Cholinergic-induced Ca\(^{2+}\) signaling in interstitial cells of Cajal from the guinea pig bladder

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Johnston L, Carson C, Lyons AD, Davidson RA, McCloskey KD. Cholinergic-induced Ca\(^{2+}\) signaling in interstitial cells of Cajal from the guinea pig bladder. Am J Physiol Renal Physiol 294: F645–F655, 2008.—Acetylcholine released from parasympathetic excitatory nerves activates contraction in detrusor smooth muscle. Immunohistochemical labeling of guinea pig detrusor with anti-c-Kit and anti-VACHT demonstrated a close structural relationship between interstitial cells of Cajal (ICC) and cholinergic nerves. The ability of guinea pig bladder detrusor ICC to respond to the acetylcholine analog, carbachol, was investigated in enzymatically dissociated cells, loaded with the Ca\(^{2+}\) indicator fluo 4AM. ICC fired Ca\(^{2+}\) transients in response to stimulation by carbachol (1/10 \(\mu M\)). Their pharmacology was consistent with carbachol-induced contractions in strips of detrusor which were inhibited by 4-DAMP (1 \(\mu M\)), an M3 receptor antagonist, but not by the M2 receptor antagonist methoctramine (1 \(\mu M\)). The source of Ca\(^{2+}\) underlying the carbachol transients in isolated ICC was investigated using agents to interfere with influx or release from intracellular stores. Nifedipine (1 \(\mu M\)) or Ni\(^{2+}\) (30–100 \(\mu M\)) to block Ca\(^{2+}\) channels or the removal of external Ca\(^{2+}\) reduced the amplitude of the carbachol transients. Application of ryanodine (30 \(\mu M\)) or tetracaine (100 \(\mu M\)) abolished the transients. The phospholipase C inhibitor, U-73122 (2.5 \(\mu M\)), significantly reduced the responses. 2-Ami-noethoxydiphenylborate (30 \(\mu M\)) caused a significant reduction and Xes-tospongin C (1 \(\mu M\)) was more effective, almost abolishing the responses. Intact in situ preparations of guinea pig bladder loaded with a Ca\(^{2+}\) indicator showed distinctively different patterns of spontaneous Ca\(^{2+}\) events in smooth muscle cells and ICC. Both cell types responded to carbachol by an increase in frequency of these events. In conclusion, guinea pig bladder detrusor ICC, both as isolated cells and within whole tissue preparations, respond to cholinergic stimulation by firing Ca\(^{2+}\) transients.

confocal microscopy; c-Kit

THERE IS AN INCREASING BODY OF evidence that the urinary bladder contains, in addition to bulk smooth muscle, a population of cells with many characteristics of the interstitial cells of Cajal (ICC) which are responsible both for the generation of peristaltic activity and the transmission of signals from enteric nerves to smooth muscle cells in the gastrointestinal tract (10, 20). Studies of ICC-like cells in tissues of the urinary tract are at a comparatively early stage; however, there is substantial evidence that they are present in the urethra, ureters, and the urinary bladder (for a review, see Ref. 3). These new cells have been described as interstitial cells (IC), ICC, cells resembling interstitial cells of Cajal (ICC-like cells), or myofibroblasts; however, while urinary tract ICC share many morphological and physiological properties of gut ICC, specific functions analogous to those of gut ICC have not been attributed to the similar cells in the urinary tract. The Fifth International Symposium on Intestinal Cells of Cajal (Ireland, 2007) recommended that all such cells in the urinary tract be classified as ICC and we adopted this nomenclature in the present paper (we previously described these cells as IC or ICC-like cells).

Morphological studies on guinea pig and human bladder have identified ICC in the suburothelial/lamina propria region [lamina propria ICC (ICC-LP)], on the boundary of smooth muscle bundles in the detrusor [intramuscular ICC (ICC-IM)], and in the spaces between the muscle bundles [interbundle interstitial cells (ICC-IB)]. These ICC have been identified in the urinary bladder using antibodies to the Kit receptor, an established ICC marker, both in fixed tissue preparations and when enzymatically dispersed (16). Vimentin antibodies have also been used to successfully label ICC in bladder and have identified ICC-LP in enzymatic cell dispersals (24). While the precise physiological roles of bladder ICC have yet to be established, information on their spontaneous activity, the complement of ion channels they possess, and their ability to fire Ca\(^{2+}\) signals in response to stimulation by agonists is accumulating (17, 18, 24, 25, 28). ICC-LP, also described as myofibroblasts, have been shown to exhibit spontaneous changes in membrane potential and respond to application of exogenous ATP by firing inward currents and Ca\(^{2+}\) transients (25, 28). These cells form close connections with each other in the lamina propria region and may be involved in sensory transmission. Detrusor ICC comprising both ICC-IM and ICC-IB also exhibit spontaneous Ca\(^{2+}\) signals and show increases in intracellular Ca\(^{2+}\) concentration in response to agonist stimulation. They also possess inward L-type and non-L-type Ca\(^{2+}\) channels and K\(^{+}\) channels including large-conductance Ca\(^{2+}\)-activated K\(^{+}\) and voltage-dependent delayed rectifier.

