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Role of mitochondria in spontaneous rhythmic activity and intracellular calcium waves in the guinea pig gallbladder smooth muscle

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Balemba OB, Bartoo AC, Nelson MT, Mawe GM. Role of mitochondria in spontaneous rhythmic activity and intracellular calcium waves in the guinea pig gallbladder smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 294: G467–G476, 2008. First published November 29, 2007; doi:10.1152/ajpgi.00415.2007.—Mitochondrial Ca^{2+} handling has been implicated in spontaneous rhythmic activity in smooth muscle and interstitial cells of Cajal. In this investigation we evaluated the effect of mitochondrial inhibitors on spontaneous action potentials (APs), Ca^{2+} flashes, and Ca^{2+} waves in gallbladder smooth muscle (GBSM). Disruption of the mitochondrial membrane potential with carbonyl cyanide 3-chlorophenylhydrazone, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, rotenone, and antimycin A significantly reduced or eliminated APs, Ca^{2+} flashes, and Ca^{2+} waves in GBSM. Blockade of ATP production with oligomycin did not alter APs or Ca^{2+} flashes but significantly reduced Ca^{2+} wave frequency. Inhibition of mitochondrial Ca^{2+} uptake and Ca^{2+} release with Ru360 and CGP-37157, respectively, reduced the frequency of Ca^{2+} flashes and Ca^{2+} waves in GBSM. Similar to oligomycin, cyclosporin A did not alter AP and Ca^{2+} flash frequency but significantly reduced Ca^{2+} wave activity. These data suggest that mitochondrial Ca^{2+} handling is necessary for the generation of spontaneous electrical activity and may therefore play an important role in gallbladder tone and motility.

motility; sarcoplasmic reticulum; calcium transients; slow waves; action potentials

ALTERED GALLBLADDER MOTILITY constitutes a primary factor in the pathogenesis of gallstone and other biliary diseases (7, 41). However, the mechanisms that cause the entrainment, the discharge, and the pacing of rhythmic electrical events in the gallbladder musculature that underlie tone and motility are not fully understood. Gallbladder tone and motility depend on rhythmic membrane depolarizations in gallbladder smooth muscle (GBSM) cells that trigger action potentials (APs) and associated contractions. The APs generated by GBSM cells are elicited by Ca^{2+} influx via voltage-dependent Ca^{2+} channels (54). Compared with slow waves in gastrointestinal (GI) smooth muscle cells, GBSM APs have a shorter duration and occur at higher frequencies (~0.3–0.4 Hz), and each AP consists of a rapid upstroke depolarization, a transient repolarization followed by a plateau phase, which precede a complete repolarization (54). These features suggest differences in the basic ionic mechanisms leading to the discharge of gallbladder APs compared with the discharge of pacemaker potentials in the interstitial cells of Cajal (ICC) and slow waves and follower potentials in smooth muscle cells in the GI tract (14, 40, 46).

In smooth muscle cells, as well as other cell types, mitochondrial Ca^{2+} sequestration and release influences spatial and temporal patterns of Ca^{2+} transients in the cytoplasm (8, 9, 11, 24, 25). There is evidence that membrane currents and cytosolic Ca^{2+} oscillations correspond to mitochondrial Ca^{2+} oscillations in isolated GI and vascular smooth muscle cells (8, 9, 25). In the GI tract, smooth muscle cells are electrically coupled with a specialized cell type, the ICC, that generates rhythmic pacemaker currents that drive peristalsis and segmental contractions (14, 38–40, 52). In the ICC, mitochondrial Ca^{2+} handling is considered a key component of the pacemaker unit, which also involves the sarcoplasmic reticulum (SR) and the plasma membrane. According to the pacemaker model that has been proposed for GI smooth muscle and ICC, mitochondrial Ca^{2+} handling depletes 1,4,5-inositol trisphosphate ($InsIP_3$)-sensitive SR Ca^{2+} stores, leading to the activation of membrane nonselective cation conductances, membrane depolarization, and activation of Ca^{2+} influx via voltage-dependent Ca^{2+} channels (38, 40). Subsequently, pacemaker depolarizations are generated and these events propagate into smooth muscle cells to initiate rhythmic activity and contraction (38, 40, 50–52). The importance of mitochondrial Ca^{2+} handling is also emphasized in another pacemaker concept related to ICC that has been proposed by Suzuki et al. (44). These investigators suggest that cyclic fluctuations of mitochondrial Ca^{2+} concentration, which are driven by mitochondrial metabolic activity, underlie rhythmic SR Ca^{2+} store depletion and activation of phosphokinase C, phospholipase C and Ca^{2+}-dependent chloride channels, leading to plasma membrane depolarization, Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCC) and pacemaker activity (44). Although these models of pacemaker units differ somewhat, they both underscore the importance of the mitochondria in the generation of spontaneous activity within ICC.

Recently, we reported the presence of ICC-like cells in the guinea pig gallbladder and demonstrated that these ICC-like cells may be involved in generating rhythmic electrical activity in the guinea pig gallbladder musculature (21). Cells with the morphological features of ICC have also been reported recently in the murine (43) and human (13) gallbladders. In intact guinea pig GBSM preparations, rhythmic, spontaneous APs correspond with Ca^{2+} flashes. Ca^{2+} flashes are rapidly occurring (~1,900 μm/s), intercellular Ca^{2+} transients that represent Ca^{2+} influx via L-type Ca^{2+} channels during APs. Further-

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more, Ca^{2+} flashes are tightly synchronized in all of the GBSM cells and associated ICC-like cells of any given GBSM bundle (3, 21). Another type of Ca^{2+} transients detected in GBSM is the slower (~70 μm/s), regenerative, intracellularly propagating Ca^{2+} waves (2). Ca^{2+} waves arise from the SR via Ca^{2+} release via $InsIP_3$-sensitive receptors and occur asynchronously among the smooth muscle cells of a given bundle. The role of Ca^{2+} waves in GBSM has not yet been established; however, these events are thought to correspond with subthreshold membrane depolarizations (2). Ca^{2+} waves and SR Ca^{2+} release via ryanodine-sensitive receptors, termed Ca^{2+} sparks, play a fundamental role in GBSM rhythmic activity (2, 27, 34). These findings correspond with observations in GI tract (49, 50) and suggest that intracellular Ca^{2+} mobilization, involving both SR and mitochondria, may be essential for the generation and propagation of rhythmic electrical activity in the gallbladder as has been observed in the GI tract (15, 38, 48–52) and the urinary bladder (19).

The objective of the present study was to test the hypothesis that mitochondrial Ca^{2+} mobilization is critical for the discharge and propagation of APs and corresponding Ca^{2+} flashes as well as Ca^{2+} waves in GBSM. Our results demonstrate that, as in the GI tract and detrusor muscle in the urinary bladder, mitochondria Ca^{2+} handling is necessary for the generation of rhythmic activity and intracellular Ca^{2+} waves in GBSM bundles.

