Calcium waves in intact guinea pig gallbladder smooth muscle cells

Onesmo B. Balemba, Thomas J. Heppner, Adrian D. Boney, Mark T. Nelson, and Gary M. Mawe. Calcium waves in intact guinea pig gallbladder smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 291: G717–G727, 2006. —Intracellular Ca2+ waves and spontaneous transient depolarizations were more prevalent in FT than in isolated chronization of SR Ca2+ excitability involves PLC-dependent augmentation and syn-

gastricular Ca2+ layer [full thickness (FT)] by laser confocal imaging of intracel-

lular Ca2+ and voltage recordings with microelectrodes, respectively. Spontaneous Ca2+ waves arose most often near the center, but sometimes from the extremities, of GBSM cells. They propagated regeneratively by Ca2+-induced Ca2+ release involving inositol 1,4,5-

trisphosphate [Ins(1,4,5)P3] receptors and were not affected by TTX and atropine (ATS). Spontaneous Ca2+ waves and spontaneous transient depolarizations were more prevalent in FT than in isolated musculairs layer preparations and occurred with similar pattern in GBSM bundles. Ca2+ waves were abolished by the Ins(1,4,5)P3 receptor inhibitors 2-aminoethoxydiphenyl borate and xestospongin C and by caffeine and cyclopiazonic acid. These events were reduced by voltage-dependent calcium channels (VDCCs) inhibitors diltiazem and nifedipine, by PLC inhibitor U-73122, and by thapsigargin and ryanodine. ACh, CCK, and carbachol augmented Ca2+ waves and induced Ca2+ flashes. The actions of these agonists were inhibited by U-73122. These results indicate that in GBMS, discharge and propagation of Ca2+ waves depend on sarco(end)plasm reticulum (SR) Ca2+ release via Ins(1,4,5)P3 receptors, PLC activity, Ca2+ influx via VDCCs, and SR Ca2+ concentration. Neurohormonal enhancement of GBSM excitability involves PLC-dependent augmentation and syn-

chronization of SR Ca2+ release via Ins(1,4,5)P3 receptors. Ca2+ flashes likely reflect the activity of a fundamental unit of spontaneous activity and play an important role in the excitability of GBSM.

SMOOTH MUSCLE CAN BE DIVIDED into two types, phasic or tonic, based on the presence or absence of spontaneous rhythmic activity (17). Whereas the gallbladder is a tonic organ, gallbladder smooth muscle (GBSM) cells exhibit rhythmic spontaneous action potentials (APs) that are shorter in duration and occur at higher frequency than the slow waves of the gut (50). In contrast to gastrointestinal smooth muscle, which is ar-

gonized in sheets of smooth muscle fibers, GBSM is arranged in interdigitated bundles that are oriented in multiple directions. Rhythmic excitation can be detected in individual GBSM cells by intracellular recording or in bundles by Ca2+ imaging, but the intact organ maintains a steady level of tone rather than undergoing coordinated phasic contractions. It is possible that net tone in the gallbladder results from asynchronous, multifocal contractions of bundles throughout the wall of the organ. This concept is supported by our recent finding that Ca2+ flashes, which likely reflect Ca2+ entry in association with APs, occur simultaneously among muscle cells in a given bundle, but flash patterns among nonintersecting bundles in a given microscopic field are typically asynchronous (1, 13, 14). Ca2+ flashes are rhythmic, rapidly occurring Ca2+ transients that are caused by Ca2+ influx via voltage-gated calcium channels. These events are rapidly followed by muscle contraction (1, 13, 14). In GBSM cells, Ca2+ flashes occur almost simultaneously in all of the smooth muscle cells in a given bundle and spread within a muscle bundle at a velocity of ~1,900.0 μm/s (1).

A second form of Ca2+ transient commonly observed in intact GBSM preparations, and that is asynchronous within a given GBSM bundle, propagates within individual muscle cells at a velocity of ~68.0 μm/s (1). In other types of smooth muscle, similar Ca2+ transients are referred to as intracellular Ca2+ waves. These events are caused by the sarcoplasmic reticulum (SR) Ca2+ release via 1,4,5-trisitol trisphosphate [Ins(1,4,5)P3] receptors [Ins(1,4,5)P3Rs] (22, 24) or by local-

ized Ca2+ release via Ins(1,4,5)P3Rs that is then amplified by SR Ca2+ release via ryanodine receptors (RyRs) during Ca2+-induced Ca2+ release (CICR) (6, 13). Ins(1,4,5)P3 receptor- 

mediated SR Ca2+ release is thought to play a role in the origin and propagation of excitability (26, 40, 44–46) and cause tonic contractions or smooth muscle relaxation (7). Furthermore, excitatory agonists may induce smooth muscle contractions by initiating or augmenting Ca2+-wave activity (7, 36).

A third type of Ca2+ transient that is observed in smooth muscle is called the Ca2+ spark. Ca2+ sparks are focal, non-

propagating Ca2+ transients that are caused by SR Ca2+ release via ryanodine-sensitive receptors (32, 38). In GBSM, Ca2+ sparks activate large-conductance Ca2+-activated K+ (BK) channels to reduce GBSM excitability (38).

