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Nitrergic relaxation of the mouse gastric fundus is mediated by cyclic GMP-dependent and ryanodine-sensitive mechanisms

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1 Ryanodine-sensitive, Ca^{2+} release ('Ca^{2+} sparks') from the sarcoplasmic reticulum (SR) can activate plasmalemmal Ca^{2+}-activated K^+ channels (K_{Ca}) to cause membrane hyperpolarization and smooth muscle relaxation. Since cyclic guanosine monophosphate (cyclic GMP) can modulate Ca^{2+} spark activity, the aim of the present study was to determine if Ca^{2+} spark-like events are involved in NO-dependent, NANC relaxations to electrical field stimulation (EFS) of mouse, longitudinal smooth muscle of the gastric fundus in isolated strips contracted to ~40% of their maximum contraction.

2 NANC relaxations to EFS were almost abolished by both the NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG; 100 μM) and the guanylate cyclase inhibitor, 1-*H*-oxodiazol-[1,2,4]-[4,3-α] quinoxaline-1-one (ODQ; 10 μM). Also, ODQ abolished relaxations to the NO donor, sodium nitroprusside (SNP; 1 nM – 30 μM). NANC relaxations and SNP-evoked relaxations were both partly ryanodine (10 μM)- and nifedipine (0.3 μM)-sensitive, but in each case, the inhibitory effects of ryanodine and nifedipine were additive.

3 Apamin (1 μM), charybdotoxin (0.1 μM), iberiotoxin (0.1 μM), tetraethylammonium (TEA; 1 mM), glibenclamide (10 μM) and 4-aminopyridine (1 mM) had no effect on either NANC- or SNP-evoked relaxations, the latter of which were also unaffected by high extracellular K^+ (68 mM).

4 Caffeine (0.1 – 1 mM) caused concentration-dependent relaxations of gastric fundus which were inhibited by ryanodine but unaffected by L-NOARG.

5 Relaxation to ATP (30 μM) was abolished by nifedipine, partly inhibited by apamin and ryanodine, but was unaffected by L-NOARG.

6 In conclusion, the results of the present study show that nitrergic relaxations in the mouse longitudinal gastric fundus occur *via* a cyclic GMP-activated ryanodine-sensitive mechanism, which does not appear to involve activation of K^+ channels.
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Keywords: NANC; ryanodine; nifedipine; Ca^{2+} sparks; sarcoplasmic reticulum; K^+ channels; nitric oxide; cyclic GMP; gastric fundus

Abbreviations: ATP, adenosine 5′-triphosphate; BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; cyclic GMP, cyclic guanosine monophosphate; EFS, electrical field stimulation; ICC, interstitial cells of Cajal; K_{ATP}, ATP-sensitive K^+ channel; K_V, voltage-dependent K^+ channel; L-NOARG, N^G-nitro-L-arginine; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NOS, nitric oxide synthase; PAR, protease-activated receptor; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channel; SNP, sodium nitroprusside; SR, sarcoplasmic reticulum; STOCs, spontaneous transient outward currents; VOCCs, voltage-operated Ca^{2+} channels

Introduction

The release of Ca^{2+} from ryanodine-sensitive, intracellular microdomains in cardiac, skeletal and smooth muscle cells plays important roles in the regulation of Ca^{2+}-sensitive cellular functions (Berridge, 1996). For example, in rat posterior cerebral artery smooth muscle cells, spontaneous local Ca^{2+} release events (i.e. 'Ca^{2+} sparks') from ryanodine-sensitive Ca^{2+} stores on the sarcoplasmic reticulum (SR), activate nearby Ca^{2+}-sensitive plasmalemmal K^+ channels (K_{Ca}) (Nelson *et al*., 1995) which results in spontaneous transient outward currents (STOCs) and hyperpolarization (Nelson *et al*., 1995; Bychkov *et al*., 1997). Also, whilst Ca^{2+} sparks are unlikely to contribute directly to global intracellular calcium levels, they can indirectly regulate the activity of voltage-sensitive Ca^{2+} channels (VOCCs) which would then affect average cytosolic Ca^{2+} (Nelson *et al*., 1995; Jaggar *et al*., 1998). Therefore, factors or mechanisms which regulate the activity of Ca^{2+} sparks may be important determinants of smooth muscle tone.

