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Role of sarcoplasmic reticulum in inhibitory junction potentials and hyperpolarizations by nitric oxide donors in opossum oesophagus

[1]Francisco S. Cayabyab & [2]Edwin E. Daniel

McMaster University, Department of Biomedical Sciences, Division of Physiology and Pharmacology, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

1 Previous patch clamp studies of oesophageal circular muscle cells showed that nitric oxide (NO) modulated the opening of Ca^{2+}-activated K^+ channels involved in mediating the inhibitory junction potentials (i.j.ps). This study clarified the role of Ca^{2+} release from the superficial sarcoplasmic reticulum (SR) in the mechanism of i.j.ps or hyperpolarizing responses to NO-releasing compounds. Electrical and mechanical activities were simultaneously recorded by intracellular microelectrode or double sucrose gap techniques.

2 The NO-donors, sydnonimine (SIN-1) and sodium nitroprusside, each at 500 μM, hyperpolarized oesophageal circular muscle cells by 15-20 mV, like i.j.ps.

3 The selective inhibitors of SR Ca^{2+}-ATPase (cyclopiazonic acid 10-30 μM and thapsigargin 5 μM) and the SR Ca^{2+} release channel activator (ryanodine 30 μM) 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 i.j.p. and NO-donor hyperpolarizations, suggesting that a functional SR Ca^{2+} uptake is necessary for the response to endogenous or exogenous NO.

4 These results, along with our previous findings of the dependence of i.j.ps and NO-donor hyperpolarizations on K^+ channel activation and cyclic GMP elevation, support the hypothesis that subplasmalemmal Ca_i^{2+} elevation, via vectorial Ca^{2+} release from superficial SR toward the plasmalemma, may be an important mechanism by which NO, from NO-liberating compounds or released from inhibitory neurones induces relaxation and i.j.ps in opossum oesophagus.

Keywords: Sydnonimine (SIN-1); sodium nitroprusside; sarcoplasmic reticulum; ryanodine; cyclopiazonic acid; thapsigargin; K^+ channels; buffer barrier

Introduction

We recently demonstrated that K^+ channel opening was the major mechanism mediating the inhibitory junction potentials (i.j.ps) evoked by electrical field stimulation of non-adrenergic, non-cholinergic (NANC) nerves in opossum oesophagus and canine ileum (Christinck *et al.*, 1991; Cayabyab & Daniel, 1995). In both these preparations, nitric oxide (NO) was shown to be a mediator of the i.j.p. while vasoactive intestinal polypeptide (VIP) was demonstrated to be ineffective as a hyperpolarizing agent (Daniel *et al.*, 1983; Christinck *et al.*, 1991). Earlier studies suggested that the effect of VIP on the oesophageal circular muscle was Cl_o^--dependent (Daniel *et al.*, 1983; 1987). However, the electrophysiological basis of the i.j.p. in opossum oesophagus remains controversial (Daniel *et al.*, 1992). Increased K^+ conductance (Jury *et al.*, 1985; Christinck *et al.*, 1991; Cayabyab & Daniel, 1995) and decreased Cl^- conductance (Crist *et al.*, 1991) have both been proposed as the ionic mechanism underlying the i.j.p. Recent studies have shown that NO-releasing compounds, the organic 3-morpholino-sydnonimine-hydrochloride (SIN-1) and the inorganic sodium nitroprusside (SNP), mimic the actions of endogenously released NO (Conklin & Du, 1992; Cayabyab & Daniel, 1995). That is, these NO-donors increased membrane conductance and caused membrane hyperpolarizations which reversed near the K^+ equilibrium potential (E_K), as did the i.j.ps. These NO-donor hyperpolarizations and i.j.ps were shown to be sensitive only to quinine among all the K^+ channel antagonists tested (Jury *et al.*, 1985; Cayabyab & Daniel, 1995).

Previous studies also showed that Ca^{2+} activated K^+ (K_{Ca}) current could be triggered by Ca^{2+} release from intracellular storage sites following Ca^{2+} influx by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Zholos *et al.*, 1991). Also, studies by Stehno-Bittel & Sturek (1992) showed that K^+-channel opening in vascular smooth muscle cells is sensitive to subsarcolemmal Ca^{2+} concentrations. These results may help clarify the mechanism of opening of the unusual K^+ channels (not blocked by apamin, tetraethylammonium ion or by charybdotoxin, see Jury *et al.*, 1985; 1996; Cayabyab & Daniel, 1995) induced by the inhibitory mediator (NO) in the opossum oesophagus. NO binds to soluble guanylate cyclase to elevate guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Katsuki *et al.*, 1977; Ignarro 1990). Guanylate cyclase inhibitors inhibited both the oesophageal i.j.ps and hyperpolarizing effects of cyclic GMP-generating NO-donors (Conklin & Du, 1992; Cayabyab & Daniel, 1995). However, it is not known whether NO-generated cyclic GMP may mediate an increase in sarcoplasmic reticulum (SR) Ca^{2+} uptake and enhance the vectorial release of Ca^{2+} toward the plasmalemma by peripherally located SR to elicit relaxation and i.j.ps in oesophagus.