Immunohistochemical labeling and confocal microscopy showed that the ICC-IM and ICC-IB in the detrusor lie in close proximity to intramural nerves (16). Similar cells in the gastrointestinal tract are involved in both inhibitory and excitatory neurotransmission, acting as intermediaries in the relay of signals from nerves to smooth muscle (for a review, see Refs. 10, 20). It is unclear whether a similar situation is present in the bladder; however, it has been reported that enzymatically dispersed detrusor ICC respond to cholinergic stimulation by firing Ca\(^{2+}\) transients (16). The major excitatory innervation to the bladder is parasympathetic and activation of cholinergic nerves evokes contraction of bladder smooth muscle in the

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micturition response. Given the structural relationship between nerves and ICC in the bladder wall, it was therefore of interest to investigate the Ca\(^{2+}\) responses of detrusor ICC to cholinergic stimulation both in isolated cells and within tissue preparations.

Preliminary findings have been communicated to The Physiological Society.

**METHODS**

**Immunohistochemistry.** Guinea pigs (250–500 g) of either sex were killed by cervical dislocation in accordance with United Kingdom Animal Scientific Procedures Act (1986) and were approved by local University animal welfare and ethics committee. Bladders were removed, opened longitudinally, and the mucosa was removed by sharp dissection. Detrusor preparations were fixed in acetone (4°C), washed in PBS, blocked in 1% BSA, and incubated with primary antibodies, anti-c-Kit (rabbit), and anti-vesicular acetylcholine transferase (goat) (both from Santa Cruz Biotechnology, 1:200) for 24 h. After being washed to remove excess antibody, tissues were incubated in secondary fluorescent antibodies (Alexa 488 and Alexa 594, 1:200, Molecular Probes) for 1 h, washed in PBS, and mounted on glass slides for examination with epifluorescent microscopy. Slides were imaged with a Bio-Rad 1024 confocal/multiphoton imaging system mounted on a Nikon upright microscope using a 60× Plan Apo 1.2 numerical aperture water immersion objective. Fluorophores were excited with the 488- or 568-nm line of a krypton-argon laser, a Ti-sapphire multiphoton laser tuned to 750 nm (for DAPI imaging), and the resulting emission fluorescence was collected through appropriate filters to photomultiplier tubes. Sequential imaging of fluorophores was carried out to minimize any bleed-through.

Control experiments were carried out as follows: 1) omission of the primary antibody to control for the specificity of the secondary antibody, 2) omission of all antibodies to control for autofluorescence of the tissue, 3) anti-c-Kit with the secondary antibody for vAChT, i.e., anti-goat (irrelevant secondary control), and 4) anti-vAChT with secondary antibody for anti-c-Kit, i.e., anti-rabbit. Significant fluorescence was not observed in any of the control samples.

**Organ bath tension recordings.** After removal of the mucosa, strips of detrusor (10 × 2 × 2 mm) were mounted in organ baths, to an initial tension of 1 g. Tissues were perfused with Krebs solution at 35°C and allowed to equilibrate for 1 h. The strips typically relaxed as spontaneous contractile activity developed and this activity was maintained for several hours. Drugs were applied via the perfusion system (1–2 ml/min). Data were acquired using Intracept Chart Software and analyzed using Microsoft Excel and Prism (Graphpad) software.

**Isolated cell fluorescent microscopy.** Cells were enzymatically isolated from the detrusor region as previously described (16), loaded with 1–2 μM fluo 4AM (Molecular Probes) at room temperature, plated to the bottom of a recording chamber, and washed for at least 30 min with PSS solution before being studied with confocal microscopy. A mixed population of cells was obtained, including typical spindle-shaped smooth muscle cells and branched cells that have previously been identified as ICC using c-Kit antibodies. In addition to the bath perfusion, the cell of interest was superfused via a drug delivery system placed 200 μm away enabling the solution to be replaced with one containing a drug in less than 10 s. All experiments were carried out at room temperature. Cells were imaged with a confocal microscope (Nikon) mounted on an upright (e90i) microscope using a water-dipping objective lens (×60W Fluor numerical aperture 1.0). The fluorophore was excited at 488 nm with an argon-ion laser and the resulting emission was collected via a 515/30-nm filter to a photomultiplier tube. Data were collected as time series with acquisition rates between 1 and 10 frames/s, typically in a 512 pixel by 64 line box using Nikon EZ-C1 software. Images were analyzed with WinFluor software (Dr. J. Dempster, University of Strathclyde), Microsoft Excel and Prism (Graphpad). Statistical comparisons were made using the Student’s t-test, taking the P < 0.05 level as significant. n Refers to the number of cells and summary data are expressed as means ± SE. Data are expressed as F/F_0, where the fluorescence F of an event is expressed as a ratio of background F_0.

