Ca\textsuperscript{2+} sparks and BK currents in gallbladder myocytes: role in CCK-induced response

MÁRÍA J. POZO, GUILLERMO J. PÉREZ, MARK T. NELSON, AND GARY M. MAWE

Department of Physiology, University of Extremadura, 10071 Cáceres, Spain; and Department of Pharmacology and Department of Anatomy and Neurobiology, University of Vermont, Burlington, Vermont 05405

Received 27 July 2001; accepted in final form 17 September 2001

Pozo, María J., Guillermo J. Pérez, Mark T. Nelson, and Gary M. Mawe. Ca\textsuperscript{2+} sparks and BK currents in gallbladder myocytes: role in CCK-induced response. Am J Physiol Gastrointest Liver Physiol 282: G165–G174, 2002. –We sought to elucidate the regulation of gallbladder smooth muscle (GBSM) excitability by localized Ca\textsuperscript{2+} release events (sparks) and large-conductance Ca\textsuperscript{2+}-dependent (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 hyperpolarization (10.9 ± 1.3 mV). Because 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 contribute to the regulation of GBSM excitability and that CCK can act in part by inhibiting this pathway.

Addresses for reprint requests and other correspondence: G. M. Mawe, Given Bldg. C423, Dept. of Anatomy and Neurobiology, Univ. of Vermont, Burlington, VT 05405 (E-mail: gmawe@zoo.uvm.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
gallbladder shows both relaxing and contractile behavior during bile storage and bile flow phases. This motor activity is the result of changes in gallbladder smooth muscle (GBSM) contractility in response to excitatory or inhibitory neurotransmitters and hormones (37). However, it is not yet known whether Ca\textsuperscript{2+} spark activity plays a role in the regulation of gallbladder motility.

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

**MATERIALS 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. GBSM cells were dissociated enzymatically using a method based on that described previously for the guinea pig gallbladder (15). Briefly, the gallbladder was removed and placed in a Sylgard-coated petri dish containing cold Krebs-Henseleit solution (KHS; for composition, see Solutions and drugs). After the mucosa and the connective tissue that attaches the gallbladder to the liver were removed, 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. The tissue was then transferred to fresh ES containing 1 mg/ml BSA, 1 mg/ml collagenase, and 100 \textmu M CaCl\textsubscript{2} and incubated in this solution for 9 min at 37°C. After the digestion was finished, the tissue was washed three times using ES, and single cells were 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\textsuperscript{2+}) measurements and confocal microscopy.** Confocal images of GBSM cells were obtained using a laser scanning confocal system (Oz, Noran Instruments, Middlesex, Mountainview, CA) interfaced with an Indy workstation (Silicon Graphics, Mountainview, CA) and Intervision software. The confocal system was mounted in an inverted Diaphot microscope with a ×60 water-immersion objective (NA 1.2; Nikon). Isolated myocytes were plated in the recording chamber (vol, ~1 ml) and loaded with the Ca\textsuperscript{2+}-sensitive fluorophore fluo 4-AM by incubation in ES containing 5 \textmu M fluo 4 and 2.5 \textmu M pluronide acid for 30 min in the dark 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 wavelength >515 nm. Images were typically acquired at 120 Hz (320 × 240 pixels or 64 × 48 \mu M, 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 Interactive Data Language version 5.0.2 (Research Systems, Boulder, CO). Baseline fluorescence \(F_{0}\) was determined by averaging 20 images containing no discernable Ca\textsuperscript{2+} transients. Ratio images \(F/F_{0}\), where \(F\) is instantaneous fluorescence at a given time point were then constructed and analyzed for areas of 2.2 × 2.2 \mu m in which \(F/F_{0}\) increased rapidly. \(F/F_{0}\) vs. time traces were generated and analyzed for fluorescence increases using Origin software (Microcal Software, Northampton, MA). Ca\textsuperscript{2+} sparks were defined as local increases in fluorescence of 1.2 \(F/F_{0}\).

