Opposing roles of K⁺ and Cl⁻ channels in maintenance of opossum lower esophageal sphincter tone

YONG ZHANG, DAVID V. MILLER, AND WILLIAM G. PATERSON
Gastrointestinal Diseases Research Unit and Departments of Medicine and Physiology, Queen’s University, Kingston, Ontario, Canada K7L 5G2

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Tonic contraction is the key feature of circular smooth muscle of the lower esophageal sphincter (LES) and results in a pressure barrier at the gastroesophageal junction that prevents the reflux of gastric contents. Basal tone of the LES is primarily myogenic in origin but can be modulated by both neural and hormonal factors. Pharmacological manipulation of myogenic LES tone has obvious therapeutic importance but has been difficult to achieve, largely because the ionic mechanisms underlying LES tone remain poorly understood.

Daniel et al. (17) reported that the resting membrane potential (RMP) of opossum LES circular muscle was about −40 mV, which was relatively more positive than that of the adjacent esophageal body (about −50 mV) (14). It was proposed that this more positive RMP resulted in continuous activation of voltage-sensitive Ca²⁺ channels, and thus constant Ca²⁺ entry into the cell, which helped maintain basal LES tone. The ionic mechanisms underlying the RMP of the LES are unclear, but changes in several ionic conductances could theoretically contribute. Since the RMP is due to a major contribution of K⁺ channels in smooth muscle (7), less activity of K⁺ channels could be associated with the more positive membrane potential of LES circular muscle. In addition, one or more other channels that carry inward currents, such as nonselective cation channels (5) and Cl⁻ channels, could contribute to the more positive RMP.

It is estimated that the reversal potential of the Cl⁻ channel is between −30 mV and −20 mV (1, 2). Activation of Cl⁻ channels would allow outward movement of Cl⁻ ions to carry an inward current, resulting in the depolarization of RMP (31). Recent studies from a number of different laboratories have provided evidence that Cl⁻ channels are involved in tonic contraction of certain vascular (33) and gut (34) smooth muscles and in maintenance of RMP in tracheal smooth muscle (49). Furthermore, it has been reported that DIDS- and indanoyloxyacetic acid-94 sensitive volume-regulated channels are functionally and molecularly expressed in canine vascular and colonic smooth muscle cells (18, 46).

On the basis of the above observations, we hypothesize that a balance of K⁺ and Cl⁻ channel activity might be one of the ionic mechanisms responsible for maintenance of LES basal tone. This possibility was investigated with the use of conventional isometric tension and intracellular microelectrode recordings in LES circular smooth muscle of the opossum.

METHODS

Tissue Preparation

The protocols were approved by the Animal Care Committee of Queen’s University. Opossums (Didelphis virginiana) of either sex, weighing between 2.5 and 5 kg, were anesthetized by tail vein injection of phenobarbital sodium (40 mg/
body circular smooth muscle from the LES were also excised and prepared for intracellular recordings. In 10 animals, similar sheets of mucosa side up in a dissecting dish. With the use of a binocular microscope, the mucosa and connective tissue layers were carefully removed by sharp dissection. The LES was visible as a distinct thickening of circular muscle in the resultant tissue, located just on the gastric side of the squamocolumnar junction (35, 36). A strip of LES (with attached longitudinal muscle) of ~3 × 15 mm was excised for intracellular recordings. In 10 animals, similar sheets of esophageal body circular smooth muscle from ~2 cm above the LES were also excised and prepared for intracellular recordings.

Isometric Tension Recordings

Standard isometric tension recordings were used to study the mechanical responses. A strip was hung in a water-jacketed tissue bath containing 10 ml Krebs solution gassed with 5% CO2-95% O2 at 35°C. One end of a strip was fixed to a hook at the base of the tissue bath, and the other was tied, using a fine silk thread, to a Grass FT03 isometric force transducer that coupled to the Windaq data acquisition system (DATAQ Instruments). Signals were sampled at 100 Hz and stored in a Pentium computer for subsequent analysis. Strips were initially stretched to 140% of the unloaded length and equilibrated for at least 1 h. This degree of stretch has previously been shown to result in optimal responses (37, 45).

