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Activation of Ca^{2+}-activated Cl^- current by depolarizing steps in rabbit urethral interstitial cells

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Hollywood, M. A., G. P. Sergeant, N. G. McHale, and K. D. Thornbury. Activation of Ca^{2+}-activated Cl^- current by depolarizing steps in rabbit urethral interstitial cells. *Am J Physiol Cell Physiol* 285: C327–C333, 2003. First published April 2, 2003; 10.1152/ajpcell.00413.2002.—Interstitial cells were isolated from strips of rabbit urethra for study using the amphotericin B perforated-patch technique. Depolarizing steps to −30 mV or greater activated a Ca^{2+} current (I_{Ca}), followed by a Ca^{2+}-activated Cl^- current, and, on stepping back to −80 mV, large Cl^- tail currents were observed. Both currents were abolished when the cells were superfused with Ca^{2+}-free bath solution, suggesting that Ca^{2+} influx was necessary for activation of the Cl^- current. The Cl^- current was also abolished when Ba^{2+} was substituted for Ca^{2+} in the bath or the cell was dialyzed with EGTA (2 mM). The Cl^- current was also reduced by cyclopiazonic acid, ryanodine, 2-aminoethoxydiphenyl borate (2-APB), and xestospongin C, suggesting that Ca^{2+}-induced Ca^{2+} release (CICR) involving both ryanodine and inositol 1,4,5-trisphosphate receptors contributes to its activation.

interstitial cells; urethra; calcium-activated chloride current; calcium-induced calcium release; inositol 1,4,5-trisphosphate; ryanodine

RECENTLY, WE ISOLATED a group of cells from the rabbit urethra that are good candidates for the role of specialized pacemakers (21, 22). These were termed "interstitial cells" (IC) because they shared many features with the interstitial cells of Cajal, the pacemakers of the gastrointestinal tract (12, 27). Urethral IC generate large spontaneous transient inward currents (STICs) due to activation of Ca^{2+}-activated Cl^- currents by cyclical release of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (21, 22). Cl^- currents can also be generated by depolarizing steps under voltage-clamp conditions, although the mechanisms that link depolarization to activation of the Cl^- current have not been investigated. The most likely possibilities are that *1*) Ca^{2+} enters the cell via voltage-dependent Ca^{2+} channels (VDCC) and then activates the Cl^- channels directly, *2*) influx of Ca^{2+} via VDCC may cause Ca^{2+} release from intracellular stores that then activates the Cl^- channels (3), and *3*) depolarization may activate the Cl^- channels independently of Ca^{2+} influx by a method that putatively involves voltage-dependent production of IP_3 (10, 11, 24).

The purpose of the present study was to distinguish between these possibilities by establishing whether *1*) depolarization-induced Cl^- current required influx of external Ca^{2+}, and *2*) intracellular stores are involved in depolarization-induced activation of the Cl^- current.

METHODS

The bladder and urethra were removed from both male and female rabbits immediately after they had been killed by lethal injection of pentobarbitone. The most proximal 1 cm of the urethra was removed and placed in Krebs solution, and from this, strips were dissected for cell dispersal.

Cell dispersal. Strips (0.5 cm) of proximal urethra were cut into 1-mm^3 pieces and stored in Hanks' Ca^{2+}-free solution for 30 min before they were incubated in an enzyme medium containing (per 5 ml of Hanks' Ca^{2+}-free solution) 15 mg of collagenase (Sigma type 1a), 1 mg of protease (Sigma type XXIV), 10 mg of BSA (Sigma), and 10 mg of trypsin inhibitor (Sigma) for ~5 min at 37°C. They were then placed in Hanks' Ca^{2+}-free solution and stirred for a further 5–10 min to release both single relaxed smooth muscle cells and IC. These were placed in petri dishes containing Hanks' solution (100 μM Ca^{2+}) and stored at 4°C for use within 8 h.

Recordings were made using the amphotericin B perforated-patch method (20). After gigaseals were obtained, the series resistance fell over a 10- to 15-min period to 10–15 MΩ and remained stable for up to 1 h. Voltage-clamp commands were delivered with an Axopatch 1D patch-clamp amplifier (Axon Instruments), and currents were recorded by means of a 12-bit analog-to-digital/digital-to-analog converter (Labmaster, Scientific Solutions) interfaced to an Intel computer running pCLAMP software (Axon Instruments). During experiments, the dish containing the cells was superfused with bath solution (*solution 2*). In addition, the cell under study was continuously superfused with bath solution by means of a close delivery system consisting of a pipette (tip diameter 200 μm) placed ~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 37°C.

