npg

Role of sarcoplasmic reticulum in control of membrane potential and nitrergic response in opossum lower esophageal sphincter

[1]Yong Zhang & *,[1]William G. Paterson

[1]Gastrointestinal Disease Research Unit and Departments of Medicine, Biology and Physiology, Queen's University, Kingston, Ontario, Canada

1 We previously demonstrated that a balance of Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) and K^+ current activity sets the resting membrane potential of opossum lower esophageal sphincter (LES) circular smooth muscle at ~ -41 mV, which leads to continuous spike-like action potentials and the generation of basal tone. Ionic mechanisms underlying this basal $I_{Cl(Ca)}$ activity and its nitrergic regulation remain unclear. Recent studies suggest that spontaneous Ca^{2+} release from sarcoplasmic reticulum (SR) and myosin light chain kinase (MLCK) play important roles. The current study investigated this possibility. Conventional intracellular recordings were performed on circular smooth muscle of opossum LES. Nerve responses were evoked by electrical square wave pulses of 0.5 ms duration at 20 Hz.

2 In the presence of nifedipine (1 μM), substance P (1 μM), atropine (3 μM) and guanethidine (3 μM), intracellular recordings demonstrated a resting membrane potential (MP) of -38.1 ± 0.7 mV ($n = 25$) with spontaneous membrane potential fluctuations (MPfs) of 1–3 mV. Four pulses of nerve stimulation induced slow inhibitory junction potentials (sIJPs) with an amplitude of 6.1 ± 0.3 mV and a half-amplitude duration of 1926 ± 147 ms ($n = 25$).

3 $1H$-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific guanylyl cyclase inhibitor, abolished sIJPs, but had no effects on MPfs. Caffeine, a ryanodine receptor agonist, hyperpolarized MP and abolished sIJPs and MPfs. Ryanodine (20 μM) inhibited the sIJP and induced biphasic effects on MP, an initial small hyperpolarization followed by a large depolarization. sIJPs and MPfs were also inhibited by cyclopiazonic acid, an SR Ca^{2+} ATPase inhibitor. Specific $I_{Cl(Ca)}$ and MLCK inhibitors hyperpolarized the MP and inhibited MPfs and sIJPs.

4 These data suggest that (1) spontaneous release of Ca^{2+} from the SR activates $I_{Cl(Ca)}$, which in turn contributes to resting membrane potential; (2) MLCK is involved in activation of $I_{Cl(Ca)}$; (3) inhibition of $I_{Cl(Ca)}$ is likely to underlie sIJPs induced by nitrergic innervation.
British Journal of Pharmacology (2003) **140**, 1097–1107. doi:10.1038/sj.bjp.0705537

Keywords: Sarcoplasmic reticulum; ryanodine receptors; myosin light chain kinase; nitric oxide; Ca^{2+}-activated Cl^- channels; niflumic acid; wortmannin; lower esophageal sphincter; intracellular microelectrode recordings

Abbreviations: 4-AP, 4-aminopyridine; 8-Br-cAMP, 8-bromo-cAMP; A-9-C, 9-anthroic acid; $BK_{(Ca)}$, Ca^{2+}-activated large conductance K^+ channel; CPA, cyclopiazonic acid; $I_{BK(Ca)}$, Ca^{2+}-activated large conductance K^+ current; $I_{Cl(Ca)}$, Ca^{2+}-activated Cl^- current; IP_3, inositol 1,4,5-triphosphate; LES, lower esophageal sphincter; MLCK, myosin light chain kinase; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1-H-hexahydro-1,4-diazepine; MP, resting membrane potential; MPfs, resting membrane potential fluctuations; NANC, nonadrenergic and noncholinergic; NFA, niflumic acid; ODQ, $1H$-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PLC, phospholipase C; sIJP, slow inhibitory junction potential; SR, sarcoplasmic reticulum; TEA, tetraethylammonium

Introduction

Circular smooth muscle of lower esophageal sphincter (LES) is characterized by spontaneously generated basal tone, which creates a pressure barrier at the gastroesophageal junction to prevent reflux of gastric contents into the esophagus. The abnormal dynamics of LES function have been considered to be the most important factors in the pathogenesis of gastroesophageal reflux disease (Goyal & Paterson, 1989). Basal tone of LES is considered to be largely myogenic in origin as it can occur without neuronal and circulating factors (Goyal & Paterson, 1989). However, the ionic mechanism for this myogenic tone and its nitrergic regulation is incompletely understood.

We previously demonstrated that in opossum LES smooth muscle, the K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) enhanced, whereas the Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) blocker, niflumic acid (NFA), decreased basal tone. Conventional intracellular recordings revealed that basal tone was associated with a relatively more positive membrane potential (MP) of ~ -41 mV and ongoing, spike-like action potentials, which were potentiated by K^+ channel blockers and attenuated by $I_{Cl(Ca)}$ blockers (Zhang *et al.*, 2000). This suggests that opposing activity of K^+ and Cl^- channels set the MP at a more positive level compared to the adjacent esophageal body (Zhang & Paterson, 2002), which

in turn activates L-type Ca^{2+} channels, leading to ongoing action potentials and the generation of resting tone. Patch-clamp studies showed that delayed rectifier and Ca^{2+}-activated large conductance K^+ channels ($BK_{(Ca)}$) were the major K^+ channels in the LES (Zhang & Paterson, 2001). Furthermore, our recent observation in circular smooth muscle of opossum esophageal body suggests that basal activity of the $I_{Cl(Ca)}$, which requires myosin light chain kinase (MLCK), also contributes to resting MP, and that suppression of the $I_{Cl(Ca)}$ by nitrergic innervation leads to the slow inhibitory junction potential (sIJP). However, a mechanism for genesis of basal activity of $I_{Cl(Ca)}$ remains undefined.

Nelson *et al.* (1995) showed that a local increase in intracellular Ca^{2+}, which presumably results from spontaneous release of Ca^{2+} from the sarcoplasmic reticulum (SR), appears to mediate hyperpolarization of vascular smooth muscle *via* activation of $BK_{(Ca)}$ channels. Furthermore, ZhuGe *et al.* (1998) using freshly dissociated guinea-pig tracheal myocytes reported that depending on membrane potential, Ca^{2+} release from SR triggers either spontaneous transient inward currents (STICS) due to $I_{Cl(Ca)}$, or spontaneous transient outward currents (STOCS), *via* Ca^{2+}-activated large conductance K^+ current ($I_{BK(Ca)}$). This results in biphasic currents, with the outward phase always preceding the inward. These observations imply that spontaneous Ca^{2+} release plays an important role in the genesis of basal activity of ion channels in smooth muscle. We hypothesize that in LES circular smooth muscle, spontaneous Ca^{2+} release from the SR activates $I_{Cl(Ca)}$ *via* MLCK, which maintains resting MP at a relatively more positive level, and that inhibition of the basal activity of $I_{Cl(Ca)}$ by nitrergic innervation produces the sIJP. The current study was designed to test this hypothesis.

Methods

Tissue preparation

The protocols were approved by the Animal Care Committee of Queen's University. Opossums (*Didelphis Virginiana*) of either sex and weighing between 2.5 and 5 kg were anesthetized by tail vein injection of sodium phenobarbital (40 mg kg^{-1}). The chest and abdominal cavities were then exposed *via* a midline incision, and the lower part of esophagus and a short segment of attached stomach were removed and placed in preoxygenated Kreb's solution. The opossum was then killed by intracardiac injection of sodium phenobarbital. The lower part of esophagus and esophago-gastric junction were opened longitudinally and pinned out with mucosa side up in a dissecting dish. Using 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 (Sengupta *et al.*, 1987). A sheet of LES of about $2 \times 3 \times 15$ mm was excised for intracellular recordings.

Electrical recordings

Conventional intracellular microelectrode recording techniques were employed to study the 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, Japan). The chamber was continuously perfused at 2.1 ml min^{-1} with prewarmed and preoxygenated Kreb's solution and maintained at 35°C. Nifedipine (1 μM), atropine (3 μM), quanethidine (3 μM) and SP (1 μM) were included in the Kreb's solution. SP desensitization was tested by short-duration exposure (4 min) to additional 1 μM SP, to ensure that the excitatory effect of this agent was no longer present. The tissue was allowed to equilibrate for 2 h prior to the experiment. Glass microelectrodes were pulled using a vertical microelectrode puller (Model P-87, Sutter Instrument, U.S.A.) and filled with 3 M KCl. Microelectrode resistance was 50–70 MΩ. The microelectrode was positioned for the impalement under the guidance of the inverted microscope. The criterion for acceptance of a successful impalement was a sharp voltage drop on penetration that was maintained for at least 2 min. Silver wire electrodes, placed on either side of the muscle strip, were used to electrically stimulate intramural nerves using square wave pulses of 0.5 ms duration and 80 V generated by a Grass S88 Stimulator (Grass Instrument Co., MA, U.S.A.). Transmembrane potential was amplified and measured with an intracellular electrometer (Model IE-210, Warner Instrument Corporation, U.S.A.). An agar bridge (2% agar in 3 M KCl) was used to minimize junction potentials. MP was calibrated upon withdrawal of the microelectrode from the impaled cell. The output of the signal was displayed on an oscilloscope (Tektronix Model 5103N,Tektronix, U.S.A.) and coupled to the Axon Digidata-1200 acquisition system (Axon Instruments, U.S.A.). Data were digitized at a frequency of 500 Hz and stored in a Pentium computer for later analysis using Axon Scope 7.0 software (Axon Instruments, U.S.A.). The following parameters were used to quantitatively analyze the electrical properties of LES circular smooth muscle: (1) MP; (2) variance of MP fluctuations (MPfs) obtained by calculating the standard deviation of a 12 s MP sample; (3) amplitude of the sIJP; and (4) duration of the sIJP at 1/2 amplitude.