Electrical Recordings

Conventional intracellular microelectrode recording techniques were employed to study electrical properties of LES circular smooth muscle. The sheet of LES tissue was pinned on the silicon-coated bottom of a 2-ml electrophysiological recording chamber mounted on the stage of an Olympus IX-70 inverted microscope (Olympus). The chamber was continuously perfused at 1.6 ml/min with prewarmed and preoxygenated Krebs solution and maintained at 35°C. Tissue was allowed to equilibrate for 2 h before the experiment. Glass microelectrodes were pulled using a vertical microelectrode puller (Sutter Instrument) and filled with 3 M KCl. Microelectrode resistance was 50–70 MΩ. The microelectrode was positioned to impale a smooth muscle cell under the guidance of the inverted microscope. The criterion for acceptance of a successful impalement was a sharp voltage drop of approximately −40 mV on penetration that was maintained for at least 2 min. Transmembrane potential was amplified and measured with an intracellular electrometer (model IE-210, Warner Instrument). An agar bridge (2% agar in 3 M KCl) was used to minimize junction potentials. RMP was calibrated on withdrawal of the microelectrode from the cell. The output of the signal was displayed on an oscilloscope (Tektronix model 5103N) and coupled to the Axon Digidata-1200 acquisition system (Axon Instruments). Data were digitized at a frequency of 500 Hz and stored in a Pentium II computer for later analysis using Axon Scope 7.0 software (Axon Instruments). Intracellular recordings in the resting state were characterized by ongoing, spike-like action potentials (APs). The following parameters were used to quantitatively analyze the electrical properties of LES circular smooth muscle (see Fig. 3A): 1) RMP, measured at 0.4 s before the peak of the AP amplitude; 2) amplitude of APs, determined as voltage difference between the AP peak and the RMP; 3) maximal rise rate of AP upstroke, calculated using a time constant equal to 30 ms to determine a maximal rise rate of upstroke of AP; 4) duration of APs at 1/3 amplitude; and 5) frequency of APs.

Solutions and Drugs

The modified Krebs solution contained (mM): 118.07 NaCl, 25 NaHCO3, 11.10 D(+)-glucose, 4.69 KCl, 2.52 CaCl2, 1 MgSO4, and 1.01 NaH2PO4. Niflumic acid was purchased from ICN Biochemicals, TTX, charybdotoxin, and apamin were from Research Biochemicals, and all other materials were from Sigma. Niflumic acid, DIDS, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), and glibenclamide were dissolved in DMSO as stock solutions. Tamoxifen and nifedipine were prepared in ethyl alcohol (100%), and others were prepared in distilled water. These were diluted to final concentrations with Krebs solution. In cumulative dose-response experiments of niflumic acid, final concentration of DMSO in Krebs solution was no more than 1%. The Krebs solution containing diluted drugs was fully bubbled with 5% CO2-95% O2 to restore pH before application.

Statistical Analysis

Data are means ± SE. For tension recording studies n refers to the number of animals, whereas for intracellular recording studies n refers to the number of cells impaled. Pre- and postdrug comparisons were made with the paired Student’s t-test, and a P value of <0.05 was considered statistically significant.

RESULTS

Isometric Tension Studies

Mechanical activity. After stretch to 140% of resting length, LES strips slowly developed tone that reached a steady state of 52.9 ± 5.5% (n = 20) of KCl-induced (60 mM) maximal contraction in 1–1.5 h. The application of atropine (3 μM) plus guanethidine (3 μM) did not significantly alter the basal tone, but TTX (1 μM) increased tone by 26.1 ± 11.1% over control (n = 5, P < 0.05). Twenty-five percent of strips showed spontaneous phasic contractions, superimposed on basal tone, with a frequency of 3.6 ± 0.5/min and amplitude of 13.5 ± 2.9% of basal tone. Nifedipine (1 μM), an L-type Ca2+ channel blocker, abolished the spontaneous phasic contractions and diminished basal tone to 27.7 ± 3.8% of control (n = 4) (Fig. 1B).