METHODS

Animals and tissue preparation. Male adult guinea pigs (200–350 g) were exsanguinated under halothane or isoflurane anesthesia, according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Vermont. The abdomen was opened and the gallbladder was removed and placed in an ice-cold Krebs solution (in mM: 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 8 glucose; pH 7.4). The gallbladder was cut open from neck to base, washed to remove bile, and pinned-stretched mucosa side up in a Sylgard-coated dish (Dow Corning, Midland, MI). The mucosal layer was teased off with sharp forceps under stereoscopic microscopic observation to make whole mount preparations of muscularis propria, which divided into two to four preparations depending on the use. Preparations that were not used immediately were kept in ice-chilled HEPES or Krebs buffers for 2–4 h.

Intracellular recording. For intracellular recording, the gallbladder muscularis was cut in half to produce preparations suitable for recording. Preparations were stretched pinned in a small recording chamber (~2.5 ml volume). The recording chamber was placed onto a Nikon TMD inverted microscope (Nikon USA, Melville, NY) fitted with a Hoffman filter, and tissue was constantly superfused with heated Krebs (35–37°C) containing the myosin light chain kinase inhibitor wortmannin (0.5 μM). Individual muscle bundles were identified under a ×10 objective and impaled with sharp glass microelectrodes (80–200 MΩ) filled with 0.1 M KCl. Electrical activity and membrane potential was recorded with a negative-capacity compensation amplifier (Axoclamp 2A, Axon Instruments, Union City, CA) with bridge circuitry. Electrical activity was analyzed via PowerLab/4SP and Chart 5, v. 5.01 software (AD Instruments, Colorado Springs, CO). Each preparation was superfused for a minimum of 15 min before impalements to initiate spontaneous activity. After a basal recording period (5–10 min), drugs were applied to preparations through the superfusion buffer throughout the recording time frame. Recordings were continued for 30–40 min after application of drugs. All GBSM cells within a given bundle discharge APs at the same frequency (3); therefore, if an impalement was lost during recording, another impalement was obtained within the same muscle bundle to allow for a more continuous time frame of AP frequency. Membrane potential was determined as the difference between bath potential and cellular potential. The AP was defined as a rapid spike followed by a plateau phase, and frequency was calculated as hertz from a 1-min period at given time points during the recording.

Laser confocal imaging of Ca^{2+} transients. Laser confocal imaging of Ca^{2+} transients (Ca^{2+} waves and Ca^{2+} flashes) was performed as described previously (2, 3). Briefly, tissues were washed with HEPES buffer (in mM: 110 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES, 5 glucose, 60 sucrose; pH 7.4) and pinned out, serosal surface up, between two Sylgard blocks (1.5 cm^2). They were loaded at room temperature with 10 μM fluo-4 acetoxymethyl ester (fluo-4 AM; Invitrogen, Carlsbad, CA) in HEPES buffer containing 2.5 μg/ml pluronic acid for 1 h and then washed for 30 min to 1 h with HEPES buffer to allow for deesterification. Tissues were studied by using a 2-ml chamber maintained at 35–36°C by continuous superfusion with aerated (70% N_2-25% O_2-5% CO_2) recirculating physiological saline solution (PSS; in mM: 119 NaCl, 7.5 KCl, 1.6 $CaCl_2$, 1.2 $MgCl_2$, 23.8 $NaHCO_3$, 1.2 NaH_2PO_4, 0.023 EDTA, 11 glucose; pH 7.3). Laser confocal scanning was performed using an inverted Nikon TMD microscope (Nikon USA; ×60 water-immersion objective lens, 1.2 numerical aperture) equipped with fast-speed Noran Oz laser scanning confocal system (Noran Instruments, Madison, WI). Ca^{2+} indicator dye was illuminated with a krypton-argon laser at 488 nm. Oscillating fluctuations of cytosolic Ca^{2+} concentration in intact GBSM bundles were recorded as movies (30 images/s for 20 s, 600 images per movie) by using Prairie View 2.0 software (Prairie View Technologies, Middleton, WI). After basal activity of GBSM was recorded, tissue was continuously superfused with mitochondrial drugs for up to 35 min. Data studying the effects of the drugs were collected after 5, 15, and 25 min of exposure to these compounds to minimize photobleaching. In some cases, data were collected after 35 min.

Analysis of digital movie files. Movie files were analyzed for both the frequency of Ca^{2+} flashes and Ca^{2+} waves (Hz) by using custom software (Spark-AN) written in our laboratory (Dr. A. D. Bonev) as described previously (2, 3). The software provides a continuous readout of the intensity of defined regions and can be used to assess the frequency of Ca^{2+} flashes and Ca^{2+} waves in four to five different GBSM cells in each movie file. In addition, movies were visually assessed for the discharge and propagation of Ca^{2+} transients because in some cases tissue contractions moved the cell of interest away from the defined measurement region. Measurements of Ca^{2+} transient activity before, during, and after application of experimental compounds were obtained from the same cells. The basal frequency of Ca^{2+} flashes and Ca^{2+} waves typically ranged between 0.2–0.5 Hz. In GBSM Ca^{2+} flashes and Ca^{2+} waves occur together (2, 3, 21), therefore tissues were considered for studying Ca^{2+} flashes if the frequency of Ca^{2+} flashes was ≥0.14 Hz and for Ca^{2+} waves if the frequency of Ca^{2+} flashes was ≤0.09 Hz with frequency of Ca^{2+} waves in at least four active cells in a given bundle being ≥0.14 Hz.

Drugs. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), antimycin A mixtures and rotenone were all obtained from Sigma (St Louis, MI). Ru360, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157) oligomycin, and cyclosporin A were purchased from Calbiochem (EMD Biosciences, San Diego, CA) Ru360 was dissolved in dH_2O; antimycin A, FCCP, oligomycin, and cyclosporin A were all dissolved in absolute ethanol; CCCP, rotenone, and CGP-37157 were dissolved in DMSO. Further dilutions of each drug were performed with the superfusion buffer to the concentration stated in the text. Appropriate controls were performed with DMSO and ethanol to demonstrate that these solutions alone did not alter spontaneous activity (Fig. 1). In the present investigation we noticed that, although CGP-37157 dissolved very well in DMSO up to 100 mM, the compound fell out of solution by forming precipitations when stock solutions were being dissolved into Krebs solution. Using

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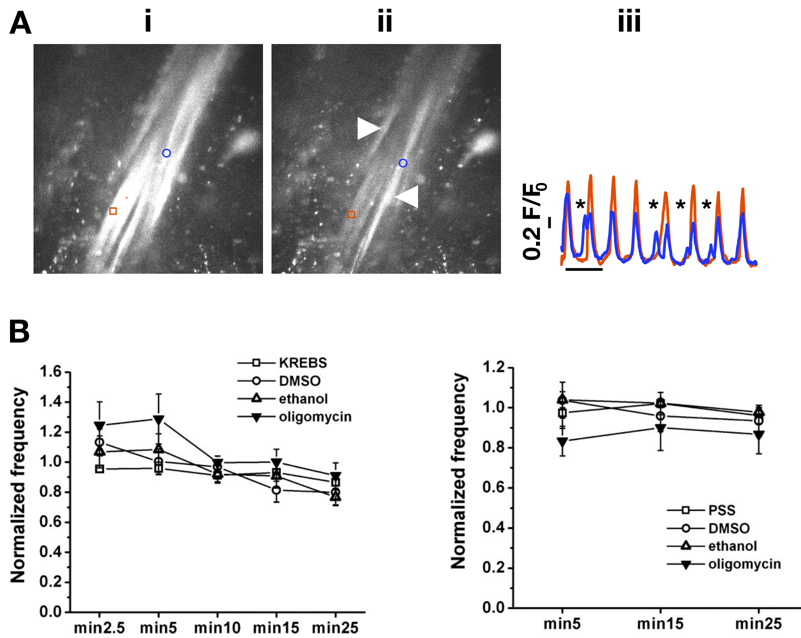