**Whole tissue confocal microscopy.** Small preparations of guinea pig bladder containing only a few smooth muscle bundles (~3 × 3 mm) were obtained by fine dissection and pinned to the Sylgard base of a recording chamber. Tissues were placed in an incubator for 30 min at 35°C before being loaded with Oregon Green BAPTA-AM (Molecular Probes; 5 μM) and pluronic (0.06%) at room temperature for 1 h (loading at 35°C often resulted in the tissue becoming overloaded with indicator). After being washed in HEPES-buffered Krebs solution for at least 30 min, tissues were imaged with a water-dipping objective lens (×60 W Fluor numerical aperture 1.0) either with a confocal microscope or an EMCCD camera (Nikon) running at 25 frames/s. The camera-based imaging system was operated with WinFluor software. Movement artifacts due to spontaneous contraction of the tissue were minimized by extensive pinning of the preparation. Analysis of images was performed using WinFluor, Microsoft Excel and Prism software. Experiments were carried out at room temperature.

**Solutions.** The solutions (in mM) were as follows: 1) Hanks solution (PSS): 130 Na\(^+\), 5.8 K\(^+\), 135 Cl\(^-\), 0.44 H_2PO_4^-\), 0.34 HPO_4^{2-}\), 4.16 HCO_3^-\), 0.4 SO_4^{2-}\), 1.8 Ca\(^{2+}\), 0.9 Mg\(^2+\), 10 HEPES, 10 AJP-Renal Physiol • VOL. 294 • MARCH 2008 • www.ajprenal.org

![Fig. 1. Immunohistochemical micrographs showing relationship between cholinergic nerves labeled with anti-vesicular acetylcholine transferase (red) and anti-c-Kit (green). Nuclei in A and B were counterstained with DAPI. Arrows show points of close contact between cholinergic nerve fibers and interstitial cells (B, C). Scale bars represent 10 μm in each case.](http://ajprenal.physiology.org/content/294/3/F646/F646.supp)
glucose, 2.9 sucrose, pH buffered to 7.40 with NaOH; 2) Krebs solution: 146.2 Na\(^+\), 5.9 K\(^+\), 133.3 Cl\(^-\), 1.2 H\(_2\)PO\(_4\), 25 HCO\(_3\), 2.5 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 11 glucose, pH was buffered to 7.4 by gassing with 95% O\(_2\)-5% CO\(_2\); and 3) HEPES-buffered Krebs solution: 125.3 Na\(^+\), 5.8 K\(^+\), 133.3 Cl\(^-\), 0.77 H\(_2\)PO\(_4\), 1.8 Ca\(^{2+}\), 1.0 Mg\(^{2+}\), 11 glucose, 10 HEPES.

Drugs. Nifedipine, atropine, methoctramine, 4-DAMP, 2-aminoethoxydiethylborate (2-APB), ryanodine, xestospongin C, and tetra-caine were all purchased from Sigma. U73122 was obtained from Tocris. Nifedipine was dissolved in ethanol; Xestospongin and U73122 were dissolved in DMSO. Dilutions were made from stock solutions such that the percentage of vehicle in the final concentration did not exceed 0.1%.

RESULTS

Immunohistochemistry. Whole mount preparations of guinea pig bladder were labeled with anti-vesicular acetylcholine transferase (vAChT) to label cholinergic nerve fibers (red), and anti-c-Kit to label ICC (green). Nuclei were counterstained with DAPI (blue). Figure 1 shows the location of ICC on the boundary of smooth muscle bundles in close proximity to cholinergic nerve fibers with points of contact highlighted by arrows. These images are typical of similar experiments in bladder tissue from at least five animals.

Muscarinic receptor-mediated contractions. Application of 1 μM carbachol caused a large contraction of the tissue and spontaneous contractions were often superimposed on the response. The effects of carbachol were repeatable after the preparations were washed for 15 min. The M₃ muscarinic receptor antagonist 4-DAMP (1 μM) reduced the carbachol response (control 1 ± 0 reduced to 0.04 ± 0.03 by the drug, n = 16 strips from 6 animals, P = 0.0001; Fig. 2A). The M₂ antagonist methoctramine (1 μM) had little effect (control 1 ± 0 reduced to 0.93 ± 0.05, P = 0.1979, n = 18 strips from 6 animals; Fig. 2B). The general muscarinic receptor antagonist atropine (1 μM) abolished the responses to carbachol (control 1 ± 0 reduced to 0.03 ± 0.01, n = 18 strips from 6 animals, P = 0.0001).

