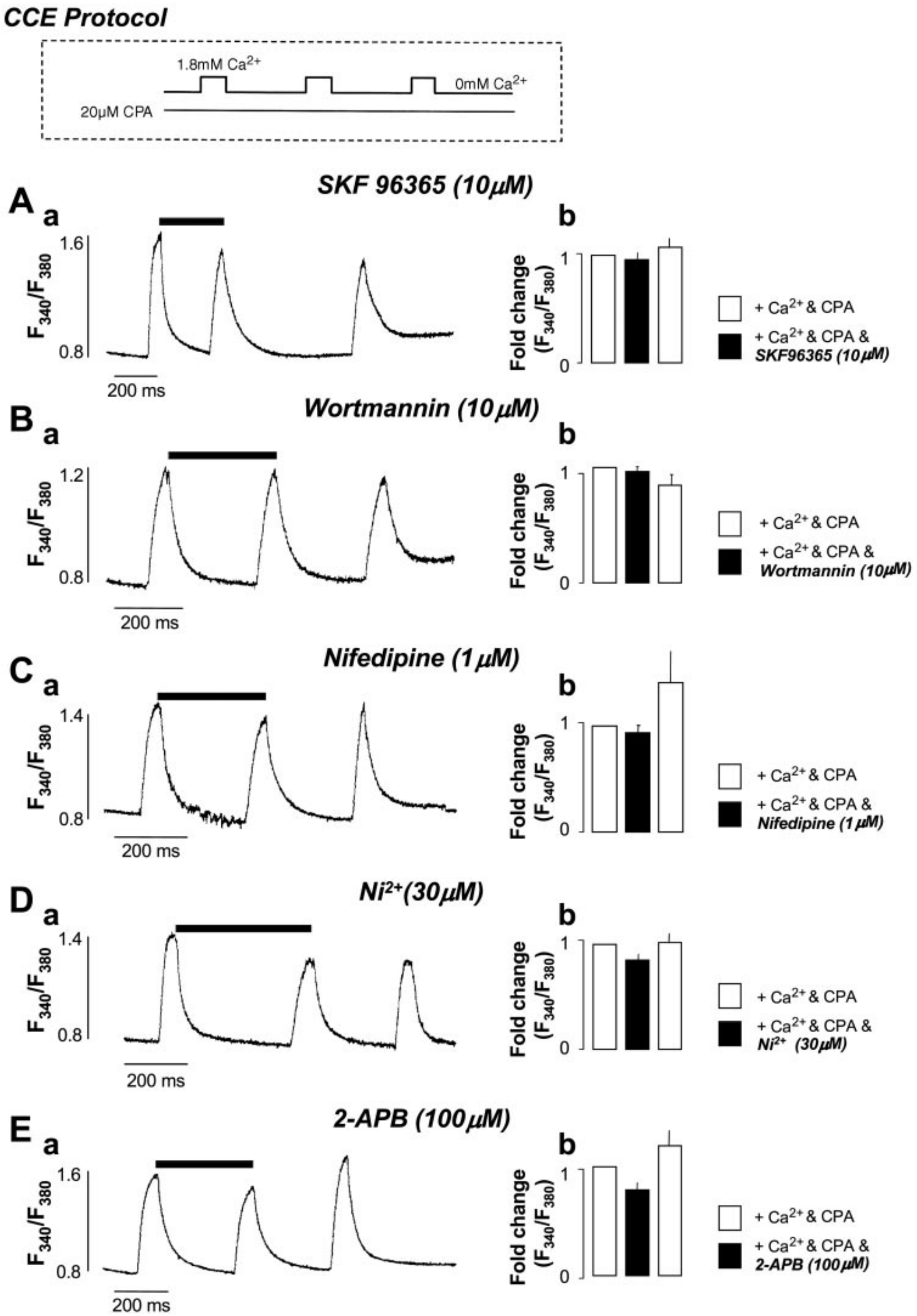

Fig. 3. Effects of putative nonselective Ca^{2+} influx inhibitors on CCE in urethral IC. *A–C*: CCE in urethral IC is not sensitive to SKF-96365 (10 μM; *A*), wortmannin (10 μM; *B*), or nifedipine (1 μM; *C*). *D*: CCE in IC is inhibited by 15% by application of 30 μM Ni^{2+}. *E*: 2-aminoethoxydiphenyl borate (2-APB; 100 μM) caused a 21% reduction in CCE. Panels *a* and *b* show representative experimental traces and summary bar charts, respectively.

