Coactivation of capacitative calcium entry and L-type calcium channels in guinea pig gallbladder

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Numerous physiological responses are mediated by changes in cytosolic Ca2+ concentration ([Ca2+]i). A variety of cellular transport systems that act to increase or remove Ca2+ from the cytosol are used by cells to tightly control this ubiquitous signal. In smooth muscle cells, many hormones and neurotransmitters activate contraction by Ca2+ release from intracellular stores and by extracellular Ca2+ influx (5). There is a clear understanding of the main mechanisms of release from sarcoplasmic reticulum (SR), mediated by channels activated by second messengers and/or [Ca2+]i, increases inositol trisphosphate (IP3) receptors and ryanodine receptors (RyRs) (6, 31). Similarly, physiological, biochemical, and biophysical features of extracellular Ca2+ entry via voltage-operated Ca2+ channels (VOCC) have been extensively characterized in gastrointestinal smooth muscle and other excitable tissues (13, 14).

However, mechanisms for Ca2+ influx are quite variable. One of the most elusive and ubiquitous systems is activation of Ca2+ entry through the plasma membrane after depletion of intracellular Ca2+ stores, a process termed capacitative Ca2+ entry (CCE) (28). The basis of this mechanism, initially observed in nonexcitable cells, is that Ca2+ concentration within the intracellular Ca2+ pools (mainly endoplasmic reticulum/SR) determines permeability of the plasma membrane to external Ca2+, so that release from the stores increases Ca2+ influx, resulting in a sustained Ca2+ plateau during stimulation. However, despite intense research in the field, the mechanism linking the fall in Ca2+ concentration to opening of plasma membrane Ca2+ channels remains highly controversial. One set of theories postulates the release of a diffusible messenger by the pools; other hypotheses claim a physical interaction between the empty stores and plasma membrane involving membrane proteins, secretory vesicles, or even cytoskeletal elements (for review see Ref. 30). Moreover, none of these models can explain all the experimental results.

Contrary to nonexcitable tissues, reports of CCE in excitable tissues are relatively scarce. A common problem in the research of CCE in excitable tissues is the prominent role of VOCC of the plasma membrane mediating Ca2+ influx in this cellular model, which introduces complications in the design and interpretation of experiments. In the few studies on this topic in excitable cells, it is common for authors to use different procedures to block VOCC to unmask the CCE-induced [Ca2+]i changes (9, 25). This has led to a full conceptual separation of CCE from voltage-operated Ca2+ influx, which is generally regarded as an entirely independent process.

Reports on the presence of CCE are much scarcer in gastrointestinal than in vascular smooth muscle. In gallbladder smooth muscle (GBSM), CCE has not been reported, although Ca2+ influx is determinant in the contractile responses: we previously showed that L-type Ca2+ channels are important in the maintenance of gallbladder resting tone (3), similar to the well-known role of extracellular Ca2+ in the contractile response to agonists (2, 3, 26, 32). In addition, we also found that, in GBSM cells, localized Ca2+ release from SR regulates plasma membrane potential by activation of Ca2+-activated K+ channels (27). Therefore, we investigated in GBSM cells the presence of CCE and the possible relation between this mechanism and activation of L-type Ca2+ channels. The aim of the present study was to test whether CCE is linked to contraction in GBSM and to explore a functional relation between CCE and VOCC.

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MATERIALS AND METHODS

Functional studies. Gallbladders, isolated from 300- to 450-g male guinea pigs after deep halothane anesthesia and cervical dislocation, were immediately placed in cold modified Krebs-Henseleit solution (K-HS; for composition see Chemicals and solutions) at pH 7.35. The gallbladder was opened by cutting along the longitudinal axis and was trimmed of any adherent liver tissue. After the preparation was washed with the nutrient solution to remove any biliary component, the mucosa was carefully dissected away and the gallbladder was cut into strips along the longitudinal axis, each strip measuring ~3 × 10 mm. On average, four strips were obtained from each guinea pig gallbladder. Each strip was placed vertically in a 10-ml organ bath filled with the nutrient solution maintained at 37°C and gassed with 95% O₂ -5% CO₂. Isometric contractions were measured using force displacement transducers that were interfaced with a Macintosh computer using a MacLab hardware unit and software. The strips were placed under an initial resting tension equivalent to a 1.5-gram load and allowed to equilibrate for 60 min, with solution changes every 20 min. Then the length of each strip was increased in 1-mm increments until a maximal response to 10 μM acetylcholine was achieved. The muscle length corresponding to the optimal preload was maintained throughout the experiments. All experiments were carried out according to the guidelines of the Animal Care and Use Committee of the University of Extremadura.