Fig. 1. Demonstration of basal Ca^{2+} transients in intact gallbladder smooth muscle (GBSM) and the effect of control solutions on rhythmic activity. *A*: illustrations of instantaneous, widespread, synchronized, Ca^{2+} flashes in all GBSM cells (*i*) and asynchronous, intracellular Ca^{2+} waves in cells indicated by arrowheads in a muscle bundle (*ii*). *iii*: Traces of fluorescence ratios from GBSM cells indicated by a box and a circle in *i* and *ii* to show that Ca^{2+} flashes (synchronized oscillations) and Ca^{2+} waves (asterisks) occur together in the cell marked by a circle. *B*: compared with Krebs solution, 0.1% ethanol and 0.1% DMSO did not significantly alter action potential (AP) frequency (*left*). Similarly, ethanol and DMSO did not alter Ca^{2+} flashes (*right*). The F/F0 ATPase inhibitor oligomycin did not alter AP or Ca^{2+} flash frequency compared with ethanol control ($P > 0.05$) for the duration of the experiment. PSS, physiological saline solution. In all illustrations, scale bars represent 4-s duration.

lower concentrations of stock solution down to 10 mM and preheating the stocks and buffers at 36–37°C did not appear to improve solubility. CGP-37157 did have greater solubility in PSS buffer.

Statistical analysis. The change in frequency of APs and Ca^{2+} transients after drug exposure was normalized to the basal frequency of the cell immediately prior to drug application. Statistical analysis (one-way ANOVA with multiple comparisons vs. control) was done using GraphPad Prism 4 (El Camino Real, San Diego, CA) or NCSS/PASS (Kaysville, UT). Data are expressed as means ± SE, and the difference was considered statistically significant at $P < 0.05$. The *n* values represent tissue preparations from different animals.

RESULTS

In the present study, we tested whether rhythmic discharge of APs, Ca^{2+} flashes, as well as generation of Ca^{2+} waves in GBSM involve mitochondrial Ca^{2+} handling by evaluating the effects of a number of mitochondrial inhibitors on these events (Tables 1–3). Rhythmic spontaneous APs and corresponding Ca^{2+} flashes as well as Ca^{2+} waves were recorded from smooth muscle bundles in the muscularis propria of the guinea pig gallbladder (Fig. 1*A*). Under basal conditions, GBSM cells had an average resting membrane potential of −51.6 ± 1.8 mV ($n = 26$) and discharged APs at a frequency of 0.30 ± 0.10 Hz. The basal Ca^{2+} flashes occurred at a similar frequency (0.26 ± 0.20 Hz; $n = 14$). These results are similar to previously published data from guinea pig gallbladder muscularis (3, 54). Compounds used in this study were dissolved in dimethyl sulfoxide (DMSO) and ethanol to aid dissolution into aqueous solutions. DMSO ($P > 0.05$; $n = 6$) and ethanol ($P > 0.05$; $n = 6$) alone did not alter basal rhythmic activity of GBSM compared with Krebs ($P > 0.05$; $n = 7$) and PSS ($P > 0.05$; $n = 6$) controls (Tables 1–2; Fig. 1*B*).

Short-term inhibition of oxidative phosphorylation does not affect rhythmic activity. Protonophores and respiratory chain inhibitors that are routinely used to study the role of mitochondria in Ca^{2+} handling and spontaneous rhythmic activity (19, 25, 50, 52) may also inhibit mitochondrial ATP production. Decreases in the ATP concentration may affect membrane electrical properties and Ca^{2+} transients owing to reduced ATP supply to Ca^{2+} pumps (e.g., through deactivation of Na-K-ATPase and Ca-ATPase). To determine the effect of a short-term decrease in ATP production on GBSM activity, we exposed gallbladder muscularis preparations to oligomycin. Oligomycin binds to proteins in the mitochondrial ATPase (F_0-F_1) complex, causing conformation changes that block the proton channel and prevent oxidative phosphorylation (32). Superfusing gallbladder preparations with oligomycin (5 μM)

Table 1. *Effect of mitochondria inhibitors on action potentials*

Drug	Normalized Frequency				
	2.5 min	5 min	10 min	15 min	25 min
Krebs	0.95±0.02	0.96±0.04	0.91±0.04	0.93±0.06	0.86±0.04
DMSO 0.1%	1.13±0.12	1.00±0.20	0.97±0.10	0.81±0.19	0.80±0.19
Ethanol 0.1%	1.07±0.11	1.08±0.10	0.92±0.06	0.91±0.08	0.77±0.05
Oligomycin (5 μM)	1.25±0.16	1.29±0.17	1.00±0.04	1.00±0.08	0.91±0.08
CCCP (1 μM)	0.98±0.28	0.38±0.28	0.08±0.05*	0.11±0.07*	0.36±0.13*
Antimycin A (10 μM)	0.73±0.14	0.39±0.09*	0.43±0.14*	0.34±0.05*	0.37±0.16*
Rotenone (10 μM)	0.71±0.21	0.42±0.08	0.50±0.11	0.54±0.11	0.46±0.11
Cyclosporin A (10 μM)	1.16±0.07	1.15±0.06	1.13±0.06	0.97±0.02	0.93±0.06

Values are means ± SE. CCCP, carbonyl cyanide 3-chlorophenylhydrazone. *Significantly different from control.

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Table 2. *Effect of mitochondria inhibitors on Ca^{2+} flashes*

Drug	Normalized Frequency		
	5 min	15 min	25 min
PSS	0.97±0.08	1.02±0.06	0.96±0.04
DMSO 0.1%	1.03±0.09	0.96±0.08	0.93±0.08
Ethanol 0.1%	1.01±0.04	1.02±0.02	0.98±0.02
Oligomycin (5 μM)	0.83±0.07	0.90±0.11	0.87±0.10
CCCP (1 μM)	0.04±0.04*	0*	ND
FCCP (1 μM)	1.40±0.42	0.03±0.03*	ND
CCCP (1 μM) + oligomycin (5 μM)	0.15±0.08*	0.06±0.06*	ND
Antimycin A (10 μM)	0.28±0.08*	0.21±0.07*	0.23±0.06*
Rotenone (10 μM)	0.36±0.16*	0.50±0.14*	0.14±0.07*
Ru360 (10 μM)	0.90±0.15	0.55±0.13*	0.42±0.07*
CGP-37157 (30 μM)	0.59±0.13*	0.57±0.07*	0.39±0.09*
Cyclosporin A (10 μM)	1.19±0.07	1.31±0.14	1.22±0.16

Values are means ± SE. PSS, physiological saline solution; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; CGP-37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one. *Significantly different from control; ND, not determined.

for a period of 25 min did not significantly alter AP ($P > 0.05$; $n = 5$) or Ca^{2+} flash ($P > 0.05$; $n = 5$) frequency in GBSM cells (Tables 1–2; Fig. 1*B*). In addition, oligomycin did not change the resting membrane potential of GBSM cells (−53.8 ± 2.8 mV control vs. −49.6 ± 2.0 mV oligomycin; $P = 0.29$, paired *t*-test). These results suggest that short-term (≤25 min) inhibition of ATP production does not alter membrane electrical activity in the GBSM.