The superficial SR is not only a significant source of Ca^{2+} for intracellular signalling, but may also act as a buffer barrier to Ca^{2+} entry as demonstrated in vascular tissues (Van Breemen & Saida, 1989; Chen *et al.*, 1992; Chen & Van Breemen, 1993). This would account for the observed delay between cytosolic Ca^{2+} elevation due to Ca^{2+} influx and activation of the contractile apparatus when the Ca^{2+} stores are empty. SR unloading and refilling and elicitation of relaxation by cyclic

[1]Present address: Department of Physiology, University of Toronto and Playfair, Neurosciences Unit, The Toronto Hospital (Western Division), 12-419 McLaughlin Pavilion, Toronto, Ontario, Canada
[2]Author for correspondence.

GMP-generating compounds require that the SR Ca^{2+}-ATPase and Ca^{2+} release channels be functional (Chen *et al.*, 1992; Chen & Van Breemen, 1993; Low *et al.*, 1993; Luo *et al.*, 1993). Prevention of the normal functioning of the buffer barrier system with the aid of agents known to modulate SR Ca^{2+} release or SR Ca^{2+}-uptake has been demonstrated to deplete Ca^{2+} stores and inhibit the outward K_{Ca} current in gastrointestinal (Sakai *et al.*, 1988; Zholos *et al.*, 1991; Suzuki *et al.*, 1992) and vascular (Stehno-Bittel & Sturek, 1992; Chen & Van Breemen, 1993; Low *et al.*, 1993) smooth muscle cells. Selective interference with superficial SR Ca^{2+} transport also causes a steady state elevation of $[Ca^{2+}]_i$, which may be due to a sustained increase in influx of Ca^{2+} from extracellular space (Mason *et al.*, 1991). In contrast when the SR Ca^{2+} pump is active to fill stores, a continuous vectorial release of Ca^{2+} from the SR lumen to the extracellular space may occur (Chen *et al.*, 1992; Chen & Van Breemen, 1993). This vectorial Ca^{2+} release depends on elevated Ca_i^{2+} levels near the plasmalemma and utilizes Na^+-Ca^{2+} exchange for Ca^{2+} extrusion from the cell (Van Breemen & Saida, 1989). These observations are consistent with the suggestion that K^+ current generation may be triggered by Ca^{2+} elevation in localized areas where the SR and plasmalemmal membranes are closely apposed (Zholos *et al.*, 1991; Chen *et al.*, 1992; Stehno-Bittel & Sturek, 1992).

Recent patch clamp studies of several gastrointestinal smooth muscle cells (Murray *et al.*, 1995; Duridanova *et al.*, 1995; Jury *et al.*, 1996) demonstrated that NO-donors enhanced K_{Ca} outward currents. These effects were prevented by inhibition of protein kinase G (Murray *et al.*, 1995), activated by cyclic GMP analogues (Duridanova *et al.*, 1995), and decreased by agents blocking SR Ca^{2+}-ATPase (Duridanova *et al.*, 1995; Jury *et al.*, 1996). These studies support the hypothesis that in tissue preparations membrane hyperpolarization induced by exogenous and endogenous NO (Christinck *et al.*, 1991; Conklin & Du, 1992; Cayabyab & Daniel, 1995) requires a functional SR Ca^{2+} pump and involves cyclic GMP activated kinase. Thus the aim of this study was to determine whether interference with SR Ca^{2+} accumulation modulates K_{Ca} channels mediating the i.j.ps and NO-donor hyperpolarizations in the intact opossum oesophagus circular muscle. Initial accounts of these data proposed that NO opened K^+ channels by enhancing SR Ca^{2+} uptake and causing an overflow of Ca^{2+} into a restricted region between the plasmalemma and superficial SR (Cayabyab *et al.*, 1993 Cayabyab & Daniel, 1994).

Methods

Adult North American opossums (*Didelphis marsupialis*) of either sex were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}). All animals were supplied by North Eastern Wildlife (New York, U.S.A.) and were in good health. The abdomen was opened along the midline, and the animal was subsequently killed by intracardiac injection of sodium pentobarbitone. These procedures were approved by the Animal Care Committee of McMaster University. A 15 cm strip of the lower oesophageal body with about 2 cm of the stomach and sphincter was later removed and stored in Krebs-Ringer solution at 25±2°C (for composition, see below).

Electrophysiological studies

A 30 mm × 2 mm strip of oesophageal body circular muscle was mounted in the double sucrose gap to monitor simultaneously the relative membrane potential, electrotonic potential, and mechanical activity. Details of this method have been described previously (Jury *et al.*, 1985; Christinck *et al.*, 1991; Cayabyab & Daniel, 1995). In brief, the preparations were allowed to equilibrate for an hour in the double sucrose gap while being superfused with Krebs-Ringer solution at 25±2°C. The strips fitted closely to the Krebs, sucrose, and KCl compartments but were not compressed; tension was recorded by a Grass FTOC3 transducer from the tissue in the Krebs compartment. Intramural non-adrenergic non-cholinergic (NANC) nerves in tissue strips were stimulated by field stimulation at supramaximal stimulus parameters (pulse rate, 30 p.p.s.; pulse duration, 0.3 ms; voltage, 40–50 V; train duration, 300 ms; (Daniel *et al.*, 1983)). With the temperature and dissection procedure used, responses to field stimulation were stable for up to 7 h (Jury *et al.*, 1985; Christinck *et al.*, 1991).