Solutions and drugs

The modified Kreb's solution contained (mM) NaCl 118.07, NaHCO$_3$ 25.00, D(+)-glucose 11.10, KCl 4.69, CaCl$_2$ 2.52, MgSO$_4$ 1.00 and NaH$_2$PO$_4$ 1.01. NFA was purchased from ICN Biochemicals Inc., ryanodine from Molecular Probes Inc. and all others from Sigma. Nifedipine, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), cyclopiazonic acid (CPA), ryanodine, NFA, 9-anthroic acid (A-9-C), 1-(5-chloronaphthalene-1-sulfonyl)-1-*H*-hexahydro-1,4-diazepine (ML-9) and wortmannin were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, and others in distilled water. These were diluted to final concentrations with Kreb's solution. Final concentration of DMSO in Kreb's solution was no more than 1%, which did not produce any effect on the electrical activity of the tissue. The Kreb's solution containing diluted drugs was fully bubbled with 5% CO$_2$ + 95% O$_2$ to restore pH prior to the application.

Statistical analysis

Data are shown as mean $\pm$ s.e. *n* refers to number of animals. If more than one cell was successfully impaled in the preparation

of an animal, parameters would be averaged. Only recordings in which a full protocol was completed in the same cell are included in the statistical analysis. Pre- and postdrug comparisons were made using the paired Student's t-test, and a P-value of <0.05 was considered statistically significant.

Results

General resting membrane electrical properties

Under the nonadrenergic and noncholinergic (NANC) conditions (atropine 3 μM and guanethidine 3 μM), nifedipine (1 μM) was used to immobilize muscle contraction and stabilize the intracellular impalements, while SP (1 μM) desensitization was used to eliminate neurokinin responses. Intracellular recordings revealed a resting MP of -38.1 ± 0.7 mV ($n=25$) that was characterized by spontaneous MPfs of 1–3 mV (Figure 1A, a). MPfs were quantitated by calculating the standard deviation

of MP sample over a 12 s recording period. The resulting variance value was 0.44 ± 0.03 mV ($n=25$). Electrical nerve stimulation with four square wave pulses (0.5 ms, 20 Hz, 80 V) evoked sIJPs with a mean amplitude of 6.1 ± 0.3 mV and a half-amplitude duration of 1926 ± 147 ms ($n=25$). ODQ (10 μM, 10 min), a specific guanylyl cyclase inhibitor, decreased the amplitude of sIJPs to 0.5 ± 0.1 mV from 6.5 ± 1.3 mV ($n=3$, $P<0.05$), but had no effect on resting MP and MPfs (Figure 1B), demonstrating that a cGMP-dependent signal cascade is involved in the sIJP. The inhibition of the sIJP by ODQ was reversed 60 min after washing out.

### Effects of caffeine, ryanodine and CPA on MP and sIJP

Caffeine has been reported to be an agonist of ryanodine receptors in the SR of different smooth muscles (Janssen & Sims, 1994; Wang et al., 1996; 1997; Cotton et al., 1997; Curtis & Scholfield, 2001). Application of caffeine causes Ca^{2+} release from SR, but with continuous application, Ca^{2+} stores
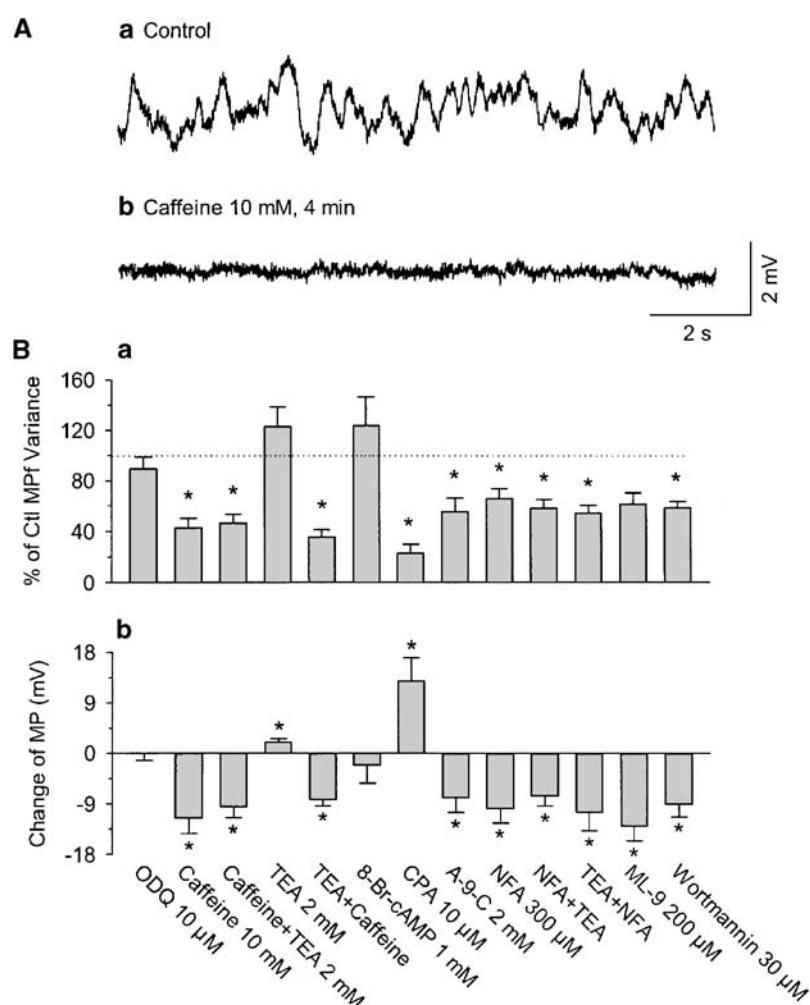
Figure 1 Effects of pharmacological intervention on MPfs and MP. A (a, b) Snapshots of raw recordings of MPfs before and after application of caffeine (10 mM). (B) Histograms demonstrated statistical analysis of effects of pharmacological intervention on MPfs (a) and MP (b) ($n=3$–8). Neither ODQ (10 μM), a specific guanylyl cyclase inhibitor, nor 8-Br-cAMP (1 mM), a membrane-permeable cAMP analog, significantly affected MPf and MP. TEA (2 mM), a $BK_{(Ca)}$ blocker, depolarized MP. SR function inhibitors (caffeine 10 mM; CPA 10 μM), $I_{Cl(Ca)}$ blockers (A-9-C 2 mM, NFA 300 μM) and MLCK inhibitors (ML-9 200 μM, wortmannin 30 μM) greatly suppressed MPfs. Both of the $I_{Cl(Ca)}$ blockers and MLCK inhibitors hyperpolarized MP. Caffeine hyperpolarized, but CPA depolarized, MP.