The solutions used were of the following composition (in mM): *1*) Hanks' Ca^{2+} free solution (cell dispersal): 141 Na^+, 5.8 K^+, 130.3 Cl^-, 15.5 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 10 dextrose, 2.9 sucrose, and 10 HEPES, pH adjusted to 7.4 with NaOH. *2*) Bath solution: 130 Na^+, 5.8 K^+, 135 Cl^-, 4.16 HCO_3^-, 0.34 HPO_4^{2-}, 0.44 $H_2PO_4^-$, 1.8 Ca^{2+}, 0.9 Mg^{2+}, 0.4

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SO_4^{2-}, 10 dextrose, 2.9 sucrose, and 10 HEPES, pH adjusted to 7.4 with NaOH. In some experiments, nominally Ca^{2+}-free conditions were created by replacing the Ca^{2+} in this solution with equimolar Mg^{2+} and adding 5 mM EGTA. *3*) Cs^+ pipette solution: 133 Cs^+, 1 Mg^{2+}, 135 Cl^-, 0.5 EGTA, and 10 HEPES, pH adjusted to 7.2 with CsOH.

The following drugs were used: caffeine (Sigma), ryanodine (Sigma), 2-aminoethoxydiphenyl borate (2-APB; Acros), cyclopiazonic acid (CPA; Calbiochem), xestospongin C (Calbiochem), and 2-nitro-4-carboxyl-*N*,*N*-diphenylcarbamate (NCDC; Sigma). Data are presented as means ± SE, and statistical differences were compared using Student's paired *t*-test, taking the $P < 0.05$ level as significant. *n* refers to the number of cells in series of experiments; a minimum of three animals was used for each data set.

RESULTS

We have previously isolated a group of cells from the rabbit urethra that are quite distinct from smooth muscle cells. Under phase-contrast microscopy, these cells are darker and thinner than smooth muscle cells and are highly branched. Under voltage clamp, they are noncontractile when subjected to 10 mM caffeine or to depolarizing pulses (21, 22). These cells also typically developed large Ca^{2+}-activated Cl^- currents and Cl^- tails in response to depolarization, which was a feature only rarely encountered in rabbit urethral smooth muscle cells (21) [it should be noted, however, that they were frequently seen in sheep urethral myocytes (4)]. One problem that occurred when systematically attempting to study depolarization-evoked currents was the fact that the majority of IC were spontaneously active and tended to fire large STICs during patch-clamp step protocols. For this reason, it was necessary to select cells with low firing rates (<4 min^{-1}) for this study. When occasional STICs occurred between depolarizing steps, the following step was rejected from the protocol and repeated later.

In the initial part of the present study, the requirement for external Ca^{2+} for evoking a Cl^- tail current was investigated and characterized. In Fig. 1*A*, Ca^{2+} current (I_{Ca}) and Cl^- tail current were evoked by stepping to −20 mV from a holding potential of −80 mV, followed by a step back to −80 mV while superfusing with normal (1.8 mM) Ca^{2+}. External Ca^{2+} was then removed by switching the superfusate to nominally Ca^{2+}-free 5 s before the next depolarizing step. This caused abolition of both I_{Ca} and the Cl^- tail. This was confirmed in a total of four experiments in which the Cl^- tails were abolished in Ca^{2+}-free conditions (control tail −1,114 ± 226 pA, tail in Ca^{2+}-free −23 ± 7 pA; $n = 4$; $P < 0.01$). In a second series of experiments, the effect of substituting Ba^{2+} for Ca^{2+} was investigated (Fig. 1*B*). In this case, the L-type Ca^{2+} current was enhanced, but the Cl^- current was greatly reduced from −670 ± 153 to −104 ± 27 pA ($n = 6$; $P < 0.01$). Finally, the requirement for Ca^{2+} was examined by dialyzing the slow Ca^{2+} buffer EGTA into the cell. In these experiments, I_{Ca} and Cl^- tail were evoked, first under perforated-patch recording conditions and then after the patch was ruptured by applying suction to the pipette (Fig. 1*C*). Pipette solution containing