Mitochondrial membrane potential is essential for GBSM rhythmic activity. The protonophores FCCP and CCCP collapse mitochondrial membrane potential (10), depolarize mitochondria, and in turn inhibit mitochondrial Ca^{2+} uptake and Ca^{2+} release in smooth muscle and ICC (8, 9, 19, 25, 50, 52). Therefore, FCCP and CCCP (1 μM) were used to study the effect of mitochondrial membrane potential on the rhythmic discharge of APs and Ca^{2+} flashes in intact gallbladder muscularis propria. FCCP and CCCP (1 μM) exhibited dramatic actions (Tables 1–2; Fig. 2). During the first 5 min, the actions of CCCP were highly variable among preparations. However, by 10 min AP frequency was greatly reduced or abolished in all GBSM cells studied ($P < 0.05$; $n = 6$). A transient hyperpolarization in membrane potential was observed in GBSM cells treated with CCCP (−51.5 ± 3.8 mV basal vs. −61.3 ± 2.8 mV CCCP 5 min, $P < 0.05$ repeated measure ANOVA; $n = 6$). This was followed by depolarization of membrane potential after 5–15 min and APs began to reappear in these GBSM cells, suggesting a possible adaptive mechanism for AP generation. The resting membrane potential returned to normal values after 25 min (−51.5 ± 3.8 mV basal vs. −52.8 ± 2.5 mV CCCP). CCCP caused a dramatic reduction in Ca^{2+} flash frequency in GBSM (Fig. 2; $P < 0.01$; $n = 4$), and although treatment with FCCP initially increased the frequency of Ca^{2+} flashes, spontaneous Ca^{2+} flashes were abolished after 15 min incubation (Fig. 2; $P < 0.01$; $n = 3$).

Inhibition of complex I or complex III of the mitochondrial electron transport chain with rotenone and antimycin A mixture, respectively, disrupts the mitochondrial membrane potential, leading to subsequent inability of the mitochondria to sequester Ca^{2+} from the cytoplasm (47). Rotenone ($n = 5$) and antimycin A ($n = 5$) significantly reduced the frequency of APs and Ca^{2+} flashes after 5 min of superfusion, a trend that continued after 25 min of exposure (Tables 1–2; Fig. 3). Suppression of Ca^{2+} flashes by rotenone and antimycin A was associated with irregular pattern of rhythmic discharges of APs and Ca^{2+} flashes. The effects of applying CCCP with oligomycin onto Ca^{2+} flashes were similar to those of CCCP (Table 2). Rotenone (−50.4 ± 4.0 mV basal vs. −49.5 ± 2.6 mV rotenone; $P > 0.05$; $n = 6$; 15 min) and antimycin A (−53.5 ± 3.7 mV basal vs. −47.2 ± 5.0 mV antimycin A; $P > 0.05$; $n = 4$; 15 min) did not significantly affect the resting membrane potential. Taken together, these findings indicate that the maintenance of the mitochondrial membrane potential is involved in generating the discharge of APs and Ca^{2+} flashes in GBSM possibly through the disruption of mitochondrial Ca^{2+} uptake and release.

Mitochondrial Ca^{2+} handling regulates rhythmic activity. Mitochondrial Ca^{2+} uptake and Ca^{2+} efflux regulates cytosolic Ca^{2+} homeostasis and SR-endoplasmic reticulum Ca^{2+} release and therefore modulate global and localized cytosolic Ca^{2+} signals (5, 8, 9, 16, 24, 25). Mitochondrial Ca^{2+} uptake occurs via the mitochondrial uniporter, which is inhibited by Ru360 (1, 25). Ru360 (10 μM) was therefore employed to study the effects of mitochondrial Ca^{2+} uptake on Ca^{2+} flashes in GBSM. We found that Ru360 gradually reduced the frequency of rhythmic Ca^{2+} flashes in GBSM during constant superfusion, an effect that was statistically significant after 25 min of treatment (Table 2; Fig. 4*A*; $P < 0.0001$; $n = 5$).

Mitochondria release Ca^{2+} via Na^+/Ca^{2+} exchangers as well as $Na^+/H^+/2Ca^{2+}$ exchangers (18, 36). The mitochondrial Na^+/Ca^{2+} exchanger inhibitor CGP-37157 has been shown to disrupt spontaneous activity within isolated gastrointestinal ICC (18). In the present study, CGP-37157 (30 μM) significantly reduced the frequency of Ca^{2+} flashes 5 min after application ($P < 0.05$; $n = 4$), and it continued to decrease during continuous superfusion ($P < 0.05$; $n = 4$; 25 min). These results suggest that mitochondrial Ca^{2+} release has an important role in the regulation of excitability of GBSM.

In addition to the exchangers, mitochondria release Ca^{2+} via membrane permeability transition pores (PTPs) in states of mitochondrial Ca^{2+} overload (4). However, emerging evidence suggests that under normal physiological conditions, PTPs release reactive oxygen species (ROS). The ROS have been shown to regulate Ca^{2+} sparks and Ca^{2+} waves in vascular

Table 3. *Effect of mitochondria inhibitors on Ca^{2+} waves*

Drug	5 min	15 min	25 min
DMSO	1.17±0.24	1.42±0.15	1.44±0.24
Ethanol	1.11±0.20	1.17±0.05	1.11±0.16
PSS	1.09±0.07	0.89±0.05	0.87±0.05
Oligomycin (5 μM)	0.81±0.10	0.69±0.10*	0.56±0.13*
Oligomycin (5 μM) + CCCP (1 μM)	0.15±0.08*	0.06±0.06*	ND
CCCP (1 μM)	0.18±0.07*	ND	ND
FCCP (1 μM)	0.94±0.35	0.0006±0.0006*	ND
Rotenone (10 μM)	0.86±0.15	0.24±0.16*	0.24±0.11*
Antimycin A (10 μM)	1.26±0.17	0.64±0.14*	0.27±0.08*
Ru360 (10 μM)	0.73±0.05	ND	0.38±0.08*
CGP-37157 (30 μM)	0.62±0.13	0.53±0.16*	0.18±0.09*
Cyclosporin A (5 μM)	0.93±0.06	0.71±0.2 (N = 2)	0.54±0.04*

Values are means ± SE. *Significantly different from control; ND, not determined.