Intracellular recording from smooth muscle. The methods used for intracellular electrophysiological recording were similar to those previously described (47). The gallbladder whole mount preparation was pinned, serosal side up, in a 3-ml tissue chamber and placed on the stage of an inverted microscope (Diaphot T200). After cell sedimentation, a gravity-fed system was used to perfuse the chamber with Na+-HEPES solution (Diaphot T200). After cell sedimentation, a gravity-fed system was used to perfuse the chamber with Na+-HEPES solution in the absence or presence of different agents. For deesterification of the dye, ≥20 min were allowed to elapse before Ca²⁺ measurements were started.

Cells were illuminated at 340 and 380 nm by a computer-controlled filter wheel (Lambda 2, Sutter Instruments) at 1–3 cycles/s, and the emitted fluorescence was selected by a 500-nm band-pass filter. The emitted fluorescence images were captured by a cooled digital charge-coupled device camera (model C-4880-91, Hamamatsu Photonics) and recorded using dedicated software (Argus-HisCa, Hamamatsu Photonics). The ratio of fluorescence at 340 nm to fluorescence at 380 nm (F₃₄₀/F₃₈₀) was calculated pixel-by-pixel and used to indicate the changes in [Ca²⁺]ᵢ. A calibration of the ratio for Ca²⁺ was performed from changes in [Ca²⁺]ᵢ. These studies are expressed as ΔF/Fo, where ΔF = 100 × F - F₀, is basal fluorescence.

Chemicals and solutions. Drug concentrations are expressed as final bath concentrations of active species. Acetylcholine chloride, BSA, caffeine, cyclopiazonic acid (CPA), choleystokinin octapeptide (CCK), dithioerythritol, EGTA, gadolinium chloride, methoxyverapamil hydrochloride (D600), pinacidil, SKF-96365, and thapsigargin (TPS) were obtained from Sigma Chemical (St. Louis, MO); 2-aminoethoxydiphenylborane (2-APB) from Tocris (Bristol, UK); fura 2-AM, di-0-C₅(3), and rhodamine from Molecular Probes (Leiden, The Netherlands); collagenase from Fluka (Madrid, Spain); and papain from Worthington Biochemical (Lakewood, NJ). Other chemicals were of analytic grade and were purchased from Panreac (Barcelona, Spain).

The composition (in mM) of the K-HS was as follows: 113 NaCl, 4.7 KCI, 2.5 CaCl₂, 1.2 K₂HPO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.5 d-glucose; the solution was equilibrated with 95% O₂-5% CO₂. The Ca²⁺-free K-HS was prepared by substitution of EGTA (1 mM) for CaCl₂. The composition (in mM) of ES for isolation of smooth muscle cells was as follows: 10 HEPES, 55 NaCl, 5.6 KCl, 80 sodium glutamate, 2 MgCl₂, and 10 d-glucose. The composition (in mM) of the Na⁺-HEPES solution was as follows: 10 HEPES, 140 NaCl, 4.7 KCI, 2 CaCl₂, 1.11 MgCl₂, and 10 d-glucose. The Ca²⁺-free Na⁺-HEPES solution was prepared by substitution of EGTA (1 mM) for CaCl₂.

Statistics. [Ca²⁺]ᵢ results are expressed as changes in absolute F₃₄₀/F₃₈₀ [ΔF(F₃₄₀/F₃₈₀)]. Contractile responses are expressed as absolute tension (in mN) or percentage of the response elicited by an initial test application of 320 nM CCK in normal K-HS, which is a submaximal concentration in our preparation. In membrane potential measurements in isolated cells, data are expressed as changes in relative fluorescence (see above). Values are means ± SE. Statistical evaluation was performed by using Student’s t-test (2-tailed). P < 0.05 was considered significant.
RESULTS