Effect of K+ and Cl− channel blockers on LES basal tone. 4-Aminopyridine (4-AP; 2 mM), a transient K+ channel blocker, increased resting tone to 83.1 ± 6.5% over basal values (n = 4, P < 0.05), whereas glibenclamide (10 μM), an ATP-sensitive K+ channel blocker, did not significantly alter basal tone (n = 4). Tetraethylammonium (TEA; 2 mM) and charybdotoxin (100 nM), large-conductance K+ channel blockers, increased basal tone over control by 32.9 ± 15.5 and 18.42 ± 5.9%, respectively (n = 4, P < 0.05). However, apamin (300 nM), a small-conductance Ca2+-activated K+ channel blocker, increased the basal tone by 9.4 ± 5.7% over control (n = 6, P > 0.05) (Fig. 1A). DIDS (500 μM) and NPPB (500 μM), nonspecific Cl− channel blockers,
deceased basal tone to 75.0 ± 7.4 and 67.3 ± 10.8%, respectively, of control (n = 4). Niflumic acid, a putative selective Ca\(^{2+}\)-activated Cl\(^{-}\) channel blocker, relaxed LES in a dose-dependent manner with an IC\(_{50}\) of 1.23 ± 0.33 \(\mu\)M (n = 9) (Fig. 2). The maximally effective concentration of niflumic acid (300 \(\mu\)M) decreased tone to 29.4 ± 5.1% of control (n = 9), which was not different from the effect induced by a maximally effective concentration of nifedipine (1 \(\mu\)M) (P > 0.05) (Fig. 4), indicating that Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels is responsible for the APs.

Intracellular recordings were also performed in 32 cells from the distal esophageal body circular muscle. None of these cells displayed spontaneous APs. RMP

**Intracellular Recording Studies**

**Electrical activity.** The electrical properties of LES circular smooth muscle are summarized in Table 1. The intracellular recordings revealed the existence of ongoing, spontaneous, spike-like APs. Two patterns of APs were observed (Fig. 3B): APs occurring on a stable RMP baseline and APs superimposed on the plateau as well as the valley of slow wave-like potentials. Slow wave-like potentials were recorded in only 2 of 24 cells and had a frequency of 3.5 ± 0.78/min (Fig. 3B). Nifedipine (1 \(\mu\)M) abolished APs and depolarized RMP from -41.3 ± 1.3 to -34.4 ± 3.4 mV (n = 3, P < 0.05) (Fig. 4), indicating that Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels is responsible for the APs.

Intracellular recordings were also performed in 32 cells from the distal esophageal body circular muscle. None of these cells displayed spontaneous APs. RMP...
was significantly more negative compared with LES cells (−50.5 ± 1.4 mV, n = 32 vs. −41.3 ± 0.9, n = 24; P < 0.05).

Effects of K⁺ channel and Cl⁻ channel blockers on electrical properties. Table 1 summarizes the effects of K⁺ and Cl⁻ channel blockers on electrical properties of LES circular smooth muscle cells. Both TEA (2 mM, n = 6) and 4-AP (2 mM, n = 4) significantly depolarized RMP and increased amplitude, maximal rate of upstroke, and frequency of APs (Fig. 5). TEA, but not 4-AP, also significantly shortened the lower 1/3-amplitude duration of APs. Time course studies of niflumic acid (300 μM) demonstrated that it gradually hyperpolarized RMP from −40.6 ± 2.3 mV to −48.1 ± 2.2 mV (n = 5, P < 0.05), decreased the amplitude and maximal rise rate of upstroke of APs, and eventually abolished the ongoing APs after ~5 min (Fig. 6). The studies of the cumulative dose response of niflumic acid yielded an IC₅₀ for hyperpolarization of 19.5 ± 2.2 μM and for inhibition of maximal rise rate of upstroke of 81.7 ± 15.9 μM (n = 5) (Fig. 7B).

