Ca^{2+} sparks and BK currents in gallbladder myocytes. Role in CCK-induced response

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ABSTRACT

We sought to elucidate the regulation of gallbladder smooth muscle (GBSM) excitability by localized Ca\(^{2+}\) release events (sparks) and large-conductance Ca\(^{2+}\)-dependent (BK) channels by determining whether sparks exist in GBSM, and if so, whether they activate BK channels. Sparks were identified in isolated GBSM loaded with fluo-4. Each spark was associated with a transient outward current, suggesting communication of ryanodine receptor-channels (RyRs) with BK channels. This was confirmed by the inhibition of outward currents with iberiotoxin (100 nM), thapsigargin (200 nM), and ryanodine (10 \(\mu\)M). In current clamp mode, the transient BK currents were associated with brief membrane hyperpolarizations (10.9 ± 1.3 mV). As transient BK currents could dampen GBSM excitability, we tested whether CCK attenuates these events. CCK (10 nM) reduced the amplitude and frequency of transient BK currents, and subsequent caffeine application restored transient BK current activity. These results support the concept that RyRs and BK channels play contribute to the regulation of GBSM excitability, and that CCK can act in part by inhibiting this pathway.

Key words: guinea pig, smooth muscle, sarcoplasmic reticulum, ryanodine receptor, thapsigargin, L-type Ca\(^{2+}\) channels, caffeine.
INTRODUCTION

Increases in global intracellular calcium concentration \([\text{Ca}^{2+}]_i\) regulate cellular processes from growth to apoptosis. In smooth muscle cells, elevations in \([\text{Ca}^{2+}]_i\) in response to neurotransmitters or hormones initiate the contractile process. Receptor activation in the plasma membrane causes either activation of non-selective cation channels or G-protein-dependent stimulation of phospholipase C and generation of inositol 1,4,5-triphosphate (IP\(_3\)) or both, depending on the neurotransmitter or hormone binding the receptor (7). The opening of non-selective cation channels induces depolarization and the consequent activation of voltage-operated calcium channels (VOCCs) (5), which causes calcium influx that collaborates to increase \([\text{Ca}^{2+}]_i\). IP\(_3\) binds to its receptor in the sarcoplasmic reticulum, a calcium channel, and induces calcium release from the SR (6). Intracellular calcium stimulates calmodulin-dependent activation of myosin light chain kinase (MLCK) to initiate contraction (39).

Despite this pro-contractile role for \([\text{Ca}^{2+}]_i\) increases, in arterial smooth muscle, localized calcium release events from ryanodine receptors (RyRs), termed calcium sparks, oppose vasoconstriction by activating BK channels (32). Thus, unlike cardiac muscle, where calcium sparks sum to cause a global calcium transient promotes contraction (10), smooth muscle sparks activate BK channels in the nearby plasma membrane (35), resulting in a membrane hyperpolarization. This hyperpolarization closes voltage-dependent Ca\(^{2+}\) channels, and, therefore, leads to a reduction of global \([\text{Ca}^{2+}]_i\) and relaxation (29). The relevance of this mechanism is supported by the observation that inhibition of BK channels with iberiotoxin leads to marked membrane depolarization and vasoconstriction (29, 32).
and reduces the actions of a variety of smooth muscle relaxants (reviewed in 25). Recently, we have shown that the targeted deletion of the gene for the $\beta_1$ subunit of the BK channel leads to a decrease in the calcium sensitivity of BK channels, a reduction in functional coupling of calcium sparks to BK channel activation, and increases in arterial tone and blood pressure (9).

In the gastrointestinal tract, where receptive relaxations of smooth muscles actively contribute to the movement of luminal contents, the presence of calcium sparks coupled to activation of outward currents has been reported (2, 3, 20, 27). Inhibitory neurotransmitters, such as ATP, cause the activation of apamin-sensitive $K^+$ channels that is mediated by localized $Ca^{2+}$ sparks, which could provide a mechanism for coupling ATP to hyperpolarization responses (inhibitory junction potentials) (2). In addition to this, inhibition of resting spark activity and/or outward currents by excitatory neurotransmitters in the gastrointestinal tract (3, 4) induces a decrease in spark events and/or outward currents, which could lead to contraction. The gallbladder shows both relaxing or contractile behaviors during bile storage and bile flow phases. This motor activity is the result of changes in gallbladder smooth muscle contractility in response to excitatory or inhibitory neurotransmitters and hormones (37). However, it is not yet known whether calcium spark activity plays a role in the regulation gallbladder motility.

In this study, we provide the first evidence of calcium sparks in GBSM cells, and we demonstrate functional coupling between $Ca^{2+}$ sparks and BK channel activation. Our data indicate that RyRs are the main source of spontaneous $Ca^{2+}$ transients, and that basal $Ca^{2+}$ influx through L-type calcium channels is necessary for full activation of BK channels. Cholecystokinin (CCK), a contractile agonist of GBSM, causes inhibition of spontaneous
transient BK currents, suggesting that Ca\(^{2+}\) sparks play a physiological role in the regulation of gallbladder excitability.