are eventually depleted. Caffeine (10 mM) hyperpolarized MP by 11.6 ± 2.7 mV ($n = 6$, $P < 0.05$) and abolished MPfs and the sIJP (amplitude of sIJP 0.3 ± 0.3 mV *versus* control of 5.6 ± 0.5 mV, $n = 6$, $P < 0.05$) 5 min after application (Figures 1 and 2A). The inhibitory effects reached a peak within a minute and reversed completely within 5 min of washing. In arteriolar and gallbladder smooth muscle, it was previously reported that Ca^{2+} release from SR activated $BK_{(Ca)}$ channels, resulting in membrane hyperpolarization (Nelson *et al.*, 1995; Pozo *et al.*, 2002). We therefore used TEA, a putative $BK_{(Ca)}$ channel blocker, to test this possibility in consecutive experiments. In the presence of caffeine (10 mM), TEA produced only slight depolarization (2.1 ± 1.0 mV; $n = 3$). Administration of TEA alone induced MP depolarization of 2.2 ± 0.5 mV over control and potentiated the sIJP amplitude

from 7.3 ± 0.6 to 10.6 ± 1.1 mV ($n = 8$, $P < 0.05$) (Figure 2C and D), but did not affect MPfs (Figure 1B). However, the concomitant application of caffeine (10 mM) still abolished MPfs and the sIJP, and hyperpolarized MP by 8.2 ± 1.2 mV ($n = 5$), a degree of hyperpolarization that was not significantly different from that induced by caffeine alone. Caffeine has also been reported to be a potent inhibitor of cAMP phosphodiesterase in different tissues (Rivedal & Sanner, 1985; Riley & Barclay, 1990; Tesarik *et al.*, 1992; Pozo *et al.*, 2002), resulting in intracellular cAMP accumulation, which activates protein kinase C-dependent signal cascade. A membrane-permeable cAMP analog, 8-bromo-cAMP (8-Br-cAMP), was used to exclude this possibility. Bath application of 8-Br-cAMP (1 mM) for up to 10 min did not evoke any measurable alterations of electrical parameters, but TEA
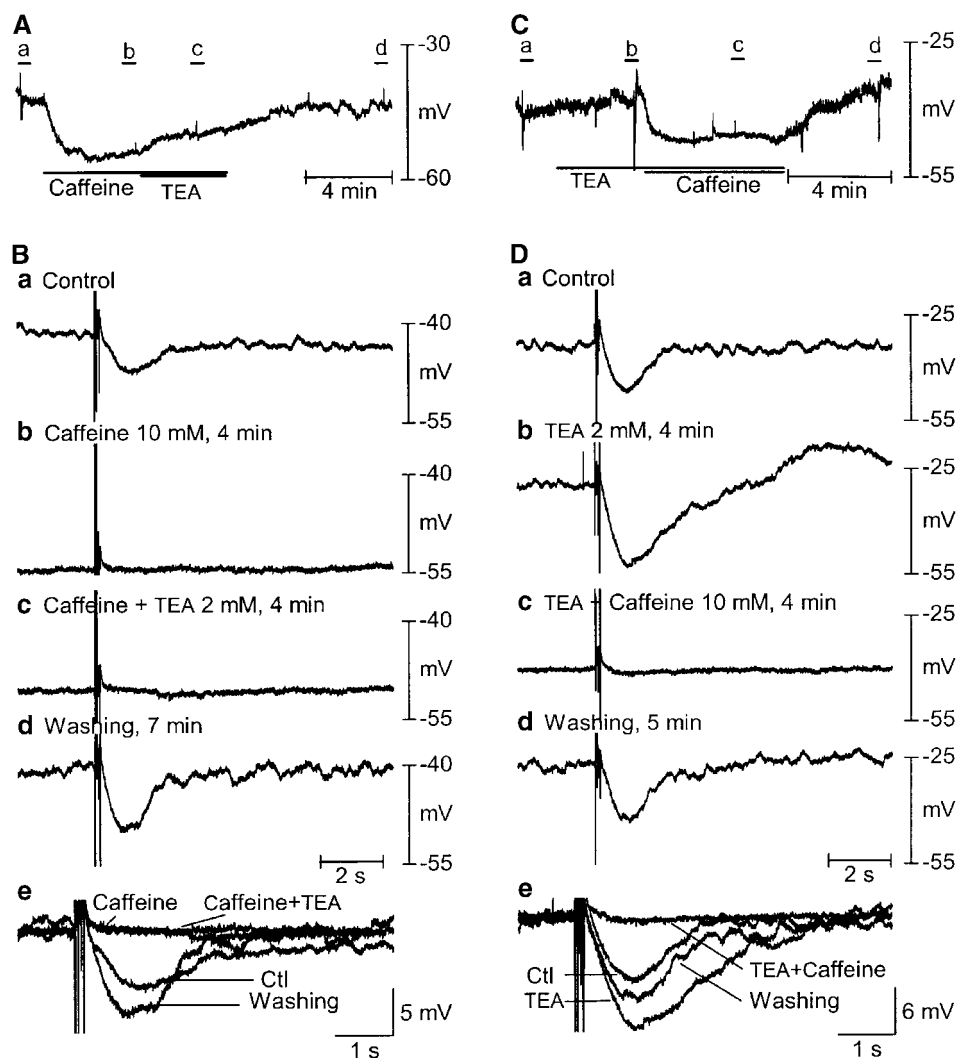
Figure 2 Effects of combined application of caffeine and TEA on the sIJP. sIJPs were evoked by four square wave electrical pulses of 0.3 ms duration at 20 Hz. (A, C) Original recordings of combined application of caffeine (10 mM) with TEA (2 mM) (A) or *vice versa* (C). (B, D) Original recordings labelled in (A, C) before (a), during (b, c) and after (d) combined application of caffeine and TEA, respectively. (e) Superimposed sIJPs using the periods prior to nerve stimulation as guides before, during and after application of caffeine and TEA for better comparison of sIJPs. Caffeine produced MP hyperpolarization, inhibited MPfs and abolished the sIJP. The effects of caffeine reached a peak in ~1 min and stayed steady until the termination of the application. The alterations of electrical properties fully recovered 5–10 min after washout of caffeine. Concomitant application of TEA could not restore the alterations of electrical properties. However, application of TEA alone depolarized MP by 2.2 ± 0.5 mV *versus* control ($n = 8$, $P < 0.05$) and significantly augmented the sIJP. Hyperpolarization of MP and inhibition of MPfs and the sIJP induced by caffeine were not affected by preapplication of TEA (2 mM).

(2 mM) significantly potentiated the sIJP in the presence of 8-Br-cAMP ($n = 3$, Figure 1B).

Ryanodine is reported to be a putative agonist of ryanodine receptors in the SR (Fill & Copello, 2002), which locks the channel in a slow-gating subconductance state. Bath application of ryanodine (20 μM) produced biphasic effects on MP, namely an initial small hyperpolarization of 2.3 ± 0.1 mV over control ($n = 3$, $P < 0.05$) in 4–4.5 min, followed by a large depolarization of 8.6 ± 2.5 mV ($n = 3$, $P < 0.05$), which reached a steady state in 20–25 min (Figure 3A). The sIJP was decreased from 4.2 ± 0.4 to 2.6 ± 0.1 mV ($n = 3$, $P < 0.05$) and the MPf variance declined from 0.32 ± 0.08 to 0.20 ± 0.02 mV ($n = 3$, $P > 0.05$) 20 min after ryanodine. CPA has been demonstrated to be a specific Ca^{2+} ATPase inhibitor of the SR, and continuous application also depletes Ca^{2+} stores in the SR (Cohen et al., 1999; Ethier et al., 2001; Ng & Gurney, 2001; Albert & Large, 2002). Application of CPA (10 μM) depolarized MP by 12.9 ± 4.1 mV ($n = 3$, $P < 0.05$) and abolished MPfs and the sIJP 15 min after bath application (Figure 3B). The effects of CPA reached the maximum in 5 min, but did not recover up to 30 min after washing.

Inhibitory effects of $I_{Cl(Ca)}$ blockers on sIJP

Two putative $I_{Cl(Ca)}$ blockers, A-9-C and NFA, were used to test the role of $I_{Cl(Ca)}$ in the sIJP. The maximal A-9-C effect (2 mM) was reached within 5–10 min, when MPfs were abolished and the sIJP was significantly suppressed. This effect could be reversed within 10–15 min of washing out. MP was hyperpolarized by 7.9 ± 2.6 mV and the amplitude of the sIJP was inhibited to 3.4 ± 0.6 mV from 5.9 ± 0.7 mV ($n = 4$, $P < 0.05$, Figures 1B and 4). NFA had a similar time course of action as A-9-C. NFA at a concentration of 300 μM, which was reported to be the maximal effective concentration in circular smooth muscle of esophageal body (Zhang & Paterson, 2002), abolished MPfs, hyperpolarized MP by 9.8 ± 2.6 mV versus control and decreased the amplitude of the sIJP from 6.7 ± 0.4 to 1.9 ± 0.3 mV ($n = 5$, $P < 0.05$). In the presence of NFA (300 μM), TEA (2 mM) produced MP depolarization of only 3.2 ± 1.8 mV ($n = 3$) (Figure 5A and B). However, in the presence of TEA, the concomitant application of NFA (300 μM) still abolished MPfs, hyperpolarized MP by 10.5 ± 3.3 mV and decreased the amplitude of the sIJP from 8.9 ± 0.5 to 2.1 ± 0.8 mV ($n = 4$, $P < 0.05$) (Figure 5C and D), suggesting that $BK_{(Ca)}$ channels do not contribute to the NFA-induced MP hyperpolarization. It has also been reported that NFA inhibits cation channels of rabbit SA node myocytes (Accili & DiFrancesco, 1996). A possible contribution of cation channels to MPfs, MP and the sIJP was tested by bath application of gadolinium, a putative nonselective cation channel blocker (Yang & Sachs, 1989; Zou et al.,