DISCUSSION

The major novel findings of the current study are that 1) compared with circular smooth muscle from the esophageal body, LES circular smooth muscle displays ongoing, spontaneous, spike-like APs and has a more

Table 1. Electrical properties of spontaneous action potentials of LES and effects of K⁺, Cl⁻, and Ca²⁺ channel blockers

<table>
<thead>
<tr>
<th></th>
<th>MP, mV</th>
<th>Amplitude, mV</th>
<th>Maximum Rise Rate, V/ms</th>
<th>Duration, ms</th>
<th>Frequency, min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>General, n = 24</td>
<td>−41.3 ± 0.9</td>
<td>19.9 ± 1.0</td>
<td>0.271 ± 0.023</td>
<td>201.9 ± 16.8</td>
<td>43.0 ± 2.7</td>
</tr>
<tr>
<td>4-AP, n = 4</td>
<td>−43.5 ± 2.3</td>
<td>21.7 ± 2.2</td>
<td>0.311 ± 0.055</td>
<td>138.8 ± 14.6</td>
<td>30.5 ± 5.0</td>
</tr>
<tr>
<td>2 mM</td>
<td>−39.3 ± 2.5</td>
<td>23.0 ± 2.9</td>
<td>0.353 ± 0.062</td>
<td>164.0 ± 37.4</td>
<td>53.9 ± 4.6</td>
</tr>
<tr>
<td>TEA, n = 6</td>
<td>−43.4 ± 1.5</td>
<td>20.4 ± 1.3</td>
<td>0.221 ± 0.028</td>
<td>174.7 ± 30.8</td>
<td>41.8 ± 8.1</td>
</tr>
<tr>
<td>Control</td>
<td>−37.6 ± 1.9</td>
<td>22.5 ± 1.2*</td>
<td>0.305 ± 0.032*</td>
<td>115.2 ± 25.3*</td>
<td>66.5 ± 9.9*</td>
</tr>
<tr>
<td>2 mM</td>
<td>−40.6 ± 2.3</td>
<td>21.0 ± 2.9</td>
<td>0.288 ± 0.042</td>
<td>201.5 ± 44.1</td>
<td>41.3 ± 1.5</td>
</tr>
<tr>
<td>Control</td>
<td>−39.3 ± 1.3</td>
<td>21.1 ± 1.3</td>
<td>0.292 ± 0.053</td>
<td>199.7 ± 41.6</td>
<td>40.0 ± 1.7</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>−41.2 ± 0.1</td>
<td>22.3 ± 0.8</td>
<td>0.338 ± 0.049</td>
<td>255.2 ± 52.1</td>
<td>40.0 ± 1.1</td>
</tr>
<tr>
<td>1 μM</td>
<td>−41.7 ± 0.1</td>
<td>22.1 ± 0.9</td>
<td>0.285 ± 0.006</td>
<td>243.0 ± 87.0</td>
<td>46.5 ± 4.3</td>
</tr>
<tr>
<td>10 mM</td>
<td>−43.3 ± 0.2*</td>
<td>22.8 ± 1.1</td>
<td>0.327 ± 0.039</td>
<td>263.4 ± 44.1</td>
<td>47.8 ± 7.8</td>
</tr>
<tr>
<td>30 μM</td>
<td>−43.8 ± 1.6*</td>
<td>19.3 ± 2.4*</td>
<td>0.285 ± 0.035</td>
<td>274.7 ± 54.5</td>
<td>49.2 ± 9.9</td>
</tr>
<tr>
<td>100 μM</td>
<td>−45.1 ± 1.8*</td>
<td>11.8 ± 2.7*</td>
<td>0.125 ± 0.048*</td>
<td>331.8 ± 52.8</td>
<td>38.4 ± 1.1</td>
</tr>
<tr>
<td>300 μM</td>
<td>−48.1 ± 2.2*</td>
<td>22.0 ± 1.5</td>
<td>0.255 ± 0.029</td>
<td>258.8 ± 44.4</td>
<td>44.8 ± 11.2</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>−41.3 ± 1.3</td>
<td>22.0 ± 1.5</td>
<td>0.255 ± 0.029</td>
<td>258.8 ± 44.4</td>
<td>44.8 ± 11.2</td>
</tr>
<tr>
<td>Control</td>
<td>−34.4 ± 1.0*</td>
<td>22.0 ± 1.5</td>
<td>0.255 ± 0.029</td>
<td>258.8 ± 44.4</td>
<td>44.8 ± 11.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. LES, lower esophageal sphincter; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; NA, niflumic acid. *P < 0.05.
positive RMP; 2) resting tone of the opossum LES is increased by K⁺ channel blockers and decreased by Cl⁻ channel blockers, and this corresponds to the opposing effects of these agents on RMP and spontaneous APs; and 3) changes in LES tone appear to correlate better with alterations in spontaneous APs than with RMP, in that niflumic acid and nifedipine, which both profoundly decrease resting LES tone, markedly inhibit spontaneous APs yet have opposing effects on RMP.