**MATERIAL AND METHODS**

*Cell isolation.* All procedures were reviewed and approved by the Office of Animal Care Management at the University of Vermont. Guinea pigs (250-350 g) were euthanized by halothane overdose and then exsanguinated. Gallbladder smooth muscle (GBSM) cells were dissociated enzymatically using a method based on that described for the guinea pig gallbladder (15). Briefly, the gallbladder was removed and placed in a Sylgard-coated petri dish containing cold Krebs-Henseleit solution (K-HS; for composition see Solutions and drugs). After removing the mucosa and the connective tissue that attaches the gallbladder to the liver, the gallbladder was cut into small pieces and incubated for 35 min at 37 °C in enzyme solution (ES, for composition see Solutions and drugs) supplemented with 1 mg/ml BSA, 1 mg/ml papain and 0.5 mg/ml dithioerythritol. Then, the tissue was transferred to fresh ES containing 1mg/ml BSA, 1 mg/ml collagenase and 100 µM CaCl\(_2\) and incubated in this solution for 9 min at 37 °C. After finishing the digestion, the tissue was washed three times using ES, and the single cells isolated by passing the muscle pieces through the tip of a fire-polished glass Pasteur pipette several times. The resultant cell suspension was kept in ES at 4 °C until use, generally within 6 h. All experiments were performed at room temperature (22 °C).

*Local \([Ca^{2+}]\) measurements and confocal microscopy*

Confocal images of gallbladder smooth muscle cells were obtained using a laser scanning confocal system (Oz, Noran Instruments, Middleton, WI) interfaced with an Indy workstation
(Silicon Graphics, Mountainview, CA) and Intervision software. The confocal system was mounted in an inverted Diaphot microscope with a x60 water immersion objective (NA 1.2; Nikon). Isolated myocytes were plated in the recording chamber (~1 ml volume) and loaded with the Ca\textsuperscript{2+} sensitive fluorophore fluo 4-AM by incubation in ES containing 5 µM fluo-4 and 2.5 µg/ml pluronic acid in the dark for 30 min at room temperature. Cells were subsequently washed for 30 min with fresh physiological Ca\textsuperscript{2+} bath solution (BS, for composition see Solutions and drugs) to remove fluo-4 from the extracellular fluid and to allow for fluo-4 deesterification. Cells were illuminated with a krypton-argon laser at 488 nm, and emitted light was collected with the confocal photomultiplier tube at wavelengths >515 nm. Images were typically acquired at 120 Hz (320 x 240 pixels or 64 x 48 µM, 8.33 ms/image) for 20 s. Experimental data were stored on compact discs for later analysis.

Image data were analyzed with custom software written by Dr. Adrian Bonev (University of Vermont) using IDL 5.0.2 (Research Systems, Boulder, CO). Baseline fluorescence Fo is the determined by averaging 20 images containing no discernable Ca\textsuperscript{2+} transients. Ratio images (F/Fo) were then constructed and analyzed for 2.2 x 2.2 µm areas where F/Fo increased rapidly. F/Fo vs. Time traces were generated and analyzed for fluorescence increases using Origin software (Microcal Software, Northhampton, MA). Ca\textsuperscript{2+} sparks were defined as local increases in fluorescence of 1.2 F/Fo.

Electrophysiology

Ionic currents were measured in isolated muscle cells using the whole cell, perforated-patch configuration of the patch-clamp technique (23). The cell suspension was transferred to an experimental chamber made with a glass coverslip and mounted on the stage of an inverted microscope (Nikon). After cell sedimentation, the chamber was perfused, using a gravity-fed
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system, with BS (for composition see *Solutions and drugs*). The pipette solution (for composition see *Solutions and drugs*) also contained amphotericin B (100 µg/ml). Cells were perfused with bathing solution throughout the experiment.

For cells used in this study cell capacitance was 29.0 ± 0.7 pF and series resistance was 27.6 ± 0.8 MΩ. Currents were recorded using an Axopatch 200A amplifier (Axon instruments, Foster City, CA) filtered a 1 kHz and digitized at 4 KHz. Characterization of voltage dependence of transient outward currents was performed holding cells at -40, -20 and -10 mV. In some cells, the current clamp configuration was used to record changes in membrane potential generated by the ionic currents. For pharmacological characterization a holding potential of -20 mV was selected and a 4 min period before adding the drugs was used for analysis as control data. To determine the effect of a given drug, a 4-min period within the steady state of the effect was analyzed. Transient outward currents were analyzed using Mini Analysis (Synaptosoft) with an amplitude threshold of three times the unitary BK channel current for guinea pig GBSM at the given holding potential (40).

**Simultaneous current and Ca\(^{2+}\) measurements**

To examine the temporal relationship between Ca\(^{2+}\) sparks and BK channel activation, Ca\(^{2+}\) sparks and whole cell currents, at a holding potential of -20 mV, were measured using the methods described above. A trigger source output on the confocal microscope was used to align the fluorescence and electrical records. These data were analyzed as described above.