The solutions used contained (mM): Krebs-Ringer solution; NaCl 115.5, KCl 4.16, $NaHCO_3$ 21.9, NaH_2PO_4 1.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.2, gassed at all times with 95% O_2–5% CO_2; KCl solution; KCl 160, sucrose solution, 270 mM of extra pure sucrose (Merck, Germany and/or BDH Inc., Canada). The isotonic sucrose solution used in the double sucrose gap had a conductivity below 2 $\mu S\ cm^{-1}$. Note that the values of hyperpolarizations or i.j.ps. from the double sucrose gap are attenuated by 25–30% due to short circuiting in the sucrose gap (Daniel *et al.*, 1983; 1987). The membrane potential changes measured with the double sucrose gap were confirmed by the conventional intracellular microelectrode recording technique.

Microelectrode studies were conducted at 35±2°C. Tissue strips (1 mm × 20 mm) were cut parallel with the circular muscle fibres and were pinned to the floor of the Abe-Tomita 5 ml organ chamber. A detailed description of intracellular recordings has been described previously (Cayabyab & Daniel, 1995). Briefly, a small region (1 mm × 1 mm) of the tissue selected to record the intracellular electrical activity was well isolated with fine pins, and the whole tissue was superfused at all times with Krebs solution at a rate of 3 ml min^{-1}. The tissue was equilibrated for 1 h before the intracellular electrical activity was recorded simultaneously with contractile responses. Glass microelectrodes filled with 3 M KCl had resistances ranging from 35 to 90 MΩ. Membrane potential changes were measured by a standard electrometer (World Precision Instruments C-700), displayed on a dual beam oscilloscope (Tektronix D13: 5A22N differential amplifier; 5B12N dual time base), and recorded on 0.25 inch magnetic tape with a Hewlett-Packard recorder and on chart paper (Gould 2200). Electrical field stimulation was achieved by a pore-type silver electrode in contact with the tissue on one side of the strip, and a silver ground electrode on the other side. Stimuli from a Grass S88 stimulator were delivered through a stimulus isolation unit (Grass SIU5). Field stimulation parameters used were similar to those used in the double sucrose gap.

Solutions and drugs

The sydnonimine SIN-1 (3-morpholino-sydnonimine-hydrochloride) and sodium nitroprusside (SNP) were used as cyclic GMP-generating NO sources (Feelisch & Noack, 1987; Kukovets *et al.*, 1991) to confirm that the actions of these compounds mimic the actions of the endogenous i.j.p. mediator. SIN-1 was a generous gift from Dr Rudolf Kunstmann (Cassella AG). SIN-1 was prepared by dissolving in dimethyl sulphoxide (DMSO) to give a 1 M stock solution and was stored in the dark at −22°C at all times.

To manipulate the Ca^{2+} status of these stores and to evaluate the effect of depletion of Ca^{2+} storage sites on K^+ channel opening, we have used agents that have been shown to interfere with the SR function: the selective inhibitors of Ca^{2+}-ATPase, cyclopiazonic acid (CPA; Goeger *et al.*, 1988; Seidler *et al.*, 1989; Suzuki *et al.*, 1992; Pasyk *et al.*, 1995) and thapsigargin (TSG; Jackson *et al.*, 1988; Thastrup *et al.*, 1990) and the selective agonist of SR Ca^{2+} release channels, ryanodine (Sutko *et al.*, 1985; Sakai *et al.*, 1988; Zholos *et al.*, 1991).

To affect the buffering capacity of internal Ca^{2+} stores, either of the selective SR Ca^{2+}-ATPase inhibitors (CPA at 30 μM or TSG at 5 μM) or the SR Ca^{2+} release channel activator (ryanodine at 30 μM) was added to the superfusing medium before exposure to NO-donors. The individual con-

centrations used are the 'standard' maximal concentrations of these compounds used for functional studies (Sakai *et al.*, 1988; Suzuki *et al.*, 1992; Low *et al.*, 1993; Luo *et al.*, 1993) or biochemical studies (Goeger *et al.*, 1988 Seidler *et al.*, 1989; Darby *et al.*, 1993). Ryanodine and TSG were superfused for 45–60 min while CPA was superfused for only 30 min, so that membrane potentials became stable and returned to control values before addition of the NO-donor compounds. In experiments in which drugs were given, all necessary control and experimental values were obtained before, during and usually after washout of drug effects.

SNP and CPA (dissolved in DMSO), were purchased from Sigma Chemical Co. (California, U.S.A.). Ryanodine (dissolved in methanol) and TSG (dissolved in DMSO) were obtained from Research Biochemicals International (R.B.I.) (U.S.A.).

Statistical analysis

Data are expressed as arithmetic mean ± s.e.mean. Two-tailed Student's *t* test (paired or unpaired) was used for statistical analysis of data involving the effects of antagonists on the inhibitory junction potentials, electrotonic potentials, relative membrane potential changes, and electrophysiological effects of SIN-1 or SNP. The number of animals used was denoted by *n*: significances were denoted by $*P<0.05$, $**P<0.01$, $***P<0.001$, and NS ($P>0.05$).