10 μM SKF-96365 had no significant effect on CCE in these cells ($n = 10$, $P > 0.05$). The myosin light chain kinase inhibitor WT was recently shown to be an effective blocker of CCE in smooth muscle cells isolated from rabbit cerebral arterioles (5); therefore, we tested whether CCE in rabbit urethra IC was similarly affected. Figure 3*B* shows that CCE was not significantly decreased by application of 10 μM WT ($P > 0.05$, $n = 6$). Nifedipine, in addition to blocking L-type Ca^{2+} channels, has also been shown to inhibit store-operated Ca^{2+} entry in rabbit arteriolar smooth muscle cells (4); therefore, we investigated whether sensitivity to nifedipine was a characteristic of CCE in urethral IC. However, the data shown in Fig. 3*C* demonstrate that CCE was not significantly affected by application of 1 μM nifedipine ($n = 7$, $P > 0.05$). To further characterize CCE in these cells we also tested the effect of 30 μM Ni^{2+}. Results from these experiments are shown in Fig. 3*D*. In eight cells application of 30 μM Ni^{2+} caused a small but significant reduction in the amplitude of CCE in IC by 15 ± 6% ($P < 0.05$).

The membrane-permeant IP_3 receptor inhibitor 2-APB (14) was recently shown to block store-operated Ca^{2+} entry in several cell types (3, 11, 22). Our recent studies (26), however, showed that caffeine responses were unaffected by 2-APB in urethral IC, suggesting that stores can refill normally. We

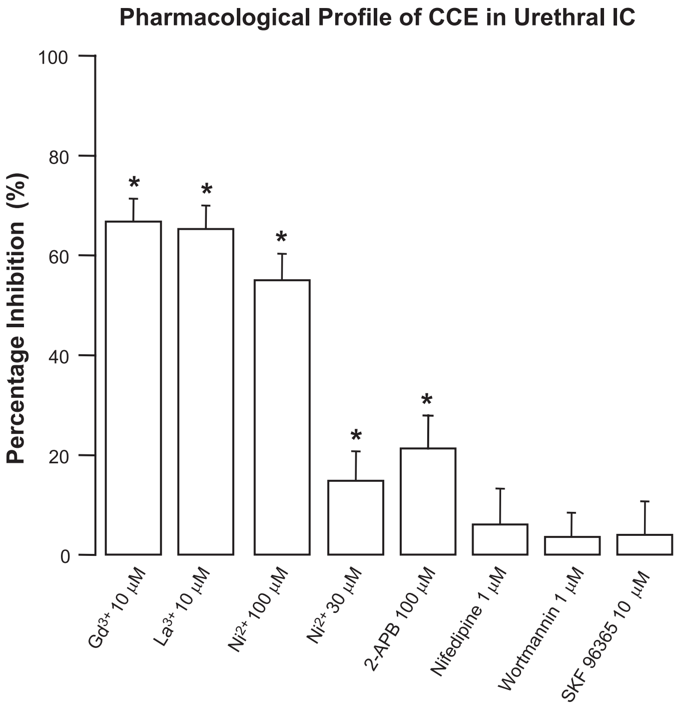

Fig. 4. Pharmacological profile of CCE in IC. Data show the mean % inhibition of CCE in urethral IC produced by Gd^{3+} (10 μM), La^{3+} (10 μM), Ni^{2+} (100 and 30 μM), 2-APB (100 μM), nifedipine (1 μM), wortmannin (10 μM), and SKF-96365 (10 μM).

therefore performed experiments to test the effect of 2-APB on CCE in urethral IC directly. The results shown in Fig. 3*E* demonstrate that 2-APB produced a modest reduction in the amplitude of CCE in urethral IC. In eight cells CCE was reduced by 21 ± 7% by 100 μM 2-APB ($P < 0.05$).

A summary bar graph of the inhibitory effect of all these agents on CCE is plotted in Fig. 4. This pharmacological profile demonstrates that CCE in urethral IC was inhibited by Gd^{3+} (10 μM), La^{3+} (10 μM), and Ni^{2+} (100 μM) and, to a lesser extent, by 2-APB (100 μM) and Ni^{2+} (30 μM). However, it was not inhibited by SKF-96365 (10 μM), nifedipine (1 μM), or WT (10 μM).