**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. The bath solution (BS) was replaced by SBS (for composition, see Solutions and drugs). Single cell patch-pipettes were prepared from borosilicate glass capillaries (World Precision Instruments) and polished to a tip diameter of 1–2 \mu m. Patch pipettes were filled with a solution with the following composition (in mM): 140 Cs-gluconate, 10 CsCl, 2 MgCl\textsubscript{2}, 0.5 CaCl\textsubscript{2}, 10 Hepes, pH 7.4 adjusted with CsOH. Single cell recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). The patch pipette was connected to a MacLab system consisting of a MacLab hard-drive (AD Instruments, Colorado Springs, CO) and a Digidata 1440A data acquisition module (Axon Instruments). The data were acquired using pClamp 10 software (Axon Instruments) and filtered at 1 kHz and digitized at 4 kHz. The reversal potential of transient outward currents was measured using the Mini Analysis program (Synaptosoft) with an amplitude threshold of 200 \mu V. The data were analyzed as described above.

**Dissection and contraction recording of guinea pig GBSM strips.** Gallbladders were isolated from 300- to 450-g male guinea pigs following deep halothane anesthesia and cervical dislocation and immediately placed in cold KHS (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 being washed 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 measured ~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% O\textsubscript{2}-5% CO\textsubscript{2}. Isometric contractions were measured using force-displacement transducers connected to a MacLab system consisting of a MacLab hard-
ware unit and software application that runs on the Macintosh computer. The strips were placed under an initial resting tension equivalent to a 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 ACh occurred. 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 ACh (10 μM) was achieved. The optimal preload length was maintained throughout the duration of the experiments.

The effects of thapsigargin (1 μM) and tetraethylammonium chloride (TEA; 1 mM) on the resting tone were assayed by adding these agents at the stated concentrations to the organ bath. To check the effects of 2-aminoethoxydiphenylborate (2-APB) on Ca^{2+} entry through L-type Ca^{2+} channels, KCl (60 mM) was assayed in the absence or presence of 50 μM of 2-APB.

**Methods**

The solution was (in mM) 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, with pH 7.4. In other experiments containing (in mM) 110 potassium aspartate, 30 KCl, 2.2 MgCl₂, 10 D-glucose, and 10 HEPES, with pH adjusted to 7.3 with NaOH. The KHS used in this study 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 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. ACh chloride, amphetamine B, caffeine, dithioerythritol, EGTA, nifedipine, methoxysperamid hydrochloride (D-600), thapsigargin, and TEA were from Sigma Chemical (St. Louis, MO). 2-APB was from Calbiochem (La Jolla, CA), and fluo 4 and pluronic acid were from Molecular Probes (Leiden, The Netherlands). Collagenase was from Fluka (Madrid, Spain), papain was from Worthington Biochemical (Lakewood, NJ), and ryanodine was from L.C. Laboratories.

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

**Statistics.** Contractile responses are expressed in absolute values (in mN) and/or as the percentage of the maximal response elicited by ACh (10 μM). Values are expressed as means ± SE where applicable. Data were compared using two-tailed Student’s t-tests, where appropriate. *P* < 0.05 was considered statistically significant.

**Results**

Identification of Ca^{2+} sparks in GBM. Gallbladder myocytes loaded with the Ca^{2+}-sensitive indicator fluo 4 produced spontaneous transient elevations in [Ca^{2+}], which are color coded as indicated by the bar. Colored box shows the region of the cell in which changes in fluorescence are indicated in B. B: time course of changes in relative fluorescence in the 2.2 × 2.2-μm area indicated in A. *Corresponding spark.

**Characterization of transient outward currents.** In smooth muscle cells, Ca^{2+} increases corresponding to events, from 9 cells). Figure 1 shows pseudocolor images corresponding to the life cycle of a spark in a GBSM cell and the image corresponding to the boxed region. Frequently, the Ca^{2+} transients were clustered into groups consisting of multiple events (Figs. 1 and 2).