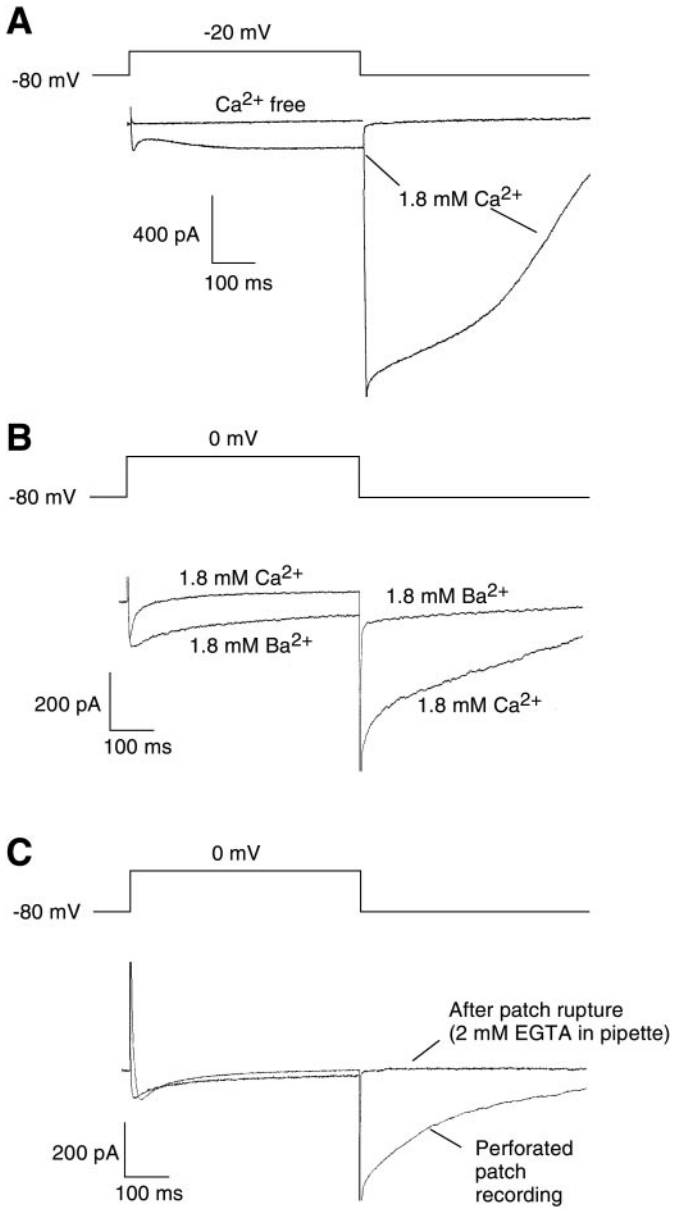

Fig. 1. Ca^{2+} dependence of the Cl^- currents evoked by depolarization. *A*: a test step to −20 mV followed by a step to −80 mV evoked a Ca^{2+} current (I_{Ca}), Cl^- current, and a Cl^- tail when the cell was superfused with 1.8 mM Ca^{2+}. These currents were abolished by brief (<5 s) exposure to nominally Ca^{2+}-free superfusate. *B:* a step to 0 mV and then to −80 mV evoked I_{Ca} and a Cl^- tail current. Equimolar Ba^{2+} substitution for Ca^{2+} in the superfusate enhanced I_{Ca} and blocked the tail. *C:* step to 0 mV and then to −80 mV evoked I_{Ca} and a Cl^- tail current in the perforated-patch configuration. The patch was then ruptured, and the voltage steps were repeated after 20-s dialysis of the cell interior with pipette solution containing 2 mM EGTA. This procedure abolished the Cl^- tail while having little effect on I_{Ca}.

EGTA (2 mM) was allowed to dialyze into the cell for 20 s before the step to 0 mV was repeated. This abolished the Cl^- tail current while having little effect on the amplitude of I_{Ca}. It is unlikely that the disappearance of the tail current was due to "run down," because, in our experience, run down of the Cl^- current in whole cell recording occurs gradually over 5–10 min after

rupture of the patch in parallel with run down of I_{Ca}. In these experiments, the current disappeared after only 20 s of dialysis after patch rupture and I_{Ca} was maintained at this time. In six cells, rupture of the patch and dialysis of 2 mM EGTA reduced the tail current from −688 ± 326 to −13 ± 4 pA ($P < 0.05$). We previously reported that nifedipine blocked the Cl^- currents evoked by depolarization (22). Together with the present results, these data suggest that depolarization-induced activation of the Cl^- channels is absolutely dependent on Ca^{2+} influx. It is unlikely, therefore, that voltage-dependent activation of the Cl^- channels, voltage-dependent production of IP_3 (17), or physical coupling of L-type Ca^{2+} channels to ryanodine receptors (c.f., skeletal muscle, Ref. 25) play any role in the activation of the Cl^- channels in these cells.