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Sodium nitroprusside (SNP) has been shown to cause cyclic GMP-dependent increases in Ca^{2+} spark frequency and K_{Ca}-dependent outward currents in rat posterior cerebral artery myocytes as well as ryanodine-sensitive, K_{Ca}-dependent vasodilatation in the intact pressurized rat cerebral artery (Porter *et al*., 1998). Such findings suggest that nitric oxide (NO)-induced increases in cyclic GMP are able to mediate vasodilatation *via* ryanodine-sensitive, Ca^{2+} spark-dependent pathways. Since NO acts as a NANC inhibitory neurotransmitter in the gut including the mouse gastric fundus (Mashimo *et al*., 1996) in which ryanodine-sensitive, small conductance K_{Ca} (SK_{Ca})-dependent relaxation has been reported in response to protease-activated receptor (PAR) stimulation (Cocks *et al*., 1999), the aim of the present study was to determine whether similar ryanodine-sensitive mechanisms are involved in NANC relaxation in this preparation. We found that NO-dependent, NANC relaxations in response to electrical field stimulation (EFS) of intramural nerves in the mouse longitudinal gastric fundus were mediated by cyclic GMP-dependent mechanisms and like those to SNP, involved a ryanodine-sensitive

mechanism. In contrast to the responses to PAR agonists, however, these nitrergic relaxations were not due to K^+ channel activation, which indicates that ryanodine-sensitive Ca^{2+} release in response to NO in this gastrointestinal tissue can evoke smooth muscle relaxation by activation of other Ca^{2+}-sensitive mechanisms.

Methods

Tissue preparation

Mice (Male, Balb/C, 20–30 g) were killed by cervical dislocation. The fundus of the stomach was cut into two longitudinal strips (2 mm wide by 10 mm long) with Auerbach's plexus and some of the underlying circular smooth muscle intact. Each strip was suspended by silk ties in a 7 ml organ bath containing warm (37°C), carbogenated (95% O_2, 5% CO_2) isotonic Krebs solution (concentrations in mM; Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.0). One tie was attached to a micrometer-adjustable glass support leg and the other to a force-displacement transducer (model FT03, Grass Instruments, U.S.A.) to record changes in isometric force which were amplified (BMRI, Melbourne, Australia) and displayed on two channel chart recorders (W&W Scientific instruments, Basel, Switzerland). Some strips were positioned through a pair of platinum ring electrodes for electrical field stimulation (EFS) of intramural nerves. Strips were allowed to equilibrate for a period of 30 min prior to being stretched to an optimal passive force of 1 g. Once the tissues attained baseline levels of resting passive force for 30–45 min, maximum contractions (F_{max}) in response to acetylcholine (ACh; 30 μM) were determined before the tissues were thoroughly washed with Krebs solution and allowed to return to their resting levels of passive force.

NANC inhibitory responses

Strips of gastric fundus were exposed to guanethidine (30 μM), atropine (1 μM) and propranolol (1 μM) to block adrenergic and cholinergic responses and thus impose NANC recording conditions. Each strip was then contracted to ~40% of its F_{max} by titrating the concentration of the stable thromboxane A_2-mimetic, U46619 (0.5–50 nM). Once a steady level of active force to U46619 was obtained, a single response to EFS (1 pulse or 0.5–5 Hz, 0.1 ms duration, 30–60 V, 10 s using S44 stimulators; Grass Instruments, MA, U.S.A.) was obtained. The U46619 was then washed out and the tissues allowed to return to resting baseline levels of active force. Some strips were then incubated for 30–45 min with antagonists of NO and other putative mediators and mechanisms of NANC relaxations. These included N^G-nitro-L-arginine (L-NOARG; 100 μM), 1-*H*-oxodiazol-[1,2,4]-[4,3-α] quinoxaline-1-one (ODQ; 10 μM), nifedipine (0.3 μM), ryanodine (10 μM), apamin (1 μM), charybdotoxin (0.1 μM), iberiotoxin (0.1 μM), tetraethylammonium (1 mM), glibenclamide (10 μM) and 4-aminopyridine (1 mM). Time-matched controls were left untreated. Irrespective of the drug treatment, all tissues were then re-contracted to their respective 40% F_{max} levels of active force with U46619 and the responses to EFS repeated.

SNP and ATP

Responses to the NO donor, SNP (1 nM–30 μM) and ATP (30 μM) were obtained in tissues contracted to ~40% F_{max} with either carbachol or U46619. Tissues were then washed with Krebs solution, allowed to return to baseline levels of active force and either left untreated (time-matched controls), or were treated with similar antagonists used to characterize NANC responses. To determine whether other K^+ channels, not inhibited by the selective K^+ channel inhibitors, were involved in the relaxation to SNP, tissues were treated with high K^+ (68 mM)-containing Krebs solution (Drummond & Cocks, 1996). Due to its ability to depolarize smooth muscle and thereby cause near-maximal contraction and to depolarize nerve terminals to cause neurotransmitter release, 68 mM K^+ was used in combination with nifedipine (0.3 μM) and L-NOARG (100 μM) to prevent functional antagonism and release of NO from nerves. After 30–45 min, tissues were again contracted to ~40% F_{max} with carbachol or U46619 and subsequently exposed to cumulatively increasing half-log molar concentrations of SNP or single concentrations of ATP (30 μM). In preliminary experiments it was determined that the different types of constrictor used to contract the tissue did not affect either the sensitivity or maximum responses to SNP, ATP and caffeine.

Caffeine

Cumulative concentration-responses to caffeine (0.1–1 mM) were obtained in tissues equilibrated as described above and contracted to ~40% F_{max} with carbachol. Some tissues were incubated for 30 min with either ryanodine (10 μM) or L-NOARG (100 μM) before responses to caffeine were obtained.