**Dissection and contraction recording of guinea pig gallbladder smooth muscle strips**

Gallbladders were isolated from 300-450 g male guinea pigs following deep halothane anesthesia and cervical dislocation, and immediately placed in cold K-HS (for composition see *Solutions and drugs*). Animals were handled in accordance with the guidelines laid down by the
Animal Care and Use Committee of the University of Extremadura. The gallbladder was opened by cutting along the longitudinal axis and trimmed of any adherent liver tissue. After washing with the nutrient solution to remove any biliary component, the mucosa was scraped off and the gallbladder was cut into strips along the longitudinal axis, each strip measuring approximately 3 x 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 connected to a MacLab system consisting of a MacLab hardware unit and software application which run on the Macintosh computer. The strips were placed under an initial resting tension equivalent to 1.5 g load and allowed a 60-min period for equilibration, during which time the nutrient solution was changed every 20 min. After that period, the length of each strip was increased at a rate of 1 mm each time until a maximal response to acetylcholine. The muscle length corresponding to the optimal preload was then determined by increasing the length of each strip in increments of 1 mm until a maximal response to acetylcholine (10 µM) was achieved. The optimal preload length was maintained throughout the duration of the experiments.

The effects of thapsigargin (1 µM) and tetraethylammonium (TEA, 1 mM) on the resting tone was assayed by addition of these agents at stated concentration to the organ bath. To check the effects of 2-APB on Ca²⁺ entry through L-type Ca²⁺ channels, 60 mM KCl was assayed in the absence or presence of 50 µM of 2-APB.

Solutions and drugs

The Krebs-Henseleit solution (K-HS) used in this study contained (in mM): 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃ and 11.5 D-glucose. This solution had a
final pH of 7.35 after equilibration with 95% CO₂-5% O₂. Enzyme solution (ES) used to disperse cells was made up of (in mM) 10 HEPES, 55 NaCl, 5.6 KCl, 80 Na-Glutamate, 2 MgCl₂, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The bath solution (BS) used in patch-clamp studies and in simultaneous recording ones contained (in mM) 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 D-glucose and 10 HEPES, with pH adjusted to 7.4 with NaOH. The pipette solution (PS) used in patch-clamp experiments contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES and 0.05 EGTA, with pH adjusted to 7.2 with NaOH.

Drugs were obtained from the following sources: acetylcholine chloride, amphotericin B, caffeine, dithioerythritol, EGTA, nifedipine, methoxyverapamil hydrochloride (D-600), thapsigargin and tetraethylammonium chloride (TEA) were from Sigma Chemical Co. (St Louis, MO, USA), 2-APB was from Calbiochem (La Jolla, CA, USA), fluo-4 and pluronic acid were from Molecular Probes (Molecular Probes Europe, Leiden, Netherlands), collagenase was from Fluka (Madrid, Spain); papain was from Worthington Biochemical Corporation (Lakewood, NJ, USA), ryanodine was obtained from L.C. Laboratories.

Stock solutions of 2-APB and thapsigargin were prepared in dimethyl sulfoxide (DMSO). The solutions were diluted so that the final concentration of DMSO in the recording chamber was ≤ 0.1% v/v.

**Statistics**

Contractile responses are expressed in absolute values (mN) and/or as percentage of the maximal response elicited by ACh (10 µM). Values are expressed as means ± SEM where applicable. Data were compared using two-tailed Student’s t-tests, where appropriate. \( P < 0.005 \) was considered statistically significant.
RESULTS

Identification of local calcium transients (Ca\textsuperscript{2+} sparks) in GBSM.

Gallbladder myocytes loaded with the calcium sensitive indicator fluo-4 produced spontaneous transient elevations in intracellular calcium concentration. The spatial and temporal characteristics of these events suggested that they correspond to Ca\textsuperscript{2+} sparks that have been described in other types of smooth muscle (25, 32), as well as skeletal (28) and cardiac muscle (10). Spark frequency was of 0.9 ± 0.3 Hz with an average of 1.8 ± 0.4 spark sites per cell. Increases in the relative fluorescence (F/Fo, see Material and Methods) of 1.55 ± 0.02 were detected (n = 127 spark events, from 9 cells). Figure 1 shows pseudocolor images corresponding to the life cycle of a spark in a GBSM cell and the trace of the fluorescence measurements corresponding to the region marked by the colored box. Frequently, the Ca\textsuperscript{2+} transients were clustered into groups consisting of multiple events (see Fig 1 and Fig 2).