To test whether activation of the Cl^- current also involved release of intracellular Ca^{2+}, the effects of a variety of drugs that are known to interfere with intracellular Ca^{2+} stores were examined. Figure 2*A* shows the effect of CPA, an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump. In these experiments, current-voltage (*I-V*) relationships were evoked by stepping protocols that involved holding the cell at −60 mV and then stepping to a series of potentials from −80 to +50 mV. In the control, this protocol evoked a series of inward Ca^{2+} currents that could be resolved on steps to −40 through to +30 mV (Fig. 2*A*). Slower activating Cl^- currents could also be observed. These became fully activated after 100 to 300 ms and were inward at −40 to −10 mV and outward at +10 to +50 mV. On stepping down to −80 mV, Cl^- tail currents could be observed. In the example shown in Fig. 2*A*, CPA reduced the Cl^- currents without affecting I_{Ca}. [In these experiments, it was confirmed that the stores had emptied by showing that the caffeine-evoked Cl^- current was abolished by CPA (data not shown).] Figure 2*B* shows an *I-V* plot for I_{Ca} that was obtained by measuring the peak I_{Ca} at the beginning of each step. Figure 2*C* shows *I-V* plots for the Cl^- current, obtained by measuring the current at the end of each step, when most of the I_{Ca} had inactivated. These data confirm that CPA blocked a large component of Cl^- current ($P < 0.05$; $n = 4$) without reducing the L-type Ca^{2+} current.

In another series of experiments, the effect of a high concentration of ryanodine (30 μM) was determined. This concentration would normally be expected to

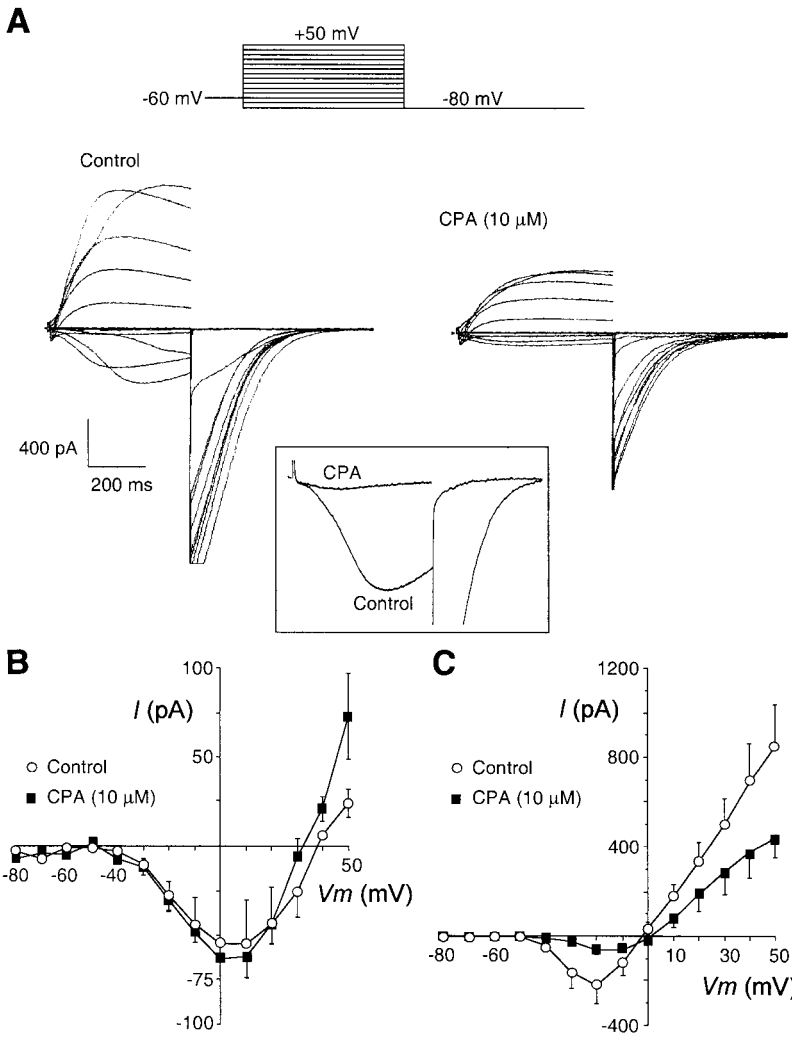

Fig. 2. Activation of the Cl^- current by depolarizing steps is amplified by Ca^{2+} release from intracellular stores. *A*: families of currents elicited by the stepping protocol indicated at *top*. CPA reduced the Cl^- currents without affecting the I_{Ca}. *Inset* shows currents evoked by steps to −20 mV before and after CPA (scale ×2). *B*: summary current-voltage (*I-V*) relationships for I_{Ca} (measured within the first 50 ms of the test step) before and after CPA (10 μM; $n = 4$). *C*: summary *I-V* relationships for the Cl^- current (measured at the end of the 500-ms test step) in the same cells.