Muscarinic receptor-mediated Ca\(^{2+}\) transients in isolated ICC. Cells with the typical branched morphology of ICC were selected for the following series of experiments. These were

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**Fig. 2.** A: tension recordings from guinea pig detrusor strips showing contractile response to carbachol (1 μM). Arrow heads indicate application of carbachol. This was significantly reduced by the M₃ receptor antagonist 4-DAMP (1 μM). Summary data are shown for n = 6 tissues. B: carbachol-induced contraction was not blocked by the M₂ antagonist methoctramine (1 μM) but was abolished by atropine (1 μM). Summary data for methoctramine (n = 7) and atropine (n = 6) are shown in the graph. *Statistical significance.
previously identified as ICC by labeling with c-Kit antibodies (16). Application of 0.1, 1, or 10 μM carbachol to isolated ICC induced an increase in intracellular [Ca\(^{2+}\)], although lower concentrations sometimes induced oscillatory activity rather than eliciting individual Ca\(^{2+}\) transients. One micromolar carbachol was selected for the following experiments as this was used in the mechanical experiments described above and in our previous published work on isolated ICC. These responses were repeatable and comprised large transients (F/F\(_0\) 2.36 ± 0.02) lasting 2.5 ± 0.19 s (n = 27). Previous work showed that the carbachol responses were sensitive to the muscarinic blocker atropine; this finding was confirmed in the present investigation with atropine (1 μM) blocking carbachol responses in eight cells; control amplitude F/F\(_0\) was reduced from 2.5 ± 0.72 to 0.03 ± 0.007 (P = 0.011). The identity of the muscarinic receptors mediating the carbachol-induced Ca\(^{2+}\) transients was further examined using the pharmacological antagonists of M\(_2\) and M\(_3\) receptors used in the contractile experiments above. Exposure of cells to 4-DAMP (1 μM) inhibited the carbachol responses (Fig. 3A) by 90% (n = 7, \(P = 0.004\)), with complete inhibition seen in five cells (Fig. 3B). Methoctramine (1 μM; Fig. 3B) reduced the carbachol responses (n = 11); however, this effect was somewhat variable with little change seen in five cells, whereas reductions were seen in six cells. Overall, the mean control amplitude was reduced from 2.4 ± 0.17 to 1.73 ± 0.25 (P = 0.005; Fig. 3B).

**Role of Ca\(^{2+}\) influx.** The source of Ca\(^{2+}\) underlying the carbachol-induced transients was first investigated using agents to interfere with Ca\(^{2+}\) influx. Influx via voltage-dependent L-type Ca\(^{2+}\) channels was investigated using the L-type Ca\(^{2+}\) channel blocker nifedipine (Fig. 4A). The amplitude of carbachol-induced transients was reduced by 1 μM nifedipine from 3.32 ± 0.41 to 2.18 ± 0.25, \(P = 0.001\), n = 14. There was no effect on basal Ca\(^{2+}\). Ni\(^{2+}\) (30 μM), a blocker of other Ca\(^{2+}\) channels, including T-type (Fig. 4A), reduced transient amplitude from 2.32 ± 0.4 to 1.26 ± 0.26, \(P = 0.017\), n = 7 and a higher concentration (100 μM) had a greater effect (1.75 ± 0.30 to 0.2 ± 0.1, \(P = 0.028\), n = 4). A reduction in basal Ca\(^{2+}\) was seen with 100 μM Ni\(^{2+}\). Removal of external Ca\(^{2+}\) (Ca\(^{2+}\) substituted by Mg\(^{2+}\)) lowered the baseline (1.02 ± 0.02 to 0.62 ± 0.07) and reduced the transient amplitude from 2.29 ± 0.26 to 1.68 ± 0.32 (\(P = 0.04\), n = 7; Fig. 4B).