Data and statistics

All peak relaxations were calculated as a percentage reversal of the initial level of active force. Normalized cumulative concentration-relaxation curves to SNP were analysed with sigmoidal non-linear regressions to obtain the mean sensitivities (pEC_{50}) (Graphpad Prism, version 1.0). These and maximum relaxation values (R_{max}) were statistically compared by either unpaired *t*-tests or analysis of variance (ANOVA) with multiple comparisons using the Tukey-Kramer method where appropriate. To determine the relative effectiveness of treatments against SNP-evoked responses (i.e. to compare whether the inhibition by ryanodine and nifedipine in combination was greater than to ryanodine alone), the difference in pEC_{50} (fold-shift) between control and treatment for each group of paired responses was statistically compared between each treatment group. A P value <0.05 was taken to indicate a significant difference.

Drugs and materials

Acetylcholine chloride (ACh), adenosine 5′-triphosphate (ATP), 4-aminopyridine (4-AP), carbachol chloride, guanethidine sulphate, N^G-nitro-L-arginine (L-NOARG), L-propranolol hydrochloride, (Sigma, MO, U.S.A.); apamin, charybdotoxin, glyburide (glibenclamide), iberiotoxin, 1-*H*-oxodiazol-[1,2,4]-[4,3-*a*] quinoxaline-1-one (ODQ), nifedipine, ryanodine, tetraethylammonium chloride (TEA), tetrodotoxin, 1,5,5-hydroxy-11-(epoxymethano) prosta-5Z, 13E-dienoic acid (U46619) (Sapphire Bioscience, N.S.W., Australia); atropine sulphate, 1,3,7 trimethylxanthine (caffeine; Research Biochemicals International, U.S.A.). Stock solutions (10 mM) of guanethidine, atropine, propranolol, apamin (0.1 mM), caffeine (100 mM), TEA (100 mM), 4-AP (100 mM) and tetrodotoxin (1 mM) were prepared in distilled water. L-NOARG (100 mM) was prepared in $NaHCO_3$ (1 M). Charybdotoxin (10 μM) and

iberiotoxin (10 μM) were prepared in a buffer solution containing the following composition (0.1% BSA, 100 mM NaCl, 10 mM Tris and 1 mM EDTA; pH 7.5). ODQ (10 mM) and glibenclamide (10 mM) were prepared in dimethyl-sulphoxide (DMSO; 100%). U46619 (1 mM), ryanodine (10 mM) and nifedipine (10 mM) were dissolved in absolute (100%) ethanol. Further dilutions of all stock solutions were in distilled water. Isotonic solutions of Krebs containing 68 mM KCl were prepared by substituting NaCl for KCl in normal Krebs solution.

Results

Role of NO in NANC relaxation to EFS

EFS (5 Hz, 0.1 ms duration, 30–60 V, 10 s) evoked fast onset relaxations (R_{max}, 68.4±2.1%, $n=49$) which recovered rapidly following cessation of the stimulus. These NANC relaxations were abolished by tetrodotoxin (1 μM, $n=4$; data not shown) and were reproducible after 30 min (R_{max} of time-matched control relaxations, 69.3±6.0%, $n=6$; Figure 1). Responses to EFS were almost abolished by either L-NOARG (100 μM; R_{max}, 15.0±5.3%, $n=6$; Figure 1) or ODQ (10 μM; R_{max}, 9.8±8.7%, $n=5$; Figure 1). A similar near maximum inhibition of relaxations to 1 pulse EFS and of frequencies 0.5 Hz–5 Hz was obtained with the combined treatment of L-NOARG (100 μM) and ODQ (10 μM) (data not shown).

Effect of ion channel inhibitors on NANC relaxations to EFS

The responses to EFS (5 Hz) were significantly ($P<0.01$) reduced by nifedipine (R_{max}, 48.8±2.4%, $n=6$; Figure 2). Also, although higher concentrations of U46619 (0.05–0.1 μM) were required to contract the tissue to ~40% F_{max} in the presence of nifedipine, the same concentrations of U46619 in the absence of nifedipine did not inhibit the relaxation to EFS (data not shown).

Apamin (1 μM; R_{max}, 66.4±6.9%, $n=6$), charybdotoxin (0.1 μM; R_{max}, 69.7±7.8%, $n=4$), iberiotoxin (0.1 μM; R_{max}, 74.1±6.3%, $n=5$), TEA (1 mM; data not shown, $n=3$), 4-AP (1 mM; data not shown, $n=3$) and glibenclamide (10 μM; data not shown, $n=3$) all had no effect on the relaxation to EFS.