1999; Kirber et al., 2000). Gadolinium (500 μM) did not produce measurable changes in electrical properties up to 10 min after bath application.
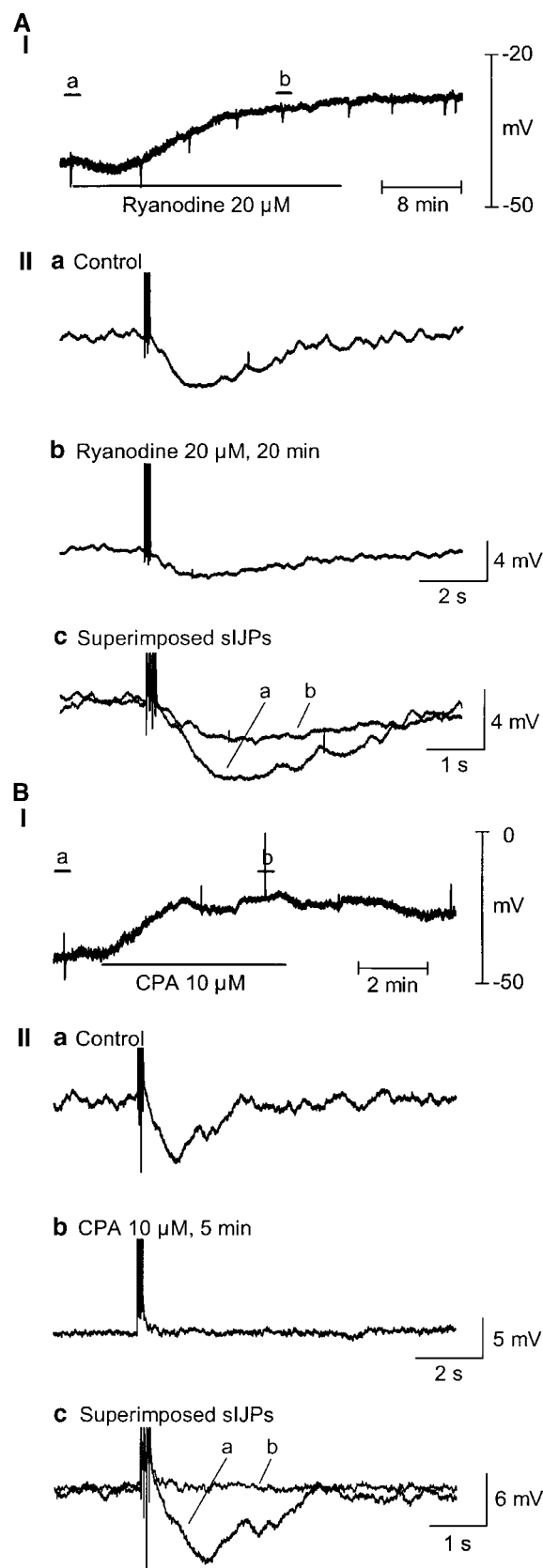
Figure 3 An example of the sIJP induced by nerve stimulation in the presence of ryanodine and CPA. (A.I) Original 40 min recording. (A.II) (a) Control sIJP; (b) ryanodine 20 μM, 20 min; (c) overlapped sIJPs. (B.I) Original recording. (B.II) (a) Control sIJP; (b) CPA 10 μM, 5 min; (c) overlapped sIJPs. Ryanodine produced biphasic effects on MP, initial small hyperpolarization followed by large depolarization, and inhibited the sIJP and MPfs, while CPA induced large depolarization and abolished the sIJP and MPfs. The effects of ryanodiine and CPA were irreversible.
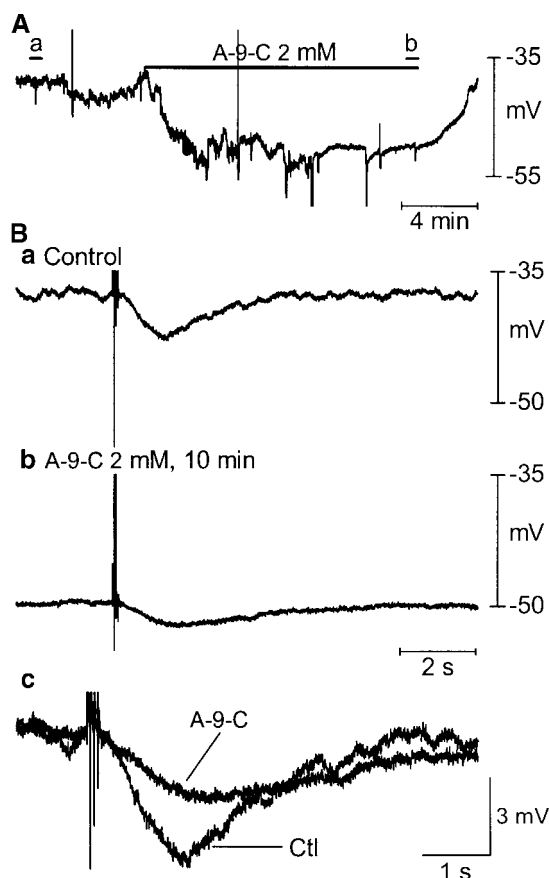
Figure 4 Effects of A-9-C on the sIJP. (A) Original recording showed that A-9-C hyperpolarized MP and abolished MPfs. Large up- and downward deflections are nerve stimulation artifacts. (B) sIJP displayed in expanded timescale. The sIJP was greatly suppressed.

Inhibitory effects of MLCK inhibitors on electrical properties

We previously demonstrated the possible involvement of MLCK in activation of the $I_{Cl(Ca)}$ and generation of the sIJP in circular smooth muscle of esophageal body (Zhang & Paterson, 2002). This possibility was also tested in the current studies by application of ML-9, an inhibitor of MLCK and protein kinase A and C, and wortmannin, a specific inhibitor of PI-3 kinase and MLCK. Time course studies showed that both inhibitors had maximal effects in about 5–10 min. The effect of ML-9 was reversible 15–17 min after washing out, but the effect of wortmannin was not reversible for up to 60 min after washing out. This is consistent with our previous studies (Zhang & Paterson, 2002). MPfs were inhibited by either ML-9 (200 μM) or wortmannin (30 μM). ML-9 and wortmannin hyperpolarized MP by 13.6 ± 2.6 and 9.1 ± 2.2 mV *versus* control, and significantly decreased the amplitude of the sIJP from 6.7 ± 0.5 and 5.4 ± 0.4 to 1.4 ± 0.5 and 1.2 ± 0.3 mV ($n = 5$, $P < 0.05$, Figure 6), respectively.

Discussion

The major findings of the current study are that in the opossum LES circular smooth muscle (1) spontaneous MPfs of

1–3 mV are recorded, which are suppressed by either depletion of SR Ca^{2+} stores, inhibition of the $I_{Cl(Ca)}$ or inhibition of MLCK; (2) inhibition of guanylyl cyclase abolishes the sIJP, but has no effect on MP and MPfs; (3) either depletion of SR Ca^{2+} stores, inhibition of the $I_{Cl(Ca)}$ or inhibition of MLCK hyperpolarizes MP by 9–12 mV and nearly abolishes the sIJP. These data support our hypothesis that spontaneous Ca^{2+} release from SR activates the $I_{Cl(Ca)}$ *via* a Ca^{2+}-dependent signal cascade in which MLCK is pivotal. Basal activity of the $I_{Cl(Ca)}$ maintains resting MP at relatively more positive levels and inhibition of the basal activity of the $I_{Cl(Ca)}$ by nitrergic innervation may produce the sIJP, leading to LES relaxation.

Role of SR in control of MP and LES basal tone

We previously provided evidence that basal activity of the $I_{Cl(Ca)}$ contributes to the resting MP of LES (Zhang et al., 2000), but the mechanisms underlying this basal $I_{Cl(Ca)}$ activity were unclear. Spontaneous Ca^{2+} release from the SR *via* the opening of ryanodine-sensitive Ca^{2+}-release channels has recently been proposed to play an important role in the regulation of membrane channel activity in cardiac (Cheng et al., 1993; 1995), skeletal (Valdivia et al., 1995) and smooth (Fay, 1995; Nelson et al., 1995) muscle cells. In stretched rat cerebral arterioles (Nelson et al., 1995; Jaggar et al., 1998) and guinea-pig gallbladder myocytes (Pozo et al., 2002), spontaneous Ca^{2+} release briefly hyperpolarizes cell membranes by activation of $BK_{(Ca)}$ channels, producing a relaxation. In guinea-pig tracheal smooth muscle cells, spontaneous Ca^{2+} transients activate either K^+ or Cl^- channels, depending on membrane potential (ZhuGe et al., 1998). Because the reversal potential of Cl^- conductance is between -30 and -20 mV in smooth muscle (Aickin & Brading, 1982; 1983), activation of the $I_{Cl(Ca)}$ would depolarize MP under the physiological conditions.