**Intracellular Ca\(^{2+}\) stores.** The possibility that carbachol was releasing Ca\(^{2+}\) from internal stores was initially addressed using experiments involving caffeine and carbachol. Initial application of carbachol caused a large Ca\(^{2+}\) transient and subsequent application of 10 mM caffeine also elicited an increase in [Ca\(^{2+}\)]. Exposure to carbachol after an initial application of caffeine, on the other hand, failed to elicit a further Ca\(^{2+}\) transient (Fig. 5A). Summary data for both experiments are shown in Fig. 5B. These experiments suggested that carbachol was releasing Ca\(^{2+}\) from internal stores and that

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**Fig. 3.** Ai: typical morphology of bladder interstitial cell. Aii–iii: time series fluorescence micrograph showing Ca\(^{2+}\) response to 1 μM carbachol which was blocked by the M\(_3\) antagonist 4-DAMP (1 μM). Traces show intensity time series of responses to carbachol and the effects of 4-DAMP and the M\(_3\) antagonist methoctramine (1 μM). Fluorescence (F) of an event is expressed as a ratio of background (F\(_0\)). CCh, carbachol. B: graph summarizing the effect of 4-DAMP and methoctramine on 7 and 11 cells, respectively. *Statistical significance.
either the release was from ryanodine-sensitive stores or that carbachol was acting on a common IP3/ryanodine store. Application of cyclopiazonic acid (CPA; 10 μM) caused a gradual reduction in the amplitude of the carbachol transients until they were abolished (Fig. 5C), indicating that release of Ca2+ from internal stores was a major source. Mean amplitude in five cells was reduced from 3.12 ± 0.32 to 0.006 ± 0.004, P = 0.001. Basal Ca2+ was generally elevated in the presence of CPA, although this was not statistically significant.

**Ca2+ release from IP3-sensitive stores.** The contribution of Ca2+ release from IP3 intracellular stores was initially investigated using 2-APB, a drug often used to block release from IP3-sensitive stores. Thirty micromolar 2-APB (Fig. 6A) reduced the carbachol-induced transients from 2.41 ± 0.37 to 1.92 ± 0.38, n = 6, P = 0.02. Increasing the concentration of 2-APB to 100 μM was more effective, reducing mean transient amplitude from 2.34 ± 0.31 to 1.53 ± 0.22, P = 0.001, n = 6. However, in some of the cells tested, application of 100 μM 2-APB caused a marked increase in basal Ca2+ making it impossible to assess its effect on the carbachol responses. Another agent widely used to block IP3 receptors, xestospongicin-C (1 μM), consistently blocked the carbachol responses (Fig. 6B) from 1.89 ± 0.39 to 0.06 ± 0.06 (n = 6, P = 0.006) with little effect on basal Ca2+. The effect of inhibition of phospholipase C was tested using U-73122 (2.5 μM). This agent blocked the carbachol transients (Fig. 6C) with no effect on basal Ca2+ in six cells (1.91 ± 0.39 to 0.11 ± 0.07, P = 0.005).

**Contribution of Ca2+ release from ryanodine-sensitive stores.** Ryanodine and tetracaine were used to assess the effect of blocking ryanodine receptors on the carbachol-induced transients. Ryanodine (30 μM) abolished the carbachol responses
The depletion of the Ca$^{2+}$ stores. The effects of ryanodine were irreversible. The overall reduction of the carbachol response by caffeine (Caf; 10 mM) could evoke a further response when applied immediately after. Pretreatment with caffeine, however, prevented carbachol from evoking a Ca$^{2+}$ transient. Pretreatment with caffeine, however, prevented carbachol from evoking a Ca$^{2+}$ transient. Ionomycin, in the continued presence of ryanodine, was addressed by treating the cells with ryanodine for 60 s (as for the experiments described above) and then applying ionomycin (1 μM) in the absence of external Ca$^{2+}$ to empty the stores. Ionomycin, in the continued presence of ryanodine, caused a substantial release of Ca$^{2+}$ indicating that ryanodine treatment had not emptied the stores (Fig. 7B). Similarly, exposure of cells to tetracaine (100 μM) abolished the carbachol responses (2.03 ± 0.57 to 0.01 ± 0.01, n = 8, P = 0.009) without changing basal Ca$^{2+}$. As others showed that tetracaine may block L-type Ca$^{2+}$ channels (23), the experiments were repeated in external Ca$^{2+}$-free conditions (in this set of experiments, the overall reduction of the carbachol response by Ca$^{2+}$-free was not statistically significant, perhaps indicating that there was variability in the contribution of Ca$^{2+}$ influx in some cells). The example shown in Fig. 7C shows that tetracaine still abolished the carbachol responses ruling out an effect via Ca$^{2+}$ channels and indicating that it was working by blocking ryanodine receptors.

**Cholinergic stimulation of ICC in whole tissue preparations.** Whole tissue preparations of guinea pig bladder containing a few smooth muscle bundles were loaded with a Ca$^{2+}$ indicator and imaged using a confocal microscope. ICC were readily identified on the boundary of the bundles or in the interstitial spaces between bundles, both by their location and their branched morphology, consistent with our previous work (16, 17). The loading of cells with fluorescent indicator enabled their morphology to be examined and cells of interest identified. To confirm that we were correctly identifying ICC, we labeled several of the loaded preparations with c-Kit antibody and Alexa 594 secondary antibody. Figure 8Ai shows that the cells on the edge of the bundle which we identified as ICC were labeled with c-Kit, whereas the smooth muscle cells were not labeled. We did not label ICC with antibodies in the following Ca$^{2+}$ imaging experiments to avoid any interference with the cellular physiology.