It has been proposed that in smooth muscle Ca^{2+} sparks serve as local Ca^{2+} signals, where the first of these views suggests that in the gallbladder, similar to other smooth muscle preparations (2, 21, 27, 32, 35), Ca^{2+} release from SR in the form of sparks activates K⁺ 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 (correlation coefficient = 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 transient currents and some small sparks generated large current transients.

**Characterization of transient outward currents.** In smooth muscle cells, Ca^{2+} increases corresponding to

Fig. 1. Identification of Ca^{2+} sparks in gallbladder smooth muscle (GBSM) cells. A: original sequence of 3-dimensional confocal images, obtained at times indicated, of an entire smooth muscle cell, depicting the time course of the fractional increase in fluorescence (F/F₀), where F is the instantaneous fluorescence at a given time point and F₀ is baseline fluorescence) and the decay of a typical spark. Images are color coded as indicated by the bar. Colored box shows the region of the cell in which changes in fluorescence are indicated in B. B: time course of changes in relative fluorescence in the 2.2 × 2.2-μm area indicated in A. *Corresponding spark.

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Ca2+ sparks and BK currents in gallbladder SPARKS AND BK CURRENTS IN GALLBLADDER

Fig. 2. Ca2+ sparks generate transient large-conductance Ca2+-dependent (BK) currents in GBSM cells. A: original recordings of whole cell membrane currents and Ca2+ sparks from a GBSM cell held at −20 mV. Each Ca2+ spark is associated with a simultaneous BK current. Small-amplitude BK currents are not associated with 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). ○, BK currents below the level used for evaluation of transient BK currents that caused detectable Ca2+ sparks.

spark events have been shown (25) to activate transient outward currents, facilitating a feedback mechanism that opposes contraction. Therefore, we next characterized the transient outward currents in GBSM and their dependence on Ca2+ 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 Ca2+-dependent K+ (SKCa) channels also contribute to transient outward currents (2). To determine which types of K+ channels are responsible for these currents in GBSM, we usediberiotoxin (100 nM), which selectively blocks BK channels (17, 33). 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 three of five 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 Ca2+ sparks.

When GBSM cells were studied in current clamp conditions, we detected transient membrane potential hyperpolarizations (Fig. 4). The mean hyperpolarization was 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 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 strips. At this concentration, TEA blocks BK channels (33). In these studies, TEA induced a contraction of 3.67 ± 0.55 mN (n = 7) in gallbladder strips, which was reduced by 89.3 ± 5.8% when tested in the presence of 10 μM methoxyverapamil.

Ca2+ dependence of BK currents. To verify that Ca2+ sparks lead to the activation of BK channels in GBSM, we next investigated whether SR Ca2+ release is necessary for spontaneous transient BK currents and which SR channels are responsible for this Ca2+ release. First, gallbladder myocytes were treated with thapsigargin (200 nM), which blocks the SR Ca2+-ATPase, depleting 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 ± 0.53 to 0.03 ± 0.03 Hz, n = 4, P < 0.05, Fig. 5B), indicating that BK channels are activated by SR Ca2+ release.