It has been proposed that, in smooth muscle, Ca\textsuperscript{2+} sparks serve as local Ca\textsuperscript{2+} signal to activate BK channels in the surface of membrane (reviewed in 25). To examine this hypothesis in GBSM, whole cell membrane currents and [Ca\textsuperscript{2+}]\text{,i} were measured simultaneously in myocytes loaded with fluo-4. To minimize disruption of the cell’s cytoplasm whole cell currents were measured using the perforated patch approach of the patch-clamp technique (23). Figure 2A illustrates simultaneous electrical and fluorescence recordings from a 20 s scan of a GBSM cell held at -20 mV. Each Ca\textsuperscript{2+} spark was associated with a transient activation of outward current. The close temporal relationship between Ca\textsuperscript{2+} sparks and outward currents suggests that in the gallbladder, similar to other smooth muscle preparations (2, 21, 27, 32, 35) Ca\textsuperscript{2+} release from SR in form of sparks activates K\textsuperscript{+} channels in the surface membrane. Unlike vascular and
urinary bladder smooth muscle cells (21, 35), the spark amplitude was not closely related to the amplitude of the transient BK currents ($r^2 = 0.17$) (Fig. 2B). Thus, small amplitude outward currents were not associated with sparks above the detection threshold. Moreover, some large sparks were associated with small current transients and some small sparks generated large current transients.

**Characterization of the transient outward currents**

In smooth muscle cells, $\text{Ca}^{2+}$ increases corresponding to a spark events have been shown to activate transient outward currents, which facilitates a feed-back mechanism that opposes to contraction (25). Therefore, we next characterized the transient outward currents in GBSM and their dependence on $\text{Ca}^{2+}$ release as spark events. The holding potential for these studies was -20 mV.

These transient currents have previously been shown to be due to the activation of BK channels (4, 21, 32, 35), although in colonic myocytes the small conductance calcium-dependent potassium (SK$_{Ca}$) channels also contribute to transient outward currents (2). To determine which type of potassium channels are responsible for these currents in our tissue, we used the selective blocker of BK channels (17, 33), iberiotoxin (100 nM). In GBSM cells, iberiotoxin had a potent and rapid inhibitory effect on the transient currents, causing a 98 % reduction of transient currents ($n = 5$, Fig 3). In the first minute after application, there was a dramatic decrease in current activity in all cells tested, and in 3 out of 5 cells, iberiotoxin at 100 nM caused a total abolition of the currents. These results suggest that, in the GBSM, BK channels are the primary target for $\text{Ca}^{2+}$ sparks.

When GBSM cells were studied in current clamp conditions, we detected transient membrane potential hyperpolarizations (Fig. 4). The mean hyperpolarization was of 10.9 ± 1.3
mV (n = 801 events, from 6 cells), although hyperpolarizations up to 20 mV were observed in all the cells studied. The maximal hyperpolarization recorded was of 37.5 mV. To investigate whether the transient BK currents and associated membrane hyperpolarizations influence GBSM tension, we tested the effects of TEA (1 mM) on GBSM muscle strips. At this concentration, TEA blocks BK channels (33). In these studies, TEA induced a $3.67 \pm 0.55$ mN (n = 7) contraction of gallbladder strips, which was reduced by $89.3 \pm 5.8\%$ when it was tested in the presence of 10 µM methoxyverapamil.

**Calcium dependence of BK currents**

To verify that Ca$^{2+}$ sparks lead to the activation of BK channels in GBSM, we next investigated whether SR Ca$^{2+}$ release is necessary for spontaneous transient BK currents, and which channels in the SR were responsible for this Ca$^{2+}$ release. First of all, gallbladder myocytes were treated with thapsigargin (200 nM), which blocks the SR Ca$^{2+}$-ATPase, resulting in the depletion of SR stores. Figure 5A shows an original record of BK currents from a cell before and after treatment with thapsigargin. Thapsigargin reduced the BK current frequency by 96 % (from $1.82 \pm 0.53$ Hz to $0.03 \pm 0.03$ Hz, n = 4, $P < 0.05$, Fig 5B), indicating that BK channels are activated by SR Ca$^{2+}$ release.

To assess whether release of Ca$^{2+}$ from RyRs contributes to the activation of BK channels, a low concentration of the RyR channel activator, caffeine, was applied in the bathing solution. A typical response to 250 µM caffeine is shown in figure 5C. Caffeine caused a significant increase in the frequency of transient BK currents ($0.57 \pm 0.18$ Hz vs. $1.54 \pm 0.50$ Hz in the absence or presence of caffeine, respectively, $P < 0.001$, n = 6, Fig. 5D). This increase in frequency was associated with an increase in the amplitude of these currents ($34.7 \pm 7.9$ pA vs.
48.6 ± 8.3 pA, n = 6, P < 0.05), which may be due to the increase in the multiple events recorded during caffeine treatment.

To test whether RyR channels mediate Ca\(^{2+}\) sparks, and consequently activate BK currents, GBSM cells were treated with ryanodine (10 µM) at a concentration that inhibits RyRs (25, 32, 36). Figure 5E shows an original recording of BK currents in a single GBSM cell before and after ryanodine treatment. Within 15-20 min of application, ryanodine caused a marked reduction in transient BK current activity. At steady state, ryanodine reduced BK current frequency by 86% (from 2.33 ± 0.54 to 0.18 ± 0.05 Hz, n = 6, P < 0.01, Fig. 5F).