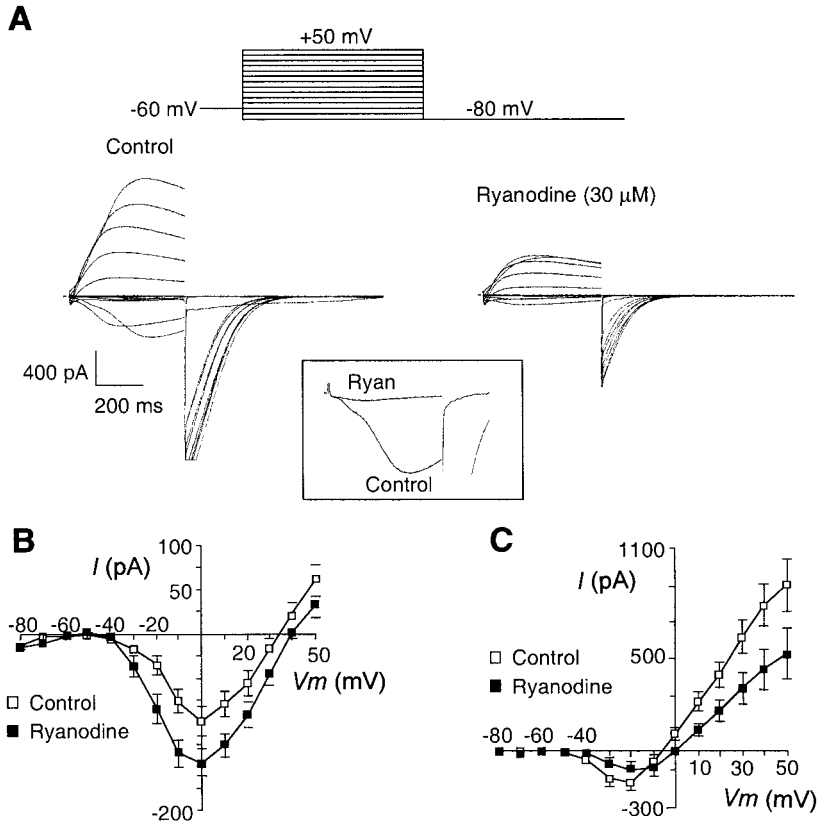

Fig. 3. Effect of ryanodine on depolarization-evoked Cl^- currents. *A*: families of currents elicited by the protocol indicated at *top*. *Inset* shows currents evoked by steps to −20 mV before and after ryanodine (scale ×2). Ryanodine (30 μM) reduced the Cl^- currents. *B*: summary *I-V* relationships for the I_{Ca} current before and after ryanodine (30 μM; $n = 11$). *C:* summary *I-V* relationships for the Cl^- current in the same cells.

block the ryanodine receptors (RYR), rather than to lock them open in a subconductance state (5, 23). Because ryanodine binds to the RYR in the open state, all cells in this series were subjected to repeated doses of caffeine in the presence of ryanodine until the caffeine-induced Cl^- current completely disappeared (generally after four exposures to caffeine at 80-s intervals). A previously acquired *I-V* relationship in each of these cells was then compared with the *I-V* obtained in the presence of ryanodine. Figure 3*A* shows that the effect of ryanodine was to reduce, but not abolish, the Cl^- currents. Summary data in Fig. 3*C* confirm that ryanodine reduced the Cl^- currents ($P < 0.05$; $n = 11$; measured at the end of the 500-ms test pulses) despite the fact that there was a moderate enhancement of I_{Ca} (Fig. 3*B*; $n = 11$; $P < 0.05$).