The aforementioned findings are not easily reconciled with the results of the current study. The resting MP recorded in LES circular smooth muscle is ~ -41 mV (Zhang et al., 2000) in the normal state and ~ -38.1 mV in the presence of atropine, guanethidine, nifedipine and SP desensitization. Depletion of SR Ca^{2+} stores by continuous application of caffeine abolishes MPfs and hyperpolarizes MP. These effects are unlikely to be due to opening of $BK_{(Ca)}$ channels, as they were not affected by preapplication of the $BK_{(Ca)}$ channel blocker TEA (2 mM) (Figure 2). Caffeine has been reported to be a potent cAMP phosphodiesterase inhibitor, resulting in intracellular cAMP accumulation (Rivedal & Sanner, 1985; Riley & Barclay, 1990; Tesarik et al., 1992; Sinha et al., 1993). However, our finding that 8-Br-cAMP, a membrane-permeable cAMP analog, had no effect on MP or MPfs precludes this mechanism as playing a role in the observed findings. On the other hand, the observations that TEA only produced MP depolarization of ~ 2 mV and 8-Br-cAMP had no measurable effect imply that $BK_{(Ca)}$ is not a major contributor to resting MP.

Ryanodine has been reported to have different effects in different muscles. It produces rigid paralysis in skeletal muscle but flaccid paralysis in cardiac muscle, whereas in smooth muscles it produces depolarization/excitation (Knot et al., 1998; Herrera et al., 2000; Haddock & Hill, 2002). In the current study, ryanodine (20 μM) initially hyperpolarized MP. Continuous application then depolarized MP significantly,
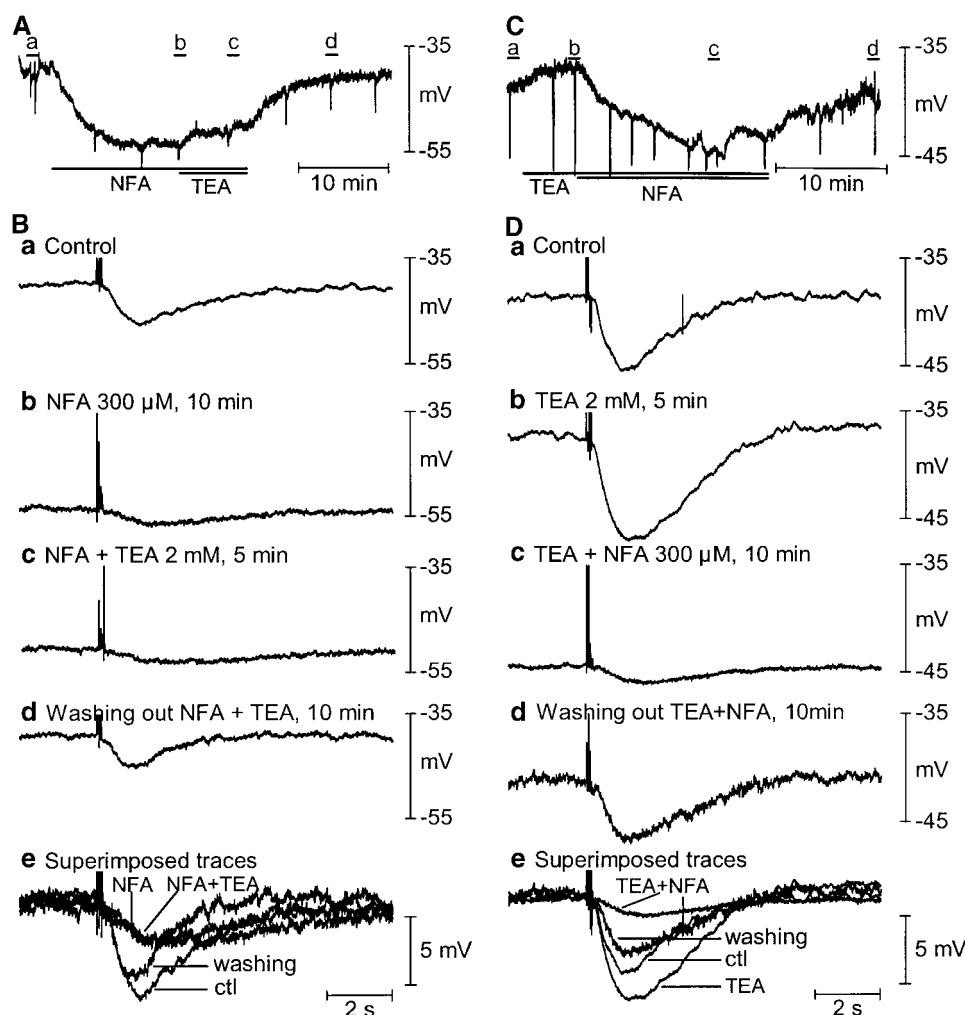
Figure 5 Example of the inhibitory effects of NFA on membrane electrical properties. (A) Raw recordings demonstrated that NFA ($300\,\mu$M) significantly hyperpolarized MP and inhibited MPfs. Concomitant application of TEA ($2\,$mM) only depolarized MP by $3.2\pm1.8\,$mV ($n=3$, $P<0.05$). (B) Snapshots of sIJPs labelled in (A) in expanded timescale. NFA significantly suppressed sIJP, which was not restored by TEA. (C) Recording demonstrating that application of TEA depolarized MP by $2.2\pm0.5\,$mV *versus* control ($n=8$, $P<0.05$). In the presence of TEA, NFA still produced MP hyperpolarization and inhibition of MPfs, which were no different from that produced by NFA alone. (D) sIJPs from (C) in expanded timescale. Application of TEA augmented the sIJP. However, NFA-induced MP hyperpolarization and inhibition of MPfs and the sIJP were not affected by preapplication of TEA, suggesting that inhibitory effects of NFA were not due to nonspecific actions, such as opening of $BK_{(Ca)}$.

which reached a steady state in $20-25\,$min (Figure 3A). This biphasic MP response is consistent with our preliminary tension recordings; that is, ryanodine induced an initial slight decrease, followed by a significant increase in basal tone (data not shown). However, these results are difficult to interpret. We speculate that the initial small hyperpolarization may be due to either activation of $BK_{(Ca)}$ or diminution of the $I_{Cl(Ca)}$, and that the large depolarization may be similar to that produced by CPA. Depletion of Ca^{2+} stores by CPA, *via* inhibition of Ca^{2+} ATPase in the SR, also abolishes MPfs, but in contrast to caffeine, CPA significantly depolarizes MP (Figures 1B and 3B), which is similar to the second phase of the observed ryanodine effect. This suggests that activation of nonselective cation channels by CPA may be predominant (Trepakova *et al.*, 2001).

Taken together, the current findings suggest that spontaneous Ca^{2+} release from the SR mainly activates the $I_{Cl(Ca)}$, which in turn results in a relatively more positive resting MP. This interpretation is further supported by the experiments in

which the $I_{Cl(Ca)}$ blockers NFA and ML-9 inhibit MPfs and produce comparable MP hyperpolarization. It is also consistent with a recent study (Suzuki *et al.*, 2003) demonstrating that A-9-C reduces membrane noise and hyperpolarizes MP in mouse gastric antrum smooth muscle.

Previous studies have reported that NFA has nonspecific effects, including inhibition of Ca^{2+} channels and Ca^{2+}-dependent contractile processes (Kato *et al.*, 1999), opening of $BK_{(Ca)}$ channels (Large & Wang, 1996) and blockade of nonselective cation channels (Accili & DiFrancesco, 1996). However, such nonspecific mechanisms cannot explain the observed abolition of MPfs and membrane hyperpolarization in our study because (1) intracellular recordings were conducted in the presence of nifedipine, thus L-type Ca^{2+} channels were already blocked; (2) abolition of MPfs and membrane hyperpolarization was not affected by preapplication of TEA, and the alterations of membrane electrical properties were not restored by concomitant administration of TEA (Figure 5A); (3) gadolinium, a nonselective cation

channel blocker (Zou *et al.*, 1999), did not produce any measurable changes of electrical properties. In addition, in cerebral artery, cation channels were not affected by NFA at

concentrations of up to $100\,\mu M$ (Nelson *et al.*, 1997; Welsh *et al.*, 2000). More recent studies by Piper *et al.* (2002) demonstrated dual effects of NFA on the $I_{Cl(Ca)}$ in acutely dissociated smooth muscle cells of rabbit pulmonary artery. NFA inhibited the outward part of the $I_{Cl(Ca)}$ with intracellular Ca^{2+} concentrations fixed at $500\,nM$ and $1\,\mu M$. Interestingly, NFA enhanced the inward part of the $I_{Cl(Ca)}$. In the LES, the opening of Cl^- channels would conduct inward currents, as recorded MP is $\sim -38.1\,mV$. If this were the case in the tissues currently studied, it would be predicted that NFA would produce MP depolarization. In our studies, NFA actually hyperpolarized MP by $\sim 10\,mV$ over control (Figure 5).