To explore a possible role of IP3, we examined the effects of the membrane permeable inhibitor of the IP3 receptor channels (31), 2-APB, on the spontaneous transient BK currents in GBSM cells. As shown in figure 5G, the presence of 50 µM 2-APB in the bathing solution did not induce any significant change in the frequency of transient BK currents (Fig. 5H). 2-APB (30 µM) did not alter high K+ (60 mM)-induced contractions of gallbladder strips (79.4 ± 11.5 vs. 80.4 ± 6.6% of ACh-induced response in the absence and presence of 50 µM 2-APB, respectively, n = 10, P > 0.05). Taken together, these results indicate that BK currents are caused by brief Ca\(^{2+}\) release events through RyRs in the SR.

**Voltage-dependence of BK currents**

To investigate the voltage-dependence of transient BK currents, GMSM cells were held at different potentials using the perforated-patch configuration of whole cell voltage clamp (see Material and Methods). As shown in figure 6, the frequency and amplitude of BK current was voltage dependent, which is consistent with the voltage dependence previously demonstrated for BK channel activity in other smooth muscle cell types (4, 12, 13, 21). Membrane potential depolarization from −40 mV to −20 mV increased transient BK current frequency by ~3.7 fold,
from 0.37 ± 0.08 Hz to 1.4 ± 0.31 Hz ($P < 0.05$; $n = 1429$ events from 11 cells), and increased current amplitudes of these events by ~2.5 fold, from 14.6 ± 0.7 pA to 36.7 ± 4.6 pA ($P < 0.01$) (Fig. 6). When the holding potential was increased to –10 mV, the frequency of current transients was similar to that recorded at –20 mV (1.41 ± 0.30 Hz; $n = 1324$ events from 11 cells, Fig. 6), but the amplitude increased (58.3 ± 6.1 pA; $P < 0.01$ vs. –20 mV; Fig. 6).

**Calcium entry through L-type Ca$^{2+}$ channels is critical to maintain BK currents**

In cardiac muscle, Ca$^{2+}$ influx through voltage activated Ca$^{2+}$ channels activates RyRs allowing Ca$^{2+}$-induced Ca$^{2+}$ release (as sparks events), which is essential for muscle contraction (30). The communication between these Ca$^{2+}$ channels and RyRs is less clear in smooth muscle, where either dependence or independence of Ca$^{2+}$ sparks or BK currents on Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels have been reported (2, 4, 26). The increase in frequency and amplitude observed in the BK currents at more depolarized holding potentials, as described above, is consistent with a role for VOCCs. To examine the role of VOCCs, we evaluated the effects of the L-type channel blocker, nifedipine (0.5 and 1 µM) on transient BK currents in GBSM cells held at –20 mV. Nifedipine at 0.5 µM and 1 µM caused similar reductions in the frequency of current transients (2.50 ± 1.21 to 0.87 ± 0.61 Hz for 0.5 µM nifedipine, 79% inhibition, $n = 4$, $P < 0.05$; and 2.45 ± 0.89 to 0.42 ± 0.15 Hz for 1 µM nifedipine, 81% inhibition, $n = 5$, $P < 0.05$ vs. control, Fig. 7B). Nifedipine did not modify current amplitude (46.2 ± 13.8 vs. 47.4 ± 15.2 pA for 0.5 µM nifedipine, $n = 4$, $P = 0.702$; and 51.2 ± 5.6 vs. 53.4 ± 7.2 pA for 1 µM nifedipine, $n = 5$, $P = 0.649$). When nifedipine effect had reached the steady-state, application of caffeine (250 µM) induced an increase in both the frequency and amplitude of the currents (see Fig 7A), indicating that the stores still had sufficient Ca$^{2+}$ to induce BK channel activation.
CCK inhibits BK currents

In the gallbladder, cholecystokinin (CCK)-induced contraction involves the release of Ca\(^{2+}\) from intracellular stores as well as calcium influx through L-type calcium channels (1, 34, 38). As the mechanisms responsible for Ca\(^{2+}\) influx in GBSM have not yet been explored, and increased PKC activity suppresses BK currents in vascular smooth muscle (8), we tested whether CCK altered transient BK currents in GBSM. As demonstrated in figure 8, CCK (10 nM) reduced both the frequency and amplitude of these currents (3.2 ± 0.8 vs. 1.2 ± 0.5 Hz, 62% of inhibition, \(P < 0.005\) for frequency and 42.2 ± 5.4 vs. 30.0 ± 4.1 pA, 23% of inhibition, \(P < 0.01\) for amplitude; \(n = 13\) cells for both). In cases in which CCK caused a complete inhibition of transient BK currents, the currents did not reappear until 12.5 ± 3.2 min (\(n = 3\)) following washout of CCK. Caffeine (1 mM) added after CCK treatment induced a burst of transient currents, indicating that the Ca\(^{2+}\) stores were not depleted (\(n = 3\)).
DISCUSSION