Results

Effects of CPA and TSG on i.j.p. and SIN-1 or SNP-induced membrane hyperpolarization

To evaluate the role played by the SR in the genesis of the i.j.p., we used CPA to inhibit the SR pump. In the double sucrose gap, superfusion of CPA 10 μM for 30 min caused modest membrane depolarization (3.7±0.6 mV, $n=9$, $P<0.05$), and reduced both the i.j.p. amplitude (control 12.3±1.1 vs. treated 8.2±1.0 mV, $n=9$, $P<0.001$) and electrotonic potentials (control 10.8±1.4 vs. treated 5.8±0.5 mV, $n=5$, $P<0.05$). The effects of 30 μM CPA on these parameters were more pronounced, and this higher CPA concentration was used for subsequent studies. A typical double sucrose gap recording of the effects of CPA 30 μM is shown in Figure 1, where CPA produced a membrane depolarization of about 10 mV (peak) which was often accompanied by spike potentials and lasted about 10 min (Figure 1a). The initial peak depolarization to CPA was also accompanied by an increased i.j.p. with reduced duration (Figure 1a). This was followed by a repolarization to near control level and a reduction or sometimes abolition of the i.j.ps after 30 min of exposure. CPA 30 μM reduced both the i.j.p. durations to 74.1±4.8% ($n=18$, $P<0.001$) of control values (typically 7–10 s) and i.j.p. amplitudes from 13.8±0.8 mV to 5.4±0.8 mV ($n=18$, $P<0.001$) as observed in the double sucrose gap (Figure 1). Figure 1b shows that CPA by itself persistently increased the membrane conductance as reflected by significantly reduced electrotonic potentials (control 12.4±1.1 mV vs. treated 4.5±0.9 mV, $n=9$, $P<0.001$) even in the absence of membrane depolarization. In our earlier study on opossum oesophagus (see Figure 4 in Cayabyab & Daniel, 1995), we showed that the NO-donors, SIN-1 and SNP each at 500 μM, elicited hyperpolarizations which were unaffected by the presence of antagonists to neural transmitters. In the double sucrose gap, prolonged exposure to 30 μM CPA reduced the hyperpolarizations induced by both SIN-1 and SNP, as summarized in Figure 2.

With intracellular recordings, CPA 30 μM elicited about 10 mV peak depolarization (i.e., from −52.8±1.0 mV to −41.9±2.1 mV; $n=8$ paired observations, $P<0.01$) which was often accompanied by spike action potentials and large contractions after 7–10 min of exposure. These spikes, when

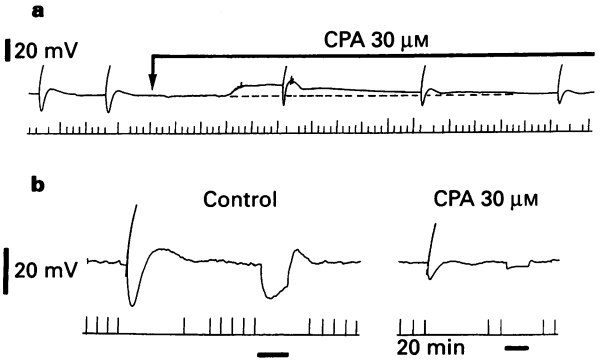

Figure 1 (a) Cyclopiazonic acid (CPA) 30 μM inhibited the i.j.ps (inhibitory junction potentials) after prolonged exposure (15–20 min) in the double sucrose gap. Note that in this preparation, the CPA depolarization recovered 15 min after onset of CPA infusion. The upward deflections before i.j.ps are the stimulus artifacts from electrical field stimulation. (b) A double sucrose gap recording from a preparation different from that of (a). CPA decreased the electrotonic potential and inhibited the voltage decay. Upward deflections are stimulus artifacts preceding i.j.ps as in (a). Note that after 20 min in 30 μM CPA, the membrane potential returns to near the control level. Recording speeds were varied as shown by the vertical bars under electrical traces. The interval between these bars change with recording speed (the interval between bigger bars was 1 min and that between smaller bars was 15 sec). Thus all the i.j.ps in (a) were recorded at the same speed, but those in (b) were recorded 5 times faster. Solid bars under time trace represent the duration of the hyperpolarizing stimulus evoking the electronic potentials.

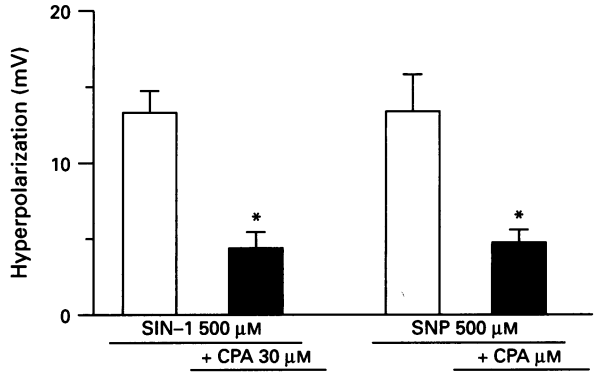

Figure 2 Summaries of double sucrose gap studies of the significant inhibitory effects of cyclopiazonic acid (CPA) 30 μM on the 3-morpholino-sydnonimine hydrochloride (SIN-1) and sodium nitroprusside (SNP)-induced hyperpolarization are shown. SIN-1 or SNP were added after 25–30 min of exposure to CPA; $n=5$ in each case. *refers to the probability that CPA had caused significant changes, $P\leqslant 0.05$.

present, disappeared or were reduced in frequency after 15–20 min when the membrane repolarized to near the control levels (i.e., control resting membrane potential of −52.8±1.0 mV; after CPA −54.9±2.5 mV; $n=8$ paired observation, $P>0.05$). The i.j.p. was reduced in amplitude and duration after 30 min in the presence of CPA as shown in Figure 3, and subsequent addition of 500 μM SIN-1 produced little or no detectable membrane potential changes (i.e., only an insignificant hyperpolarization to −57.5±2.7 mV, $n=5$, $P>0.05$). These observations confirm the results obtained from the double sucrose gap regarding the inhibitory effects of CPA on exogenous NO-induced hyperpolarizations (see Figure 2). A summary of intracellular recordings showing CPA suppression of i.j.ps (from 8 animals) and hyperpolarizations induced by SIN-1 (from 5 animals) is shown in Figure 6a).