The origin and propagation of excitation in GBSM is not well understood. To elucidate myogenic and neurogenic con-

tractile activity of the gallbladder, it is important to gain a thorough understanding of the Ca2+ release events that could contribute to myogenic contractions and how excitatory ago-

nists affect these events. The goal of this study was to charac-

terize Ca2+ waves in GBSM, examine cellular mechanisms underlying their generation and propagation, and determine how they are related to Ca2+ flashes and membrane potential. We also sought to understand how Ca2+ waves are modulated by PLC and excitatory agonists to gain a better understanding of their possible role in excitation-contraction coupling. The results presented here suggest that SR Ca2+ release via Ins(1,4,5)P3 receptors plays a critical role in the elementary events leading to Ca2+ waves, Ca2+ flashes, spontaneous transient depolarizations (STDs), and APs. The generation and
propagation of Ca\textsuperscript{2+} waves depend on SR Ca\textsuperscript{2+} release via Ins\textsubscript{(1,4,5)}P\textsubscript{3} -sensitive receptors, and these events are augmented by excitatory agonists. These findings indicate that Ca\textsuperscript{2+} release via Ins\textsubscript{(1,4,5)}P\textsubscript{3} receptors in the form of Ca\textsuperscript{2+} waves promotes excitability of GBSM.

**MATERIALS AND METHODS**

*Animals, sampling, and dissections.* Adult (250 – 350 g) guinea pigs of either sex were used for this study. Guinea pigs were killed ethically by using halothane or isoflurane anesthesia and exsanguinations in accordance to the guidelines of the Animal Care and Use Committee of the University of Vermont. The abdominal cavity was opened by midline incision, the gallbladder was removed and put into ice-chilled modified Krebs solution that was composed of (in mM) 121 NaCl, 5.9 KCI, 2.5 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 25 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, and 8 glucose; pH 7.4. The gallbladders were cut open from the end of the cystic duct to the base of the organ and subdivided into two approximately equal pieces (~2 × 2.5 cm). These tissues were rinsed with ice-chilled Krebs solution and pinned stretched mucosal-side up in a dish lined with Sylgard 184 elastomer (Dow Corning, Midland, MI). Dissections were undertaken in ice-chilled Krebs solution, which was changed every 8–10 min. Whole mounts used in the present investigation either contained an intact mucosal layer [full thickness (FT)] or were without mucosal layer [mucosalis propria (MP)]. Whole mounts of MP were obtained by teasing off mucosal layer with sharp forceps under stereoscopic microscopic observation. Tissues used for laser confocal imaging to study Ca\textsuperscript{2+} waves were ~1 × 1.5 cm. In some cases, data related to Ca\textsuperscript{2+} waves that are presented in this study were acquired with tissue samples from animals that were used to collect data pertaining to Ca\textsuperscript{2+} flashes presented in a previous publication (1), but different tissue samples were used for the two studies.

*Confocal imaging of Ca\textsuperscript{2+} events.* Gallbladder whole mounts were rinsed with HEPES buffer [in mM] 110 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1.0 MgCl\textsubscript{2}, 20 HEPES, 5 glucose, 60 sucrrose; pH 7.4] and pinned stretched serosal surface up between two Sylgard blocks. The preparations were loaded with 10 μM fluo-4 AM in HEPES buffer containing 0.5 μM/mL pluronic acid and thereafter washed with HEPES buffer to allow for deesterification. Loading (1 h) and washing (30 min to 1 h) were done at room temperature. Whole mounts were gently mounted serosal side facing the coverslip of the recording chamber (2 ml) that was maintained at 35–36°C by continuous superfusion with aerated (95% O\textsubscript{2}-5% CO\textsubscript{2}) recirculating physiological saline solution [PSS; (in mM) 119 NaCl, 7.5 KCl, 1.6 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 23.8 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 0.023 EDTA, and 11 glucose; pH 7.3] at a rate of 3 ml/min. Tissues were then equilibrated for 15–20 min to 1 h, during which multiple fields of observation (n ≥ 20) were examined by using an inverted microscope (Nikon, TMD). Smooth muscle bundles were visualized by ×10 objective using Hoffman modulation contrast

*Analysis of movie files.* Movie files were analyzed off line for the frequency of Ca\textsuperscript{2+} waves using custom software written in our laboratory (Dr. A. D. Bonev). Briefly, rectangular boxes of ~1.1–3.2 μm empirically determined to minimize the signal-noise ratio were put on images of GBSM cells (Figs. 1 and 2) that were in sharp focus and displayed intense Ca\textsuperscript{2+} waves and or Ca\textsuperscript{2+} flashes. Measurements of Ca\textsuperscript{2+}-wave frequency were taken at a fixed position of a given GBSM cell (Figs. 1 and 2). Baseline fluorescence (F\textsubscript{0}) was established by averaging 10 images (of the 600 in a movie) that did not have any Ca\textsuperscript{2+} activity. Movies were visually assessed when normalized ratio images were being reconstructed as traces of F/F\textsubscript{0} (Figs. 1 and 2) and tables of frequency, amplitude, image number, and decay maxima were generated. Data were imported into Microsoft Office Excel, and where applicable, variability between frequencies of Ca\textsuperscript{2+} waves (Hz) was reduced by normalizing each data set. Data were normalized by dividing the frequency obtained at each of the specified 5-min time intervals during drug exposure by the basal (control) frequency value of Ca\textsuperscript{2+} waves obtained before applying the drug. The usual basal Ca\textsuperscript{2+} wave frequency ranged between 0.2 and 0.5 Hz. Normalization of basal Ca\textsuperscript{2+}-wave frequency resulted into a value of 1. Similarly, the normalized frequency values had higher than the normal frequency values when Ca\textsuperscript{2+}-wave activity increased like after application of excitatory agonists, and they are lower where the events were inhibited or abolished. The normalized frequency data are ratios and therefore have no units.