The purpose of this investigation was to establish whether localized increases in $[\text{Ca}^{2+}]_i$ occur in GBSM, and if so, how they are generated and whether they lead to the activation of transient $K^+$ currents. In smooth muscle, $\text{Ca}^{2+}$ sparks are primarily caused by the coordinated opening of a cluster of RyRs in the SR (19, 41) and activate a number of BK channels to cause macroscopic BK currents (32). A link between $\text{Ca}^{2+}$ sparks and transient BK currents has been confirmed by simultaneous optical and electrical measurements in these cells (35). Localized $\text{Ca}^{2+}$ release events, termed $\text{Ca}^{2+}$ puffs, that are mediated by IP$_3$ receptor-operated channels, have been reported in colonic smooth muscle (3), where these events also regulate membrane $\text{Ca}^{2+}$-dependent potassium channels (2, 3).

In the current study, we provide the first demonstration that localized increases in $[\text{Ca}^{2+}]_i$ occur in GBSM. These increases in $[\text{Ca}^{2+}]_i$ are transient and do not lead to an elevation in global $[\text{Ca}^{2+}]_i$ ($\text{Ca}^{2+}$ sparks). $\text{Ca}^{2+}$-dependent conductances are likely targets for localized $[\text{Ca}^{2+}]_i$ elevations. The $\text{Ca}^{2+}$ sparks in GBSM are caused by opening of ryanodine-sensitive $\text{Ca}^{2+}$ release channels (RyR) in the SR.

*Calcium sparks activate BK currents in GBSM cells*

In GBSM, BK channels appeared to be the primary $K^+$ channel type activated by calcium sparks, since the transient outward currents were inhibited by 98% by the specific blocker of BK channels (17, 33), iberiotoxin. However, in colonic myocytes, local $\text{Ca}^{2+}$ transients stimulate both BK channels and small conductance calcium-dependent channels (SK$_{Ca}$) (2). In gallbladder, we have found that the selective inhibitor of SK$_{Ca}$ channels, apamin, has no effect on muscle strip tension, or resting membrane potential or action potential properties (Pozo,
Nelson and Mawe, unpublished observations), suggesting that SK\textsubscript{Ca} channels do not play a prominent role in the regulation of gallbladder tone.

The Ca\textsuperscript{2+} spark-activated transient BK current in GBSM caused a transient hyperpolarization up to 37 mV, similar to that described for arterial myocytes (18). Taking into account the high frequency of BK currents, even in resting conditions, spontaneous Ca\textsuperscript{2+} sparks and the resultant hyperpolarizations could decrease GBSM excitability by decreasing the open-state probability of VOCCs. Consistent with this hypothesis, inhibition of BK currents with the blocker of K\textsuperscript{+} channels tetraethyl ammonium (TEA), at a dose that inhibits BK channels (1 mM) (17), induced a methoxyverapamil-sensitive contraction of gallbladder strips.

In the current study, while a temporal coupling between Ca\textsuperscript{2+} and spontaneous transient BK currents was observed, the correlation between spark and BK current amplitude was not very high. In other systems, including urinary bladder, vascular and colonic smooth muscle, a strong correlation between spark and BK current amplitudes has been reported (2, 21, 36). However, a weak correlation between outward current and spark amplitude has been reported in cells from the stomach muscularis of Bufo Marinus (41) and feline esophageal smooth muscle (27). It has been proposed that if \([\text{Ca}^{2+}]\) in the spark micro-domain rapidly reaches the steady-state, the open probability for K\textsuperscript{+} channels (\(P_0\)) will be one. Therefore, many of the BK channels would be saturated with Ca\textsuperscript{2+}, and correlation between spark and BK amplitudes would not be observed. Alternatively, the density of BK channels could be quite variable, which would also affect the apparent correlation. We found that the transient BK current amplitude increased with membrane potential depolarization in a manner consistent with an increase in the K\textsuperscript{+} driving force. If, during a spark, the BK channels were not maximally activated with spark Ca\textsuperscript{2+}, then the elevation in transient BK current amplitude with membrane potential depolarization should increase more than expected for simple changes in the driving force for K\textsuperscript{+}. The amplification
of the effect of membrane depolarization on transient BK current amplitude is caused by the increase in the apparent Ca\(^{2+}\) sensitivity of the BK channel caused by membrane depolarization (see reference 2). Therefore, our results are consistent with the model that Ca\(^{2+}\) sparks maximally activate nearby BK channels in GBSM.

**Voltage dependence of BK currents**

Consistent with the voltage dependence that has been established for single BK channels (12, 13), the amplitude of transient BK currents in GBSM was enhanced at depolarized voltages. This increase probably involves the increased K\(^{+}\) driving force at these voltages. Membrane potential depolarization also increased Ca\(^{2+}\) spark frequency, which was dependent on Ca\(^{2+}\) entry through VOCC. This elevation in Ca\(^{2+}\) entry would increase cytoplasmic and SR Ca\(^{2+}\), both of which elevate Ca\(^{2+}\) spark frequency (21, 26).