TSG, another selective inhibitor of the SR pump, was shown to have effects similar to those of CPA. Figure 4 (re-

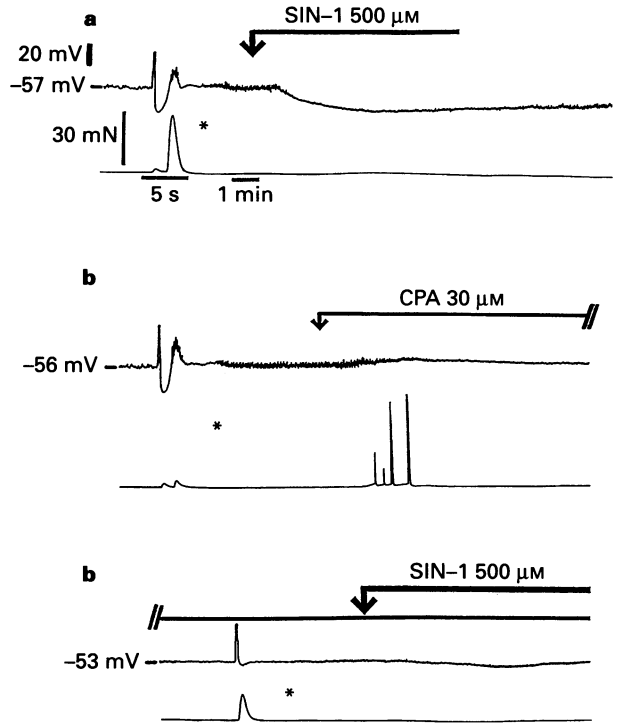

Figure 3 Effect of 30 μM cyclopiazonic acid (CPA) on the inhibitory junction potential (i.j.p.) and 3-morpholino-sydnonimine-hydrochloride (SIN-1) hyperpolarization as observed with microelectrodes. Each i.j.p. is preceded by the upward delection from the stimulus artifact during electrical field stimulation and is followed by an 'after-depolarization' as seen in (a) and (b). Voltage and tension calibrations appear as vertical bars at left of (a). Asterisks indicate a change in paper trace speed. Tissue strips were perfused with normal Krebs for 1 h following the first SIN-1 stimulation (a), and were subsequently incubated with 30 μM CPA for 30 min before the second SIN-1 stimulation.

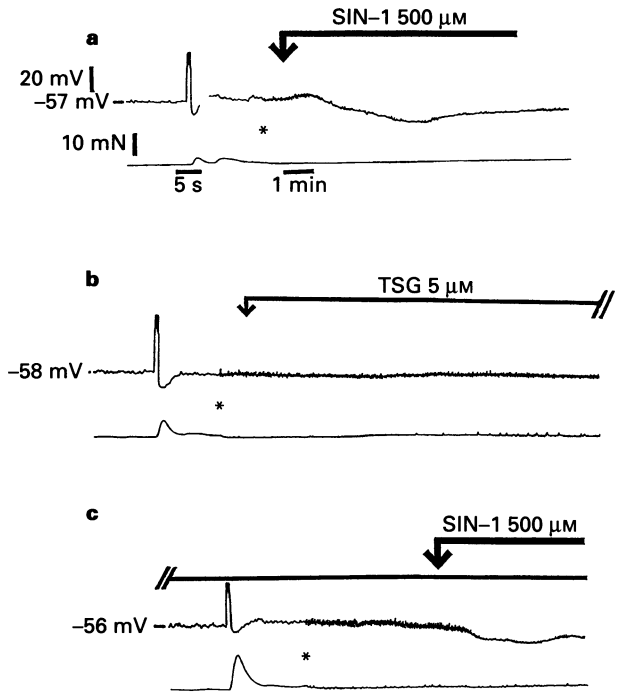

Figure 4 Effect of thapsigargin (TSG) 5 μM on the inhibitory junction potential (i.j.p.) and 3-morpholino-sydnonimine-hydrochloride (SIN-1) hyperpolarization as observed with microelectrodes. Recordings as in Figure 3. Voltage and tension calibrations appear as vertical bars at left of (a). Asterisks indicate a change in paper trace speed. Tissue strips were perfused with normal Krebs for 1 h following the first SIN-1 stimulation (a), and were subsequently incubated with 5 μM TSG for 45–60 min before the second SIN-1 stimulation.

presentative of 4 experiments from different animals) shows the inhibitory effects of TSG 5 μM on the i.j.p. and SIN-1-induced hyperpolarization as observed with intracellular recordings. Relative to CPA, the onset of effect of TSG was slower (see Figure 4b and the magnitude of effect on membrane potentials and associated contractile activity was smaller. TSG caused a modest depolarization (6.0 ± 2.3 mV, $n=4$, $P>0.05$) and inhibited the i.j.p. after 45 min and before the onset of SIN-1 infusion (Figure 4c). Figure 6b shows histograms summarizing the inhibitory effects of TSG on the i.j.p. and SIN-1 hyperpolarization.