*Intracellular recording.* Gallbladder preparations were stretched on Sylgard in a recording chamber serosal side up and placed on the stage of an inverted microscope (Nikon, TMD). Smooth muscle bundles were visualized by ×10 objective using Hoffman modulation contrast

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**Fig. 1.** Images demonstrating the origin and propagation of Ca\textsuperscript{2+} waves and traces of Ca\textsuperscript{2+} fluorescence ratios (F/F\textsubscript{0}) from a movie file of 600 images acquired by Ca\textsuperscript{2+} imaging. Traces were generated from gallbladder smooth muscle (GBSM) cells in areas indicated by a boxes and a circle. Ca\textsuperscript{2+} waves originating near the center a smooth muscle cell (picture at 5,400 s) propagated in opposite directions as shown by subsequent pictures. Notice the back of the wave in a picture after 7,433 s. Traces of F/F\textsubscript{0} from areas indicated by a box and a circle showing that spontaneous Ca\textsuperscript{2+} waves in GBSM bundle are multiple and asynchronous events.
optics (Modulation Optics, Greenvale, NY). The preparations were continuously superfused at a rate of 8–10 ml/min with the modified aerated (95% O2-5% CO2) Krebs solution. Temperature in the recording chamber was maintained between 35°C and 36°C. Glass micro-electrodes used for intracellular recording were filled with 2 M KCl and had input tip resistances of 70–150 MΩ. A negative-capacity compensation amplifier (Axoclamp 2A, Axon Instruments, CA) with bridge circuitry was used to record electrical activity. Traces of APs (amplitude, slope, duration, membrane potential) were done as changes of the frequency and membrane potential by using PowerLab/4SP and Chart 5, V.5.01 software (AD instruments). Measurements of APs (amplitude, slope, duration, membrane potential) were described earlier (50).

In these experiments, the extent of stretch was not measured. Samples that were not immediately loaded with fluo 4-AM or used for intracellular recording were kept in ice-chilled HEPES or Krebs buffers, respectively, for 3–6 h.

Statistical analysis (Student’s t-test) was done using GraphPad Prism 4. Data were expressed as the means ± SE, and the difference was assumed statistically significant at P ≤ 0.05; n indicates the number of experiments using preparations from different animals.

Chemicals and drugs. Chemicals and drugs used in the present study include fluo-4 AM, and pluronic acid (F-127) (Molecular Probes-Invitrogen, Eugene, OR); thapsigargin, ryanodine (Calbiochem, San Diego, CA); PLC inhibitor [1-{6-(17β-3-methoxyestr-1,3,5(10)-trien-17-ylamino hexyl)}-1H-pyrole-2,5-dione (∆-73122)], xestospongin C (Cayman, Ann Arbor, MI), and CCK octapeptide (Bachem, Torrance, CA). Other drugs were 2-aminoethoxydiphenyl borate (2-APB), acetylthiocholine iodide (ACh), caffeine, diltiazem hydrochloride, EDTA, MgCl2·6H2O, sucrose, glucose, KCl, NaHCO3, sodium phosphate monobasic, potassium phosphate monobasic, HEPES, dimethyl sulfoxide (DMSO), cyclopiazonic acid (Sigma-Aldrich, St. Louis, MO), NaCl, and CaCl2 (Fischer Scientific-Acros Organic, Oxford, PA). Caffeine, ACh, and diltiazem were dissolved in double-distilled water. Nifedipine was dissolved in ethanol. All other drugs were dissolved in DMSO.

RESULTS

Properties and patterns of spontaneous Ca2+ waves in GBSM. Ca2+ waves and membrane potential were studied in 201 whole mounts (n = 160 FT and n = 41 MP from 127 animals). Unless otherwise noted, all measurements reported here were made from FT whole mounts. In GBSM, multiple, asynchronous Ca2+ waves occurred in an individual GBSM cell. Ca2+ waves were spontaneously discharged most often from a location near the center of a given muscle cell and propagated toward opposite extremities (Figs. 1, 2, and 3, A–C, and movies 1 and 2). Ca2+ waves were sometimes induced by similar events in adjacent cells, and in these cases, they propagated from the extremities toward the center. Ca2+ waves propagated slowly within a given GBSM cell. We have previously reported that the velocity of Ca2+ waves is ~70 μm/s (1). In the current study, we found that the amplitude of Ca2+ waves did not diminish after propagating as far as ~40–50 μm from the origin (2.2 ± 0.1 F/F0 vs. 2.2 ± 0.1 F/F0; n = 11, P = 0.9), indicating that these events were regenerative. Within a given cell, the extent of the propagation of Ca2+ waves was variable, with some Ca2+ waves propagating along the entire cell and others propagating shorter distances. Propagation of Ca2+ waves was instantaneously obliterated by collisions between converging Ca2+ waves.

Ca2+ waves occurred asynchronously among muscle cells within a given smooth muscle bundle. Ca2+–wave activity was either multifocal, i.e., involving few bundles, or widespread among GBSM bundles in a given preparation. Within GBSM bundles, Ca2+–waves could occur without Ca2+ flashes. In other bundles, Ca2+ waves occurred asynchronously along with synchronized Ca2+ flashes (movie 2). Unlike Ca2+ flashes, the frequency, intensity, and prevalence of Ca2+ waves...
varied between GBSM cells in a given muscle bundle. Qualitative assessment showed that Ca\textsuperscript{2+}/H\textsubscript{11001} waves occurring among Ca\textsuperscript{2+}/H\textsubscript{11001} flashes had higher intensity and propagated faster. The increase of Ca\textsuperscript{2+}-wave frequency that took place during equilibration from room temperature to 36°C and during the exposure to excitatory agonists gave rise to Ca\textsuperscript{2+} flashes.