Effect of ryanodine on NANC relaxations to EFS

Ryanodine (10 μM) contracted the mouse gastric fundus from both baseline levels of active force (0.88±0.1 g, $n=6$) and in strips already contracted to ~40% F_{max} (0.34±0.1 g, $n=5$). In strips of gastric fundus in which ryanodine (10 μM) was incubated for 30 min before contraction to ~40% F_{max},

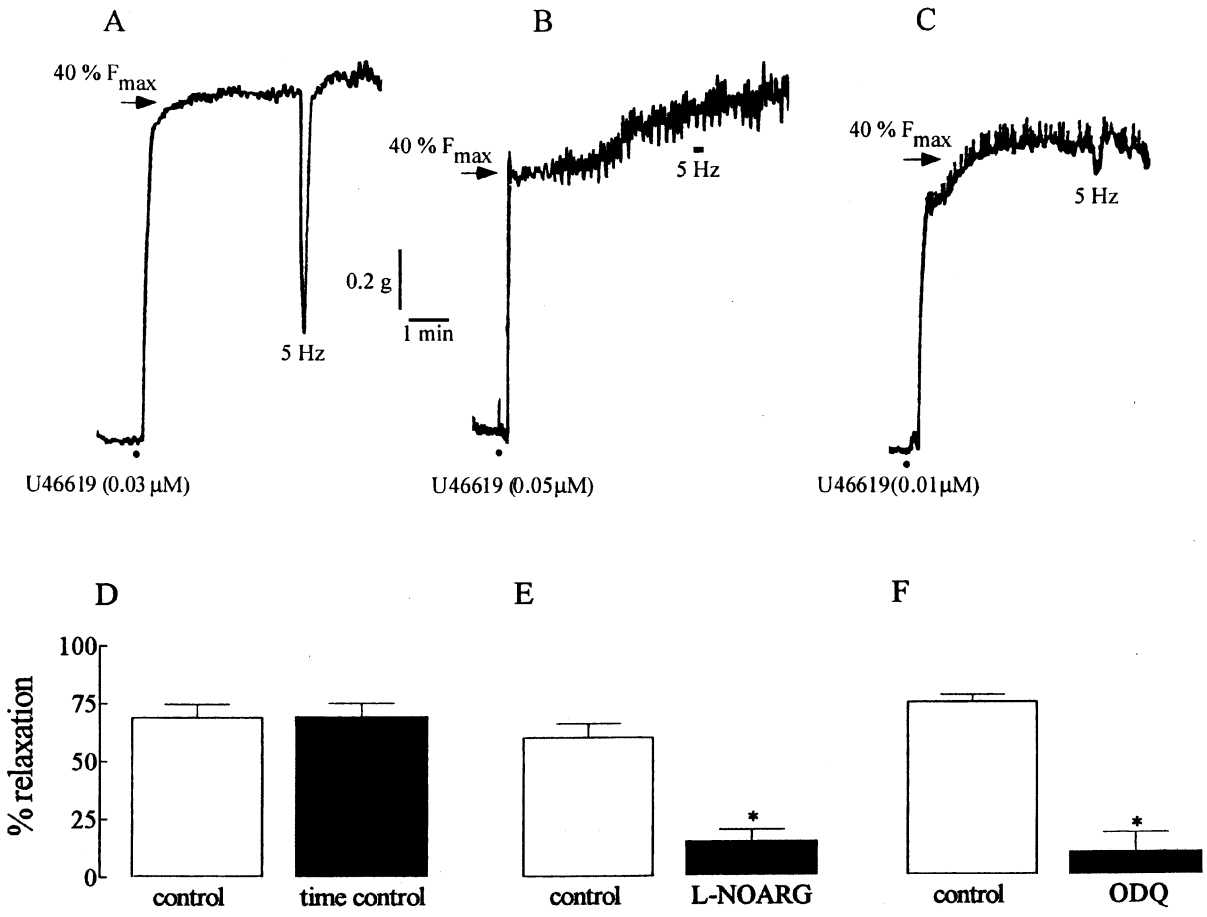

Figure 1 Demonstration that NANC responses to electrical field stimulation (5 Hz, 0.1 ms duration, 30–60 V for 10 s) of enteric inhibitory nerves in U46619-contracted strips of mouse isolated longitudinal gastric fundus are mediated by NO and cyclic GMP. Digitized original chart recordings showing the second responses obtained in (A) the absence (time control), or in the presence of either (B) N^G-nitro-L-arginine (L-NOARG; 100 μM) or (C) ODQ (10 μM) in separate preparations. The vertical (force; *g*) and horizontal (time; min) scale bars apply to all recordings. (D–F) corresponding mean data of responses depicted in (A–C) expressed as per cent reversal of the U46619-induced contraction. The open columns represent the first control responses (control) and the solid columns the second time control or treatment responses. Values (mean±s.e.mean, $n=5–6$). (*) denotes a significant difference from time control ($P<0.05$).

relaxation to EFS was significantly ($P<0.001$) reduced (R_{max}, $20.5\pm6.5\%$, $n=6$) compared with untreated control tissues (Figure 2). In the presence of nifedipine (0.3 μM) and ryanodine (10 μM; $n=6$), EFS-induced relaxations were abolished (Figure 2) and this degree of inhibition was significantly different from those observed with each inhibitor alone (ANOVA; $P<0.05$; Figure 2).