Effects of (ryanodine) on the i.j.p. and SIN-1-induced membrane hyperpolarization

To demonstrate further the dependence of NO-mediated hyperpolarizations on the state of intracellular Ca^{2+} stores, we used ryanodine 30 μM, believed to be a selective agonist of the SR Ca^{2+} release channels (Zholos *et al.*, 1991; Sakai *et al.*, 1988), to deplete the stores. Figure 5 (representative of 4 experiments from different animals) shows intracellular recordings of the effects of ryanodine on the i.j.p. and SIN-1-induced hyperpolarization. Ryanodine caused insignificant depolarization (3.5 ± 0.3 mV, $n=4$, $P>0.05$) after about 45 min of pre-incubation and before SIN-1 was added. Ryanodine caused a modest but significant reduction of the i.j.p. and SIN-1-induced hyperpolarizations, as summarized in Figure 6c.

Discussion

This study shows that the NO-mediated i.j.ps and hyperpolarizations induced by NO-donors depend upon the status of

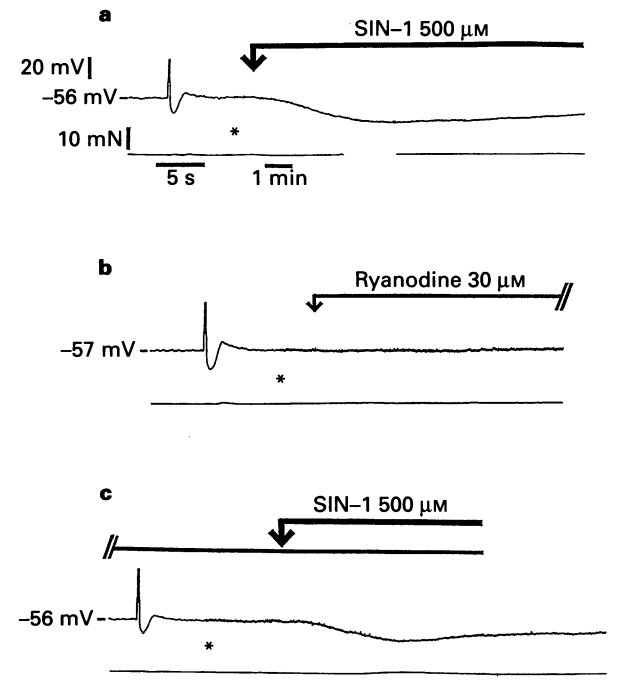

Figure 5 Effect of 30 μM ryanodine on the inhibitory junction potential (i.j.p.) and 3-morpholino-sydnonimine-hydrochloride (SIN-1) hyperpolarization as observed with microelectrodes. Recordings as in Figure 3. Voltage and tension calibrations appear as vertical bars at left of the first panel. Asterisks indicate a change in paper trace speed. Tissue strips were perfused with normal Krebs for 1 h following the first SIN-1 stimulation (a), and were subsequently incubated with 30 μM ryanodine for 45–60 min before the second SIN-1 stimulation.

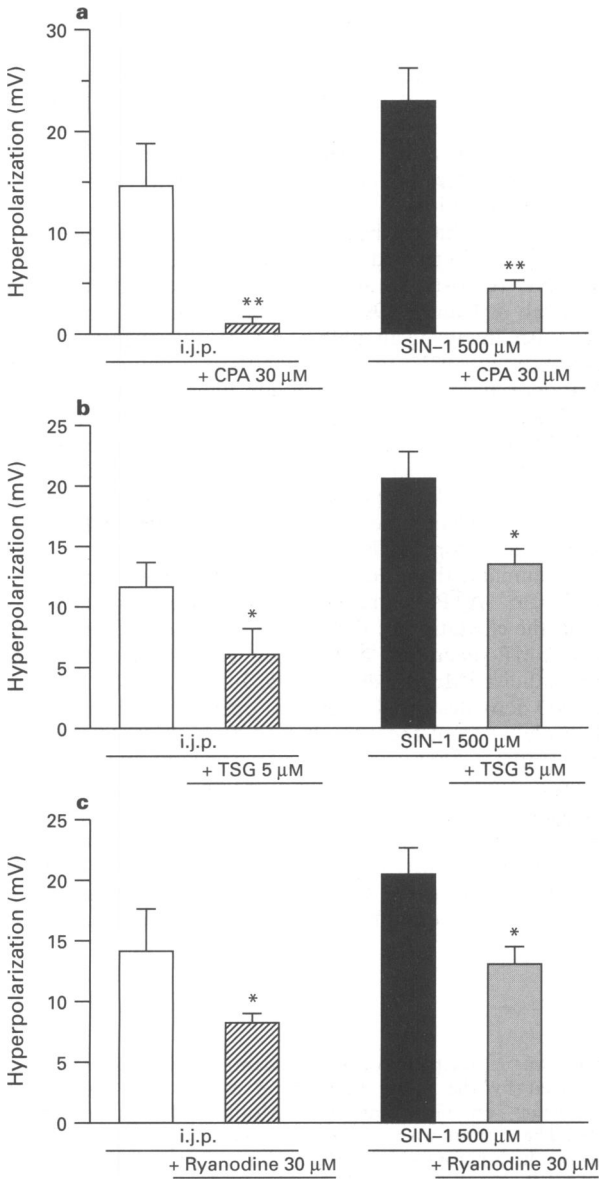

Figure 6 Summary of significant inhibitory effects of cyclopiazonic acid (CPA) 30 μM (a), thapsigargin (TSG) 5 μM (b), and ryanodine 30 μM (c) on both the inhibitory junction potential (i.j.p.) and the sydnonimine (SIN-1) hyperpolarization as measured with intracellular recordings. See details of times of drug infusions in Figures 3–5. In (a) $n=8$ for i.j.ps and 5 for SIN-1. $^{**}P \leqslant 0.01$, CPA significantly reduced both the i.j.ps and SIN-1-induced hyperpolarizations. In (b) $n=4$ for all means; $^{*}P \leqslant 0.05$, significant inhibition of hyperpolarizations evoked by endogenous or exogenous nitric oxide occurred from TSG. In (c) $n=4$ in all cases; *as in (b).