An additional set of experiments was then performed to investigate whether the agents that affected CCE also affected Ca^{2+} entry in the absence of store depletion. These experiments were performed in a manner similar to that described above, with the exception that CPA was omitted. Under these conditions, La^{3+}, Gd^{3+}, and Ni^{2+} did not significantly affect the amplitude of the Ca^{2+} influx transient caused by addition of 1.8 mM Ca^{2+} after incubation in Ca^{2+}-free medium ($P > 0.05$, Fig. 5). These data indicate that these agents did not affect basally active Ca^{2+} influx and were only effective when Ca^{2+} stores were depleted.

Effect of CCE inhibitors on spontaneous electrical activity in urethral IC. Urethral IC develop STICs when voltage-clamped at −60 mV because of activation of Ca^{2+}-activated Cl^- channels (25). It has also been shown that these events rely on release of Ca^{2+} from IP_3- and ryanodine-sensitive stores (26). Experiments were therefore performed to investigate whether these events are also dependent on CCE. Freshly dispersed IC were voltage-clamped at −60 mV, using patch pipettes filled with solution containing CsCl as previously described by Sergeant et al. (25, 26). The effects of the CCE inhibitors Gd^{3+} (10 μM), La^{3+} (10 μM), and Ni^{2+} (100 μM) are shown in Fig. 6. Cells were exposed to these agents for durations of 2 min, which was enough time to inhibit CCE as shown in Fig. 2. However, it is clear from Fig. 6 that these agents did not abolish STICs in IC. Under control conditions STICs occurred at a frequency of 6 ± 4 min^{-1} compared with 5.3 ± 2.7 min^{-1} in the presence of 10 μM La^{3+} ($P > 0.05$). The mean amplitude of these events was also not significantly affected by 10 μM La^{3+}: −457 ± 124 pA under control conditions compared with −420 ± 131 pA in the presence of the drug. Gd^{3+} was similarly ineffective. Figure 6*B* shows that the mean frequency of STICs before addition of 10 μM Gd^{3+} was 12 ± 2 min^{-1} vs. 11 ± 2 min^{-1} in the presence of the drug ($n = 10$, $P > 0.05$). STIC amplitude was also not significantly affected by 10 μM Gd^{3+}: −324 ± 122 pA under control conditions and −346 ± 128 pA in the presence of Gd^{3+} ($n = 10$, $P > 0.05$). Application of 100 μM Ni^{2+}, however, caused a small but significant reduction in STIC frequency from 14 ± 3 min^{-1} to 10 ± 2 min^{-1} in the presence of the drug ($n = 10$, $P < 0.05$). However, Ni^{2+} did not significantly affect STIC amplitude. Under control conditions the mean amplitude of STICs was −344 ± 106 pA compared with −327 ± 106 pA in solution containing 100 μM Ni^{2+} ($n = 10$, $P > 0.05$; Fig. 6*C*).

DISCUSSION

CCE refers to a Ca^{2+} influx pathway that is triggered by depletion of intracellular Ca^{2+} stores. Although CCE was traditionally thought to be activated during sustained elevations in Ca^{2+} induced by PLC-coupled neurotransmitters or hormones (1, 22), it is now recognized that CCE is also important for sustaining repetitive Ca^{2+} oscillations after agonist stimulation (2, 31). CCE is particularly well suited to this role, as it can activate and deactivate in coordination with each Ca^{2+} oscillation, providing an elegant means for refilling of Ca^{2+} stores by the amount of Ca^{2+} released during one cycle (24, 31).

A recent study by Torihashi et al. (32) suggested that pacemaker activity in ICC cultured from the murine small intestine is dependent on store-operated Ca^{2+} entry. They showed that removal of extracellular Ca^{2+} leads to cessation of spontaneous Ca^{2+} oscillations, suggesting that they are dependent on Ca^{2+} influx. This effect was not due to inhibition of L- or T-type Ca^{2+} channels, as nifedipine (1 μM) and Ni^{2+} (50 μM) did not affect the activity. The possibility that CCE was involved came from the observations that Ca^{2+} oscillations were inhibited by the putative CCE inhibitor SKF-96365 (4 μM) and that ICC were immunopositive for TRPC4 proteins, which had previously been described as store-operated Ca^{2+} channels in adrenal and endothelial cells (6, 20). The idea that a similar pathway could be responsible for sustaining spontaneous activity in urethral IC was prompted by the observations of Johnston et al. (12) that spontaneous Ca^{2+} oscillations were dependent not only on release of Ca^{2+} from intracellular stores but also on the extracellular concentration of Ca^{2+}. Oscillations immediately ceased on addition of Ca^{2+}-free medium and doubled in frequency when the concentration of Ca^{2+} was increased from 1.8 to 3.6 mM. This sensitivity to external Ca^{2+} implied a role for Ca^{2+} influx in mediating the activity, possibly by refilling the depleted Ca^{2+} stores.