Effect of ion channel inhibitors on relaxations to SNP

In strips of gastric fundus contracted to $\sim40\%$ F_{max} (Figure 3), SNP (0.001–30 μM) caused concentration-dependent relaxations (pEC_{50}, 7.36 ± 0.11; R_{max}, $109.3\pm1.1\%$, $n=56$) which were reproducible after 30 min (pEC_{50}, 7.57 ± 0.13; R_{max}, $109.7\pm1.9\%$, $n=4$) and abolished by ODQ (10 μM;

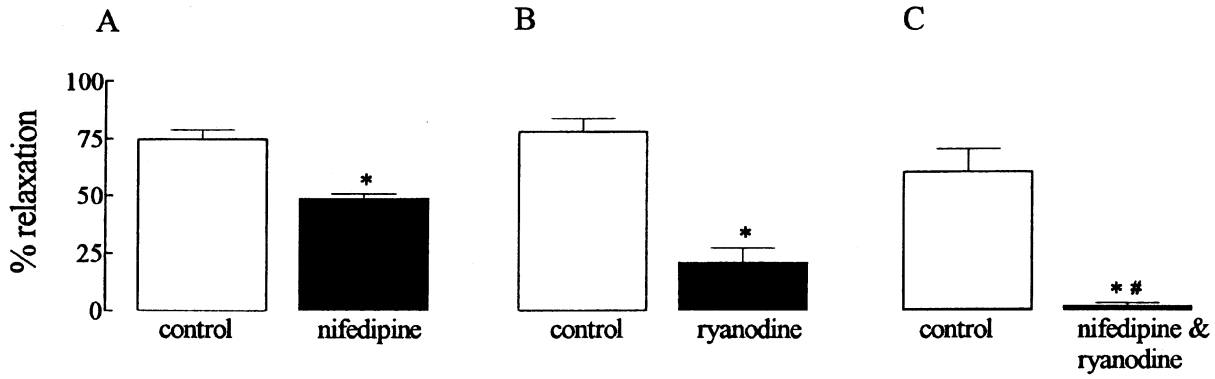

Figure 2 The effect of (A) nifedipine (0.3 μM), (B) ryanodine (10 μM) and (C) a combination of nifedipine (0.3 μM) and ryanodine (10 μM) on NANC responses to electrical field stimulation (5 Hz, 0.1 ms duration, 30–60 V for 10 s) in strips of mouse isolated longitudinal gastric fundus. Recording conditions were as described in Figure 1. The open columns represent the first control responses (control) and the solid columns the second treatment responses. Values (mean$\pm$s.e.mean, $n=6$ strips) are expressed as per cent reversal of the initial contraction to U46619. (*) denotes a significant difference ($P<0.05$) from control and (#) that the degree of inhibition to a combination of nifedipine and ryanodine was significantly greater (ANOVA; $P<0.05$) than that observed to either ryanodine or nifedipine alone.

