ERG $K^+$ channels modulate the electrical and contractile activities of gallbladder smooth muscle

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Submitted 6 August 2002; accepted in final form 30 October 2002

GALLBLADDER DISEASE IS TYPICALLY accompanied by decreased gallbladder motility (18). However, the currents that underlie gallbladder smooth muscle (GBSM) activity remain subjects of investigation. Intracellular voltage recordings from intact guinea pig GBSM have revealed characteristic spontaneous action potentials that occur with a frequency of ~0.3 Hz (27). These myogenic action potentials have four distinct components: from a resting membrane potential of ~40 to ~50 mV, the cells exhibit a rapidly depolarizing and repolarizing spike; this is followed immediately by a more slowly developing and declining plateau (27). The spike results from rapid activation and inactivation of L-type voltage-dependent calcium channels, whereas 4-aminopyridine-sensitive voltage-dependent $K^+$ channels ($K_C$ channels) contribute to repolarization of both the spike and the plateau (27). Nevertheless, repolarization still occurs despite $K_C$ channel blockade, thus indicating that other conductances play an important role in repolarizing the plateau of these action potentials.

Although cardiac muscle is histologically and physiologically distinct from smooth muscle, this tissue also displays action potentials with characteristic spike and plateau phases (16). Repolarization of the plateau in cardiac cells involves at least two conductances, rapidly activating ($I_{Kr}$) and slowly activating delayed rectifier $K^+$ currents (13). Molecular studies have revealed that $I_{Kr}$ involves the product of the ether-a-go-go-related gene ($erg1$) (21). The ERG1 protein is a member of the $K_C$ family of $K^+$ channels whose unique gating properties can allow it to pass more current during repolarization than depolarization (19). Heterologous expression of ERG1 together with the accessory protein, Min-K-related protein (also called KCNE2), results in currents almost identical to native $I_{Kr}$ (1, 24). Moreover, both $I_{Kr}$ and ERG1 currents are blocked by similar concentrations of the methanesulfonanilide drug E-4031 (10, 28). A growing number of other drugs has also been found to block these currents. For example, the gastrointestinal prokinetic drug cisapride can block ERG1 channels (14) in addition to its action as an agonist of 5-HT4 receptors (3, 12). Cisapride has also been studied in vivo and ex vivo as a potential prokinetic drug in gallbladder (8, 23). However, most such work has interpreted its action to be via 5-HT4 receptors.

ERG1 has also been suggested to function in the regulation of smooth muscle activity in the rat stomach and in the circular muscle of the opossum esophagus (2, 17). We set out to determine whether ERG1 channels are expressed in GBSM, whether inhibition of these channels alters spontaneous electrical activity of GBSM, and whether blockade of ERG1 channels af-
fects basal tone and/or receptor-activated contractions of gallbladder muscle strips.

**METHODS**

_Tissue preparation._ Adult guinea pigs of either sex were anesthetized with isoflurane and killed by exsanguination. This method has been reviewed and approved by the Institutional Animal Care and Use Committee. The abdominal cavity was opened, and the gallbladder was removed and transferred to cold, oxygenated (95% O₂-5% CO₂) Krebs solution (in mM: 121 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, and 8 glucose) as previously described (27). Briefly, under a dissecting microscope, the gallbladder was opened from the cystic duct to the base, then stretched and pinned mucosa-side up on a Sylgard 184 elastomer (Dow Corning, Midland, MI)-coated dish, and the mucosa was gently removed. These preparations of GBSM were then used for intracellular recording, immunohistochemistry, or RNA isolation as described below or further processed to isolate smooth muscle cells for RNA isolation. For cell isolation, the tissue was rinsed in dispersion medium (in mM: 10 HEPES, 10 glucose, 6 KCl, 55 NaCl, 80 Na-glutamate, and 2 MgCl₂), cut into smaller pieces, and incubated with agitation for 34 min at 37°C in dispersion medium containing 1 mg/ml papain and 1 mg/ml dithioerythritol. Tissues were then transferred to dispersion medium containing 1 mg/ml collagenase and 0.1 mM CaCl₂ for 10 min at 37°C with agitation. Pieces of tissue were triturated with a blunt-tipped pipette, and the resulting suspension was placed on the stage of an inverted microscope where ~100–150 isolated gallbladder smooth muscle cells were collected.