These results suggest that RYR contributed to the activation of the Cl^- current by depolarizing steps. The possibility that IP_3 receptors (IP_3R) also contributed was considered. Initially, this was tested with the IP_3 receptor blocker xestospongin C. An example of its effect is shown in Fig. 4, where currents were evoked by stepping to −20 mV and then to −80 mV. This protocol evoked I_{Ca} at the beginning of the sweep, followed by a large Cl^- current that had, in this example, a biphasic pattern of activation consisting of an early phase (beginning after ~10 ms) and a late phase (beginning after ~150 ms). On repolarization to −80 mV, a large tail current was recorded. Xestospongin C had little effect on I_{Ca} in this experiment but blocked the late phase of the Cl^- current, as well as the tail. In

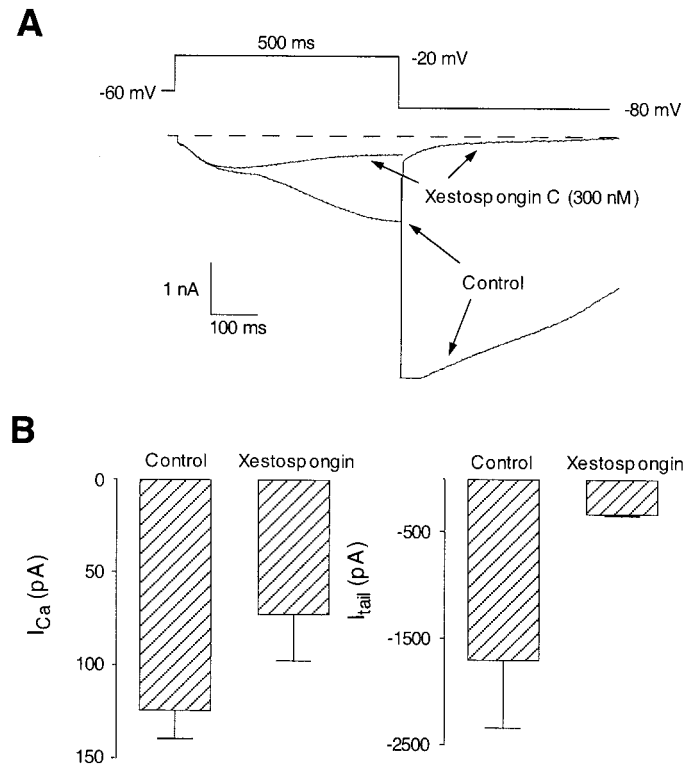

Fig. 4. Effect of xestospongin C. *A:* under control conditions, a step to −20 mV evoked a Ca^{2+} current, followed by a Cl^- current that activated in 2 phases. On stepping down to −80 mV, there was a large Cl^- tail. The steps were then repeated in the presence of xestospongin C, which blocked the second phase of the Cl^- current and the Cl^- tail. *B:* summary of the effect of xestospongin C (300 nM) on I_{Ca} and the Cl^- tail current in 6 cells.

six experiments, xestospongin C reduced the Cl^- tails from $-1{,}695 \pm 635$ to -331 ± 121 pA ($P < 0.05$). However, xestospongin C also had a variable effect on I_{Ca}. In two of the six experiments, there was no effect on Ca^{2+} current, but in the other four there was a variable degree of block. Overall, the mean effect was to reduce I_{Ca} from -124 ± 15 to -72 ± 25 pA ($P < 0.05$; $n = 6$). To further assess the involvement of IP_3R, the effect of 2APB was examined. We have previously found this drug to be a good discriminator between RYR- and IP_3-R-mediated responses (21). Figure 5*A* shows a typical example were 2-APB (100 μM) reduced the Cl^- currents without affecting I_{Ca}. Summary data in Fig. 5*B* show that 2-APB had no effect on I_{Ca} throughout the voltage range ($n = 7$), but it effectively reduced Cl^- current in the same cells (Fig. 5*C*; $P < 0.05$; $n = 7$). Figure 5*D* shows the effect of 2-APB in combination with CPA on the inward Cl^- currents evoked by steps to -20 mV ($n = 4$), where it is clear that the addition of 2-APB after CPA had no further effect. This suggests that CPA can deplete the store that is sensitive to blockade with 2-APB. We also tested the effect of a third drug, NCDC, a blocker of phospholipase C. NCDC (100 μM) effectively reduced the Cl^- tails (from -821 ± 227 to -352 ± 193 pA; $n = 5$; $P < 0.05$) but also reduced L-type Ca^{2+} current by a similar proportion (from -111 ± 37 to -31 ± 28 pA; $n = 5$; $P < 0.02$).

DISCUSSION

The results of this study suggest that, in rabbit urethral IC, the Ca^{2+}-activated Cl^- currents evoked by depolarizing steps were dependent on influx of extracellular Ca^{2+}, and this effect was enhanced by Ca^{2+} release from intracellular stores by CICR. The evidence for a role for CICR is that the Cl^- currents were reduced in amplitude *1*) after depletion of the stores with CPA or *2*) after blockade of either RYR or IP_3R with agents that are well known to block these receptors. The effects on Cl^- current could not be attributed to a reduction in I_{Ca}, because this was either unchanged or enhanced after treatment with CPA, 2-APB, or ryanodine. Even in the case of xestospongin C, in which a variable reduction in I_{Ca} was observed, the Cl^- current was greatly reduced in some examples where there was no effect on I_{Ca}. We therefore conclude that Ca^{2+} stores play a significant part in the activation of the Cl^- current by depolarization as a result of CICR due to Ca^{2+} influx.