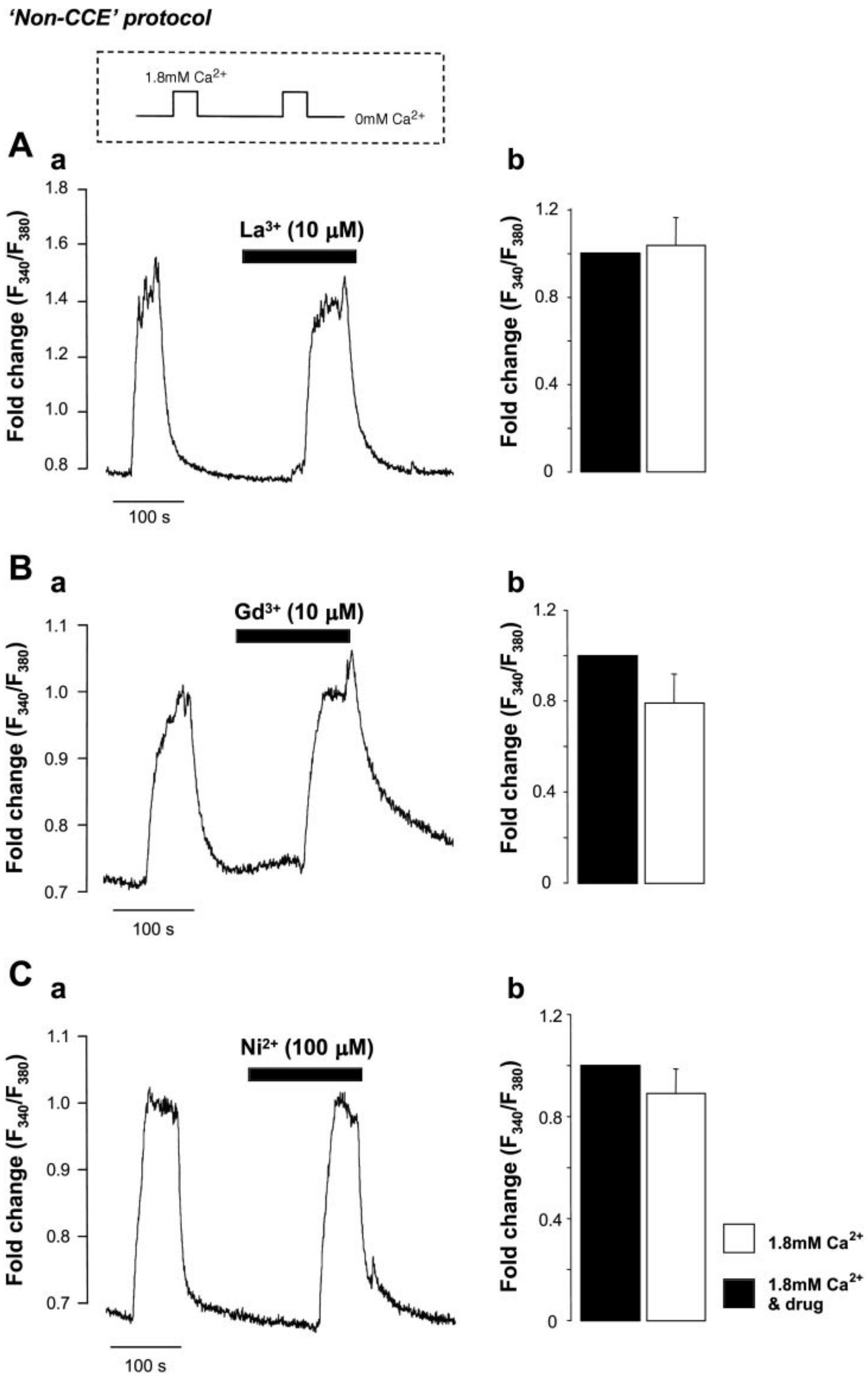

Fig. 5. Effect of the CCE inhibitors La^{3+} (10 µM; *A*), Gd^{3+} (10 µM; *B*) and Ni^{2+} (100 µM; *C*) on "non-CCE" evoked in urethral IC. In the absence of store depletion, application of 1.8 mM Ca^{2+} to isolated IC evokes a Ca^{2+} transient referred to as non-CCE. The CCE inhibitors La^{3+} (10 µM), Gd^{3+} (10 µM) and Ni^{2+} (100 µM) had no significant effect on the amplitude of Ca^{2+} transients evoked in this fashion (see Fig 6, *A*, *B*, and *C*, respectively). Panels *a* and *b* show representative experimental traces and summary bar charts, respectively.