Adult mice of either sex were anesthetized with CO₂ and exsanguinated, as approved by the Institutional Animal Care and Use Committee. The mucosa was removed, and RNA was immediately extracted from the muscularis layer, as described below.

Normal human gallbladder specimens were obtained from liver transplant donors at the Royal Victoria Hospital of the McGill University Medical Center. This protocol was approved by the Institutional Review Board for Human Research. The muscularis was dissected from 0.5-mm² specimens, placed in RNAlater (Ambion, Austin, TX), and stored at 4°C for up to 2 wk before RNA extraction.

_RT-PCR._ RNA from GBSM tissues or isolated GBSM cells was purified with Tri-Reagent (Sigma, St. Louis, MO) using the manufacturer’s protocol. For isolated cells, 240 μl polyinosinic acid (Sigma-Aldrich, St. Louis, MO) was added before RNA purification as a carrier to visualize the precipitate. Reverse transcription was performed with Superscript (Invitrogen, Carlsbad, CA). Primers were designed spanning 242–251-bp segments of ERG1 from guinea pig, mouse, and human (guinea pig, accession no. U75211, nucleotides (nt) 29–48 [5'-tgtgctcatcagccgcttgtc-3'] and nt 279–260 [5'-ggcagccgactccggcctgga-3']; mouse, accession no. AF012868, nt 1583–1602 [5'-gttgctcatcagccgctgtc-3'] and nt 1832–1813 [5'-atggagacacgtccgacgga-3']; human, accession no. U04270, nt 1433–1452 [5'-gggctcatcagccgctgtc-3'] and nt 1674–1655 [5'-ccagccgtccagctgtgtaggga-3']). The thermal cycler program for PCR amplification consisted of 1 min each at 96°C, 61°C, and 72°C for 35–50 cycles and a final extension at 72°C for 10 min. Products were resolved on 1% agarose gels and visualized with 1 μg/ml ethidium bromide.

_Immunohistochemistry._ Tissues for immunohistochemistry were fixed with 0.1 M sodium phosphate buffer containing 2% paraformaldehyde and 0.2% picric acid overnight at 4°C. After tissues were washed with PBS, they were preincubated for 1 h at room temperature with PBS + 0.1% Triton X-100 + 4% normal goat serum (PBS-Triton-NGS). They were then transferred to PBS-Triton-NGS with rabbit anti-ERG1 (1:50; Sigma) and mouse anti-PGP9.5 (1:400 each, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. After 3 more rinses (PBS, 5 min each), tissues were rinsed briefly in water and mounted on a slide with Citifluor (Citifluor, London, UK)._ Intracellular recording._ GBSM preparations were pinned out in a Sylgard-lined recording chamber with recirculating, oxygenated Krebs solution (10–12 ml/min) and placed on the stage of an inverted microscope (Nikon Diaphot). Smooth muscle bundles were visualized at ×200 with Hoffman Modulation Contrast optics (Modulation Optics, Greenvew, NY). Temperature was maintained at 35–37°C. Wortmannin (500 nM) was added to inhibit tissue contractions without altering excitability (4). Global excitability was then assessed, and the bath solution was changed to 2 M KCl and had resistances from 60 to 110 MΩ. Transmembrane potential was measured with an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) and was recorded using MacLab hardware and software (AD Instruments, Castle Hills, Australia). All drugs were added directly to the bathing solution.

_Muscle strip studies._ GBSM preparations were typically dissected into four strips each. Strips were placed vertically in a 10-ml organ bath filled with oxygenated (95% O₂-5% CO₂) nutrient solution (in mM: 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.5 glucose) maintained at 37°C. Isometric contractions were measured using force displacement transducers interfaced with a Macintosh computer using MacLab hardware and software. Strips were placed under an initial resting tension equivalent to a 1.5-g load and allowed to equilibrate for 1 h with solution changes every 20 min. The effects of E-4031 were studied by addition of the drug to the organ bath in the presence of 1 μM TTX and 10 μM atropine to minimize potential neural actions of the drug. For experiments with bethanechol, atropine was not present and tension measurements were initially recorded at successively higher concentrations of bethanechol. The bethanechol was then washed out of the tissue before addition of either 500 nM or 1 μM E-4031, and tension measurements were repeated with each concentration of bethanechol.