CCE is present in GBSM cells. TPS is a specific inhibitor of the SR/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump widely used to deplete intracellular Ca\(^{2+}\) stores (34). Figure 1 illustrates the effect of acute application of 1 \(\mu\)M TPS on isolated GBSM cells. In the absence of extracellular Ca\(^{2+}\), TPS caused a transient increase in \([\text{Ca}^{2+}]_i\), \(0.075 \pm 0.011 \Delta(F_{340}/F_{380})\), \(n = 12\) that lasted for several minutes (average time to recovery = 278.8 \pm 33.5 s, \(n = 12\); Fig. 1A), as expected if there was a continuous Ca\(^{2+}\) leak from the pool. In contrast, in the presence of extracellular Ca\(^{2+}\), application of TPS induced a \([\text{Ca}^{2+}]_i\) increase that reached a sustained plateau \(0.064 \pm 0.01 \Delta(F_{340}/F_{380})\), \(n = 8\); Fig. 1B) in 250 \pm 49.5 s, indicating a continuous influx of external Ca\(^{2+}\) after depletion of the intracellular stores.

These data could indicate the existence of CCE in GBSM cells. To further test this possibility, Ca\(^{2+}\) stores were depleted by application of TPS in a Ca\(^{2+}\)-free medium, and then external Ca\(^{2+}\) was reintroduced. Restoration of extracellular Ca\(^{2+}\) induced a sustained \([\text{Ca}^{2+}]_i\) increase \(\Delta(F_{340}/F_{380}) = 0.062 \pm 0.006, n = 13\); Fig. 2A), indicative of enhanced permeability to extracellular Ca\(^{2+}\). Although TPS is generally regarded as a specific inhibitor of SR Ca\(^{2+}\) pumps, we also used other compounds to deplete internal stores, to avoid possible TPS side effects. In cells pretreated with CPA, a chemically unrelated inhibitor of the SERCA pump, Ca\(^{2+}\) reintroduction induced a plateau of 0.069 \pm 0.007 \(\Delta(F_{340}/F_{380})\) (\(n = 10\); Fig. 2C).

A different approach to deplete internal stores is to activate Ca\(^{2+}\) release through RyRs. Therefore, we used a mixture of ryanodine (to lock the RyRs in the open state) and caffeine (which activates RyRs), which resulted in an increase in \(\Delta(F_{340}/F_{380})\) of 0.062 \pm 0.006 (\(n = 8\)) on reintroduction of Ca\(^{2+}\). A similar pattern was also observed when depletion was evoked with 10 nM CCK \(\Delta(F_{340}/F_{380}) = 0.051 \pm 0.007, n = 8\); Fig. 2B), which has been shown to release Ca\(^{2+}\) from intracellular stores in GBSM by activating IP\(_3\) receptors (16, 32).

A potential drawback of the use of TPS to activate CCE is the possibility that inhibition of reuptake into the stores could reduce cytosolic Ca\(^{2+}\) buffering, so that influx of external Ca\(^{2+}\) would induce a strong \([\text{Ca}^{2+}]_i\) increase without an actual increase in Ca\(^{2+}\) entry (9, 36, 37). To avoid this artifact, a Ca\(^{2+}\)-free solution containing EGTA was used to repeat the
protocol in the absence of TPS. This simple protocol is useful only if the Ca\textsuperscript{2+} content of the stores has a rapid turnover (due to superficial location and/or a high rate of exchange with the cytosol) so that it suffices to deplete the intracellular Ca\textsuperscript{2+} stores and, eventually, leads to CCE activation. To assess the temporal course of intracellular Ca\textsuperscript{2+} store depletion, CCK was applied at different times after withdrawal of external Ca\textsuperscript{2+}. Figure 3A shows that application of 10 nM CCK immediately after Ca\textsuperscript{2+} removal induced a transient [Ca\textsuperscript{2+}], peak followed by a rapid recovery toward resting levels, indicating fast emptying of the Ca\textsuperscript{2+} stores (in this Ca\textsuperscript{2+}-free solution, the response is due to release only from the SR). This Ca\textsuperscript{2+} transient progressively decayed when the delay between Ca\textsuperscript{2+} removal and CCK application was increased. After 20 min of perfusion with Ca\textsuperscript{2+}-free solution, the response to CCK was eliminated, indicating that the intracellular Ca\textsuperscript{2+} stores were depleted because of the presence of Ca\textsuperscript{2+} leak in nonstimulated GBSM cells. Consistent with this concept, when Ca\textsuperscript{2+}-free perfusion was followed by restoration of external Ca\textsuperscript{2+}, cells showed a clear capacitative behavior, although the plateau was smaller than in TPS-treated cells [\(\Delta F_{340/F380} = 0.046 \pm 0.004, n = 13, P < 0.05\) vs. TPS treatment; Fig. 3B].