**L-type Ca\(^{2+}\) channels are critical to maintain BK currents in GBSM**

Our data suggest that Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is essential to maintain BK currents in the gallbladder, since nifedipine and membrane potential hyperpolarization reduced the frequency of these currents. Activation of L-type voltage channels by depolarization increases the frequency and amplitude of Ca\(^{2+}\) sparks in vascular (26) and urinary bladder smooth muscle (21), which increases the amplitude and frequency of BK currents. The increase in spark activity could be due to elevations in local [Ca\(^{2+}\)] and the concomitant increase in RyRs open probability (22) or increases in SR load (42). In cardiac muscle, Ca\(^{2+}\) sparks are induced by membrane potential-dependent entry of Ca\(^{2+}\) through sarcolemmal L-type Ca\(^{2+}\) channels at the transverse tubules (30). RyR channels are positioned in junctional SR elements within short
distances (~20 nM) of voltage-dependent Ca\(^{2+}\) channels in the T-tubes, and high local [Ca\(^{2+}\)] is in the level required for significant RyR channel activation (for review see 16). Although smooth muscle cells lack the transverse tubular membrane system, L-type Ca\(^{2+}\) channels co-localize with junctional SR (14), and Ca\(^{2+}\) entry through these channels would increase in the microdomain of RyRs increasing their probability of opening. However, Collier et al. (11) have provided evidence that VOCC and RyRs are distant since there is a considerable time lag between activation of VOCC and subsequent increases in spark probability. It is also possible that the rise in average cytoplasmic Ca\(^{2+}\) is sufficient to activate Ca\(^{2+}\) sparks. On the other hand, the reduction of BK current amplitude exerted by nifedipine pretreatment could be due to a decrease in SR luminal [Ca\(^{2+}\)].

**CCK inhibits BK currents**

In many types of smooth muscle, excitatory agonists have been shown to act at least in part by inhibiting K\(^{+}\) conductances. In vascular and gastrointestinal smooth muscle, excitatory agonists that, like CCK, mediate their responses through PLC and PKC activation, cause reductions in the amplitude and frequency of Ca\(^{2+}\) sparks (24), and activators of PKC decrease the activity of Ca\(^{2+}\) sparks and BK currents through an inhibitory effect on RyR channels (3, 8). In the present study we found that CCK caused a reduction in the amplitude and frequency of transient BK currents. These findings indicate that reducing the activation of BK channels, which are presumably active under resting tone conditions, contributes to the excitatory effect of CCK.

The CCK-induced reduction of BK currents in the gallbladder could reflect a reduction in Ca\(^{2+}\) sparks associated with a decrease in the Ca\(^{2+}\) sensitivity of RyR channels caused by PKC. It is unlikely that a reduction in SR load decreases BK current activity, because subsequent
application of caffeine immediately restored transient BK currents, indicating that SR maintains enough Ca\(^{2+}\) to generate Ca\(^{2+}\) sparks through RyR channels in the presence of CCK.

In the colon, agonist-induced inhibition of transient BK currents is thought to involve an inhibitory effect of Ca\(^{2+}\) entry through receptor-operated cation channels on IP\(_3\) receptor-operated Ca\(^{2+}\) events (3). This mechanism is unlikely to be involved in the GBSM response to CCK, since transient BK currents are RyR-mediated and since CCK-induced gallbladder contraction is mediated, in part, by Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (1, 1, 34, 38). Moreover, in our study, suppression of Ca\(^{2+}\) influx by nifedipine caused a reduction in BK currents, indicating a significant role for Ca\(^{2+}\) influx in spark activity and BK currents, whereas, in colon, these authors reported no effects for nicardipine when tested neither in control situations nor under stimulation with ACh (3).

**Concluding remarks**

In summary, this study provides the first evidence of the presence of local Ca\(^{2+}\) transients or Ca\(^{2+}\) sparks in gallbladder smooth muscle cells. These Ca\(^{2+}\) sparks, which are mainly due to the activation of RyR in the SR, are potentially coupled to activation of BK channels. The spontaneous transient activation of BK currents causes transient membrane hyperpolarizations, providing a Ca\(^{2+}\)-mediate mechanism to decrease excitability. Inhibition of these events by the excitatory agonist CCK indicates that decoupling between Ca\(^{2+}\) sparks and BK channels can also contribute to the increased excitability in response to these agonists.

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Figure 1.- Identification of Ca\textsuperscript{2+} sparks in GBSM cells.