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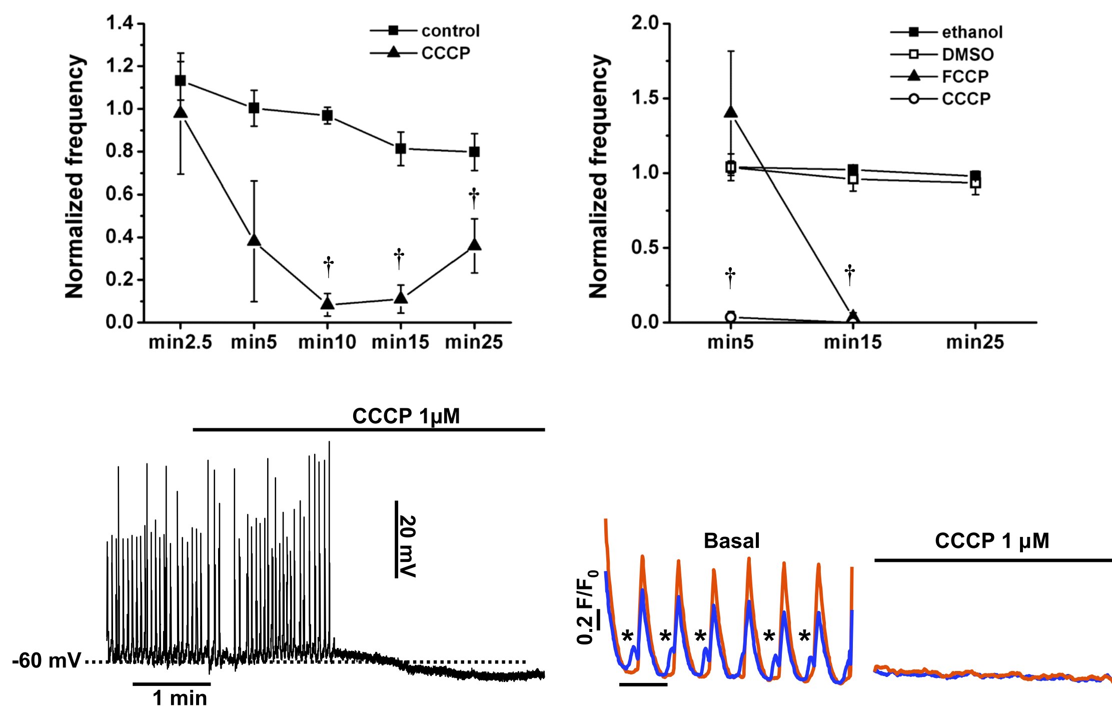

Fig. 2. Effects of mitochondria membrane potential uncouplers on APs and Ca^{2+} flashes. Compared with vehicles solutions [carbonyl cyanide 3-chlorophenylhydrazone (CCCP) vs. DMSO; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) vs. ethanol], disrupting mitochondrial membrane potential with CCCP (1 μM) was initially variable but dramatically reduced APs after 10 min superfusion (*left*). There was a tendency of APs to bounce back after 15–25 min although this was still significantly different than control ($n = 6$). Example of rapid elimination of APs by CCCP is shown in the trace on *bottom left*. CCCP ($n = 4$) and FCCP ($n = 3$) had comparable effects on Ca^{2+} flashes (*right*). Asterisks beside synchronous traces of Ca^{2+} flashes (*bottom right*) show Ca^{2+} waves, which were also eliminated by CCCP. Traces represent simultaneous recordings from separate cells in the same muscle bundle. †Significantly different from control ($P < 0.05$).

smooth muscle (1, 4, 5). We evaluated the involvement of PTPs in Ca^{2+} flash and Ca^{2+} wave activities in intact GBSM preparations by using cyclosporin A (5 and 10 μM), which inhibits PTPs by binding cyclophilin (12). Cyclosporin A (5 μM) did not alter the frequency of Ca^{2+} flashes ($P > 0.05$; $n = 4$; 25 min). Similarly, a higher concentration (10 μM) did not affect the frequency of APs (Table 1; $P > 0.05$; $n = 4$) or Ca^{2+} flashes (Table 2; $P > 0.05$; $n = 5$). These findings suggest that PTPs do not influence the spontaneous, rhythmic discharge of APs and Ca^{2+} flashes in GBSM under basal conditions.

Effect of mitochondrial calcium handling on intracellular Ca^{2+} waves in GBSM. Mitochondrial Ca^{2+} uptake regulates cytosolic Ca^{2+} concentration in the microdomains between mitochondria and SR arising from $InsIP_3$ receptor ($InsIP_3R$)-mediated Ca^{2+} release (16, 24). We have recently shown that SR Ca^{2+} release via $InsIP_3Rs$ causes asynchronous, intracellular Ca^{2+} waves in GBSM (2). Ca^{2+} waves along with Ca^{2+} flashes and SR Ca^{2+} release via $InsIP_3Rs$ appear to be involved in the discharge of rhythmic APs and Ca^{2+} flashes in intact GBSM. The loss of Ca^{2+} waves indicates depletion of SR Ca^{2+} content as well as reduction in PLC (hence $InsIP_3$) activity (2). In the next series of experiments we sought to determine the effect of mitochondrial Ca^{2+} handling on Ca^{2+} waves within intact GBSM preparations (Table 3).

Short-term inhibition of oxidative phosphorylation. To determine the effect of a short-term decrease in ATP production on Ca^{2+} waves, we examined the action of ATP synthase (F_0-F_1) inhibitor oligomycin on the frequency of Ca^{2+} waves for 5–25 min. Oligomycin (5 μM) did not alter the frequency of Ca^{2+} waves up to 10 min after application ($P > 0.05$; $n = 5$). However, after 15 min of superfusion the frequency of Ca^{2+} waves was significantly reduced although not abolished and this effect continued after 25 min ($P < 0.05$; $n = 5$; 25 min). These results are in contrast to the lack of effect of oligomycin on Ca^{2+} flashes and APs described above, suggesting that Ca^{2+} waves are more sensitive to ATP levels perhaps via the effects of reduced ATP levels on sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump and reduced SR Ca^{2+} loading.

Mitochondrial membrane potential and electron transport chain uncouplers abolish Ca^{2+} waves. The effect of the protonophores CCCP and FCCP (1 μM each) on Ca^{2+} waves was evaluated by using intact GBSM preparations. In addition, CCCP was studied in the presence of oligomycin to determine whether oligomycin would augment the actions of protonophores. CCCP significantly reduced the frequency of Ca^{2+} waves within 5 min ($P < 0.05$; $n = 3$) and eliminated Ca^{2+} waves after 10–15 min. The same activity pattern was shown by CCCP-oligomycin mixture (Table 3; $P < 0.05$; $n = 3$). FCCP did not reduce the frequency of Ca^{2+} waves within 5 min and in some cases increased frequency; however, it eliminated Ca^{2+} waves after 10–15 min of superfusion (Table 3; Fig. 5*A*; $P < 0.001$; $n = 3$).