Another approach to characterize Ca\textsuperscript{2+} influx without interference from intracellular buffering of Ca\textsuperscript{2+} is the use of Ba\textsuperscript{2+} as a surrogate for Ca\textsuperscript{2+} influx (8, 46). Ba\textsuperscript{2+} binds to fura 2 and is permeable through Ca\textsuperscript{2+} channels but cannot be transported by SERCA pumps; therefore, it is insensitive to changes in buffering by SR uptake (15). This means that evaluation of its intracellular concentration during Ca\textsuperscript{2+} readmission provides an estimate of CCE unaffected by side effects of TPS or other store-depleting protocols in SR buffer. Figure 4 shows that, after depletion of the stores with TPS for 30 min, application of 2 mM Ba\textsuperscript{2+} induced a sustained plateau in \(\Delta F_{340/F380}\) (0.203 ± 0.029, n = 9). When we corrected this plateau for the increase induced by a previous control pulse of Ba\textsuperscript{2+} \(\Delta F_{340/F380} = 0.154 ± 0.02\), the magnitude of the CCE-induced influx was similar to that in Ca\textsuperscript{2+} restoration experiments \(\Delta F_{340/F380} = 0.049 ± 0.009\). Therefore, TPS enhanced Ba\textsuperscript{2+} influx, demonstrating that, under experimental conditions, loss of Ca\textsuperscript{2+} buffering by blockers of the SERCA pump does not result in overestimation of CCE.

Data from the methods used in the present study to release Ca\textsuperscript{2+} from intracellular stores indicate that TPS, CPA, and ryanodine + caffeine pretreatments were effective protocols. However, caffeine has been shown to have multiple cellular effects related to inhibition of phosphodiesterase activity, which could make interpretation of our data difficult. The other SERCA pump inhibitor used in this study, CPA, caused a similar CCE, but the irreversibility of TPS action made TPS the drug of choice to elicit CCE. Although CCK also induced a clear CCE, this approach could involve a noncapacitative component linked to receptor-operated Ca\textsuperscript{2+} influx or to the generation of arachidonic acid by this hormone (4, 22), introducing uncertainty into the interpretation of results.

**Characterization of Ca\textsuperscript{2+} entry pathways in response to store depletion.** Once it was established that isolated GBSM cells display a CCE response, we studied the nature of the capacitative pathway following a protocol involving depletion of the pools by 30 min of pretreatment with TPS followed by treatment with two pulses of extracellular Ca\textsuperscript{2+} separated by 20 min. The [Ca\textsuperscript{2+}], plateaus induced by the Ca\textsuperscript{2+} pulses were repetitive in all the cells examined (second plateau was 97.8 ± 3.3% with respect to the first pulse, n = 9; Fig. 5A), indicating that this value could be used as an index of CCE. Therefore, the interval between the two Ca\textsuperscript{2+} applications was used to apply...
different Ca\(^{2+}\) channel inhibitors, so that each experiment served as its own control.

In several systems, 2-APB inhibited store-operated Ca\(^{2+}\) influx without affecting VOCC (18), and experiments with excised membrane patches demonstrated that capacitative stores are a direct target for this drug (7). Given the importance of L-type Ca\(^{2+}\) channels in our model (3, 26), we tested any possible effect of 2-APB in GBSM contraction evoked by

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### Fig. 5. Inhibition of store-activated Ca\(^{2+}\) entry by 2-aminoethoxydiphenylborane (2-APB), Gd\(^{3+}\), and L-type channel blockers.