Similar to spontaneous Ca\textsuperscript{2+}/H\textsubscript{11001} flashes and APs, the generation of spontaneous Ca\textsuperscript{2+}/H\textsubscript{11001} waves did not appear to involve a neural mechanism because they were not affected by application of a solution containing the Na\textsuperscript{2+}/H\textsubscript{11001} receptor blocker TTX (2 \textmu M) and muscarinic receptor antagonist ATS (1 \textmu M; vehicle: 1.15 ± 0.2, n = 6 vs. TTX-ATS: 0.9 ± 0.07, n = 5; P = 0.2; 25 min; Fig. 4A). Interestingly, the frequencies of Ca\textsuperscript{2+} waves and Ca\textsuperscript{2+}/H\textsubscript{11001} flashes (0.18 ± 0.01 Hz, n = 49 vs. 0.21 ± 0.03 Hz, n = 18; P = 0.4) were comparable (Fig. 4B).

**Ca\textsuperscript{2+} waves and STDs are most prevalent in preparations with intact mucosa.** In our initial studies of Ca\textsuperscript{2+} waves in GBSM, we observed that Ca\textsuperscript{2+} waves were more frequently encountered in preparations with intact mucosa compared with MP alone. Therefore, we compared Ca\textsuperscript{2+} waves and transmembrane potential in GBSM whole mounts with or without the mucosal layer intact (FT vs. MP whole mounts). Sponta-

Fig. 3. Illustration of properties of Ca\textsuperscript{2+} waves in GBSM. A: schematic drawing demonstrating that Ca\textsuperscript{2+} waves originate near the center, and each discharge gives rise to 2 Ca\textsuperscript{2+} waves that propagate regeneratively in the opposite directions, or sometimes Ca\textsuperscript{2+} waves originate from the extremities. B and C: summary data showing that the velocity and amplitude of Ca\textsuperscript{2+} waves do not change during propagation.

Fig. 4. Summary data showing that inhibition of neural activity with TTX and atropine (ATS) do not affect Ca\textsuperscript{2+} waves (A). The frequency of Ca\textsuperscript{2+} waves and Ca\textsuperscript{2+}/H\textsubscript{11001} flashes is comparable (B); however, Ca\textsuperscript{2+} waves propagate slower than Ca\textsuperscript{2+}/H\textsubscript{11001} flashes (C), and leaving the mucosal layer on the preparations increased prevalence of Ca\textsuperscript{2+} waves (D). FT, full-thickness preparations; MP, muscularis propria preparations. **P ≤ 0.01, and ***P ≤ 0.001.
neous Ca\textsuperscript{2+} waves were abundant and widespread in FT whole mount preparations, and the frequency of Ca\textsuperscript{2+} waves was higher in these preparations compared with MP alone (FT: 0.2 ± 0.01 Hz, n = 23 vs. MP: 0.15 ± 0.01 Hz, n = 21; P = 0.004; Fig. 4D).

In intracellular recording studies, STDs were observed in FT and MP and occurred irregularly between APs with a pattern similar to that of Ca\textsuperscript{2+} waves and Ca\textsuperscript{2+} flashes (Fig. 5A; see also Fig. 7B). STDs in GBSM comprised all spontaneous subthreshold depolarizations that did not trigger a regenerative spike on the plateau of the AP.

STDs ranged in amplitude from just above the background noise level (~1 mV) to ~15 mV, and similar to Ca\textsuperscript{2+} waves, STDs were most frequently encountered in FT compared with MP preparations (FT: 0.1 ± 0.02 Hz vs. MP: 0.01 ± 0.01 Hz, n = 12; P < 0.0002). The duration of STDs did not differ between FT and MP preparations (FT: 1.0 ± 0.06 s vs. MP: 1.2 ± 0.1 s, n = 12; P = 0.1). In an effort to determine the type of Ca\textsuperscript{2+} events displayed in tissues that exhibited both STDs and APs, Ca\textsuperscript{2+} imaging studies were conducted in bundles in which STDs and APs had been detected by intracellular recording and injected with Lucifer yellow or and 1,1’-didodecyl 3,3’,3’-indocarbocyanine perchlorate as a means of relocalization. GBSM bundles exhibiting APs with intermittent STDs displayed both Ca\textsuperscript{2+} flashes and Ca\textsuperscript{2+} waves.

There appears to be a relationship between the STDs and APs in GBSM cells, and depolarizations leading to suprathreshold events appeared to result from entrained STDs. The average slope (STDs: 0.005 ± 0.0007 vs. AP: 0.007 ± 0.001 mV/s, n = 12; P = 0.1) and duration (STDs: 1.1 ± 0.09 s vs. AP 1.0 ± 0.06 s, n = 12; P = 0.3) of the upstroke depolarization of the STDs and APs were comparable. Furthermore, large STDs with amplitudes of >10 mV often gave rise to small spikes (13.7 ± 0.6 mV; n = 10; Fig. 5A).

We also compared APs in FT and MP preparations. APs had a larger amplitude in FT preparations (FT: 69.1 ± 1.0 mV, n = 12; P = 0.0009) as well as a larger overshoot (FT: 18.9 ± 1.6 mV vs. MP: 11.5 ± 2.0 mV, n = 12; P = 0.01; Fig. 5, A–D). Furthermore, the duration of the AP was shorter in FT preparations (FT: 1.2 ± 0.09 s vs. MP: 1.9 ± 0.09 s, n = 12; P < 0.0001), and the resting membrane potential was higher (FT: −52.6 ± 0.8 mV vs. MP: −46.7 ± 1.6 mV, n = 12; P = 0.003; Fig. 5E). Despite these differences, the frequency (FT: 0.3 ± 0.02 Hz vs. MP: 0.3 ± 0.02 Hz, n = 12; P = 0.5) and the average slope of the upstroke depolarization of the AP (FT: 0.005 ± 0.0006 mV/s vs. MP: 0.007 ± 0.001 mV/s, n = 12; P = 0.2) were not significantly different. Taken together, these findings suggest that the electrical activity of MP and its associated Ca\textsuperscript{2+} mobilization events are influenced by chemical agents and/or current spreading from cells in the inner layers of the gallbladder wall.