The mitochondrial respiratory chain inhibitors antimycin A (10 μM) and rotenone (10 μM) did not significantly reduce the frequency of Ca^{2+} waves within the first 5 min, but they markedly reduced or eliminated Ca^{2+} waves after 25 min of constant superfusion (Table 3; antimycin A: Fig. 5*B*; $P < 0.001$; $n = 8$; rotenone: Fig. 5*C*; $P < 0.001$; $n = 7$). These

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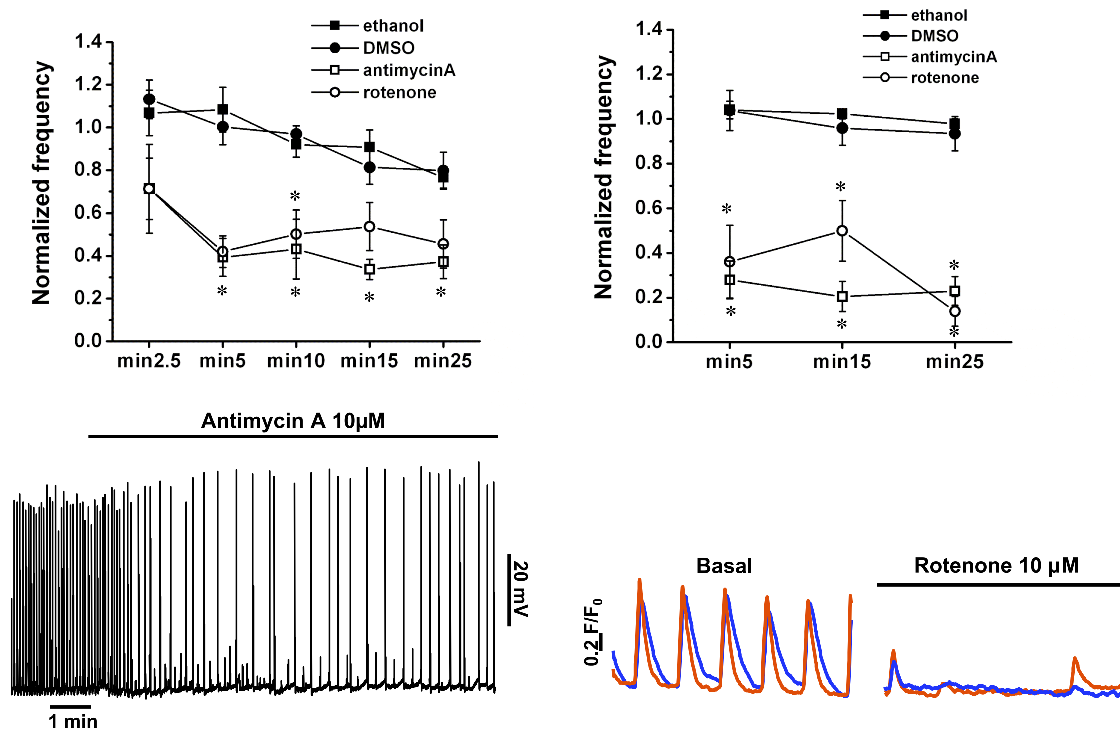

Fig. 3. Effect of the mitochondrial respiratory chain uncouplers on APs and Ca^{2+} flashes. The inhibitors of the electron transport chain I and III, rotenone (10 μM) and antimycin A mixtures (10 μM), respectively, either highly reduced or abolished the discharge of action APs (*top left*) and Ca^{2+} flashes (*top right*) compared with controls. Example of the effects of antimycin on APs is demonstrated in the *bottom left* trace. Example of rotenone effect on Ca^{2+} flash frequency is demonstrated in the *bottom right* traces representing simultaneous recordings from separate cells in the same muscle bundle. *Significantly different from control ($P < 0.05$).

results indicate that the effects of mitochondrial membrane potential and electron transport chain uncouplers on the frequency of Ca^{2+} waves are similar to those on APs and Ca^{2+} flashes.

Role of mitochondrial Ca^{2+} handling on Ca^{2+} waves. To elucidate the role of mitochondrial Ca^{2+} handling on Ca^{2+} waves in GBSM, we evaluated the effects of Ru360 and CGP-37157 that modulate mitochondrial Ca^{2+} uptake and Ca^{2+} release (1, 6, 18, 23) on Ca^{2+} waves. The mitochondrial Ca^{2+} uptake inhibitor, Ru360 (10 μM), reduced the frequency of Ca^{2+} waves as early as 5 min after application ($P < 0.05$; $n = 5$) and continued to reduce the frequency of Ca^{2+} waves with exposure time (Table 3; Fig. 6*A*; $P < 0.001$; $n = 5$; 25 min). The Na^{+}/Ca^{2+} exchanger blocker, CGP-37157 (30 μM), did not affect Ca^{2+} waves during the initial 5-min superfusion ($P > 0.05$; $n = 4$) but greatly reduced or eliminated Ca^{2+} waves after 25 min (Table 3; $P < 0.05$; $n = 4$). The results described above suggest that mitochondrial Ca^{2+} handling is important for $InsIP_3$-mediated SR Ca^{2+} release in GBSM.

Mitochondrial membrane transition pores are involved in Ca^{2+} waves. In this study, inhibiting mitochondrial PTPs with 5–10 μM cyclosporin A did not alter APs and Ca^{2+} flash discharge. Likewise, at 1 μM cyclosporin A did not affect Ca^{2+} waves ($n = 3$) but 5 μM reduced the frequency of Ca^{2+} waves after 25 min (Table 3; $P < 0.05$; $n = 3$). These findings suggest that PTPs may influence the discharge of Ca^{2+} waves in GBSM.

DISCUSSION

The purpose of this investigation was to elucidate the role of mitochondrial Ca^{2+} handling in the rhythmic activity in GBSM. We provide evidence that mitochondrial Ca^{2+} handling is involved in the rhythmic discharge of APs and Ca^{2+} flashes and is essential for Ca^{2+} wave activity in GBSM. This conclusion is based on our findings that mitochondrial membrane potential and electron transport uncouplers and inhibitors of mitochondria calcium handling pathways abolished or reduced rhythmic activity and Ca^{2+} transients in GBSM.

In GBSM, short-term inhibition of mitochondria ATP synthase (F_0-F_1) did not affect rhythmic activity, suggesting that rapid actions of mitochondrial inhibitors were independent of ATP depletion. This is consistent with previous findings in other types of smooth muscle (5, 25, 50, 52). In contrast, FCCP and CCCP, which depolarize the mitochondrial transmembrane potential (10), dramatically reduced and eventually abolished spontaneous activity in GBSM, suggesting that maintenance of the mitochondrial membrane potential is essential for rhythmic activity. The importance of mitochondria to regulate rhythmic activity and Ca^{2+} waves in GBSM was also revealed by using the respiratory chain complex inhibitors antimycin A and rotenone, which disrupt the mitochondrial proton gradient, leading to collapse of the mitochondrial membrane potential (47). These compounds inhibited rhythmic activity and Ca^{2+} waves in GBSM, which is consistent with previous reports in GI (50, 52) urinary bladder muscularis (19) as well as vascular tissues (5, 45, 53).