A.- Original sequence of three-dimensional confocal images, obtained when indicated, of an entire smooth muscle cell illustrating the time course of the fractional increase in fluorescence (F/Fo, where F is the instantaneous fluorescence at a given time point and Fo is baseline fluorescence) and the decay of a typical spark. The images are color coded as indicated by the bar. Colored box shows the region of the cell whose changes in fluorescence are represented in B. B.- Time course of changes in relative fluorescence in the 2.2 x 2.2 μm area included in the colored box. * Spark shown in A.
Figure 2.- Ca²⁺ sparks generate transient BK currents in GBSM cells

A.- Original recordings of whole cell membrane currents and Ca²⁺ sparks from a GBSM cell held at –20 mV. Every Ca²⁺ spark is associated with a simultaneous BK current. Small amplitude BK currents are not associated to detectable Ca²⁺ sparks. B.- Plot of relationships between BK current and Ca²⁺ spark amplitudes. The correlation between both parameters is very low (correlation coefficient 0.17). In green, BK currents below the level used for evaluation of transient BK currents that caused detectable Ca²⁺ sparks.
Figure 3.- Spontaneous transient outward currents in GBSM cells are caused by activation of BK channels.

A.- Original recording of whole cell currents in a GBSM cell held at –20 mV before and after the treatment with the blocker of BK channels, iberiotoxin (100 nM). To better appreciate the inhibition caused by iberiotoxin, some parts of the recordings are shown in expanded scales. B.- Summarized data of the effect of iberiotoxin on the frequency of the whole cell currents. Histograms are means ± S.E.M. n = 5 cells. P < 0.05 vs. control.
Figure 4. BK currents hyperpolarize GBSM cells

_A._ BK currents in a GBSM cell recorded by voltage clamp configuration of the patch-clamp technique. The holding potential was –10 mV. _B._ Changes in membrane potential associated to these currents when switched to current clamp configuration. This cell had a resting membrane potential of –21 mV.
Figure 5.- Spontaneous transient BK currents in GBSM cells depend on ryanodine receptor (RyR)-mediated Ca\(^{2+}\) release from the sarcoplasmic reticulum

A.- Original recording of whole cell currents before and after the treatment with the SR Ca\(^{2+}\) ATPase inhibitor, thapsigargin (200 nM), showing the inhibition of the current activity exerted by thapsigargin. B.- Summarized data of the effect of thapsigargin on the frequency of the whole cell currents. Histograms are means ± S.E.M. n = 4 cells. C.- Original recording of whole cell currents before and after activation of RyRs by caffeine (250 µM), showing the increase in the current activity induced by this treatment. D.- Summarized data of the effect of caffeine on the frequency of the whole cell currents. Histograms are means ± S.E.M. n = 6 cells. E.- Original recording of whole cell currents before and after application of 10 µM ryanodine, a dose that blocks RyRs. Ryanodine treatment reduced BK currents. F.- Summarized data of the effect of ryanodine on the frequency of the whole cell currents. Histograms are means ± S.E.M. n = 6 cells. G.- Original recording of whole cell currents before and after the treatment with the IP3 receptor antagonist 2-APB (50 µM). This treatment induced no significant modification of the frequency of the transients but the amplitude of them was decreased. H.- Summarized data of the effect of 2-APB on the frequency of the whole cell currents. Histograms are means ± S.E.M. n = 6 cells. The duration of the treatments is indicated by the solid bars. All experiments were performed at a holding potential of –20 mV. * P < 0.05; ** P < 0.01 vs. control.
Figure 6. Membrane potential dependence of transitory BK currents in GBSM cells.

A. Original traces showing recordings of transitory BK currents in one cell held at –10, –20 and –40 mV. Note that frequency and amplitude of the events increase at higher holding potentials.

B. Summarized data for frequency and amplitude of BK currents at the different holding potentials. Histograms represent mean ± SEM, n = 10 cells for holding potential of –40 and n = 11 cells for holding potentials of –20 and –10 mV. * P < 0.05 vs –40 mV; ** P < 0.01 vs. –40 mV.
Figure 7.- L-type Ca\textsuperscript{2+} currents are necessary to maintain BK currents.

A.- Representative trace of whole cell currents in a GBSM cell before and after the blockade of L-type Ca\textsuperscript{2+} channels with 1 µM nifedipine. Once the effect of nifedipine was stable, cells were exposed to caffeine (250 µM) to activate Ca\textsuperscript{2+} release through RyRs, which induced an increase in the BK currents recorded. B.- Summarized data of the effect of nifedipine (0.5 and 1 µM) on the frequency and amplitude of the whole cell currents. Histograms are means ± S.E.M. n = 4 and 5 cells for 0.5 and 1 µM nifedipine, respectively. The duration of the treatments is indicated by the solid bars. All experiments were performed at a holding potential of –20 mV.
Figure 8.- CCK inhibits BK currents

Original recording showing the CCK-induced inhibition of whole cell BK currents. Application of 1 mM caffeine immediately after CCK treatment induced a rapid burst of transitory currents, indicating that the caffeine-sensitive stores were not empty B and C.

Summarized data of the effects of CCK on the frequency (B) and amplitude (C) of the whole cell currents. Histograms are means ± S.E.M. n = 14 cells. The duration of the treatments is indicated by the solid bars. All experiments were performed at a holding potential of –20 mV. * P < 0.05; ** P < 0.01 vs. control. In the cell shown in A, CCK caused a 44.3% decrease in frequency, and a 48.6% decrease in the amplitude of transient outward currents.