_Drugs._ Stock solutions of wortmannin (1 mM; Sigma-Aldrich) and cisapride (1 mM; Research Diagnostics, Flanders, NJ) were prepared in dimethylsulfoxide. TTX (0.5 mM; Sigma-Aldrich) was dissolved in ethanol. Stock solutions of E-4031 (1 mM; Sigma-Aldrich), atropine (0.2 mM; Sigma-Aldrich), diltiazem (50 mM; Sigma-Aldrich), and bethanechol (1 mM; Sigma-Aldrich) were prepared in water.

_Data analysis._ Bursts were defined as excitatory events with extra spikes superimposed on the plateau and/or closely spaced action potentials that failed to remain repolarized for at least 0.5 s (see below). Duration of bursts was measured at the membrane potential halfway between the previous resting potential and the first plateau potential. Events were considered to be part of a single burst when the membrane potential did not remain below the halfway point for at least 0.5 s. Measurements of spike frequency were not possible, because the spikes often became less pronounced during prolonged bursts. Action potential frequency was not a useful description, because it was increased when bursts consisted
of closely spaced action potentials and reduced when the plateau phase was prolonged. The measurements at a given drug concentration began 1 min after addition of the drug for a 4- to 5-min interval before addition of the next highest concentration. Least-squares nonlinear regression analysis of bursting data was used to create sigmoidal dose-response curves and calculate EC50 values with Prism v. 3.0a for Macintosh GraphPad Software (La Jolla, CA). Basal tension values were expressed in milliNewtons. Bethanechol-induced contractions were expressed as percentages of the maximal response. Each concentration-response curve was analyzed to calculate the EC50 by using GraphPad Prism software.

Data are expressed as means ± SE, and statistical comparisons were made with ANOVA or two-tailed t-test, as appropriate.

RESULTS

ERG1 expression in guinea pig GBSM. RT-PCR was used to test for expression of erg1 RNA using primers that span a 251-bp segment of the guinea pig erg1 gene from the S1 to the S3 transmembrane domains. Primers that span similar-sized segments of the comparable region in human and mouse erg1 were also used to confirm its expression in other species. Expression of erg1 was found in guinea pig heart and the smooth muscle layers of the gallbladder as well as in human and mouse gallbladder (Fig. 1A). The localization of the transcripts in smooth muscle cells from guinea pig gallbladder was confirmed by RT-PCR of RNA extracted from isolated GBSM cells (Fig. 1A).

Immunohistochemistry was used to examine the distribution of ERG1 protein in whole mount preparations of the guinea pig gallbladder. These preparations consisted of the serosa, smooth muscle, and blood vessels as well as associated ganglia and nerves. ERG1 immunoreactivity was detected in bundles of muscle cells in the guinea pig GBSM (Fig. 1B). Labeled fibroblast-like cells were also sometimes observed on the serosal surface (not shown). Ganglia, nerve fibers, and blood vessels were not labeled in these preparations (Fig. 1C and not shown). When the primary antibody was omitted, no labeling was seen (not shown).

Effects of ERG1 blockers on the electrical activity of GBSM. Intracellular recordings were made from 21 GBSM cells in 21 preparations, with a mean resting membrane potential of −44 ± 1 mV. The properties of GBSM cells that were impaled in this study were comparable with those reported in previous studies (27). Each spontaneous action potential comprised four phases: a rapid depolarization upstroke, a transient repolarization downstroke, a plateau phase, and a complete repolarization back to the resting membrane potential. Spontaneous action potentials typically occurred at a frequency of 0.3–0.4 Hz, although in some cells the normal low-frequency pattern of individual action potentials was occasionally disrupted by high-frequency “bursts” of action potentials (see below).