Inhibiting voltage-dependent Ca\textsuperscript{2+} channels reduces Ca\textsuperscript{2+} wave activity. In GBSM, Ca\textsuperscript{2+} influx via voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) determines the level of cytosolic...
Ca\(^{2+}\) and the Ca\(^{2+}\) content of the SR (1, 29). We evaluated the response of Ca\(^{2+}\) waves to VDCC blockers diltiazem (Dzm; 50 μM) and nifedipine (Nifed; 10 μM). Both Dzm (vehicle: 1.1 ± 0.06, n = 5 vs. Dzm: 0.6 ± 0.2, n = 5; P = 0.03; 25 min) and nifedipine (vehicle: 1.4 ± 0.2, n = 6 vs. nifedipine: 0.4 ± 0.2, n = 4; P = 0.01; 25 min) reduced the frequency of Ca\(^{2+}\) waves, respectively (Fig. 6, A and B). These results are consistent with Ca\(^{2+}\) influx maintaining the source of Ca\(^{2+}\) waves (SR Ca\(^{2+}\) content).

The roles of Ins(1,4,5)P\(_3\)R and RyR-mediated SR Ca\(^{2+}\) release on Ca\(^{2+}\)-wave activity. Our understanding of Ins(1,4,5)P\(_3\)R-mediated SR Ca\(^{2+}\)-release events has been advanced by the use of Ins(1,4,5)P\(_3\)R inhibitors, namely 2-APB, xestospongin C, and heparin (9, 24, 44, 45). In GBSM, blocking Ins(1,4,5)P\(_3\)Rs with 2-APB abolished CCK-induced elevation of intracellular Ca\(^{2+}\) (27), and both 2-APB and xestospongin C abolished Ca\(^{2+}\) flashes (1). 2-APB (100 μM) and xestospongin C (5 μM) were used to evaluate the effect of Ins(1,4,5)P\(_3\)R inhibition on Ca\(^{2+}\) waves in GBSM. Compared with vehicle, 2-APB rapidly reduced the frequency of Ca\(^{2+}\) waves (vehicle: 1.4 ± 0.2, n = 6 vs. 2-APB: 0.2 ± 0.09, n = 10; P < 0.0001; 10 min) and abolished these events 5–20 min after exposure. Likewise, xestospongin C rapidly reduced the frequency of Ca\(^{2+}\) waves and then abolished these events (n = 3; see Fig. 7, A, B, and F).

In other types of smooth muscle, propagation of Ca\(^{2+}\) waves by CICR involves SR Ca\(^{2+}\) release via RyRs (6, 9, 13) or Ins(1,4,5)P\(_3\)R (6, 9, 13, 23, 24). The cross-talk between the Ins(1,4,5)P\(_3\) and RyR receptors is crucial for G protein agonist-mediated actions (22, 25). In GBSM, Ins(1,4,5)P\(_3\) and RyR receptors share the same store (27), and Ca\(^{2+}\) sparks (RyRs) have an inhibitory action on excitability (38). Because Ca\(^{2+}\) waves have not been directly studied, we sought to investigate whether RyRs play a role in the generation and/or the propagation Ca\(^{2+}\) waves in GBSM by using ryanodine that blocks these receptors. Prolonged (25 min) incubation with ryanodine (20 μM) reduced frequency (vehicle 1.4 ± 0.2, n = 6 vs. RyR: 0.5 ± 0.1, n = 10; P = 0.002; 25 min) of Ca\(^{2+}\) waves (Fig. 7, C and F).

Caffeine sensitizes RyRs to Ca\(^{2+}\), causing increased intracellular Ca\(^{2+}\) release via RyRs (10, 20). Unlike ryanodine, which locks RyRs in an open or closed state (20), caffeine (3 mM) abolished Ca\(^{2+}\) waves (vehicle: 1.1 ± 0.06, n = 5 vs. caffeine: 0.09 ± 0.05, n = 4; P < 0.0001; 5 min) shortly after exposure (Fig. 7, D and F). These findings suggest that in GBSM, the generation and propagation of Ca\(^{2+}\) waves by CICR depend on Ca\(^{2+}\) release via Ins(1,4,5)P\(_3\)Rys and require functional RyRs in addition to P\(_3\)Rs. RyRs could either participate in the Ca\(^{2+}\) waves or in the maintenance of SR Ca\(^{2+}\) to be released by Ins(1,4,5)P\(_3\)Rs.