the internal Ca^{2+}-stores as well as the pumps and Ca^{2+} release channels that maintain them. Two selective inhibitors of the SR Ca^{2+}-ATPase (CPA and TSG) and the selective agonist (ryanodine) of the SR Ca^{2+} release channels were shown to suppress i.j.ps and NO-donor hyperpolarizations. Since these hyperpolarizations involve opening of K_{Ca} channels (Cayabyab & Daniel, 1994; 1995; Murray *et al.*, 1995; Jury *et al.*, 1996), the question arises: how does Ca^{2+} uptake into intracellular stores contribute to K^+-channel opening? One interpretation of these results is in terms of the existence of a superficial buffer barrier (SBB) (hypothesis proposed by Van Breemen & Saida, 1989) with the property that, when Ca^{2+} stores near the plasmalemma are empty, entering Ca^{2+} is rapidly sequestered in them, delaying or preventing Ca^{2+} from reaching contractile elements. Moreover, when the SR Ca^{2+} pumps are functioning to replete the stores, Ca^{2+} is suggested to be extruded vectorially toward the plasmalemma for removal from the cell, by Na^+-Ca^{2+} exchange and the plasmalemmal Ca^{2+} pump.

CPA and TSG have been demonstrated to deplete the SR, prevent SR repletion, and increase cytosolic [Ca^{2+}] (Nishimura *et al.*, 1989; Chen *et al.*, 1992; Chen & VanBreemen, 1993; Low *et al.*, 1993). This study showed that CPA and TSG inhibited the i.j.p. and NO-mediated hyperpolarization, consistent with patch clamp studies which showed that Ca^{2+} uptake into these stores was required to be functional and their loading with Ca^{2+} affected the opening of K_{Ca} channels by NO releasing agents (Duridanova *et al.*, 1995; Jury *et al.*, 1996). CPA which acts more rapidly than TSG, caused initial depolarization followed by recovery and a persistent decrease in electronic potentials (reflecting under the conditions of the double sucrose gap either a CPA-induced increase in membrane conductance (G) or an increase in longitudinal conductance, either one leading to increased shunting of the K^+ channel opening). We showed earlier (Cayabyab & Daniel 1995) that the i.j.p. mediator as well as SIN-1 and SNP mediated a membrane conductance increase (g) due to K^+ channel opening. In this study, the observed reduction in the amplitudes of i.j.ps and NO-donor hyperpolarizations may have occurred as a consequence of not only preventing SR Ca^{2+} uptake but also shunting of conductance increase produced by CPA (i.e., CPA increased G and hence reduced the ratio, $g/(g+G)$, which determines the change in potential from the resting potential – see Coburn *et al.*, 1975). This persistent increase in membrane conductance elicited by CPA may relate to Ca_i^{2+} levels and activation of several Ca^{2+}-activated currents. In this study we did not characterize the nature of regulation of transmembrane channel activity by SR Ca^{2+} depletion, but it is known that CPA activates Ca^{2+} influx pathways in various smooth muscles (Uyama *et al.*, 1993; Gonzalez De La Fuente *et al.*, 1995) as well as in non-excitable cells (Pasyk *et al.*, 1995). Earlier studies (Cayabyab & Daniel, 1993; unpublished observations) in this tissue suggested that emptying of Ca^{2+} stores can initiate Ca^{2+} or Na^+ entry through non-specific cation channels causing depolarization in Ca^{2+} containing medium. The CPA-induced membrane conductance increase was abolished in Ca^{2+}-free solutions (Cayabyab & Daniel, unpublished). This may have been more pronounced when CPA was used compared to TSG because the rapid release of calcium with the former agent overwhelmed the mechanisms of calcium removal from the cell. The increase in conductance from CPA probably contributed to a reduced hyperpolarization due to endogenous or exogenous NO. In microelectrode studies, however, the ability of CPA to inhibit i.j.ps was not similarly influenced by changes in longitudinal resistance or background membrane conductance, suggesting that its major action was independent of this effect.

Maintenance of the SBB requires vectorial Ca^{2+} release from internal stores toward the plasmalemma dependent on ongoing SR Ca^{2+}-ATPase activity. Nothing suggests that leakage of Ca^{2+} from the store after CPA is vectorial. Thus until the stores are depleted, leakage will serve to enable Ca^{2+} levels in the cell to rise. Ca^{2+} levels may later fall as the plasmalemma Ca^{2+} pump and Na^+-Ca^{2+} exchanger lower cytoplasmic Ca^{2+}, but in the case of CPA the continued elevation of conductance suggests that near the plasmalemma they remained elevated. TSG caused no clear change in membrane potential perhaps because the time course of depletion of stores was slower. It was also less effective than CPA in blocking hyperpolarization. The inhibition of SR Ca^{2+}-ATPase by CPA or TSG would reduce the Ca^{2+} buffering function of the superficial SR and inhibit the preferential Ca^{2+} release toward the plasmalemma (Van Breemen & Saida, 1989). The resultant increase in general levels of cytoplasmic Ca^{2+} levels is associated with suppression of i.j.ps (Cayabyab & Daniel, 1995) and basal and NO-increased K^+ outward currents (Duridanova *et al.*, 1995; Jury *et al.*, 1996).