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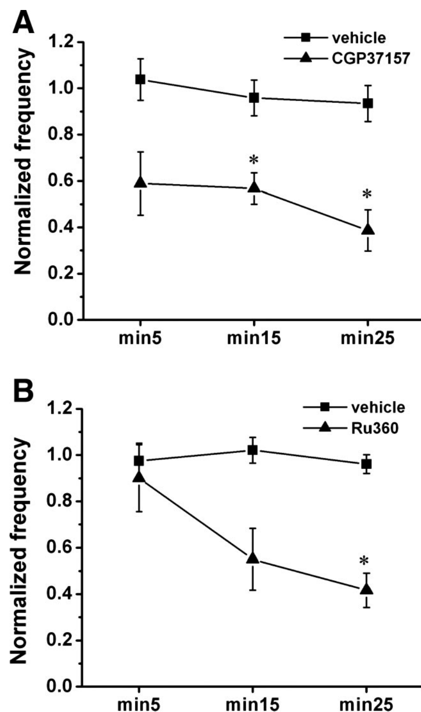

Fig. 4. Effects of mitochondria Ca^{2+} uptake inhibitor Ru360 and Na^{+}/Ca^{2+} exchanger inhibitor CGP-37157 on Ca^{2+} flashes. *A*: compared with physiological saline solution (PSS; vehicle), the mitochondria uniporter inhibitor Ru360 (10 μM) significantly reduced the discharge of Ca^{2+} flashes after 15 min superfusion. *B*: similarly, inhibiting mitochondria Ca^{2+} release via Na^{+}/Ca^{2+} exchanger with CGP-37157 (30 μM) reduced the frequency of Ca^{2+} flashes after 25 min. *Significantly different from control ($P < 0.05$).

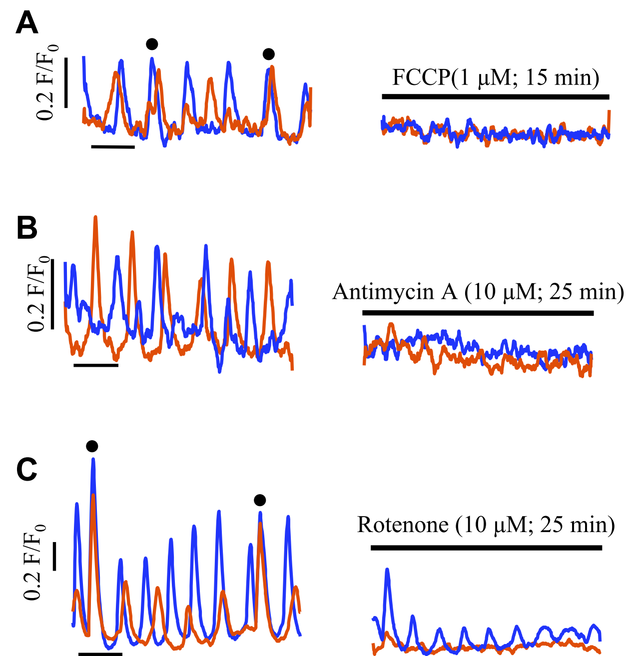

Fig. 5. Traces showing the effects of mitochondria membrane potential uncouplers and respiratory chain uncouplers on Ca^{2+} waves. Like APs and Ca^{2+} flashes, disrupting mitochondrial inner membrane potential with FCCP (1 μM) (*A*) eliminated Ca^{2+} waves after 5–15 min. Uncoupling the electron transport chains I and III, respectively, with antimycin A (10 μM) (*B*) and rotenone (10 μM) (*C*) either abolished or highly reduced the frequency of Ca^{2+} waves after 25 min. Dots in *A* and *C* show Ca^{2+} flashes that occurred with Ca^{2+} waves GBSM bundles. Note: traces represent simultaneous recordings from separate cells in the same muscle bundle.

In other types of smooth muscle cells, disrupting the ability of mitochondria to sequester Ca^{2+} causes plasma membrane depolarizations (5, 8, 9, 24, 25, 53). However, CCCP transiently hyperpolarized the resting membrane potential and eliminated APs in GBSM (5–10 min), followed by a reappearance of APs and return of the resting membrane potential to normal values after 15–25 min. In arterial smooth muscle cells, nanomolar concentration CCCP causes generation of ROS and activation of Ca^{2+}-activated K^{+} channels (53). Ca^{2+}-activated K^{+} channels are present in GBSM and are involved with reducing excitability through a hyperpolarization (28, 34, 54), which may explain the tendency for plasma membrane hyperpolarization observed in some GBSM immediately after the application of CCCP. It is also possible that CCCP and FCCP reduced APs and Ca^{2+} flashes through transient activation of other types of potassium channels known to exist in GBSM (17, 31, 34, 54).

We have previously demonstrated that in GBSM, SR Ca^{2+} release via $InsIP_3Rs$ causes the discharge and salutatory propagation of Ca^{2+} waves (2). In the present study we show that the pattern and time course of the actions of protonophores and the inhibitors of the electron transport chain on the frequency of APs, Ca^{2+} flashes, and Ca^{2+} waves are quite similar. These observations support our proposition of an association between Ca^{2+} flashes and Ca^{2+} waves in GBSM and suggest a Ca^{2+}-dependent link between the plasma membrane and SR (2, 3) as well as the mitochondria (in this study) during rhythmic activity.

In GBSM, mitochondrial Ca^{2+} uptake via the uniporters appears to be the primary link between mitochondria, the SR, and plasma membrane. This conclusion is based on our finding that Ru360, a mitochondrial uniporter inhibitor, reduced Ca^{2+} waves before its actions on Ca^{2+} flashes were observed. The model of pacemaker activity in ICC in the gut suggests that the entrainment of rhythmic activity is set in motion by interactions between the

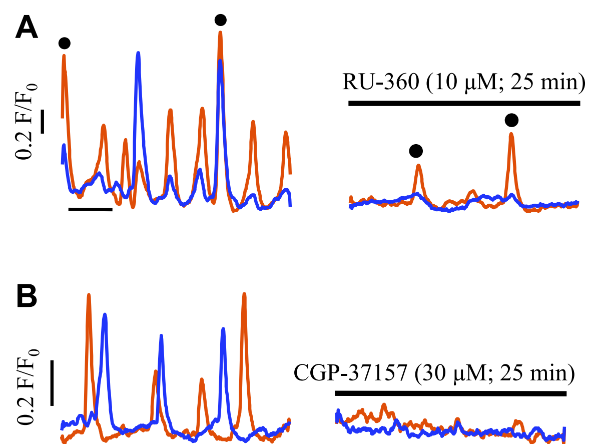

Fig. 6. Effects of mitochondria Ca^{2+} uptake inhibitor Ru360 and Na^{+}/Ca^{2+} exchanger inhibitor CGP-37157 on Ca^{2+} waves. Inhibiting mitochondria Ca^{2+} uptake via the uniporters with Ru360 (10 μM) (*A*) and mitochondria Ca^{2+} release via Na^{+}/Ca^{2+} exchangers with CGP-37157 (30 μM) (*B*) either abolished or highly reduced the frequency of Ca^{2+} waves 25 min. Dots in *A* show Ca^{2+} flashes and the resistance of Ca^{2+} flashes in an interstitial cells of Cajal-like cell to Ru360 (10 μM) after 25 min compared with GBSM cell. Note: traces represent simultaneous recordings from separate cells in the same muscle bundle.