Depleting SR Ca\(^{2+}\) stores by using SR Ca\(^{2+}\) ATPase pump inhibitors abolishes Ca\(^{2+}\) waves. In GBSM cells, intracellular Ca\(^{2+}\) depletion with SR Ca\(^{2+}\) ATPase (SERCA) pump inhibitors thapsigargin and cyclopiazonic acid (CPA) induces capacitative Ca\(^{2+}\) entry via the Ins(1,4,5)P\(_3\)Rs, VDCC, and Gd\(^{3+}\)-sensitive pathway (29). We tested the effect of SERCA pump inhibitors thapsigargin (2 μM) and CPA (10 μM) on Ca\(^{2+}\) waves in intact GBSM. Thapsigargin augmented Ca\(^{2+}\)-wave frequency and induced Ca\(^{2+}\) flashes (≥50% of the preparations) 2–5 min after application in four of six preparations. Compared with vehicle, prolonged (25 min) exposure of thapsigargin reduced the frequency of Ca\(^{2+}\) waves (vehicle: 1.4 ± 0.2, n = 6 vs. thapsigargin: 0.5 ± 0.08, n = 10; P = 0.0007; 25 min). CPA augmented Ca\(^{2+}\)-wave frequency in three of five preparations 2–5 min after application but, in contrast to thapsigargin, CPA reduced the frequency of Ca\(^{2+}\) waves (vehicle: 1.4 ± 0.2, n = 6 vs. CPA: 0.02 ± 0.02; n = 5; P = 0.004; 25 min). The difference between the actions of thapsigargin and CPA was significant (thapsigargin: 0.5 ± 0.08, n = 10 vs. CPA: 0.02 ± 0.02, n = 5; P = 0.0005; Fig. 7, E and F). Ca\(^{2+}\) flashes were seen in two of five preparations treated with CPA after 15–25 min when all Ca\(^{2+}\) waves had been inhibited. These findings suggest differences in the mechanisms of action between thapsigargin and CPA and that SR Ca\(^{2+}\) uptake via SERCA pumps is critical for optimal Ca\(^{2+}\)-wave activity.
Excitative agonists augment Ca\(^{2+}\) waves via PLC-dependent generation of Ins(1,4,5)P\(_3\). PLC-dependent production of Ins(1,4,5)P\(_3\) plays a pivotal role in the generation of Ca\(^{2+}\) waves in muscle and nonmuscle cells (4, 9, 30). Inhibiting PLC with U-73122 abolished CCK-induced contractions in the cat gallbladder (48) but did not affect basal APs in the guinea pig gallbladder (1). We tested the effects of the PLC inhibitor U-73122 (10–50 \(\mu\)M) on Ca\(^{2+}\) waves and on agonist-induced responses in intact GBSM. U-73122 (10 \(\mu\)M; \(n = 4\)) did not affect the frequency of Ca\(^{2+}\) waves in the absence of agonists.
Fig. 8. Traces of F/F₀ for Ca²⁺ waves (A) obtained by Ca²⁺ imaging and summary data (B) showing that U-73122 (25 μM) decreased the frequency of Ca²⁺ waves after 25 min. Demonstration of the effects of excitatory agonists: ACh, 50 (μM), carbachol (3 μM), and CCK (100 nM) on Ca²⁺ waves in GBSM cells. Control data were obtained immediately before exposure of drugs as shown by the dotted line and an arrow in C. Excitatory agonists augmented the frequency of Ca²⁺ waves immediately after exposure; however, the frequency of Ca²⁺ waves decreased with time after ~10 min (C and D). Compared with carbachol (3 μM) alone, pretreatment of tissues with U-73122 for 20 min abolished the augmentation of Ca²⁺ waves by carbachol (E). *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

At 25 μM, U-73122 inhibited Ca²⁺ waves (vehicle: 1.4 ± 0.2, n = 6 vs. U-73122: 0.4 ± 0.1, n = 5; P < 0.005; 25 min; Fig. 8, A and B). A higher concentration (50 μM; n = 5) induced Ca²⁺ flashes in GBSM that originally had Ca²⁺ waves alone.

We previously demonstrated that in isolated GBSM cells, CCK stimulates excitability by inhibiting Ca²⁺ sparks (38) and that carbachol and CCK augment Ca²⁺ flashes (1). In the present study, we tested the effect of carbachol (3 μM), ACh (50 μM), and CCK (10 nM) on Ca²⁺ waves. Compared with vehicle, all compounds rapidly augmented the frequency of Ca²⁺ waves during the first 2–5 min (vehicle: 1.1 ± 0.1, n = 4 vs. carbachol: 4.9 ± 1.0, n = 4; P < 0.05; vehicle 1.1 ± 0.1, n = 4 vs. ACh: 2.2 ± 0.3, n = 4; P = 0.008 and vehicle 1.1 ± 0.1, n = 4 vs. CCK: 2.2 ± 0.1, n = 3; P < 0.0001; Fig. 8, C and D). In most preparations, excitatory agonists also caused synchronization of Ca²⁺ waves in a given bundle and changed the pattern of occurrence from Ca²⁺ events to either Ca²⁺ waves and Ca²⁺ flashes occurring together or Ca²⁺ flashes without apparent Ca²⁺ waves. The transition from Ca²⁺ wave predominant form to Ca²⁺ waves and Ca²⁺ flashes or Ca²⁺ flashes alone always followed an initial increase in the frequency of Ca²⁺ waves. U-73122 inhibited the augmentation of Ca²⁺ waves by carbachol (carbachol: 4.9 ± 1.0, n = 4 vs. U-73122 + carbachol: 1.2 ± 0.2, n = 5; P = 0.006; 5 min; Fig. 8E). These results suggest that excitatory agonists augment Ca²⁺-wave activity via activation of PLC and subsequent generation of Ins(1,4,5)P₃ and increased Ins(1,4,5)P₃ -induced SR Ca²⁺ release.

**DISCUSSION**

The aims of the present investigation were to determine whether Ca²⁺ waves play a role in the excitability of GBSM and to elucidate the cellular mechanisms modulating these events. We also evaluated the influence of the presence or absence of the mucosal layer on Ca²⁺ waves and membrane potential fluctuations. The data obtained in this study demonstrate that in GBSM cells, intracellular Ca²⁺ waves arise most often near the center, but sometimes from the extremities, of GBSM cells and propagate regeneratively by Ins(1,4,5)P₃-mediated CICR. Basal and agonist-induced Ca²⁺-wave activity depends on PLC activation, Ca²⁺ influx via VDCC, SR Ca²⁺ content, and Ca²⁺ release via Ins(1,4,5)P₃ receptors. Ca²⁺ waves correlate with STDs, and excitatory agonists enhance excitability by augmenting Ca²⁺ waves. These findings suggest that Ca²⁺ waves (CICR) contribute to the fundamental unit of rhythmic electrical activity in GBSM and play a role in the maintenance of gallbladder tone interprandially and contractile activity during gallbladder emptying.