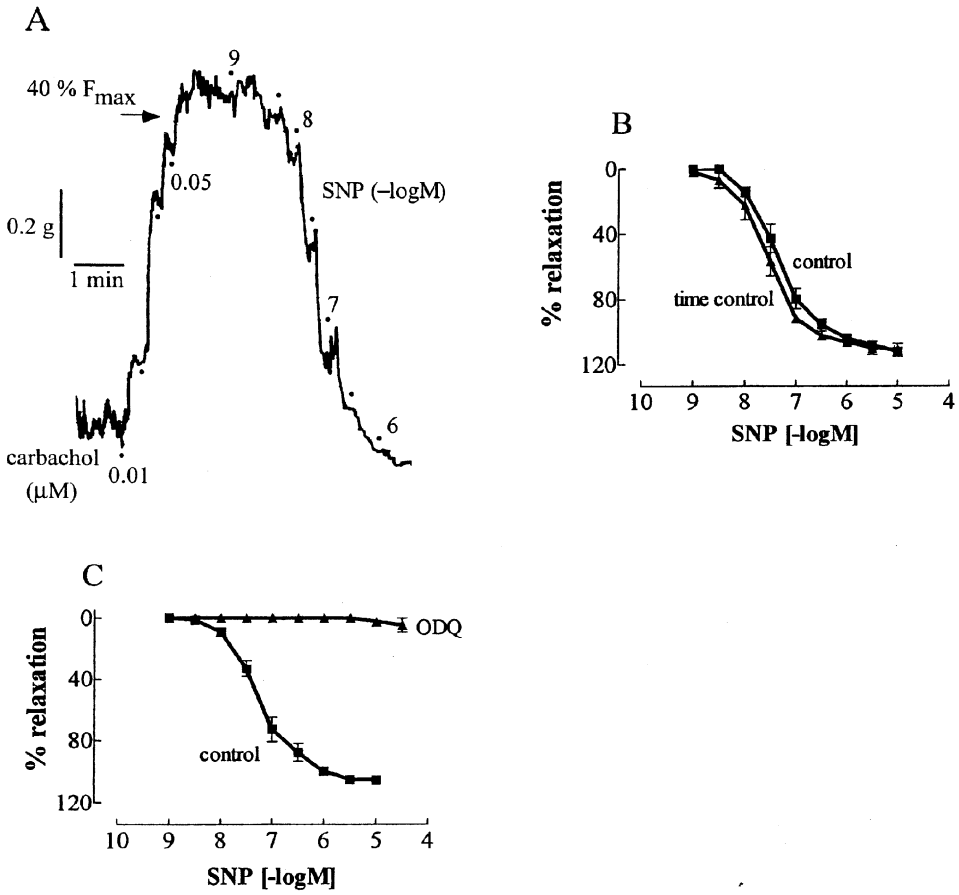

Figure 3 (A) Digitized original chart recording which shows second responses (time control) to cumulatively increasing concentrations of the NO donor, sodium nitroprusside (SNP) in strips of mouse isolated longitudinal gastric fundus contracted with carbachol. (B) and (C): Average cumulative concentration-relaxation curves to SNP showing first control (control) responses in each group and second responses obtained in the absence (time control) or presence of ODQ (10 μM), respectively. Values (mean$\pm$s.e.mean, $n=6$) are expressed as per cent reversals of the initial contraction to carbachol.

Figure 3). Nifedipine (0.3 μM) caused a small significant ($P<0.01$) decrease in the maximum relaxation (R_{max}, $98.8\pm1.5\%$, $n=5$) but no significant effect on the sensitivity (~1.5 fold shift; pEC_{50}, 7.29 ± 0.09, $n=5$) to SNP (Figure 4). Also, as for NANC relaxations, apamin (1 μM; pEC_{50}, 7.65 ± 0.13; R_{max}, $104.5\pm3.5\%$, $n=5$), charybdotoxin (1 μM; pEC_{50}, 7.52 ± 0.03; R_{max}, $107.7\pm3.2\%$, $n=3$), iberiotoxin (1 μM; pEC_{50}, 7.53 ± 0.20; R_{max}, $108\pm2.1\%$, $n=3$), TEA (1 mM; data not shown), glibenclamide (10 μM; data not shown) and 4-AP (1 mM; data not shown) all had no effect on SNP relaxations. In the presence of 68 mM K^+ and nifedipine, both R_{max} and pEC_{50} to SNP were significantly ($P<0.001$) reduced to $62.0\pm3.6\%$ ($n=6$) and 6.11 ± 0.04 (~19 fold shift), respectively. This degree of inhibition to SNP was significantly ($P<0.001$) greater than that obtained in the presence of nifedipine alone (Figure 4). However, when L-NOARG (100 μM) was added to tissues treated with nifedipine and 68 mM K^+, the relaxation to SNP (~1.9 fold shift: pEC_{50}, 7.02 ± 0.08; R_{max}, $101.6\pm3.1\%$, $n=5$; Figure 4) was not significantly different to that observed in the presence of nifedipine alone. Also, L-NOARG (100 μM) alone had no effect on responses to SNP (Figure 4).

Effect of ryanodine on relaxations to SNP

Ryanodine (10 μM) caused a significant ~3 fold reduction in sensitivity (pEC_{50}; 7.00 ± 0.11) and a small but significant inhibition of R_{max} ($98.2\pm1.8\%$, $n=5$) to SNP (Figure 5). As with NANC relaxations, combined treatment with nifedipine and ryanodine caused a similar greater decrease (ANOVA; $P<0.05$) in both the sensitivity (~13 fold shift; pEC_{50}, 6.55 ± 0.19) and maximum relaxation (R_{max}, $81.2\pm1.1\%$, $n=5$) to SNP than either nifedipine or ryanodine alone (Figure 5).

Responses to caffeine

Caffeine (0.1 – 1 mM) caused concentration-dependent relaxation of mouse gastric fundus strips with a maximum response at 1 mM of $92.7\pm3.6\%$ (Figure 6). Ryanodine (10 μM) significantly inhibited ($P<0.01$) the maximum relaxation to caffeine ($50.4\pm3.8\%$; $n=5$) but did not appear to affect sensitivity (Figure 6). By contrast, L-NOARG (100 μM) had no effect on either the sensitivity or maximum relaxation to caffeine (Figure 6).

Responses to ATP

ATP (30 μM) caused a relaxation of $55.0\pm5.4\%$ ($n=4$) which was reproducible after 30 min ($61.4\pm4.7\%$, $n=5$; Figure 7). This response was abolished by nifedipine (0.3 μM) and significantly ($P<0.05$) inhibited by ryanodine (10 μM; $16.8\pm4.3\%$, $n=4$) and apamin (0.3 μM; $41.0\pm6.3\%$, $n=4$) but not by L-NOARG (100 μM; $63.3\pm1.9\%$, $n=4$). Also, the effects of ryanodine and apamin were not additive (R_{max}, $10.5\pm2.8\%$, $n=4$; Figure 7).