It is well established that the pressure barrier between esophagus and stomach, which prevents gastroesophageal reflux, is due to tonic myogenic contraction of the LES (36). The physiological mechanisms underlying this myogenic tone are poorly understood. For years it was known that the LES was unique physiologically but not anatomically. However, more recent studies have clearly defined a number of anatomic features of this muscle that provide clues to its function. Compared with circular smooth muscle from the esophageal body, LES cells are larger, form fewer gap junction contacts with adjacent cells, and display an irregular cell surface with prominent evaginations (16, 40). Furthermore, mitochondria and smooth endoplasmic reticulum are larger in LES muscle cells, possibly reflecting their greater metabolic activity (9).

The LES also displays different contractile properties relative to circular smooth muscle from the esophageal body (43). Biancani and colleagues (6) reported that LES tone in the cat was only partially reduced in a Ca²⁺-free physiological solution or in the presence of L-type Ca²⁺ blockade, suggesting that tonic LES contraction is primarily dependent on intracellular Ca²⁺ stores. They subsequently demonstrated a unique signal transduction mechanism for maintenance of LES tone that was different from that used for phasic, acetylcholine-induced contraction (41, 42, 47). Salapatek et al. (38) recently reported that resting tone of the dog LES is dependent on continuous entry of Ca²⁺ through L-type Ca²⁺ channels, but this was from an extracellular site not readily equilibrated with the external solution in that Ca²⁺ chelators were required to demonstrate an effect. They speculated that this extracellular Ca²⁺ store may be closely associated with the plasmalemma, at or near caveolae.

Our experiments in the opossum are consistent with a major role for extracellular Ca²⁺ in the maintenance of LES tone.
of LES tone, because the L-type Ca$^{2+}$ channel blocker nifedipine caused marked reduction in resting tone of LES muscle strips. However, in the presence of maximally effective concentrations of nifedipine or niflumic acid, some residual tone remained. It is unclear whether this is due to active muscle contraction via a mechanism independent of extracellular Ca$^{2+}$ or is due to passive forces alone.