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*Fig. 5. Inhibition of store-activated Ca\(^{2+}\) entry by 2-aminoethoxydiphenylborane (2-APB), Gd\(^{3+}\), and L-type channel blockers. A: record of repetitive CCE-evoked [Ca\(^{2+}\)]\(_i\) plateaus. Cells were pretreated for 30 min with 1 \(\mu\)M TPS in Ca\(^{2+}\)-free solution before application of 2 pulses of extracellular Ca\(^{2+}\) \((n = 9)\). B-E: effects of 2-APB, Gd\(^{3+}\), nitrendipine, and pinacidil on TPS-activated CCE. After depletion of stores with TPS, cells were treated with 100 \(\mu\)M 2-APB (B), 100 \(\mu\)M Gd\(^{3+}\) (C), 1 \(\mu\)M nitrendipine (D), or 10 \(\mu\)M pinacidil (E) for 15 min before the second Ca\(^{2+}\) pulse. F: inhibition of CCE by 2-APB, Gd\(^{3+}\), methoxyverapamil (D600), nifedipine (NIF), nitrendipine (NITR), and pinacidil (PIN). Values are means ± SE of the second CCE episode expressed as percentage of control values (1st influx episode); \(n = 5-12\). \(^*P < 0.05\); \(^{**}P < 0.01\); \(^{***}P < 0.001\) vs. control (paired \(t\)-test).
repetitive application of KCl, which is exclusively mediated by L-type Ca\(^{2+}\) channels. Control application of a 60 mM KCl pulse induced an increase in isometric tension of 13.7 ± 1.7 mN, and after 20 min of treatment with 100 μM 2-APB, the second KCl-induced contractile response was not significantly different (12.8 ± 1.4 mN, n = 10). However, this compound inhibited CCE in a concentration-dependent manner (Fig. 5, B and E), reducing the second [Ca\(^{2+}\)] 

Another functional feature of CCE in various cell types is the sensitivity to divalent and trivalent cations (reviewed in Ref. 29). Gd\(^{3+}\), in addition to inhibiting stretch-activated channels, inhibits CCE in several systems, including vascular smooth muscle cells (9). Figure 5C shows inhibition of CCE in response to 100 μM Gd\(^{3+}\) (51.0 ± 5.6%, n = 12). A possible problem with Gd\(^{3+}\) is its ability to block VOCC in some systems (13). However, we ruled out this possibility in our model checking isometric contraction in response to 60 mM KCl, which was unaffected by Gd\(^{3+}\) (7.7 ± 1.36 vs. 8.47 ± 1.87 mN, n = 6). Together with the effect of 2-APB, this result closely resembles the previously reported behavior of CCE channels in other tissues.

We also used SKF-96365, a compound frequently used to block CCE or receptor-operated Ca\(^{2+}\) entry in other cell types (20, 38). However, this compound failed to inhibit CCE in our experimental conditions. Moreover, it induced an unexplained enhancement of the influx-associated [Ca\(^{2+}\)] 

The data described above suggest that “classical” capacitative channels are operative in GBSM in response to depletion of Ca\(^{2+}\) stores. The concept of capacitative entry involves only operation of non-voltage-operated channels. However, L-type Ca\(^{2+}\) channels play a dominant role in this cell type, in response to stimulation and in resting conditions (2, 3, 26). Therefore, we evaluated whether L-type Ca\(^{2+}\) channels are somehow activated by the emptying of the stores. To accomplish this, we used L-type Ca\(^{2+}\) channel blockers at concentrations effective in blocking the response to KCl in our preparation (3). Figure 5D shows that 1 μM nifedipine inhibited TPS-activated CCE. A similar effect was also detected with another dihydropyridine, nifedipine, at 1 μM and for 10 μM D600, a chemically unrelated L-type channel blocker. The apparent attenuation of CCE in the presence of these inhibitors was similar to that achieved by 2-APB or Gd\(^{3+}\) : 45.96 ± 6.2% (n = 9) for 1 μM nifedipine, 35.9 ± 5% (n = 4) for 1 μM nifedipine, and 34.6 ± 6.4% (n = 12) for 10 μM D600 (Fig. 5F). When 2-APB and L-type Ca\(^{2+}\) channel blockers were added simultaneously, the percent inhibition was even greater [77.6 ± 3.8% (n = 9) for nifedipine and 61.7 ± 20.1% (n = 2) for D600], although the effect was not additive (Fig. 5F).