It has also been reported that NFA can affect skeletal muscle ryanodine receptor channels, with either activation or inhibition being produced depending on the channel subtype and concentration of NFA used (Oba, 1997). Whether this also applies to smooth muscle ryanodine receptors, which are quite distinct from those in skeletal muscle (Fill & Copello, 2002), is unknown. Nevertheless, we cannot fully exclude the possibility that some of the observed effects of NFA were *via* a nonspecific action on ryanodine receptors.

The processes of signal transduction in activation of the $I_{Cl(Ca)}$ remain unclear. Ca^{2+}-dependent calmodulin kinase II is proposed to be involved in the regulation of the $I_{Cl(Ca)}$ in vascular smooth muscle (Greenwood *et al.*, 2001) and in guinea-pig ileum circular smooth muscle (He *et al.*, 1999). Our recent observation suggests that MLCK is pivotal to the activation of the $I_{Cl(Ca)}$ in circular smooth muscle of opossum esophageal body (Zhang & Paterson, 2002). In the current study, the observation that MLCK inhibitors ML-9 and wortmannin suppress the MPfs and hyperpolarize MP to a similar degree to that induced by the $I_{Cl(Ca)}$ blockers and the depletion of Ca^{2+} stores in SR, further supports a role for MLCK in activation of the $I_{Cl(Ca)}$. Wortmannin has also been reported to inhibit PI-3 kinase (Arcaro & Wymann, 1993). However, it is less likely that PI-3 is involved in the regulation of $I_{Cl(Ca)}$, because MP, MPfs and the sIJP in opossum esophageal body are not affected by LY 294002 ($10\,\mu M$), a specific PI-3 kinase inhibitor (Zhang & Paterson, 2002). The effects of wortmannin on MP appear to be different in circular smooth muscle of esophageal body and LES. Wortmannin ($30\,\mu M$) produces MP hyperpolarization by $\sim 2\,mV$ in opossum esopageal body (Zhang & Paterson, 2002), while it hyperpolaizes MP by $\sim 9.1\,mV$ in LES (Figure 6C). The reason for this difference is unknown. A greater degree of hyperpolarization may imply greater activity of the $I_{Cl(Ca)}$ in LES than in esophageal body. In addition, the $9.1-13\,mV$ of membrane hyperpolarization produced by the $I_{Cl(Ca)}$ blockers, MLCK inhibitors and depletion of SR Ca^{2+} stores, strongly suggests that the $I_{Cl(Ca)}$ is responsible for at least this degree of voltage in MP of LES smooth muscle.

A number of previous studies have reported that MLCK inhibitors may affect activity of other ion channels, including
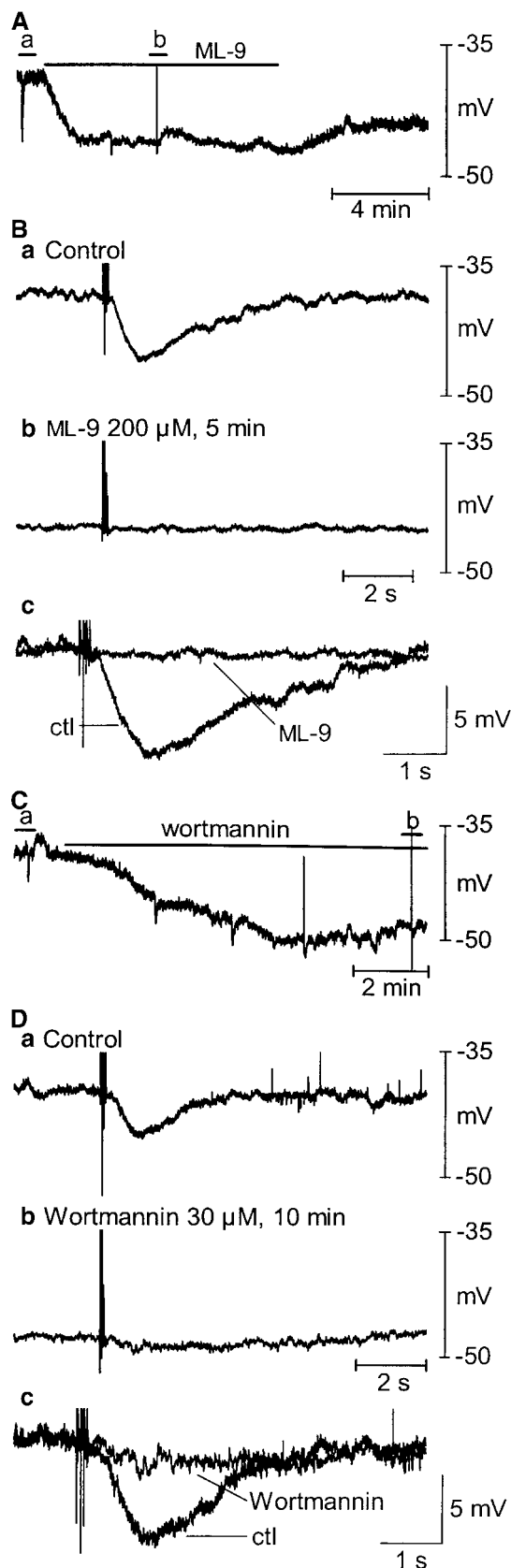
Figure 6 Inhibitory effects of the MLCK inhibitors ML-9 ($200\,\mu M$) and wortmannin ($30\,\mu M$). (A) ML-9 induced reversible MP hyperpolarization. (B) (a,b) sIJPs before and 5 min after application of ML-9, which abolished MPfs and the sIJP. (B) (c) Superimposed sIJP before and after ML-9. (C) Wortmannin produced MP hyperpolarization that was not reversed after washout. (D) Snap views of sIJPs labelled in (C) demonstrated that wortmannin abolished MPfs and the sIJP.

reduction of the M-currents in bullfrog sympathetic neurons (Akasu et al., 1993) and nonselective cation currents in gastric myocytes (Kim et al., 1997) and rabbit portal vein (Aromolaran et al., 2000). The current studies confirm our previous report of an interaction between MLCK and the $I_{Cl(Ca)}$. However, the physiological significance of this interaction remains unclear. It is likely that MLCK serves as one of the link points between channel activity and the contractile element process.

Nitrergic regulation

It is well established that intrinsic nitrergic neurons provide the predominant inhibitory innervation to the LES of several species (Jury et al., 1985; Allescher et al., 1988; Goyal, 1989; Yamato et al., 1992; Conklin et al., 1993; Preiksaitis et al., 1994; Preiksaitis & Diamant, 1995; Paterson et al., 1992; Kim et al., 1999). However, the ionic mechanisms that underlie nitrergic inhibition remain unclear. It was previously proposed that NO relaxed GI smooth muscle by either opening of K^+ channels or closing of Ca^{2+}-activated Cl^- channels (Sanders & Ozaki, 1994; Goyal, 2000). Indeed, patch-clamp studies demonstrated that NO and NO donors activate several kinds of K^+ channels in arterial (Bolotina et al., 1994; Yuan et al., 1996), colonic (Koh et al., 1995) and esophageal smooth muscle (Jury et al., 1996), and that these channels are sensitive to either TEA, apamin or 4-AP. Unfortunately, the NO-mediated sIJP is not blocked by any of the classic K^+ channel blockers in esophageal smooth muscle. We previously showed that NFA and A-9-C, putative $I_{Cl(Ca)}$ blockers, abolished the sIJP in circular smooth muscle of opossum esophageal body (Zhang & Paterson, 2002), supporting the hypothesis that suppression of the $I_{Cl(Ca)}$ by nitrergic innervation mediates the sIJP. The current studies in LES confirm the inhibitory effects of $I_{Cl(Ca)}$ blockers on sIJP, and are consistent with a recent study (Suzuki et al., 2003) in mouse antrum in which A-9-C was reported to inhibit the sIJP. It is less likely that inhibition of sIJP by NFA is due to its previously reported nonspecific actions, such as BK channel opening or blockade of cation channels, as abolition of the sIJP was not affected by either preapplication or concomitant application of TEA or gadolinium (see above). Interestingly, application of TEA potentiates the sIJP. This may be due to increased smooth muscle excitability and/or more neurotransmitter release by TEA (Jiang et al., 2001).

Jury et al. (2001) recently reported that in canine LES circular smooth muscle, NFA ($60 \mu M$) caused near-complete inhibition of basal tone, yet did not prevent electrical field stimulation (nitrergic innervation)-induced LES relaxation when tone was restored by addition of carbachol to the bath.

This observation suggests that Cl^- channels are not involved in NO-mediated LES relaxation. However, we previously demonstrated that in opossum esophageal body, the IC_{50} of NFA for inhibition of sIJP is $\sim 80 \mu M$, while NFA completely abolished sIJP at concentrations of $300 \mu M$ (Zhang & Paterson, 2002). The discrepant findings in these two studies likely relate to the different concentrations of NFA used. Nevertheless, in the presence of either of caffeine, CPA, NFA and wortmannin, there remains a residual sIJP with amplitude varying between 0.3 and 1.9 mV (versus ~ 6.1 mV in control cells). It is possible that the opening of unidentified K^+ channels is responsible for this residual MP hyperpolarization induced by nitrergic innervation.