To assess whether release of Ca2+ from RyRs contributes to the activation of BK channels, a low concentration of the RyR channel activator caffeine was applied in the BS. A typical response to 250 μM caffeine is shown in Fig. 5C. Caffeine caused a significant increase in the frequency of transient BK currents (0.57 ± 0.18 vs. 1.54 ± 0.50 Hz in the absence and presence of caffeine, respectively, P < 0.05, n = 6, Fig. 5D). This increase in frequency was associated with an increase in the amplitude of these currents (34.7 ± 7.9 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 Ca2+ sparks and consequently activate BK currents, we treated GBSM cells 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 inositol 1,4,5-trisphosphate (IP3), we examined the effects of the membrane-permeable inhibitor of IP3 receptor channels (31), 2-APB, on the spontaneous transient BK currents in GBSM cells. As shown in Fig. 5G, the presence of 50 μM 2-APB in the BS did not induce any significant change in the frequency of transient BK currents (Fig. 5H). 2-APB (50 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). 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, GBSM cells were held at different potentials using the perforated-patch configuration of whole cell voltage clamp (see MATERIALS AND METHODS). As shown in Fig. 6, the frequency and amplitude of BK current were 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 to −20 mV increased transient BK current frequency by ∼3.7-fold, from 0.37 ± 0.08 to 1.4 ± 0.31 Hz (P < 0.05; n = 1,429 events from 11 cells), and increased current amplitudes of these events by ∼2.5-fold, from 14.6 ± 0.7 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 = 1,324 events from 11 cells, Fig. 6), but the amplitude increased (58.3 ± 6.1 pA; P < 0.01 vs. −20 mV; Fig. 6).

**Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels is critical to maintaining BK currents.** In cardiac muscle, Ca\(^{2+}\) influx through voltage-activated Ca\(^{2+}\) channels activates RyRs, allowing Ca\(^{2+}\)-induced Ca\(^{2+}\) release (as spark 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 has 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 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 the effect of nifedipine reached steady state, the application of caffeine (250 μM) induced an increase in both the fre-
Fig. 5. Spontaneous transient BK currents in GBSM cells depend on ryanodine receptor (RyR)-mediated Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). A: original recording of whole cell currents before and after 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. Values are means ± SE; 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. Values are means ± SE; 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. Values are means ± SE; n = 6 cells. G: original recording of whole cell currents before and after treatment with the inositol 1,4,5-trisphosphate receptor antagonist 2-aminoethoxydiphenylborate (2-APB; 50 μM). This treatment induced no significant modification of the frequency of the transients but the amplitude was decreased. H: summarized data of the effect of 2-APB on the frequency of the whole cell currents. Values are means ± SE; n = 6 cells. The treatment duration 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.

The purpose of this investigation was to establish whether localized increases in [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, 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 Ca\(^{2+}\) sparks and transient BK currents has been confirmed

CCK inhibits BK currents. In the gallbladder, CCK-induced contraction involves the release of Ca\(^{2+}\) from intracellular stores as well as Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (1, 34, 38). Because the mechanisms responsible for Ca\(^{2+}\) influx in GBSM have not yet been explored and increased protein kinase C activity suppresses BK currents in vascular smooth muscle (8), we tested whether CCK altered transient BK currents in GBSM. As demonstrated in Fig. 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 ± 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) after 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 [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, 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 Ca\(^{2+}\) sparks and transient BK currents has been confirmed
by simultaneous optical and electrical measurements in these cells (35). Localized Ca\(^{2+}\)/H\(^{11001}\) release events, termed Ca\(^{2+}\)/H\(^{11001}\) puffs, that are mediated by IP\(_{3}\) receptor-operated channels, have been reported (3) in colonic smooth muscle, where these events also regulate membrane Ca\(^{2+}\)/H\(^{11001}\)-dependent K\(^{+}\)/H\(^{11001}\) channels (2, 3).

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

**Ca\(^{2+}\) sparks activate BK currents in GBSM cells.** In GBSM, BK channels appeared to be the primary K\(^{+}\) channel type activated by Ca\(^{2+}\) sparks, since the transient outward currents were inhibited 98% by iberiotoxin, the specific blocker of BK channels (17, 33). However, in colonic myocytes, local Ca\(^{2+}\) transients stimulate both BK and SK Ca channels (2). In the gallbladder, we have found that apamin, the selective inhibitor of SK\(_{Ca}\) channels, has no effect on muscle strip tension, resting membrane potential, or action potentials.

**Fig. 6.** Membrane potential dependence of transitory BK currents in GBSM cells. *A*: original traces showing recordings of transitory BK currents in 1 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 different holding potentials. Values are means ± SE; 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.