At no time were the actions of CPA or TSG to raise cytoplasmic Ca^{2+} levels associated with membrane hyperpolar-

ization. Thus Ca^{2+} elevation near K^+ channels was apparently insufficient to open them. Moreover, the residual i.j.ps and NO-induced hyperpolarizations observed in this study in the presence of CPA or TSG suggest that NO has an additional action to open K_{Ca} channels, either by acting directly on the channels (Bolotina *et al.*, 1994) or indirectly by activating guanylate cyclase and protein kinase G (Conklin & Du, 1992; Cayabyab & Daniel, 1995; Murray *et al.*, 1995). The role of vectorial Ca^{2+} transport toward the plasmalemma may be to maintain Ca^{2+} levels near K_{Ca} channels sufficient to permit NO to activate them. In this case, the inhibitory effects of CPA or TSG would result from the consequences of generalized elevation of cytoplasmic Ca^{2+} levels. These possible mechanisms of action of NO are supported by the present results but require further investigation with more appropriate techniques to establish and discriminate.

Recent patch clamp studies of whole cell outward currents recorded from isolated cells of the oesophageal body circular muscle demonstrated that NO-donors enhanced these K_{Ca} outward currents (Murray *et al.*, 1995; Jury *et al.*, 1996). The observed reduction of these outward currents by CPA and by the K^+ channel blocker quinine (Jury *et al.*, 1996) is consistent with observations made from tissue preparations showing that the i.j.ps and NO-donor hyperpolarizations were inhibited by CPA (this study) and by quinine (Jury *et al.*, 1985; Cayabyab & Daniel, 1995). Furthermore, a similar suppression of K_{Ca} current by Ca^{2+}-ATPase blocking agents has been obtained in smooth muscle cells from other regions of the gastrointestinal tract (Suzuki *et al.*, 1992; Duridanova *et al.*, 1995).

The results with ryanodine (which like TSG did not significantly affect the membrane potential) can also result from interference with the buffer barrier function and release of Ca^{2+} into the cytosol. Ryanodine may make the Ca^{2+} stores leaky, which then raises Ca^{2+} levels in the cytosol and inhibits the NO-induced hyperpolarization. It has not been established by direct measurement in this tissue whether ryanodine opens CICR channels, holds them in a subconductance state, or closes them in smooth muscle. In either case, it reduces hyperpolarization to NO, and this could result from the decreased vectorial Ca^{2+}-release and leakage of Ca^{2+} from the SR. The ability of ryanodine to inhibit NO-induced hyperpolarization was less than that of CPA or TSG, probably because the induced Ca^{2+} leak occurring by CICR mechanism makes a smaller contribution to decreasing buffer barrier function. Nevertheless, our observation that ryanodine inhibited the NO-mediated i.j.ps and NO-donor hyperpolarizations are consistent with previous studies (Sakai *et al.*, 1988; Zholos *et al.*, 1991) which showed that ryanodine depletes intracellular Ca^{2+} stores and suppresses K_{Ca} current in other gastrointestinal smooth muscles. Previously we reported (Cayabyab & Daniel, 1995) that the NO-mediated i.j.p. and NO-donor hyperpolarizations were not affected by an ATP-sensitive K^+ channel antagonist, but whether CPA, TSG, or ryanodine affects ATP-sensitive K^+ channels or other ionic channels needs further investigation.

In the oesophageal body, methylene blue was effective in inhibiting the i.j.ps and hyperpolarizing responses to SIN-1 and SNP (Christinck *et al.*, 1991; Conklin & Du, 1992; Cayabyab & Daniel, 1995). This inhibition could occur by a mechanism involving an inhibition of soluble guanylate cyclase (Katsuki *et al.*, 1977; Ignarro, 1990) and a reduction in cyclic GMP-dependent Ca^{2+} pumping into internal stores (Twort & Van Breemen, 1988; Lincoln & Cornwell, 1991; Karczewski *et al.*, 1992). However, Ca^{2+} pump inhibition which results from the generation of superoxide anions by methylene blue (Grover *et al.*, 1992), is also a possible mechanism for the inhibitory effect of methylene blue on NO-mediated hyperpolarizations and SR Ca^{2+} store refilling.

In summary, these findings suggest that the functioning of the SR Ca^{2+}-ATPase and the SR Ca^{2+} release channels play a role in the elicitation of the i.j.p. or hyperpolarizing effects of cyclic GMP-generating NO-donors. Recent data from patch clamp studies (Murray *et al.*, 1995; Jury *et al.*, 1996) showing that NO activates the K_{Ca} channel opening in circular muscle cells isolated from the oesophageal body are consistent with the hypothesis that enhanced sequestration of Ca^{2+} in the presence of NO-donors enhances loading of the superficial SR and vectorial SR Ca^{2+} release toward a restricted domain near the plasmalemma. Thus, locally higher subsarcolemmal $[Ca^{2+}]_i$ may facilitate the activation of quinine-sensitive K^+ channels by NO underlying the NO-mediated hyperpolarizations and i.j.ps in opossum oesophagus (Cayabyab & Daniel, 1995).

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