To test whether activation of L-type Ca\(^{2+}\) channels after depletion of the stores involved a voltage-dependent mechanism, i.e., depolarization of the plasma membrane, we treated smooth muscle cells throughout TSP treatment and Ca\(^{2+}\) restorations with 10 μM pinacidil to clamp membrane potential at negative potentials via activation of ATP-dependent K\(^{+}\) channels (Fig. 5E). This protocol resulted in an inhibition of CCE by 53 ± 5.7% (n = 15), which is similar to the inhibition caused by nifedipine (Fig. 5F). Nifedipine did not cause further decrease in pinacidil-resistant Ca\(^{2+}\) entry (n = 11 cells).

**CCE induced contraction in gallbladder strips.** CCE has been disputed as a physiological process by some authors, who postulate that it must be considered an experimental phenomenon because of supra-physiological levels of stimulation used in the currently used protocols (33). In a less extreme view, it has also been reported that, in smooth muscle, CCE is not always associated with contraction (9), and it has been proposed that extracellular Ca\(^{2+}\) entering across the capacitative pathway is diverted from the contractile machinery. Therefore, we used isometric recording of gallbladder muscle strips to test whether the capacitative behavior described above is associated with GBSM contraction. After depletion of the stores by incubation with 1 μM TPS in Ca\(^{2+}\)-free medium for 30 min, reintroduction of extracellular Ca\(^{2+}\) induced a sustained contraction (26.7 ± 3.6 mN, 82.9 ± 4.2% of the response to a previous control 320 nM CCK application, n = 6; Fig. 8A). This effect was observed in all the assayed preparations and was also present when the content of the stores was released by simple incubation in Ca\(^{2+}\)-free solution (16.2 ± 2.9 mN, 61.3 ± 7.4% of the control 320 nM CCK response, n = 8) or
by CCK stimulation (17.5 ± 3.9 nM, 74.0 ± 2.3% of the 320 nM CCK response, n = 6; Fig. 8C). This pattern is similar to that observed in the [Ca\(^{2+}\)]\(i\) experiments.

If the capacitative channels activated by emptying of the stores in GBSM are related to contraction, then CCE-induced contraction should be sensitive to L-type channel blockers and 2-APB, similar to the [Ca\(^{2+}\)]\(i\) changes evoked by CCE. Thus we tested the effects of 2-APB, D600, and nitrendipine. Both types of inhibitors significantly impaired the influx-associated contraction (Fig. 8C), strongly resembling the behavior of capacitative [Ca\(^{2+}\)]\(i\) changes. The contraction during CCE in the presence of 2-APB, D600, and nitrendipine was significantly reduced compared with control experiments (Fig. 8C), ranging from 56.2 ± 5.2% of the response to 320 nM CCK for 50 μM 2-APB (n = 6) to 33.8 ± 4.6% of the response to 320 nM CCK in the case of 10 μM D600 (n = 6). When a combination of 100 μM 2-APB and D600 or nitrendipine was applied, the inhibitory effects were additive, and the CCE-induced contraction was nearly abolished: 1.73 ± 0.7% of the control 320 nM CCK response for 2-APB + 10 μM D600 (n = 4, P < 0.01) and 3.81 ± 1.8% of the control 320 nM CCK response for 2-APB + 1 μM nitrendipine (n = 6, P < 0.001). These findings indicate that CCE through two different channels is functionally linked to contraction in GBSM cells.

**DISCUSSION**

The findings reported here demonstrate that depletion of Ca\(^{2+}\) stores activates two pathways for Ca\(^{2+}\) entry in GBSM cells: a capacitative pathway as well as voltage-dependent Ca\(^{2+}\) channels. This study also provides original evidence for a link between depletion of intracellular stores, activation of L-type Ca\(^{2+}\) channels, and GBSM contraction. To our knowledge, this is the first report of the presence of CCE in GBSM.

Furthermore, this is the first demonstration of agonist-induced [Ca\(^{2+}\)]\(i\) signals in GBSM cells, although we and other authors have described mobilization of inositol lipids (45) and intracellular Ca\(^{2+}\) stores in contractility studies (3, 26, 32).

CCE has been reported in other types of smooth muscle such as vascular (1, 9) or uterine (43) smooth muscle. In the gastrointestinal tract, reports are scarce, including lower esophageal sphincter (39), gastric antrum (42), colon (19), ileum (12), and anococcygeus smooth muscle (40, 41). In any case, much of this evidence is indirect, in that it is based on contractility studies [e.g., ileum (25)] or on the status of second-messenger filling state (19), rather than on direct [Ca\(^{2+}\)]\(i\) determinations. Our study is one of the few direct characterizations of CCE in gastrointestinal muscle. This process has been also thoroughly studied in anococcygeus smooth muscle, where Ca\(^{2+}\) currents in response to store depletion have been characterized (40, 41).