**Fig. 7.** L-type Ca\(^{2+}\) currents and tyrosine phosphorylation 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\(^{2+}\) channels with 1 \(\mu\)M nifedipine. Once the effect of nifedipine was stable, cells were exposed to caffeine (250 \(\mu\)M) to activate Ca\(^{2+}\) release through RyRs, which induced an increase in the BK currents recorded. *B*: summarized data of the effect of nifedipine (0.5 and 1 \(\mu\)M) on the frequency and amplitude of the whole cell currents. Values are means ± SE; n = 4 cells for 0.5 \(\mu\)M nifedipine and n = 5 cells for 1 \(\mu\)M nifedipine. Experiments were performed at a holding potential of -20 mV.
potential properties (Pozo, Nelson, and Mawe, unpublished observations), suggesting that SK_{Ca} channels do not play a prominent role in the regulation of gallbladder tone.

The Ca^{2+} spark-activated transient BK current in GBSM caused a transient hyperpolarization up to 37 mV, similar to that described previously for arterial myocytes (18). Taking into account the high frequency of BK currents, even in resting conditions, spontaneous Ca^{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 K^{+} channel blocker TEA, at a dose that inhibits BK channels (1 mM) (17), induced a methoxyverapamil-sensitive contraction of gallbladder strips.

In the current study, although a temporal coupling between Ca^{2+} and spontaneous transient BK currents was observed, the correlation between spark and BK currents was not very high. In other systems, including the urinary bladder and colonic smooth muscle, a strong correlation between spark and BK current amplitudes has been reported (12, 13). 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 [Ca^{2+}]_{i} in the spark microdomain rapidly reaches steady state, the open probability for K^{+} channels will be 1. Therefore, many of the BK channels would be saturated with Ca^{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^{+} driving force. If, during a spark, the BK channels are not maximally activated with spark Ca^{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^{+}. 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 (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 increases cytoplasmic and SR Ca^{2+}, both of which elevate Ca^{2+} spark frequency (21, 26).

L-type Ca^{2+} channels are critical to maintaining BK currents in GBSM. Our data suggest that Ca^{2+} influx through L-type Ca^{2+} channels is essential to maintaining 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 RyR open probability (22) or increases in SR load (42). In cardiac muscle, Ca^{2+} sparks are induced by mem-

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Fig. 8. CCK inhibits BK currents. A: original recording showing the CCK-induced inhibition of whole cell BK currents. CCK caused a 44.3% decrease in frequency and a 48.6% decrease in the amplitude of transient outward currents. Application of 1 mM caffeine (Caff) 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. Values are means ± SE; n = 13 cells. The treatment duration is indicated by the solid bars. All experiments were performed at a holding potential of -20 mV. *P < 0.05, **P < 0.005 vs. control.
brane 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 transverse tubules, and high local [Ca\(^{2+}\)] is in the level required for significant RyR channel activation (for review, see Ref. 16). Although smooth muscle cells lack the transverse tubular membrane system, L-type Ca\(^{2+}\) channels colocalize 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 phospholipase C and protein kinase C activation reduce the amplitude and frequency of Ca\(^{2+}\) sparks (24), and activators of protein kinase C 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 protein kinase C. 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-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, because transient BK currents are RyR mediated and 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 the colon no effects were reported (3) for nicardipine when tested under control conditions or during ACh stimulation.

**Concluding remarks.** In summary, this study provides the first evidence of the presence of local Ca\(^{2+}\) transients or Ca\(^{2+}\) sparks in GBSM 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\(^{2+}\)-mediated 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.

This work was supported by National Institutes of Health Grants NS-26985, DK-45410 (G. M. Mawe), HC-44455, HL-63722, and DK-53822 (M. T. Nelson) and Ministry of Education and Science of Spain Grant DGES-PB97-0370 (M. J. Pozo).

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