The contribution of CICR to the activation of Cl^- currents has been assessed in several smooth muscle types (3, 6, 9, 15, 18). In guinea pig trachea and rabbit coronary artery myocytes, the Cl^- tail current was reduced by caffeine and CPA, whereas neither of these drugs significantly affected the tail current in the rat or rabbit portal vein (6, 18), suggesting that activation

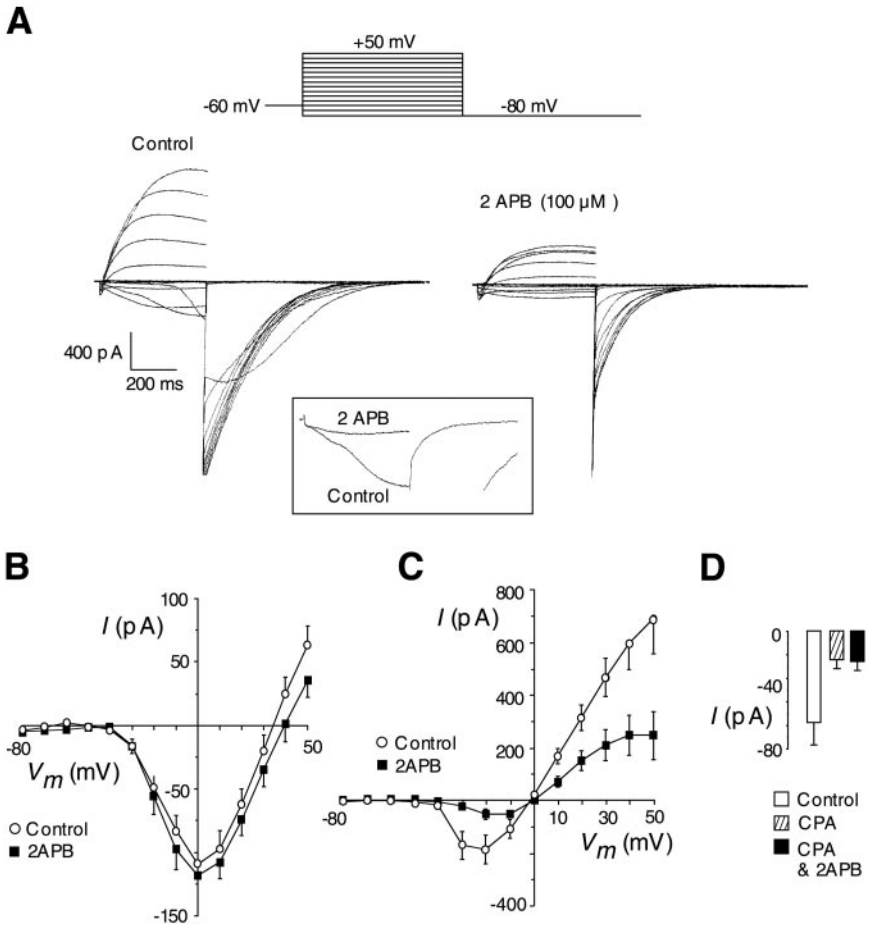

Fig. 5. Effect of 2-aminoethoxydiphenyl borate (2-APB). *A*: families of currents elicited by the protocol indicated at *top*. *Inset* shows currents evoked by steps to -20 mV before and after 2-APB (scale ×2). 2-APB reduced the Cl^- current without affecting I_{Ca}. *B*: summary *I-V* relationships for I_{Ca} before and after 2-APB (100 μM; $n = 7$). *C*: summary *I-V* relationships for the Cl^- current in the same cells. *D:* effect of CPA (10 μM) alone and in combination with 2-APB (100 μM) on Cl^- current evoked by steps to -20 mV ($n = 4$).

of Cl^- currents by CICR varies between cell types. The role of RYR in the activation of Ca^{2+}-activated Cl^- current has been elegantly demonstrated in bladder myocytes where it was shown that a depolarization-induced Cl^- tail current was activated by widespread CICR throughout the cell (3). Interestingly, in this preparation, the tail currents sometimes had a complex shape, reflecting the spread of a Ca^{2+} wave along the cell. Similarly, in the present study, Cl^- currents in rabbit IC often had complex kinetics during control conditions (see Figs. 2–5), but these became simpler in the presence of drugs that interfered with intracellular Ca^{2+} stores. It seems likely that the simpler kinetics mirrored only Ca^{2+} influx, whereas the complex kinetics recorded under control conditions depended both on Ca^{2+} influx and the spread of CICR throughout the cell.