The amplitude of the [Ca\(^{2+}\)]\(i\) increases evoked by capacitative Ca\(^{2+}\) influx was rather small in GBSM compared with other cell models, particularly nonexcitable cells (e.g., pancreatic acinar cells, a model commonly used in our laboratory under experimental conditions similar to this study). However, the amplitude of capacitative [Ca\(^{2+}\)]\(i\) signals in other smooth muscle types is rather small (9, 44), and we show here that even strong agonist stimulation induces only moderate increases in the ratio of fura 2 fluorescence (Fig. 3). Moreover, in GBSM, we have found a similar situation for strong stimulants such as KCl depolarization or caffeine (unpublished observations).

In this study, we have detected comparable capacitative behaviors using three different methods to deplete the stores: passive depletion by simple removal of extracellular Ca\(^{2+}\) or SERCA inhibition with TPS and CPA and active release from
stores with the IP$_3$-mediated agonist CCK and with the cocktail ryanodine-caffeine to promote Ca$^{2+}$ release through RyRs. TPS is widely used to activate CCE in different cell types. Its acute effect on resting GBSM cells (Fig. 1) and the stable and long-lasting capacitative behavior observed on subsequent application of external Ca$^{2+}$ (Figs. 2 and 5) follow the classical description of this process in other tissues (15, 30). TPS is highly specific for SERCA pumps; therefore, emptying of the stores is most likely due to spontaneous leak. The channels responsible for this leak have not been identified, although in other cells, resting levels of IP$_3$ (35) and the translocon (17) seem to participate in this passive release. The presence of this leak in GBSM SR is further supported by the clear loss of the agonist-mobilizable store within minutes after the simple procedure of Ca$^{2+}$ removal (Fig. 3A), which implies that in this cell type the turnover rate is higher and/or the size of Ca$^{2+}$ stores is smaller than in other models. This conclusion is in agreement with previous observations regarding gallbladder stores is smaller than in other models. This conclusion is in agreement with previous observations regarding gallbladder stores is smaller than in other models.

A potential complication of using inhibitors of Ca$^{2+}$ re-uptake stores is the superficial barrier hypothesis, which postulates that, in smooth muscle, Ca$^{2+}$ uptake by the SR operates as a local, subplasmalemmal buffer for Ca$^{2+}$ entering from extracellular space (36, 37). If this activity is strong enough, its blockade could even enhance the global [Ca$^{2+}$], increases induced by store depletion, which are an index for CCE. This means that inhibitors of SERCA pumps (such as TPS or CPA) could increase [Ca$^{2+}$], during reintroduction of external Ca$^{2+}$ without a real enhancement of transport from the extracellular medium. This concern can be ruled out in our experimental conditions, because capacitative entry is reproduced by two independent protocols without inhibition of Ca$^{2+}$ reuptake: 1) simple removal of external Ca$^{2+}$ and 2) influx of Ba$^{2+}$ as a surrogate for Ca$^{2+}$ entry. Another line of evidence ruling out this hypothesis is the presence of store-operated Ca$^{2+}$ inward currents in anococcygeus smooth muscle cells (40, 41).

Some authors have claimed that capacitative entry is an experimental artifact as opposed to a physiological component of Ca$^{2+}$ signals (21, 33). It has been proposed that physiological levels of stimulation activate Ca$^{2+}$ entry only via arachidonic acid-activated noncapacitative Ca$^{2+}$ channels (21, 22). Arachidonic acid metabolism plays a clear role in CCK-mediated contraction in GBSM (4), and we cannot rule out the presence of this pathway in our model, but in our experimental conditions we have observed a clear capacitative pattern in resting conditions, in the absence of agonist stimulation (TPS, CPA, and ryanodine + caffeine applications and simple removal of external Ca$^{2+}$).