After the preparations were loaded, they were washed with Krebs solution, to remove excess indicator and areas of the tissue containing ICC and smooth muscle cells were imaged at room temperature (Fig. 8Aii). Spontaneous Ca$^{2+}$ activity was typically present in both ICC and smooth muscle cells; however, the signaling patterns were distinctively different, consistent with the findings of Hashitani et al. (7). The smooth muscle cells fired regular Ca$^{2+}$ events of 2.3 ± 0.2-s duration, amplitude 0.21 ± 0.03 dF/F$0$ at a frequency of 9.2 ± 1.7/min (n = 8), whereas the ICC fired transients of 12.2 ± 2.4-s duration, amplitude 0.49 ± 1.2 dF/F$0$ less frequently at 1.3 ± 0.3/min. In addition, integrated, coordinated flashes in the whole smooth muscle bundle were encountered (amplitude F/0 0.61 ± 0.07, duration 3.5 ± 0.5 s, frequency 3.8 ± 0.9/min, n = 7). Application of carbachol initially caused contraction of the tissue so that the tissue moved outside of the field of focus. However, extensive pinning of the tissue meant that we were able to record the effects of cholinergic stimulation without movement artefacts. Figure 8B shows the results of one such experiment. Carbachol (30 μM) increased the frequency of events in smooth muscle cells from 11 ± 1/min to 21 ± 2.9/min (n = 5) and caused large Ca$^{2+}$ transients (0.34 ± 0.02 amplitude) in ICC followed by several oscillations.

**DISCUSSION**

The findings of this study demonstrate that bladder ICC fire Ca$^{2+}$ transients in response to cholinergic stimulation and investigated the sources of Ca$^{2+}$ underlying the generation of these transients. Experiments on ICC in situ demonstrated that they responded to application of carbachol in a manner consistent with freshly isolated ICC. Immunohistochemical experiments demonstrated the close proximity of cholinergic nerve fibers to ICC, providing morphological support for the hypothesis that ICC participate in cholinergic signaling in the bladder. Isolated smooth muscle cells and ICC present in the enzymatic cell suspension responded...
differently to stimulation with carbachol. While both cell types fired Ca\(^{2+}\) transients, this was accompanied by contraction in smooth muscle cells, whereas the ICC were not observed to contract, despite the large increases in intracellular Ca\(^{2+}\) that were generated. This is consistent with our previous studies on these cells where agonist stimulation or depolarization via a patch electrode did not induce contraction of ICC (16, 17).

Muscarinic receptor subtypes. M\(_2\) and M\(_3\) muscarinic receptors are present in the mammalian detrusor, with the numbers of M\(_2\) significantly exceeding those of M\(_3\) (9, 26, 31). In human and guinea pig bladder, the proportion of M\(_2\) and M\(_3\) is \(\sim 3:1\), although the M\(_3\) subtype is dominant in the excitation of smooth muscle contraction via \(G\_q/11\) activating hydrolysis of phosphoinositide, IP\(_3\) production, and elevation of intracellular Ca\(^{2+}\). Conversely, M\(_2\) receptors may indirectly enhance M\(_3\)-mediated contraction, by opposing the normal effects of \(\beta\)-adrenoceptor G protein-coupled activation of adenylyl cyclase which lead to cAMP production and bladder relaxation (1, 8, 31). In the present investigation, the contractile response of guinea pig detrusor strips to carbachol was antagonized by the M\(_3\) receptor blocker 4-DAMP and partially reduced by the M\(_2\) antagonist methoctramine. In isolated ICC, carbachol-induced Ca\(^{2+}\) transients were blocked by the general muscarinic antagonist atropine or with 4-DAMP. The M\(_3\) subtype was apparently dominant in mediating the response as blockade of M\(_2\) receptors with methoctramine caused only a small reduction. The latter experiments, however, provide evidence that M\(_2\) receptors are present on bladder ICC. A similar situation has been reported for mouse gastric ICC where 4-DAMP antagonized the carbachol-induced increase in frequency of pacemaker currents but methoctramine was less effective (11).
Ca$^{2+}$ influx. The source of Ca$^{2+}$ underlying the carbachol-induced Ca$^{2+}$ transients in isolated ICC was investigated using a number of pharmacological agents. Ca$^{2+}$ entry was clearly an important component, as blockade of membrane Ca$^{2+}$ channels with nifedipine or Ni$^{2+}$, or the removal of extracellular Ca$^{2+}$, caused a significant reduction of the carbachol response. Ca$^{2+}$ influx is an important component of the response to cholinergic stimulation in other cells. Isolated human bladder smooth muscle cells were reported to fire Ca$^{2+}$ transients in response to carbachol, which were reduced up to 40% in external Ca$^{2+}$-free conditions but unaffected by the L-type Ca$^{2+}$ channel blocker nicardipine (29). Similar Ca$^{2+}$ responses to carbachol have been seen in guinea pig detrusor smooth muscle cells (30) where carbachol also produced a membrane hyperpolarization followed by a small depolarization that occurred after the peak of the Ca$^{2+}$ transient, indicating that depolarization via activation of L-type Ca$^{2+}$ channels was not responsible for the transient. We do not currently have information on any changes in membrane potential in guinea pig bladder ICC due to carbachol stimulation; however, the reduction of our transients by nifedipine and Ni$^{2+}$ may suggest that some membrane depolarization could also be occurring.