Cayabyab & Daniel (1996) reported a role for the SR in sIJPs and hyperpolarizations induced by NO donors in opossum oesophagus. They demonstrated that the selective SR Ca^{2+}-ATPase inhibitors CPA and thapsigargin, and the SR Ca^{2+} release channel activator ryanodine caused depolarization and spontaneous contractions, which were diminished after prolonged (>30 min) incubation with these agents in Ca^{2+}-containing medium. Moreover, these agents inhibited both the sIJP and NO-donor induced hyperpolarization. They interpreted these results to indicate that functional SR Ca^{2+} uptake is necessary for the response to endogenous or exogenous NO. Our studies confirm that CPA depolarizes MP and abolishes the sIJP and MPfs. However, we believe that these results can also be explained by activation of the $I_{Cl(Ca)}$ by spontaneous Ca^{2+} release from caffeine-sensitive Ca^{2+} stores of the SR. The depletion of Ca^{2+} stores by CPA and caffeine abolishes spontaneous release of Ca^{2+} and thus basal activity of the $I_{Cl(Ca)}$. Without the $I_{Cl(Ca)}$ activity, the sIJP evoked by nitrergic innervation and hyperpolarization produced by NO donors would be prevented. The opposing effects on resting MP, that is depolarization by CPA and hyperpolarization by caffeine, may suggest the predominant effects of CPA on native cation channels activated by Ca^{2+} store depletion (Trepakova et al., 2001).

In summary, these data strongly support a role of the SR in control of resting MP and nitrergic responses in opossum LES circular smooth muscle. Spontaneous Ca^{2+} release from SR via activation of ryanodine receptors activates the $I_{Cl(Ca)}$ in which MLCK is pivotal. NO released from nitrergic nerves may inactivate the $I_{Cl(Ca)}$ via intracellular elevation of cGMP, leading to the sIJP and ensuing relaxation. The site(s) of action of NO and the nature of the interaction between the $I_{Cl(Ca)}$ and MLCK require further investigation.

References

ACCILI, E.A. & DIFRANCESCO, D. (1996). Inhibition of the hyperpolarization-activated current (if) of rabbit SA node myocytes by niflumic acid. *Pflugers Arch.*, **431**, 757–762.

AICKIN, C.C. & BRADING, A.F. (1982). Measurement of intracellular chloride in guinea-pig vas deferens by ion analysis, 36chloride efflux and micro-electrodes. *J. Physiol.*, **326**, 139–154.

AICKIN, C.C. & BRADING, A.F. (1983). Towards an estimate of chloride permeability in the smooth muscle of guinea-pig vas deferens. *J. Physiol.*, **336**, 179–197.

AKASU, T., ITO, M., NAKANO, T., SCHNEIDER, C.R., SIMMONS, M.A., TANAKA, T., TOKIMASA, T. & YOSHIDA, M. (1993). Myosin light chain kinase occurs in bullfrog sympathetic neurons and may modulate voltage-dependent potassium currents. *Neuron*, **11**, 1133–1145.

ALBERT, A.P. & LARGE, W.A. (2002). Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. *J. Physiol.*, **544**, 113–125.

ALLESCHER, H.D., BEREZIN, I., JURY, J. & DANIEL, E.E. (1988). Characteristics of canine lower esophageal sphincter: a new electrophysiological tool. *Am. J. Physiol.*, **255**, G441–G453.

ARCARO, A. & WYMANN, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.,* **296** (Part 2), 297–301.

AROMOLARAN, A.S., ALBERT, A.P. & LARGE, W.A. (2000). Evidence for myosin light chain kinase mediating noradrenaline-evoked cation current in rabbit portal vein myocytes. *J. Physiol.,* **524** (Part 3), 853–863.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature,* **368,** 850–853.

CAYABYAB, F.S. & DANIEL, E.E. (1996). Role of sarcoplasmic reticulum in inhibitory junction potentials and hyperpolarizations by nitric oxide donors in opossum oesophagus. *Br. J. Pharmacol.,* **118,** 2185–2191.

CHENG, H., FILL, M., VALDIVIA, H. & LEDERER, W.J. (1995). Models of Ca^{2+} release channel adaptation. *Science,* **267,** 2009–2010.

CHENG, H., LEDERER, W.J. & CANNELL, M.B. (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. *Science,* **262,** 740–744.

COHEN, R.A., WEISBROD, R.M., GERICKE, M., YAGHOUBI, M., BIERL, C. & BOLOTINA, V.M. (1999). Mechanism of nitric oxide-induced vasodilatation: refilling of intracellular stores by sarcoplasmic reticulum Ca^{2+} ATPase and inhibition of store-operated Ca^{2+} influx. *Circ. Res.,* **84,** 210–219.

CONKLIN, J.L., DU, C., MURRAY, J.A. & BATES, J.N. (1993). Characterization and mediation of inhibitory junction potentials from opossum lower esophageal sphincter. *Gastroenterology,* **104,** 1439–1444.

COTTON, K.D., HOLLYWOOD, M.A., MCHALE, N.G. & THORNBURY, K.D. (1997). Ca^{2+} current and Ca^{2+}-activated chloride current in isolated smooth muscle cells of the sheep urethra. *J. Physiol.,* **505,** 121–131.

CURTIS, T.M. & SCHOLFIELD, C.N. (2001). Nifedipine blocks Ca^{2+} store refilling through a pathway not involving L-type Ca^{2+} channels in rabbit arteriolar smooth muscle. *J. Physiol.,* **532,** 609–623.

ETHIER, M.F., YAMAGUCHI, H. & MADISON, J.M. (2001). Effects of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells. *Am. J. Physiol.,* **281,** L126–L133.

FAY, F.S. (1995). Calcium sparks in vascular smooth muscle: relaxation regulators. *Science,* **270,** 588–589.

FILL, M. & COPELLO, J.A. (2002). Ryanodine receptor calcium release channels. *Physiol. Rev.,* **82,** 893–922.

GOYAL, R.K. (1989). Muscarinic receptor subtypes. Physiology and clinical implications. *New Engl. J. Med.,* **321,** 1022–1029.

GOYAL, R.K. (2000). Targets of enteric motor neurones: smooth muscle cells. *Gut,* **47** (Suppl. 4), iv38–iv39.

GOYAL, R.K. & PATERSON, W.G. (1989). Esophageal motility. In: *Hand Book of Physiology.* ed. Schultz, S.G., Wood, J.D. & Rauner, B.B. pp. 865–908. Bethesda, MD: Oxford University Press.

GREENWOOD, I., LEDOUX, J. & LEBLANC, N. (2001). Differential regulation of Ca^{2+}-activated Cl^- currents in rabbit arterial and portal vein smooth muscle cells by Ca^{2+}-calmodulin-dependent kinase. *J. Physiol.,* **534,** 395–408.

HADDOCK, R.E. & HILL, C.E. (2002). Differential activation of ion channels by inositol 1,4,5-trisphosphate (IP_3)- and ryanodine-sensitive calcium stores in rat basilar artery vasomotion. *J. Physiol.,* **545,** 615–627.

HE, X.D., AKBARALI, H.I. & GOYAL, R.K. (1999). Modulation of resting potential and nitrergic slow IJP by CAM kinase II and MAP kinase in guinea-pig ileum circular muscle. *Gastroenterology,* **166** (Suppl. 4), A1004.

HERRERA, G.M., HEPPNER, T.J. & NELSON, M.T. (2000). Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am. J. Physiol.,* **279,** R60–R68.

JAGGAR, J.H., STEVENSON, A.S. & NELSON, M.T. (1998). Voltage dependence of Ca^{2+} sparks in intact cerebral arteries. *Am. J. Physiol.,* **274,** C1755–C1761.

JANSSEN, L.J. & SIMS, S.M. (1994). Spontaneous transient inward currents and rhythmicity in canine and guinea-pig tracheal smooth muscle cells. *Pflugers Arch.,* **427,** 473–480.

JIANG, Z.G., SI, J.Q., LASAREV, M.R. & NUTTALL, A.L. (2001). Two resting potential levels regulated by the inward-rectifier potassium channel in the guinea-pig spiral modiolar artery. *J. Physiol.,* **537,** 829–842.

JURY, J., BOEV, K.R. & DANIEL, E.E. (1996). Nitric oxide mediates outward potassium currents in opossum esophageal circular smooth muscle. *Am. J. Physiol.,* **270,** G932–G938.

JURY, J., JAGER, L.P. & DANIEL, E.E. (1985). Unusual potassium channels mediate nonadrenergic noncholinergic nerve-mediated inhibition in opossum esophagus. *Can. J. Physiol. Pharmacol.,* **63,** 107–112.