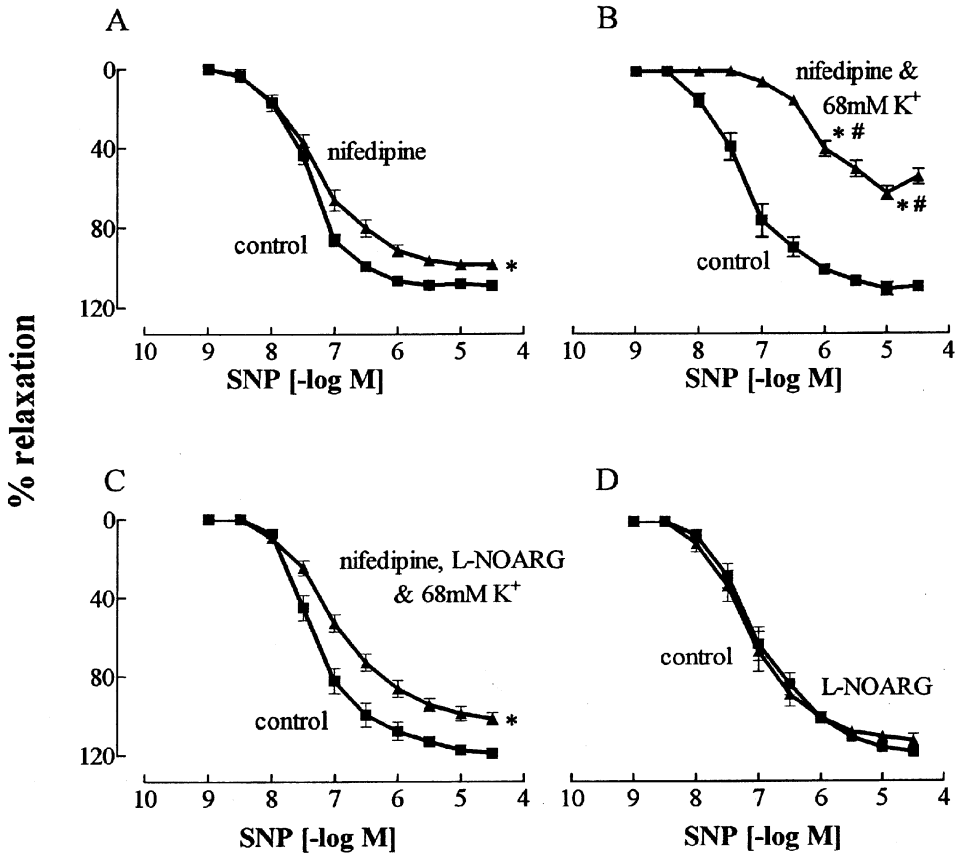

Figure 4 The effect of non-selective inhibition of K^+ channels with high extracellular K^+ on relaxation to SNP in strips of mouse isolated longitudinal gastric fundus contracted with carbachol. Average cumulative concentration-relaxation curves to SNP showing the first control responses in each group (control) and the second responses obtained in the presence of either (A) nifedipine (0.3 μM), (B) a combination of nifedipine (0.3 μM) and 68 mM K^+, (C) a combination of nifedipine (0.3 μM), 68 mM K^+ and L-NOARG (100 μM) or (D) L-NOARG (100 μM). Values (mean$\pm$s.e.mean, $n=6$) are expressed as per cent reversal of the initial contraction to carbachol. (*) denotes pEC_{50} and R_{max} values significantly different from controls ($P<0.05$) and # that the degree of inhibition to a combination of nifedipine and 68 mM K^+ was significantly greater than that observed to nifedipine alone.