Application of carbachol after prior exposure to caffeine (at a concentration to deplete the internal store) failed to elicit a Ca$^{2+}$ transient, whereas pretreatment with carbachol did not prevent further Ca$^{2+}$ release by caffeine. This contrasts with findings in mouse bladder smooth muscle cells where pretreatment with caffeine similarly reduced the amplitude of the carbachol-induced transient (22). In guinea pig bladder ICC, it would seem that carbachol causes release from intracellular stores which are also sensitive to caffeine. Further support for the internal stores being a major source of Ca$^{2+}$ for the agonist-induced signals came from experiments.
with CPA (SR Ca\(^{2+}\)-ATPase blocker) which caused elevation of [Ca\(^{2+}\)]\(_i\), and additionally blocked the carbachol responses. Release of Ca\(^{2+}\) from intracellular stores could potentially occur by at least two pathways; classical activation of muscarinic receptors coupled to G proteins activating phospholipase C leading to production of diacylglycerol and IP\(_3\) which would cause release of Ca\(^{2+}\) from the IP\(_3\)-sensitive store, or Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the intracellular stores.

**IP\(_3\)-sensitive stores.** Evidence that carbachol was acting through the IP\(_3\) signal transduction pathway to release Ca\(^{2+}\) was provided by experiments using drugs to interfere with various stages of the pathway. U-73122, an inhibitor of phospholipase C (21), consistently reduced the carbachol responses without affecting basal Ca\(^{2+}\). Xestospongin-C, used to block IP\(_3\) receptors (5), significantly reduced the Ca\(^{2+}\) signals and while 2-APB, used to block IP\(_3\)-mediated Ca\(^{2+}\) release (for a review, see Refs. 2, 15), was less effective, it also caused a significant reduction. Taken together, our findings indicate that the cholinergic Ca\(^{2+}\) transients are at least partly mediated by release from IP\(_3\)-sensitive stores. We interpret these results with a degree of caution, however, as both Xestospongin C and 2-APB have been shown to have other effects including inhibition of store-operated channels and blockade of the SERCA pump (2, 4, 6, 12, 19). In our experiments, the carbachol responses were not abolished but significantly reduced by 2-APB (30 and 100 \(\mu M\)), although there were occasions where 2-APB caused a steady increase in basal Ca\(^{2+}\), making it impossible to assess any effect on the carbachol response.

**Ryanodine-sensitive Ca\(^{2+}\) stores.** Evidence for CICR from ryanodine-sensitive stores in response to carbachol stimulation came from experiments using ryanodine which causes depletion of the ryanodine-sensitive store, or tetracaine, a local anesthetic which blocks ryanodine receptors. In mouse bladder smooth muscle cells, carbachol transients were reduced but not blocked by ryanodine (22), whereas in the present study, ryanodine provided complete inhibition. Ryanodine or tetracaine inhibited the Ca\(^{2+}\) response to carbachol and on the majority of occasions, a small component due to Ca\(^{2+}\) influx was generally not evident. This could be explained by the influx component being inhibited as a result of the elevation of intracellular Ca\(^{2+}\) that occurred in the presence of ryanodine, leading to Ca\(^{2+}\)-induced inactivation of Ca\(^{2+}\) current. Previous work on bladder ICC characterized voltage-dependent Ca\(^{2+}\) channels which undergo Ca\(^{2+}\)-induced inactivation (18). Moreover, there is some evidence that in addition to blocking ryanodine receptors, tetracaine could directly block L-type channels (23), perhaps explaining why this drug blocked all of the carbachol response under conditions in which influx could occur. If the hypothesis that carbachol normally causes Ca\(^{2+}\) influx via L-type channels which in turn activates CICR from the ryanodine-sensitive store is correct, then application of tetracaine could initially prevent influx by blocking Ca\(^{2+}\) channels which would limit CICR as well as blocking the ryanodine receptors which are responsible for CICR.