**Ca²⁺ waves link with Ca²⁺ flashes and APs to augment excitability of GBSM.** We have previously shown that SR Ca²⁺ release via RyRs (Ca²⁺ sparks) reduces excitability of GBSM (38). Recently, we reported that we can detect Ca²⁺ flashes, Ca²⁺ waves, and Ca²⁺ sparks in intact GBSM and that inhibition of SR Ca²⁺ mobilization via Ins(1,4,5)P₃Rs abolishes Ca²⁺ flashes and APs (1). One goal of the present investigation was to determine the relationship between Ca²⁺ waves and
Ca\(^{2+}\) flashes. Results obtained in this study indicate that Ca\(^{2+}\) waves and Ca\(^{2+}\) flashes (or APs) have many properties in common: 1) similar to Ca\(^{2+}\) flashes or APs, constitutive Ca\(^{2+}\) waves are not initiated via a neural mechanism; 2) both Ca\(^{2+}\) waves and Ca\(^{2+}\) flashes are abolished by inhibiting SR Ca\(^{2+}\) release via Ins\((1,4,5)\)P\(_3\)Rs, by high concentration of caffeine, and they are reduced by the RyR blocker ryanodine; 3) the temporal pattern of Ca\(^{2+}\) waves and Ca\(^{2+}\) flashes are comparable as the frequency of Ca\(^{2+}\) waves and Ca\(^{2+}\) flashes are comparable; 4) both basal Ca\(^{2+}\) waves and basal Ca\(^{2+}\) flashes (1) are augmented or induced and synchronized in a similar fashion by excitatory agonists involving a PLC-P3-dependent mechanism with an agonist-induced increase of Ca\(^{2+}\)-wave frequency preceding augmentation and/or induction of Ca\(^{2+}\) flashes; and 5) there is a relationship between increased frequency of Ca\(^{2+}\) waves and induction of Ca\(^{2+}\) flashes.

A relationship also appears to exist between Ca\(^{2+}\) waves and STDs. Ca\(^{2+}\) waves and STDs occur with a similar pattern and prevalence in FT and MP preparations. Furthermore, GBSM bundles exhibiting APs with intermittent STDs also display both Ca\(^{2+}\) flashes and Ca\(^{2+}\) waves, and Ca\(^{2+}\) waves and STDs appear to be equally affected by hyperpolarization caused by muscosal agents. Taken together, these observations suggest that although Ca\(^{2+}\) waves and STDs are not exactly the same, they are interrelated and Ca\(^{2+}\) waves may underlie STDs, supporting the proposition that Ca\(^{2+}\) release via Ins\((1,4,5)\)P\(_3\)Rs promotes excitability of GBSM cells (1). These findings suggest that STDs may arise from Ca\(^{2+}\)-mediated interaction among pacemaker units comprising the SR, mitochondria, and plasma membrane as shown in intestinal interstitial cells of Cajal (ICC) and smooth muscle cells (18, 39). They indicate that, similar to ICC (47), SR Ins\((1,4,5)\)P\(_3\)Rs couples to VDCC in GBSM.

The duration and the rate of the upstroke depolarization of STDs and APs are comparable indicating that in GBSM, APs may arise from the summation of STDs. A similar phenomenon has been reported for slow-wave APs in the ICC and smooth muscle cells in the gastrointestinal (GI) tract (18, 44, 45) and guinea pig urethra smooth muscle (11). These new findings in the gallbladder suggest a tight association of Ca\(^{2+}\) waves and STDs with Ca\(^{2+}\) flashes and APs, respectively.

The data presented here indicate that there is a strong correlation among Ca\(^{2+}\) waves, Ca\(^{2+}\) flashes, STDs, and APs, and these findings suggest that Ca\(^{2+}\) mobilization via Ins\((1,4,5)\)P\(_3\)Rs contributes to the rhythmic depolarization of GBSM. In the ICC in the GI tract, Ca\(^{2+}\) release from internal stores initiates pacemaker currents. Ca\(^{2+}\) influx via dihydropyridine-resistant channels occurring during the upstroke depolarization causes sustained plateau of the slow wave action potentials (18, 40). It is likely that Ins\((1,4,5)\)P\(_3\)R-mediated Ca\(^{2+}\) release at multiple sites corresponds with primary pacemaker currents and that the summation of asynchronous Ca\(^{2+}\) waves corresponds to STDs. Furthermore, it is likely that Ca\(^{2+}\) influx via VDCC is involved in the entrainment of STDs (50). The findings from this study correspond with those of the ICC-smooth muscle model of myogenic activity in the GI tract (18, 40, 45, 46). However, the question of whether pacemaker activity in GBSM involves a specialized pacemaker cell, such as ICC, or whether it is an intrinsic property of GBSM has not been resolved.

In other types of smooth muscle and ICC, Ca\(^{2+}\) influx via sodium-calcium exchanger has been proposed to be responsible for triggering spontaneous depolarizations underlying the origin of rhythmic activity in smooth muscle (26) by causing SR Ca\(^{2+}\) release. It has been proposed that SR Ca\(^{2+}\) mobilization triggers spontaneous inward currents to cause depolarization (11, 12, 18, 26, 44–46) via activation of chloride channels (12, 15, 16, 26, 42) or nonselective cation channels (NSCC) (10, 34, 35). We recently reported the existence of a steady-state nonselective Na\(^{+}\) receptors in GBSM (37) and that activation of store depletion-activated channels may play a role in the excitability of GBSM (1, 29). Whether in GBSM, Ca\(^{2+}\) waves activity involves chloride channels or NSCC or a different mechanism to enhance excitability or couple to STDs remains to be examined.