JURY, J., PATEL, M., BOWES, T. & DANIEL, E.E. (2001). Actions of putative chloride channel blocking agents on canine lower esophageal sphincter (LES). *Can. J. Physiol. Pharmacol.,* **79,** 1007–1014.

KATO, K., EVANS, A.M. & KOZLOWSKI, R.Z. (1999). Relaxation of endothelin-1-induced pulmonary arterial constriction by niflumic acid and NPPB: mechanism(s) independent of chloride channel block. *J. Pharmacol. Exp. Ther.,* **288,** 1242–1250.

KIM, C.D., GOYAL, R.K. & MASHIMO, H. (1999). Neuronal NOS provides nitrergic inhibitory neurotransmitter in mouse lower esophageal sphincter. *Am. J. Physiol.,* **277,** G280–G284.

KIM, Y.C., KIM, S.J., KANG, T.M., SUH, S.H., SO, I. & KIM, K.W. (1997). Effects of myosin light chain kinase inhibitors on carbachol-activated nonselective cationic current in guinea-pig gastric myocytes. *Pflugers Arch.,* **434,** 346–353.

KIRBER, M.T., GUERRERO-HERNANDEZ, A., BOWMAN, D.S., FOGARTY, K.E., TUFT, R.A., SINGER, J.J. & FAY, F.S. (2000). Multiple pathways responsible for the stretch-induced increase in Ca^{2+} concentration in toad stomach smooth muscle cells. *J. Physiol.,* **524,** 3–17.

KNOT, H.J., STANDEN, N.B. & NELSON, M.T. (1998). Ryanodine receptors regulate arterial diameter and wall $[Ca^{2+}]$ in cerebral arteries of rat *via* Ca^{2+}-dependent K^+ channels. *J. Physiol.,* **508** (Part 1), 211–221.

KOH, S.D., CAMPBELL, J.D., CARL, A. & SANDERS, K.M. (1995). Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. *J. Physiol.,* **489** (Part 3), 735–743.

LARGE, W.A. & WANG, Q. (1996). Characteristics and physiological role of the Ca^{2+}-activated Cl^- conductance in smooth muscle. *Am. J. Physiol.,* **271,** C435–C454.

NELSON, M.T., CHENG, H., RUBART, M., SANTANA, L.F., BONEV, A.D., KNOT, H.J. & LEDERER, W.J. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science,* **270,** 633–637.

NELSON, M.T., CONWAY, M.A., KNOT, H.J. & BRAYDEN, J.E. (1997). Chloride channel blockers inhibit myogenic tone in rat cerebral arteries. *J. Physiol.,* **502,** 259–264.

NG, L.C. & GURNEY, A.M. (2001). Store-operated channels mediate Ca^{2+} influx and contraction in rat pulmonary artery. *Circ. Res.,* **89,** 923–929.

OBA, T. (1997). Niflumic acid differentially modulates two types of skeletal ryanodine-sensitive Ca^{2+}-release channels. *Am. J. Physiol.,* **73,** C1588–C1595.

PATERSON, W.G., ANDERSON, M.A. & ANAND, N. (1992). Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation, and esophageal distention. *Can. J. Physiol. Pharmacol.,* **70,** 1011–1015.

PIPER, A.S., GREENWOOD, I.A. & LARGE, W.A. (2002). Dual effect of blocking agents on Ca^{2+}-activated Cl^- currents in rabbit pulmonary artery smooth muscle cells. *J. Physiol.,* **539,** 119–131.

POZO, M.J., PEREZ, G.J., NELSON, M.T. & MAWE, G.M. (2002). Ca^{2+} sparks and BK currents in gallbladder myocytes: role in CCK-induced response. *Am. J. Physiol.,* **282,** G165–G174.

PREIKSAITIS, H.G. & DIAMANT, N.E. (1995). Phasic contractions of the muscular components of human esophagus and gastroesophageal junction *in vitro*. *Can. J. Physiol. Pharmacol.,* **73,** 356–363.

PREIKSAITIS, H.G., TREMBLAY, L. & DIAMANT, N.E. (1994). Cholinergic responses in the cat lower esophageal sphincter show regional variation. *Gastroenterology,* **106,** 381–388.

RILEY, B.B. & BARCLAY, S.L. (1990). Conditions that alter intracellular cAMP levels affect expression of the cAMP phosphodiesterase gene in *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 4746–4750.

RIVEDAL, E. & SANNER, T. (1985). Caffeine and other phosphodiesterase inhibitors are potent inhibitors of the promotional effect of TPA on morphological transformation of hamster embryo cells. *Cancer Lett.,* **28,** 9–17.

SANDERS, K.M. & OZAKI, H. (1994). Excitation–contraction coupling in gastrointestinal smooth muscles. In: *Pharmacology of Smooth Muscle*. ed. Szekeres, L. & Papp, J.Gy. pp. 331–404. Berlin: Springer Verlag.

SENGUPTA, A., PATERSON, W.G. & GOYAL, R.K. (1987). Atypical localization of myenteric neurons in the opossum lower esophageal sphincter. *Am. J. Anat.,* **180,** 342–348.

SINHA, S., KUMAR, P. & LALORAYA, M. (1993). Methyl xanthine and altered biomembrane dynamics: demonstration of protein mobility and enzyme inhibition by caffeine in sperm model system. *Biochem. Mol. Biol. Int.,* **31,** 1141–1148.

SUZUKI, H., WARD, S.M., BAYGUINOV, Y.R., EDWARDS, F.R. & HIRST, G.D. (2003). Involvement of intramuscular interstitial cells in nitrergic inhibition in the mouse gastric antrum. *J. Physiol.,* **546,** 751–763.

TESARIK, J., MENDOZA, C. & CARRERAS, A. (1992). Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertil. Steril.,* **58,** 1185–1190.

TREPAKOVA, E.S., GERICKE, M., HIRAKAWA, Y., WEISBROD, R.M., COHEN, R.A. & BOLOTINA, V.M. (2001). Properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. *J. Biol. Chem.,* **276,** 7782–7790.

VALDIVIA, H.H., KAPLAN, J.H., ELLIS-DAVIES, G.C. & LEDERER, W.J. (1995). Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. *Science,* **267,** 1997–2000.

WANG, Q., AKBARALI, H.I., HATAKEYAMA, N. & GOYAL, R.K. (1996). Caffeine- and carbachol-induced Cl^- and cation currents in single opossum esophageal circular muscle cells. *Am. J. Physiol.,* **271,** C1725–C1734.

WANG, Q., WANG, Y.X., YU, M. & KOTLIKOFF, M.I. (1997). Ca^{2+}-activated Cl^- currents are activated by metabolic inhibition in rat pulmonary artery smooth muscle cells. *Am. J. Physiol.,* **273,** C520–C530.

WELSH, D.G., NELSON, M.T., ECKMAN, D.M. & BRAYDEN, J.E. (2000). Swelling-activated cation channels mediate depolarization of rat cerebrovascular smooth muscle by hyposmolarity and intravascular pressure. *J. Physiol.,* **527,** 139–148.

YAMATO, S., SPECHLER, S.J. & GOYAL, R.K. (1992). Role of nitric oxide in esophageal peristalsis in the opossum. *Gastroenterology,* **103,** 197–204.

YANG, X.C. & SACHS, F. (1989). Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science,* **243,** 1068–1071.

YUAN, X.J., TOD, M.L., RUBIN, L.J. & BLAUSTEIN, M.P. (1996). NO hyperpolarizes pulmonary artery smooth muscle cells and decreases the intracellular Ca^{2+} concentration by activating voltage-gated K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 10489–10494.

ZHANG, Y., MILLER, D.V. & PATERSON, W.G. (2000). Opposing roles of K^+ and Cl^- channels in maintenance of opossum lower esophageal sphincter tone. *Am. J. Physiol.,* **279,** G1226–G1234.

ZHANG, Y. & PATERSON, W.G. (2002). Role of Ca^{2+}-activated Cl^- channels and MLCK in opossum esophageal smooth muscle. *Am. J. Physiol.,* **283** (Suppl. 1), G104–G114.

ZHANG, Y. & PATERSON, W.G. (2001). Diversity of K^+ channels in circular smooth muscle of opossum lower esophageal sphincter. *Can. J. Physiol. Pharmacol.,* **79,** 608–620.

ZHUGE, R., SIMS, S.M., TUFT, R.A., FOGARTY, K.E. & WALSH, J.V.J. (1998). Ca^{2+} sparks activate K^+ and Cl^- channels, resulting in spontaneous transient currents in guinea-pig tracheal myocytes. *J. Physiol.,* **513** (Part 3), 711–718.

ZOU, H., LIFSHITZ, L.M., TUFT, R.A., FOGARTY, K.E. & SINGER, J.J. (1999). Imaging Ca^{2+} entering the cytoplasm through a single opening of a plasma membrane cation channel. *J. Gen. Physiol.,* **114,** 575–588.