The addition of 10 nM–1 μM E-4031 increased the occurrence of excitatory action potential bursts that were concentration dependent in duration (Fig. 2). These bursts included events with multiple spikes on the plateau and series of action potentials with very short intervening refractory periods. At higher drug

Fig. 1. Expression of ether-a-go-go-related gene 1 (ERG1) protein in gallbladder smooth muscle (GBSM). A: RT-PCR analysis of ERG1 expression in guinea pig heart, GBSM (37 cycles each), and isolated GBSM cells (42 cycles, 251 bp for each) as well as in GBSM from mouse (249 bp) and human (241 bp, 37 cycles each). B: ERG1-immunoreactive cells in guinea pig gallbladder muscle bundles have typical smooth muscle cell morphology. C: PGP9.5-immunoreactive nerves in the same field were not labeled with ERG1 antiserum. Scale bar = 100 μm.
concentrations, the repolarization of the plateau of action potentials with a single spike and plateau was often slowed and displayed a shoulder (Fig. 3). However, the rare occurrence of single-spike action potentials at these concentrations precluded a meaningful statistical analysis of this effect. In some tissues at higher drug concentrations, the membrane repolarized only transiently, and in some cells, bursts were followed by refractory periods without activity for several tens of seconds (not shown). In two experiments, 500 nM TTX and 5 μM atropine were added to inhibit neurogenic effects by blocking both nerves and muscarinic receptors on smooth muscle. Addition of TTX and atropine did not prevent bursting (not shown), suggesting that it resulted from direct drug effects on smooth muscle.

Analysis of both the duration of bursts and the percentage of time spent bursting as a function of E-4031 concentration, over a 4-min period at each concentration, revealed significant elevations of these parameters at 500 nM−1 μM of the drug (Fig. 2B). Moreover, the EC50 values for each parameter were similar (700−950 nM), although due to difficulties recording at higher drug concentrations, no true upper limit was established.

The addition of 50 nM−2 μM cisapride also initiated action potential bursting in a concentration-dependent manner (Fig. 4). However, whereas the percentage of time spent bursting continued to increase at the highest cisapride concentrations used, the duration of each burst reached a maximum of ~15 s (Fig. 4). Refractory periods following bursts were often observed (Fig. 4). As described above for the action of E-4031, at higher drug concentrations of cisapride, a shoulder was often present on the repolarization of the plateau of action potentials with a single spike. The addition of 500 nM TTX and 5 μM atropine also failed to affect the responsiveness to cisapride (not shown).

The bursting patterns that were observed in GBSM cells following application of ERG-channel blockers were not uniform events. In some cells, the bursts appeared to result from clustering of multiple action potentials, and in some cells, this was accompanied by an increase in the apparent resting membrane potential (not shown). To establish whether the excitatory effects of E-4031 involve an effect of ERG1-channel blockade on the resting membrane potential, in a further series of experiments, we eliminated the action potentials with 50 μM diltiazem before addition of E-4031. In most cases, a given smooth muscle cell depolarized slightly as the action potentials subsided in the presence of diltiazem. E-4031 was added after the cell had reached a stable membrane potential (≥5 min), and measurements were made from this membrane potential. At a concentration of 100 nM, E-4031 depolarized the membrane by 5 ± 1 mV, whereas 500 nM E-4031 depolarized the membrane by 8 ± 2 mV (n = 3 at each concentration). In one experiment, increasing concentrations stepwise from 10 nM to 1 μM incrementally depolarized the membrane from approximately −30 mV (diltiazem alone) to approximately −18 mV (1 μM). Moreover, washout of E-4031 in the presence of diltiazem resulted in partial repolarization of the membrane before the impalement was lost, indicating that prolonged diltiazem exposure was not responsible for the depolarization that was observed in the presence of E-4031 (not shown).
resulted in an increase in resting basal tone (E-4031 were concentration dependent. Application of E-4031 effects on guinea pig gallbladder strip contractility that under resting tone conditions, E-4031 had excitatory activity when it was present (E-4031 100 nM: 2.70 ± 0.61 vs. 3.63 ± 0.69 mN, 39.7% increase; \( P < 0.001, n = 7 \); E-4031 500 nM: 2.16 ± 0.62 vs. 3.86 ± 1.05 mN, 84.3%; \( P < 0.05, n = 5 \); E-4031 1 \( \mu \)M: 1.64 ± 0.51 vs. 3.20 ± 0.72 mN, 112.9%; \( P < 0.01, n = 5 \); Fig. 5). As demonstrated in Fig. 5, the increase in the amplitude of phasic contractions was associated with a decrease in their frequency. In some strips, E-4031 induced phasic contractions when they were not present under basal conditions (data not shown).