In the presence of nifedipine or the absence of extracellular Ca\(^{2+}\), tetracaine blocked the carbachol responses, supporting...
the view that its main mode of action was via blockade of RyR (in nifedipine alone or in Ca\(^{2+}\)-free, there would still be a carbachol-induced transient). However, it is worth noting that a study on guinea pig colonic smooth muscle cells (14) presents an important caveat when interpreting data relating to IP\(_3\)-mediated Ca\(^{2+}\) release and CICR at ryanodine receptors. It was demonstrated that tetracaine and ryanodine could reduce IP\(_3\)-mediated Ca\(^{2+}\) signals by directly affecting IP\(_3\) receptors or depleting the (shared) store of Ca\(^{2+}\), respectively. Ryanodine could block a carbachol response by opening the RyR and depleting the store therefore diminishing the Ca\(^{2+}\) available for release by IP\(_3\)-mediated mechanisms. This might explain why ryanodine apparently blocked the carbachol response in bladder ICC, i.e., an indirect effect, from depletion of the “common” or “total” cellular SR. We addressed this by applying the Ca\(^{2+}\) ionophore, ionomycin, to a cell which had been pretreated with ryanodine, an approach used by White and McGeown (27). The results indicated that the store (combination of IP\(_3\) and RyR) had not been depleted by ryanodine as ionomycin could still evoke a large transient (in the absence of external Ca\(^{2+}\)). This suggests that the blocking effect of ryanodine was not due to depletion of the total store content but that carbachol does release Ca\(^{2+}\) from ryanodine-sensitive stores.

Interaction of IP\(_3\)- and ryanodine-sensitive stores. The involvement of both ryanodine and IP\(_3\)-sensitive pathways raises the possibility that IP\(_3\) and ryanodine stores in bladder ICC are linked, or alternatively, that there is one store which can be released by either receptor type. If it is the case that the ICC contain one store, it seems that it is not emptied by the carbachol exposure, because application of carbachol immediately followed by caffeine results in Ca\(^{2+}\) release by both agents. This experiment could also be explained by there being two discrete stores and each agent acting on a different store. However, the reverse experiment showed that application of caffeine, followed by carbachol, gave rise to release from the caffeine, but not then by carbachol. If a single store scenario is correct, perhaps the caffeine challenge was sufficient to empty the store so that carbachol could not cause further release. This experiment would argue against there being two discrete Ca\(^{2+}\) stores. While this specific question cannot be satisfied in the present investigation, it is clear that there is interaction of IP\(_3\)- and ryanodine-sensitive Ca\(^{2+}\) release mechanisms.

Cholinergic stimulation of ICC in intact detrusor preparations. Intact tissue preparations loaded with Ca\(^{2+}\) indicators revealed that ICC and smooth muscle cells with distinctively different spontaneous Ca\(^{2+}\)-signaling patterns both responded to carbachol by increasing the frequency and amplitude of their signals in situ. The physiological significance of the smooth muscle response to carbachol is unclear—the major excitatory neurotransmission to the bladder is parasympathetic and one would therefore expect cholinergic agonists to increase intracellular Ca\(^{2+}\) and cause contraction. The situation is less clear in the ICC and future work will reveal whether the Ca\(^{2+}\) transients are perhaps associated with the release of a factor which influences the contractility of neighbouring smooth muscle cells. A possible scenario may be that detrusor ICC normally send inhibitory signals to the smooth muscle during bladder filling to prevent unwanted coordinated contractions and the ICC Ca\(^{2+}\) response to cholinergic stimulation (at the start of micturition) may override this inhibitory signal. The finding that detrusor ICC respond to cholinergic stimulation by firing Ca\(^{2+}\) transients is markedly different than the situation in ICC from the suburothelial region of the guinea pig bladder which have been reported to exhibit no response to exogenously applied carbachol and which therefore are unlikely to contain cholinergic receptors (30). This difference may indicate an important division of labor in these subpopulations of ICC in the guinea pig bladder with the suburothelial ICC currently understood to have a regulatory role in the sensation of bladder fullness (30) and the detrusor ICC modulating the smooth muscle activity.

In conclusion, c-Kit-positive ICC in the bladder have close structural relationships with cholinergic nerves. Freshly dispersed detrusor ICC and ICC in situ respond to cholinergic stimulation by firing Ca\(^{2+}\) transients. The source of Ca\(^{2+}\) underlying these events is partially due to influx but mainly attributable to release from intracellular stores via IP\(_3\)- and ryanodine-sensitive pathways.

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