The results of the present study, however, suggest that CCE is not involved in this process. IC were found to possess a CCE pathway characterized by sensitivity to low concentrations of La^{3+} and Gd^{3+} (10 µM) as well as relatively high concentrations of Ni^{2+} (100 µM). In contrast, Ca^{2+} entry induced in the absence of store depletion was unaffected by these agents, suggesting that IC possess a specific population of channels activated by store depletion that are inactive under resting conditions. Importantly, however, inhibition of CCE with these agents did not abolish STICs, suggesting that spontaneous activity in these cells is not completely reliant on CCE.

Perhaps this should not be surprising. Although several studies suggest that Ca^{2+} oscillations are driven by CCE (8, 13, 16), others have questioned this view. A review by Shuttleworth (29) pointed out that "if Ca^{2+} entry affects oscillation frequency by determining the rate at which stores recharge during the inter-spike interval, then inhibition of the SERCA-pump activity would be expected to slow oscillation frequency

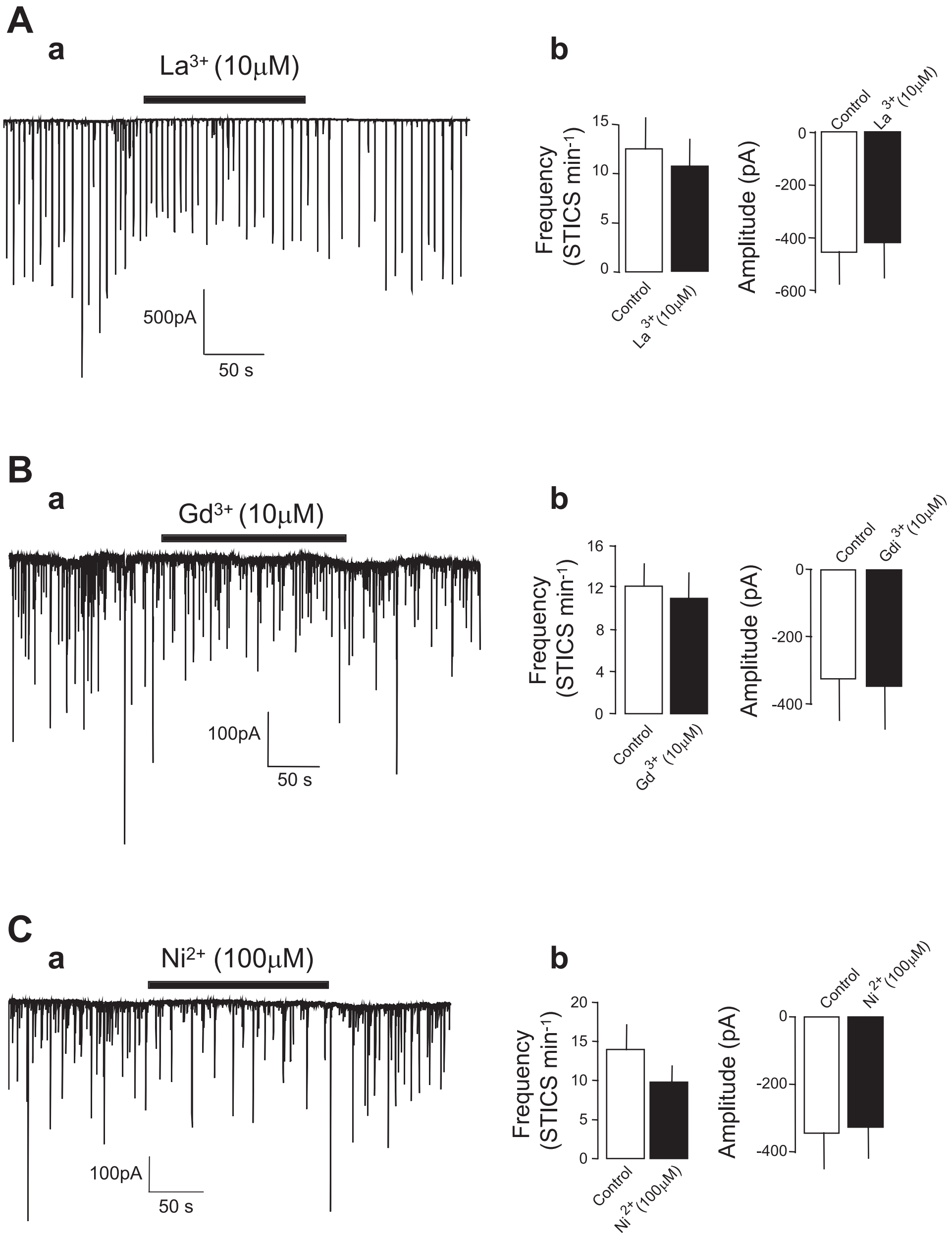

Fig. 6. Effect of the CCE inhibitors La^{3+} (10 μM; *A*), Gd^{3+} (10 μM; *B*), and Ni^{2+} (100 μM; *C*) on spontaneous transient inward currents (STICs) recorded from IC voltage-clamped at −60 mV. *A*: *a*: Recording made from a freshly dispersed IC from the rabbit urethra voltage clamped at −60 mV. This cell produced spontaneous transient inward currents that were not significantly affected by application of 10 μM La^{3+}. *b*: Summary bar charts showing that 10 μM La^{3+} does not significantly affect the mean frequency or amplitude of STICs recorded from 7 cells. *B*: representative trace from an experiment showing the effect of 10 μM Gd^{3+} on STICs recorded from an isolated IC (*a*) and summary bar charts showing the effect of 10 μM Gd^{3+} on the mean frequency and amplitude of STICs from 10 cells (*b*). Gd^{3+} does not significantly affect the mean frequency or amplitude of STICs in IC. *C*: representative trace from an experiment showing that Ni^{2+} (100 μM) caused a slight decrease in the frequency of STICs in an isolated IC (*a*) and summary data from 10 similar experiments that show that Ni^{2+} (100 μM) caused a small but significant reduction in the mean frequency of STICs recorded from 10 cells (*b*). Mean STIC amplitude was unaffected by Ni^{2+} (100 μM).