Electrophysiological properties of the LES are also distinct in that its RMP appears to be relatively more positive compared with the esophageal body (13, 17, 21, 22), although this has not been found by all investigators (10, 11). Such a relatively positive RMP has been hypothesized to contribute to myogenic tone via activation of voltage-gated Ca$^{2+}$ channels, which in turn leads to entry of extracellular Ca$^{2+}$. Studies performed in vivo using extracellular electrodes have described “spike-dependent” and “spike-independent” resting LES tone (3). The sphincter region in this study was characterized by continuous spike activity at rest that disappeared following the administration of isoproterenol or following neurogenic LES relaxation. However, there was evidence of resting tone that appeared independent of the continuous spike activity. In this in vivo study, however, it was not possible to determine whether the continuous smooth muscle spike activity was coming from the LES circular smooth muscle layer, which is responsible for LES resting pressures, or the external longitudinal muscle layer.

Our findings generally support the previous electrophysiological studies. First, we confirmed a relatively more positive RMP of LES circular smooth muscle. We also were able to record continuous and spontaneous APs from all cells, which was abolished by blockade of
L-type Ca\(^{2+}\) channels. This is similar to the studies performed in vivo but is unlike previous reports of LES intracellular recordings, in which spontaneous APs were not recorded (11, 17, 21). The reasons for this difference are unclear; however, we suspect that it relates to technical aspects of the intracellular recordings. Because it is difficult to impale and maintain intracellular recordings in actively contracting smooth muscle, many investigators delay attempts at impalement for a period of time to allow the muscle to become relatively quiescent. This might result in recordings being made at a time when spontaneous APs have fatigued. In our studies, impalements were performed after a relatively short equilibration period when the muscle still displayed spontaneous contractions.

It is interesting that nifedipine produced a marked decrease in LES tone yet resulted in relative depolarization of membrane potential. This is consistent with a more important role for spontaneous APs, as opposed to RMP per se, in the generation of resting LES tone. Depolarization of smooth muscle membrane potential by nifedipine has been reported previously (30). The exact mechanism whereby this occurs is unclear, but two mechanisms have been proposed: 1) direct blockade of voltage-dependent K\(^{-}\) channels (4, 44, 48) or 2) secondary blockade of Ca\(^{2+}\)-activated K\(^{-}\) channels. After blockade of L-type Ca\(^{2+}\) channels by nifedipine, the decreased entry of Ca\(^{2+}\) into the intracellular compartment may result in less activity of Ca\(^{2+}\) -activated K\(^{-}\) channels (32).

It is well established that K\(^{+}\) channels play an important role in the maintenance of RMP in visceral smooth muscles (7, 27, 39). Our results demonstrated the actions of K\(^{+}\) channels in the LES in that selective blockade of either large-conductance Ca\(^{2+}\)-activated K\(^{-}\) channels or transient K\(^{-}\) channels significantly depolarized LES RMP and increased tone. In addition, delayed-rectifier K\(^{-}\) channels may contribute to LES RMP, because TEA and 4-AP at the tested concentration (2 mM) may also block this kind of K\(^{-}\) channel. A functional role for Cl\(^{-}\) channels in visceral smooth muscles is less certain. It was reported that Cl\(^{-}\) channels mediated the norepinephrine-induced constriction of vascular smooth muscle (12, 28, 29) and also appeared to be involved in myogenic tone of cerebral arteries (33) and tracheal smooth muscle (20). Evidence also exists implicating activation of Cl\(^{-}\) channels in agonist-induced contraction of colonic smooth muscle (26), and closing of Cl\(^{-}\) channels might be the mechanism whereby certain inhibitory neurotransmitters cause hyperpolarization of esophageal (14) and ileal (15) smooth muscle. Our studies are the first to suggest a role for Ca\(^{2+}\)-activated Cl\(^{-}\) channels in the maintenance of myogenic tone in a gastrointestinal smooth muscle.