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mitochondria, the SR, and the plasma membrane. Mitochondrial Ca^{2+} uptake depletes Ca^{2+} from the SR, leading to activation of nonselective cation channels, membrane depolarization, and subsequent rhythmic activity and contraction (38–40, 50, 52). This view is supported by recent findings in other types of cells that mitochondrial Ca^{2+} uniporters and SR $InsIP_3Rs$ are physically coupled via macromolecular protein complexes called molecular chaperone (46). The structural association establishes microdomains that efficiently regulate mitochondrial-SR Ca^{2+} handling modalities, SR-plasma membrane protein interactions, activation of nonselective cation channels, and subsequent membrane depolarization (24, 35). In GBSM, SR Ca^{2+} release via $InsIP_3Rs$ causes Ca^{2+} waves (2) and it activates nonselective cation channels and capacitative Ca^{2+} entry, causing plasma membrane depolarization and coactivation of voltage-dependent Ca^{2+} channels (30). These mechanisms require stabilization by the cytoskeleton (29), suggesting the requirement for stable mitochondrion-SR microdomains. Collectively, our data suggest that, in GBSM, Ca^{2+} uptake (Ca^{2+} buffering) from mitochondrion-SR microdomains is essential for $InsIP_3R$-mediated SR Ca^{2+} release. This supports our proposal that, in GBSM, SR Ca^{2+} release via $InsIP_3Rs$ correlates with subthreshold membrane depolarizations and also that SR Ca^{2+} release via $InsIP_3Rs$ is fundamental for rhythmic activity (2). In addition, the findings from this study are consistent with the view that $InsIP_3Rs$-triggered Ca^{2+} oscillations underlie membrane depolarization and are key events in pacemaker activity (15).

Mitochondrial Ca^{2+} release via a Na^+/Ca^{2+} exchanger is involved in regulating rhythmic activity and SR Ca^{2+} release via $InsIP_3Rs$ in GBSM. Mitochondria release Ca^{2+} mainly via Na^+/Ca^{2+} and $Na^+/H^+/2Ca^{2+}$ exchangers (36). In isolated ICC, inhibitors of mitochondrial Na^+/Ca^{2+} exchangers abolished the pacemaking activity suggesting that mitochondrial Na^+/Ca^{2+} exchanger has an important role in pacemaking activity (18). In our study, CGP-37157 inhibited Ca^{2+} transients in GBSM, suggesting that mitochondrial Na^+/Ca^{2+} exchangers modulate gallbladder rhythmic activity and tone. In vascular smooth muscle cells, blockade of the mitochondrial Na^+/Ca^{2+} exchangers with CGP-37157 activates Ca^{2+} sparks (5), indicating that mitochondrial Na^+/Ca^{2+} exchangers may modulate Ca^{2+} sparks and $K_{Ca^{2+}}$ channel activity (34) or other potassium channels (17, 31, 34, 54) in GBSM. In GBSM, a spontaneously active, Na^+-dependent, steady-state nonselective cation conductance is required to maintain plasma membrane potential and generation of APs (33). In addition, Na^+ influx is necessary for the discharge of Ca^{2+} flashes and Ca^{2+} waves (O. B. Balemba and G. M. Mawe, personal observations), indicating that Na^+-dependent nonselective cation conductance is also essential for intracellular Ca^{2+} transients to occur, hence essential for mitochondrion-SR Ca^{2+} handling via Na^+/Ca^{2+} exchangers. It possible that mitochondria inhibitors including Ru360 and CGP-37157 acted in part by inhibiting VDCC activity internally since mitochondria Ca^{2+} handling has been proposed to modulate Ca^{2+} concentration in the microdomains of L-type Ca^{2+} channels (37).

In our initial efforts to understand the role of mitochondrial PTPs in the rhythmic activity of the gallbladder we found that cyclosporin A, the inhibitor of PTPs, did not affect Ca^{2+} flashes, but it inhibited Ca^{2+} waves. The mechanisms of action for the differential actions are not understood. Our results are in agreement with observations of reduced Ca^{2+} sparks, K_{Ca} channel activity, and Ca^{2+} waves in vascular smooth muscle (5). Emerging evidence from studies involving vascular smooth muscle indicates that, under normal physiological conditions, PTPs modulate release of ROS, which activate $InsIP_3$- and ryanodine receptor-gated Ca^{2+} stores and Ni^{2+}-sensitive cation channels (22, 53). The roles of ROS signaling in the rhythmic activity in the gallbladder have not been studied. Cyclosporin A could have reduced affinity of $InsIP_3Rs$ (26) to $InsIP_3$ or enhanced Ca^{2+} uptake by the mitochondria and SR (42). Overall, our results suggest that, in GBSM, PTPs modulate intracellular Ca^{2+} waves through a yet-unknown mechanism.

Rhythmic activity in the gallbladder muscularis, including APs and Ca^{2+} flashes, persists when neural transmission is blocked (3, 54). Therefore, basal spontaneous activity in the gallbladder is not dependent on release of transmitters from gallbladder nerves. GBSM cells are arranged in interwoven muscle bundles that contain sparsely distributed ICC-like cells (21). In the gallbladder, ICC-like cells do not form a distinct network, and it is not always possible to identify these cells in a given field of observation. Therefore, in the present study, Ca^{2+} transients and APs were recorded in GBSM cells. It should be noted that in a previous study we have shown that rhythmic activity in GBSM cells and associated ICC-like cells is synchronized (21). Also, it appears that ICC-like cells generate the pacemaker activity in gallbladder muscularis because gap junction inhibitors eliminated activity in GBSM, but ICC-like cells continue to generate Ca^{2+} flashes. It is noteworthy that, in the present study, spontaneous activity was eliminated by treatment with mitochondrial inhibitors in the limited number of gallbladder ICC-like cells that were observed. Although it is unknown whether GBSM or ICC-like cells are affected first, this study highlights the importance of mitochondria in the generation of spontaneous activity in the gallbladder muscularis.

In conclusion, mitochondrial membrane potential and electron transport chain, Ca^{2+} uptake via uniporters, and Ca^{2+} release via the exchangers are important components of the mitochondrial machinery that play key roles in regulating Ca^{2+} handling and regulation of rhythmic activity in GBSM. Furthermore, we have previously demonstrated that VDCC and nonselective cation channels in the plasma membrane (27, 30, 33, 54) and the SR (2, 3, 27, 34) are also essential for rhythmic activity in the GBSM. Collectively, these findings indicate that the mitochondrion, SR, and plasma membrane channels constitute key components of the GBSM pacemaker. This is consistent with the necessary components proposed for the pacemaker unit of the GI tract (38, 39, 50–52).

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