In the present study, not only RYR but also IP_3R were necessary for the activation of the Cl^- currents as they were reduced by xestospongin C and 2-APB. The latter was first described as a specific blocker of the IP_3R Ca^{2+} release channel in a variety of cell types by Maruyama and colleagues (16) and has since been widely used for this purpose. Despite some evidence in nonexcitable cells that 2-APB may also block store filling currents (19), we have shown that it is a good discriminator between IP_3-sensitive and RYR-mediated responses in rabbit IC (21). Thus it abolished norepinephrine-evoked Cl^- currents but had no effect on either caffeine-evoked Ca^{2+} release or on spontaneous transient outward currents (STOCs), both of which are believed to be mediated by RYR (14). The present results therefore support the involvement of IP_3R in depolarization-induced activation of Cl^- currents.

It is interesting to speculate how both RYR and IP_3R could be involved in the activation of Cl^- current by depolarization. Certainly, the requirement for both types of receptors for inducing global Ca^{2+} increases inside the cell has many precedents (1, 2, 5). For example, Boittin et al. (2) showed that in portal vein myocytes, norepinephrine-induced Ca^{2+} waves could be blocked by dialyzing either RYR- or IP_3R-specific antibodies into the cell. They demonstrated that RYR and IP_3R were colocalized and proposed a model involving sequential activation of the receptors, where IP_3-induced Ca^{2+} release was amplified by a Ca^{2+} wave propagated by RYR (2). Similarly, amplification of IP_3R-mediated purinergic responses by RYR was observed in murine colonic myocytes (1). Recently, the idea of cooperativity between the two receptor types has been extended to spontaneous (i.e., non-agonist evoked) Ca^{2+} events (5). Gordienko and Bolton (5) showed that ryanodine blocked both spontaneous Ca^{2+} sparks and Ca^{2+} waves, whereas 2-APB and xestospongin C blocked only the latter. Their interpretation was that RYR were responsible for initiating sparks, but both RYR and IP_3R were necessary for propagation of the Ca^{2+} waves. Although the mechanism of this cooperativity was not fully elucidated, it was suggested that basal activity of phospholipase C (PLC) resulted in IP_3-induced Ca^{2+} release in the microdomain of the RYR, thus sensitizing them to CICR. Such a mechanism may also operate in rabbit urethral IC, because large spontaneous Cl^- currents were blocked by either ryanodine or blockers of IP_3R/PLC, but small spontaneous transient outward currents (mediated by RYR) were blocked only by ryanodine (21). Applying these ideas to the present study, it is possible that basal activity of PLC elevates Ca^{2+} in the vicinity of the RYR, thus sensitizing them to CICR by the Ca^{2+} that comes in during depolarization.

In conclusion, we have demonstrated that the depolarization-induced Ca^{2+}-activated Cl^- current in rabbit urethral IC is enhanced by Ca^{2+} release from stores by a CICR mechanism that requires both RYR and IP_3R. CICR may provide a mechanism for synchronizing pacemaker activity across a network of IC in the urethra. Urethral IC generate large STICs due to activation of Ca^{2+}-activated Cl^- currents (21, 22). In single IC, these currents can generate electrical slow waves that resemble the slow waves in whole tissue recorded with sharp microelectrodes (7, 8), whereas smooth muscle cells were electrically quiescent. This led us to propose that the IC act as pacemakers that drive the bulk smooth muscle (22). For this pacemaker system to work, it would be necessary for the activity of a sufficiently large group of pacemaker cells to be synchronized before they could generate enough current to drive the bulk smooth muscle cells lacking in the pacemaker mechanism. It is unlikely that sufficient synchronization could be achieved by spread of intercellular Ca^{2+} waves across the network, because their velocity is limited to <100 μm/s by the rate of Ca^{2+} diffusion (13). However, Ca^{2+}-release could be coordinated across the network if it was coupled to depolarization by CICR. Indeed, in the gastrointestinal tract, where a similar pacemaking model has been proposed, it has been shown that depolarization recruits further "spontaneous transient depolarizations" (STD) to produce the regenerative components of electrical slow waves (10, 11, 24, 26).

DISCLOSURES

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