Another possible concern regarding the physiological relevance of CCE is its relation to cell function, i.e., contraction in the case of smooth muscle. A recent study in arterial smooth muscle demonstrated that capacitative Ca$^{2+}$ influx can operate independently from contraction (9). However, we show here that in GBSM capacitative influx activated using three different experimental approaches is directly linked to contraction. This finding is consistent with a number of studies reporting a clear capacitative behavior in contractile experiments (reviewed in Ref. 12). Some of the gastrointestinal models showing CCE, such as antrum, are tonic muscles, similar to gallbladder muscle, suggesting that this influx mechanism is important for this kind of contractile activity. In addition, some authors report that CCE is probably involved in spontaneous rhythm of gastrointestinal muscle (23) and plays a role in propagation of stretch-induced Ca$^{2+}$ waves in colonic myocytes (44).

The present data indicate that filling status of the Ca$^{2+}$ stores controls at least two types of Ca$^{2+}$ channels, including VOCC. Studies of capacitative influx in excitable tissues have not focused on the role of VOCC in store depletion-induced Ca$^{2+}$ influx. Most of these studies avoid activating VOCC using a background of VOCC blockers during all the experimental procedures (9, 25) or performing voltage-clamp of the membrane (41, 42). This has led to a complete separation of the CCE concept from voltage-operated Ca$^{2+}$ influx; these events are considered by most investigators to be entirely independent processes. However, our results clearly indicate that L-type channels play an important role in the overall influx elicited by store depletion, acting as an important additional “paracapacitative” or “capacitative-like” pathway for influx. Previous reports show that Ca$^{2+}$ entry subsequent to store depletion in smooth muscle is sensitive to VOCC blockers (12) in the gastrointestinal tract and in other smooth muscle types.

The mechanism for activation of L-type channels in GBSM is likely to be mediated by a plasmalemma depolarization...
caused by store depletion; e.g., in the continuous presence of pinacidil to clamp membrane resting potential at hyperpolarizing values, there was a reduction in CCE similar to that caused by L-type Ca\(^{2+}\)/H11001 blockers. This concept is supported by our present finding of TPS-evoked depolarization, as demonstrated by two independent methods. This finding is in contrast to the previous report that, in colonic muscle, store depletion does not activate VOCC (19). Given that these authors did not determine Ca\(^{2+}\) influx but evaluated filling state of second messenger-releasable Ca\(^{2+}\) pools, it is not possible to conclude whether this discrepancy is due to tissue-specific differences or the method used to assess Ca\(^{2+}\) entry.

A possible link between store depletion and L-type Ca\(^{2+}\) channel opening could be activation of large-conductance K\(^{+}\)/H11001 channels. This channel passes hyperpolarizing spontaneous transient outward current in response to spontaneous Ca\(^{2+}\) sparks (transient localized Ca\(^{2+}\) signals) arising from intracellular store Ca\(^{2+}\) release, as shown in vascular smooth muscle (13, 24). We previously demonstrated the presence of this mechanism in GBSM cells, where depletion of internal pools by TPS leads to inhibition and eventual disappearance of hyperpolarizing spontaneous transient outward current (27). Therefore, depletion of the stores would be expected to induce plasmalemmal depolarization through this mechanism, as previously demonstrated in vascular smooth muscle (24). However, preliminary results in our laboratory show that TPS can induce further depolarization in the presence of the large-conductance K\(^{+}\) channel blocker iberiotoxin, indicating that another yet to be identified mechanism is responsible for this depolarization.

Data presented here indicate that sarcoplasmic Ca\(^{2+}\) pools and multiple plasma membrane pathways can operate as a functional unit linked to muscle contraction and refilling of the pools. Thus a decrease of the content of the stores would lead to a rapid Ca\(^{2+}\) entry by two different routes. In addition to replenishment of the stores, extracellular Ca\(^{2+}\) would also induce contraction, a meaningful sequence, given that the main agonists for Ca\(^{2+}\) release from the stores are aimed at contraction of the gallbladder.

It is possible that this functional continuum between stores, plasmalemma, and muscle contraction operates at local, subcellular domains. The fact that even the fast and small [Ca\(^{2+}\)], increases associated with sparks control the plasmalemma voltage (10, 24, 27) provides a dynamic link between Ca\(^{2+}\) stores and multiple plasma membrane Ca\(^{2+}\) channels. If agonists lead to depletion of Ca\(^{2+}\) stores, it is likely that the resultant focal activation of CCE and L-type Ca\(^{2+}\) channels would replenish the stores and initiate muscle contraction.
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