To test whether ERG channel blockade could affect receptor-operated contractions, we assayed the effects of the muscarinic agonist bethanechol in the absence and presence of E-4031. The cumulative concentration-response curve for bethanechol (10 nM–0.32 mM) was left shifted in the presence of E-4031 as indicated by the decrease in the EC\textsubscript{50} value (E-4031 500 nM: 45.6 ± 12 vs. 22.2 ± 10.1 \( \mu \)M; \( P < 0.05, n = 8 \); E-4031 1 \( \mu \)M: 62.8 ± 13.1 vs. 22.4 ± 5.47 \( \mu \)M; \( P < 0.01, n = 7 \)). In addition, an increase in the efficacy of the agonist was recorded as indicated by the increase in the maximal response (Fig. 6). The lowest concentration of the blocker that was tested (100 nM) was only able to increase the contractile response to the lowest concentrations of bethanechol (10 nM–3.2 \( \mu \)M; data not shown).

**DISCUSSION**

The current study was conducted to determine whether ERG channels are expressed in GBSM and, if so, whether they contribute to the electrical and contractile activity of the gallbladder muscularis. We have shown that erg1 transcripts are expressed in GBSM and that GBSM bundles are ERG1-immunoreactive but blood vessels, ganglia, and nerve fibers are not. Moreover, pharmacological blockade of ERG1 channels caused excitatory “bursting” of action potentials in GBSM by delaying the repolarization of the plateau phase of the action potential. This is reminiscent of the excitatory afterdepolarizations that ERG1 blockers can cause in cardiac muscle (21, 22). In fact, current spread through extensive cell coupling is thought to suppress early afterdepolarizations in cardiac cells (20, 21, 26), and so the action potential bursting observed in GBSM may reflect an exaggerated response, reflecting more limited cell coupling in this tissue. It is also likely that ERG channels contribute to the resting membrane potential in GBSM, because application of the ERG-channel blocker E-4031 led to a depolarization of smooth muscle cells. As also reported for cardiac myocytes (7, 25), E-4031 both increased basal contractility of gallbladder muscle strips and enhanced their con-

**Effect of E-4031 on contractility of GBSM strips.** Under resting tone conditions, E-4031 had excitatory effects on guinea pig gallbladder strip contractility that were concentration dependent. Application of E-4031 resulted in an increase in resting basal tone (E-4031 100 nM: 1.24 ± 0.23 mN; 500 nM: 3.12 ± 0.59 mN; 1 \( \mu \)M: 4.06 ± 0.74 mN) and in the amplitude of phasic activity when it was present (E-4031 100 nM: 2.70 ± 0.61 vs. 3.63 ± 0.69 mN, 39.7% increase; \( P < 0.001, n = 7 \); E-4031 500 nM: 2.16 ± 0.62 vs. 3.86 ± 1.05 mN, 84.3%; \( P < 0.05, n = 5 \); E-4031 1 \( \mu \)M: 1.64 ± 0.51 vs. 3.20 ± 0.72 mN, 112.9%; \( P < 0.01, n = 5 \); Fig. 5). As demonstrated in Fig. 5, the increase in the amplitude of phasic contractions was associated with a decrease in their frequency. In some strips, E-4031 induced phasic contractions when they were not present under basal conditions (data not shown).

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ttractile response to receptor activation. Together, these data indicate an important role for ERG1 channels in regulation of excitation-contraction coupling in GBSM and suggest that ERG1 channels may be potential targets of receptor-activated events in these tissues.

In the current study, the EC_{50} values for the excitatory effects of ERG-channel blockers on intact GBSM cells were in the 0.5 to 1.0 μM range. These values are substantially higher than the values reported for their inhibition of I_{Kr} in dispersed cardiac muscle cells or heterologously expressed ERG1 in cultured cells (~ 10 nM) (10, 28). This may reflect more limited access of these blockers to ERG channels in intact tissues. Alternatively, it is also possible that the molecular composition of ERG1 channels in GBSM varies from that in the heart, thus rendering GBSM less sensitive to these drugs. Indeed, splice variants of ERG1 may give rise to channels with different properties (9, 11). It is also possible that as yet unidentified subunits of ERG1 channels may differentially regulate their function. In fact, controversy still exists as to whether all of the subunits that underlie I_{Kr} have been identified (24). Further studies will be required to distinguish between these possibilities.