**Cellular mechanisms modulating Ca\(^{2+}\) waves in GBSM.** The excitability of GBSM cells is modulated by Ca\(^{2+}\) influx via VDCCs (1, 50) and intracellular Ca\(^{2+}\) mobilization (1, 27–29, 38). We evaluated the role of VDCCs in Ca\(^{2+}\) activity to determine the association among Ca\(^{2+}\) waves, Ca\(^{2+}\) flashes, or APs. Unlike Ca\(^{2+}\) flashes or APs or associated ionic currents, which are rapidly abolished by VDCCs inhibitors (1, 50), Ca\(^{2+}\) waves were gradually reduced by VDCCs inhibitors supporting the concept that Ca\(^{2+}\) influx via VDCCs is crucial for replenishing SR Ca\(^{2+}\) stores and Ca\(^{2+}\)-wave activity.

We also examined the role of Ins\((1,4,5)\)P\(_3\)Rs and RyRs in GBSM wave activity because both Ins\((1,4,5)\)P\(_3\)Rs (22, 24) and RyRs (6, 9, 13) are involved in propagation of Ca\(^{2+}\) waves in other types of smooth muscle cells. Inhibiting Ca\(^{2+}\) release via Ins\((1,4,5)\)P\(_3\)Rs abolished Ca\(^{2+}\) waves and GBSM contractions. These observations are similar to observations in GI smooth muscle cells (10, 22, 24) and support the view that SR Ca\(^{2+}\) waves are sensitive to thapsigargin and CPA in their effectiveness to trigger spontaneous depolarizations underlying the slow wave-spark activity in isolated GBSM cells (38), inhibition of SERCA pumps with thapsigargin and CPA reduced or abolished SR Ca\(^{2+}\) release, respectively, indicating that SR Ca\(^{2+}\) uptake via SERCA pumps is critical for optimal SR Ca\(^{2+}\) mobilization via both Ins\((1,4,5)\)P\(_3\)Rs and RyRs. The differences between thapsigargin and CPA in their effectiveness to abolish Ca\(^{2+}\) waves is likely due the ability of thapsigargin to rapidly depolarize GBSM, causing Ca\(^{2+}\) influx and subsequent refill of the SR Ca\(^{2+}\) stores before tangible inhibition of SERCA pumps (1, 29).

**Excitatory agonists augment excitability via Ca\(^{2+}\) waves.** PLC activation is crucial for Ca\(^{2+}\)-wave activity in smooth muscle and nonmuscle cells (9, 23, 30). The results of the present study are consistent with this view that basal and agonist-evoked augmentations of Ca\(^{2+}\) waves are sensitive to PLC inhibition. This is different from our previous findings that constitutive Ca\(^{2+}\) flashes and AP activity do not require PLC activation (1). This study shows that PLC activity via generation of Ins\((1,4,5)\)P3 plays a fundamental role in generating and sustaining basal spontaneous CICR and in agonist-
evoked augmentation of CICR to enhance excitability of GBSM. It is possible that agonist-induced Ca\(^{2+}\)-wave activity requires a global elevation of intracellular Ins(1,4,5)P\(_3\), as shown in other types of smooth muscle cells (8, 23, 24, 30), that is probably caused by depolarization because Ins(1,4,5)P\(_3\) production is voltage dependent (8). Agonist-induced augmentation of Ca\(^{2+}\) waves in GBSM decreased with time as reported in other smooth muscle cells (2). This observation has been attributed to an increased intracellular Ca\(^{2+}\) concentration (3) or a decreased Ins(1,4,5)P\(_3\) generation (33).

Mucosal factors reduce excitability without affecting Ca\(^{2+}\) wave and STD activity. In vascular smooth muscle, endothe-
lum-derived factors such as nitric oxide inhibit intracellular Ca\(^{2+}\) transients activity (41). Similarly, mucosal agents, in-
cluding ATP and nitric oxide from the urinary bladder (5, 21) and ureter (19) epithelium and prostanooids and other agents from tracheal mucosa (43), exert inhibitory actions to smooth muscle excitability. In the current study, preparations with mucosal layer intact had reduced excitability as well as prev-
alent Ca\(^{2+}\) waves and STDs. At this time, we do not know what mucosal factors contributed to this phenomenon or how they affected Ca\(^{2+}\) mobilization and excitability of GBSM. It is possible that gallbladder epithelial cells release ATP that is metabo-
lized to adenosine, which reduces the excitability of GBSM cells (31, 49). It is also possible that physiological differences between the preparations are related to tissue damage during microdissection and/or the presence of mucosal tissue reduces the extent of stretch to GBSM bundles in intact preparations compared with MP.

In conclusion, the results of the present study have revealed that in GBSM cells, the discharge and propagation of Ca\(^{2+}\) waves depend on SR Ca\(^{2+}\) release via Ins(1,4,5)P\(_3\)-Rs, SR Ca\(^{2+}\) content, as well as PLC activity. SR Ca\(^{2+}\) release via Ins(1,4,5)P\(_3\)-Rs couples to VDCC receptors to enhance excit-
ability of GBSM, and it may be crucial in the pacemaking activity in GBSM. Ca\(^{2+}\) waves likely play an important role in maintaining the basal tone as well as neurohumoral-induced stimulation of gallbladder motility and emptying. Finally, agents from gallbladder mucosa, which have not yet been identified, appear to modulate GBSM excitability.

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