by extending the time required to recharge the stores." However, previous studies by Peterson et al. (19) showed that application of the SERCA pump inhibitor thapsigargin actually decreased the interspike interval, suggesting that the rate of refilling was not aligned with the oscillation frequency. Indeed, in urethral IC, application of CPA decreased the amplitude of STICs but had little effect on their frequency (26), suggesting that spontaneous activity is not sustained by CCE. This observation, in addition to the findings of the present study, points to an alternative "non-capacitative entry" pathway as a means for sustaining Ca^{2+} oscillations. At present we have little information with regard to the nature of this pathway in urethral IC. We know that in addition to the CCE inhibitors La^{3+}, Gd^{3+}, and Ni^{2+}, STICs recorded in IC voltage-clamped at −60 mV were not inhibited by nifedipine (10 μM), suggesting that influx via L-type Ca^{2+} channels is also not involved (26).

Given the apparent lack of involvement of CCE in the generation of STICs in IC, the question arises as to what the role of CCE is in these cells. Once again, we have no definitive answer to the question; however, one possibility is that the amount by which Ca^{2+} stores are depleted during normal Ca^{2+} cycling is not sufficient to activate CCE. Such a model would be consistent with the findings of Parekh et al. (17), who concluded that activation of CCE is a threshold-dependent, all-or-nothing phenomenon. These investigators showed that intraluminal Ca^{2+} within IP_3-sensitive stores had to fall to a particular threshold to activate Ca^{2+} release-activated current (I_{CRAC}). It is possible, therefore, that a similar situation may exist in IC, although the exact physiological conditions under which this would occur are unknown. It is conceivable that stores are more fully depleted after activation of postjunctional α_1-receptors on IC, which is known to cause depletion of

IP_3-sensitive stores in these cells (28). An alternative explanation may be that CCE exists as a protective mechanism to promote uptake into stores when intraluminal Ca^{2+} levels fall (29).

In summary, the data presented in this study show that a CCE pathway is present in urethral IC. However, it appears that this pathway is not critical for pacemaking in these cells and that inhibition of CCE does not account for effects of Ca^{2+}-free medium in abolishing Ca^{2+} oscillations in IC. Further studies are needed to elucidate the exact role and molecular identity of CCE in IC as well as to investigate the Ca^{2+} influx pathways that contribute to pacemaker activity in IC.

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