Despite the induction of action potential bursting by both E-4031 and cisapride, these reagents did not act identically on GBSM. For example, whereas increasing concentrations of E-4031 continued to enhance both the percentage of bursting time and maximal burst time within the concentration range used, the maximal burst time induced by cisapride reached a plateau value of substantially shorter duration. This seems likely to reflect the broader range of targets for cisapride, because it is known to act as an agonist of 5-HT_{4} receptors to facilitate neurotransmitter release (3, 6, 12) and may have other, as yet uncharacterized, activities. Indeed, previous studies have often reported conflicting data regarding the effects of cisapride on gallbladder motility in vivo (23). Furthermore, at least one previous study has shown complex mechanical effects of cisapride on guinea pig GBSM strips that appear to involve both cholinergic and noncholinergic pathways (8). Complex electrophysiological responses of GBSM to cisapride were detected in experiments designed to test its effects on the resting membrane potential in the absence of action potentials (E. Parr and G. M. Mawe, unpublished data). These effects included transient depolarizations and hyperpolarizations that cannot easily be explained on the basis of ERG1 channel blockade alone and may have been junction potentials elicited by enhanced neurotransmitter release. Nevertheless, the excitatory effects of ERG blockers reported herein are unlikely to reflect neurogenic activity because the effects were not inhibited by addition of TTX and atropine. Furthermore, in those instances in which 5-HT_{4} receptors have been reported on smooth muscle, agonists act to relax the muscle (5, 15). In any event, the present study suggests that ERG1-channel blockade by cisapride is one more factor that affects the complex responses of the gallbladder.

The distribution of ERG channels in gastrointestinal smooth muscle has not been completely resolved. Recently, it has been reported that ERG1 expression in the rat gastrointestinal tract is primarily restricted to the stomach (17). Interestingly, those investigators also found that E-4031 depolarized isolated smooth muscle cells from the rat stomach, supporting a role for ERG1 channels in the maintenance of smooth muscle resting membrane potential. This also parallels the E-4031- and cisapride-evoked depolarizations of esophageal smooth muscle (2), and the present study indicates that this is also likely to be the case for guinea pig GBSM. On the other hand, this contrasts with cardiac preparations, in which E-4031 does not markedly alter the resting potential (25), perhaps due to the more negative resting potential in those tissues. Nevertheless, the gradual deactivation of ERG1 channels allows some current to persist following repolarization, and the decay of ERG1 currents during this interval has also been suggested to serve a potential role in the subsequent repolarization (28). Thus ERG1 might also contribute to pacing of action potentials in GBSM.

In conclusion, intact GBSM consistently generates rhythmic spontaneous action potentials that involve the openings and closings of at least two different types of ion channels, including dihydropyridine-sensitive Ca^{2+} channels and 4-aminoypyridine-sensitive K^{+} channels. The findings reported here indicate that another class of K^{+} channel, the ERG1 K^{+} channel, is present in GBSM and contributes to the contour and rhythmicity of the action potential. When ERG1 channels are pharmacologically suppressed in GBSM, the membrane is depolarized by bursts of action potentials; these changes would lead to elevated Ca^{2+} entry into

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**Fig. 6. Effects of pretreatment with E-4031 on bethanecol-evoked contractile response.** After the control curve was performed, the blocker was added during 20 min, and after the second curve was repeated in presence of the inhibitor. Both concentrations of E-4031 were able to left-shift the concentration-response curves and enhance the maximal responses. Data points indicate means from the number of experiments (n = 8 for 500 nM E-4031 and 7 for 1 μM E-4031), and vertical lines indicate SE.

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the cells. Consistent with this, ERG1-channel blockade led to increased contractility of gallbladder muscle strips. These findings support the view that ERG1 channels contribute to a steady-state modulation of GBSM electrical and contractile activities.

We thank L. Ellis and Drs. D. Manning, D. Linden, and T. Firth of the Univ. of Vermont for technical assistance, and we are also thankful to Drs. J. Tchervenkov, A. Di Carlo, and G. Tzimas of McGill Univ. for providing human gallbladder tissue for these studies.

This work was supported by National Institutes of Health Grant NS-26995 and McT Grant SAF-2001–0295.

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