The appropriate interpretation of our results rests on the selectivity of the Cl\(^{-}\) channel blockers used. Both DIDS and NPPB are known to be nonselective Cl\(^{-}\) channel blockers; therefore, it is possible that their effect on resting LES tone could be due to an action on something other than the Cl\(^{-}\) channel (25). Niflumic acid, on the other hand, is believed to be a relatively selective blocker of the Ca\(^{2+}\)-activated Cl\(^{-}\) channels. Some studies have suggested that this drug may also have nonselective effects (24). For instance, it has been shown that in certain tissues niflumic acid may activate large-conductance Ca\(^{2+}\)-activated K\(^{-}\) channels (19) and ATP-sensitive K\(^{-}\) channels (24) in vascular smooth muscle. However, such actions could not explain the results in our studies. First, glibenclamide had no effect on resting LES tone, suggesting that ATP-sensitive K\(^{-}\) channels are, at least, not active in this tissue in the resting state. Furthermore, neither TEA nor glibenclamide at concentrations that are known to block large-conductance and ATP-sensitive K\(^{-}\) channels, respectively, affected the niflumic acid-induced relaxation of LES circular smooth muscle strips.

More recent studies by Kato et al. (23) suggested that niflumic acid relaxed pulmonary arteries preconstricted by endothelin-1 via an effect that was independent of Cl\(^{-}\) channel blockade. This effect was slowly reversible and seemed unrelated to the activation of large-conductance Ca\(^{2+}\)-activated or ATP-sensitive K\(^{-}\) channels. Furthermore, in this model, niflumic acid also relaxed pulmonary arteries preconstricted by the Ca\(^{2+}\) ionophore A-23187, leading the authors to suggest that the niflumic acid effect may be via Ca\(^{2+}\) -dependent contractile processes. Such mechanisms cannot explain the LES relaxant effect of niflumic acid in our experiments because 1) the effect of niflumic acid was rapidly reversible in our experiments; 2) niflumic acid had no effect on KCl-induced LES contraction, indicating that it was not affecting LES tone via a nonspecific effect of voltage-gated Ca\(^{2+}\) channels or contractile proteins; 3) nifedipine depolarized RMP and relaxed LES, but niflumic acid hyperpolarized RMP and relaxed LES; and 4) maximal relaxation induced by niflumic acid was comparable to the maximal relaxation induced by nifedipine, suggesting that niflumic acid might inhibit LES tone by closing L-type Ca\(^{2+}\) channels via membrane hyperpolarization.

Finally, Cao et al. (8) have recently reported that maintenance of LES tone in the cat was related to release of arachidonic acid via spontaneous activity of phospholipase A\(_2\). This in turn led to formation of PGF\(_{2a}\) and thromboxane A\(_2\) via a cyclooxygenase pathway. These mediators then activated G proteins, which led to protein kinase C-dependent muscle contraction. The nonsteroidal anti-inflammatory drugs ASA and indomethacin, by blocking cyclooxygenase and hence PGF\(_{2a}\) and thromboxane A\(_2\) generation, decreased LES tone in the cat. It is unlikely that the effect of niflumic acid seen in our experiments is due to inhibition of cyclooxygenase. First, the onset and reversal of the LES relaxant effect of niflumic acid was rapid, whereas the effect of indomethacin described in the cat LES has a much slower time course. In our opossum LES strips, addition of indomethacin (3–10 \(\mu\)M) had inconsistent effects on LES tone (unpublished observations), and if relaxation was observed it was very slow to develop compared with niflumic acid. Furthermore, niflumic...
acid caused prompt membrane hyperpolarization in LES circular smooth muscle, an effect that could not be explained by inhibition of cyclooxygenase and decreased prostaglandin or thromboxane generation. However, it nevertheless is possible that second messengers derived from the cyclooxygenase pathway may be involved in activation of Ca$^{2+}$-activated Cl$^{-}$ channels.

In summary, our studies demonstrate for the first time that resting tone of the opossum LES is dependent on ongoing, spontaneous, and spike-like APs. Furthermore, the data suggest that a balance between K$^{+}$ channels and Ca$^{2+}$-activated Cl$^{-}$ channels sets RMP of LES circular smooth muscle at a more positive level. This in turn activates L-type Ca$^{2+}$ channels, leading to ongoing spontaneous APs and the generation of resting tone.

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