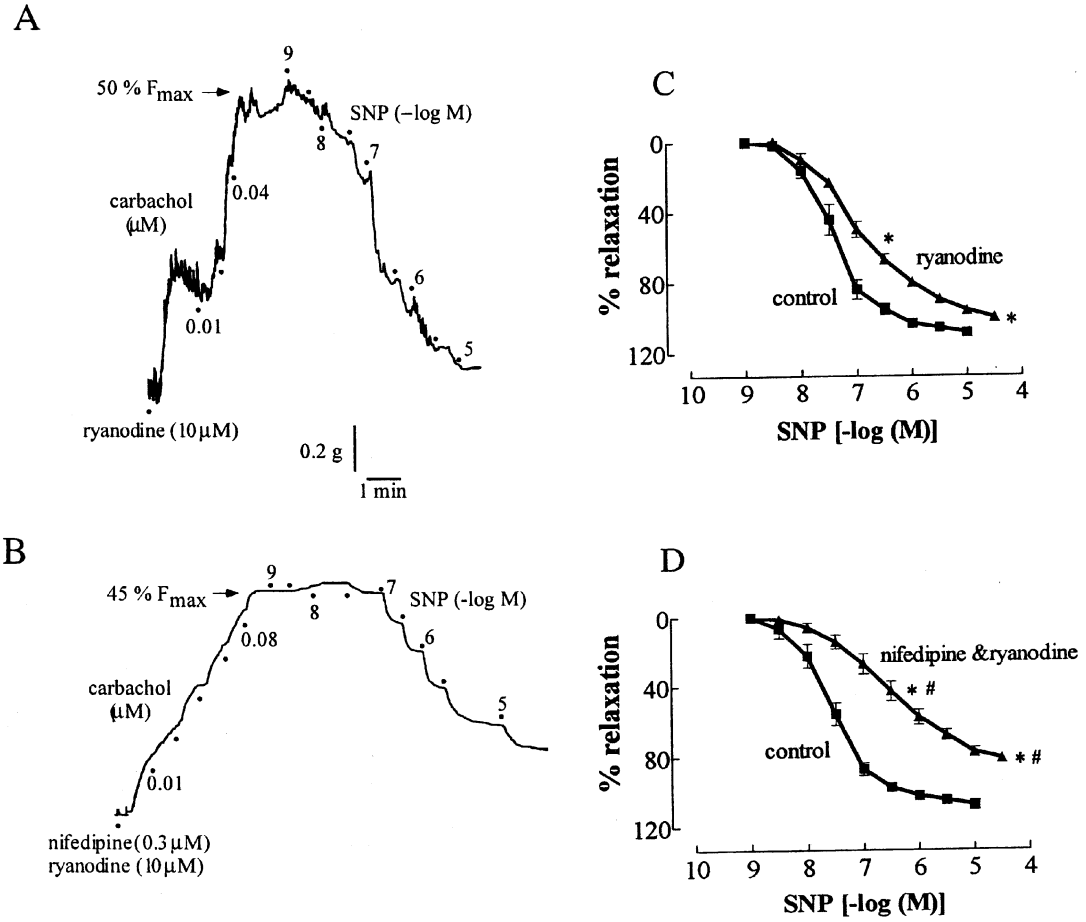

Figure 5 The effect of ryanodine (10 μM) and nifedipine (0.3 μM) on relaxation to SNP in mouse isolated longitudinal gastric fundus contracted with carbachol. Digitized original chart recordings of second responses to cumulatively increasing concentrations of SNP in the presence of (A) ryanodine (10 μM) and (B) a combination of nifedipine (0.3 μM) and ryanodine (10 μM). (C) and (D): Average cumulative concentration-relaxation curves to SNP from experiments as depicted in (A) and (B) showing first control (control) and second responses obtained in the presence of ryanodine or a combination of nifedipine and ryanodine, respectively. Values (mean $\pm$ s.e.mean, $n=6$) are expressed as per cent reversal of the initial contraction to carbachol. (*) denotes pEC_{50} and R_{max} values significantly different from controls ($P<0.05$).

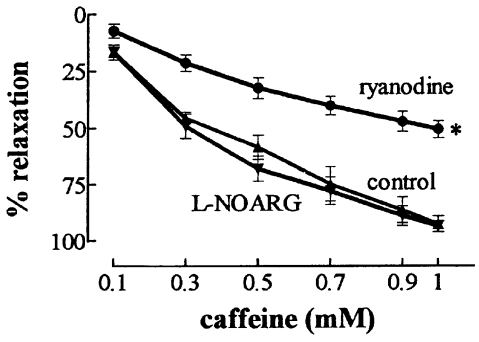

Figure 6 Concentration-relaxation curves to caffeine in carbachol-contracted strips of mouse isolated longitudinal gastric fundus in the absence (control) and presence of either ryanodine (10 μM) or L-NOARG (100 μM). Values (mean $\pm$ s.e.mean, $n=6$) are expressed as per cent reversal of the initial contraction to carbachol. (*) denotes R_{max} values significantly different from controls ($P<0.05$).

Discussion

The present study shows that NANC relaxations to EFS of enteric nerves in the longitudinal smooth muscle of the mouse gastric fundus at frequencies of stimulation $\leqslant 5$ Hz are mediated solely by NO *via* cyclic GMP-dependent and ryanodine-sensitive mechanisms. While previous reports have shown evidence for ryanodine-sensitive mechanisms and K^+ channels in the inhibitory responses to NO in gut preparations such as the opossum oesophagus (Cayabyab & Daniel, 1996), our findings here indicate that a K^+ channel-dependent mechanism is not involved in NO-mediated relaxation of the mouse longitudinal gastric fundus. Rather our data suggest that NO causes smooth muscle relaxation in this tissue *via* the activation of a novel ryanodine-sensitive mechanism.

Ryanodine-sensitive Ca^{2+} sparks have been shown to initiate relaxation of arterial smooth muscle by activation of K_{Ca} within discrete microdomains of the plasma membrane to cause hyperpolarization and closure of L-type VOCCs (Nelson *et al.*, 1995). Also, the finding that cyclic GMP increases both Ca^{2+} spark frequency and K_{Ca} activity (Porter *et al.*, 1998), indicates that hyperpolarization and relaxation in response to NO in blood vessels can involve activation of K_{Ca} *via* ryanodine-sensitive Ca^{2+} release. As shown here, NANC relaxation of gut smooth muscle due to NO and cyclic GMP, can be similarly inhibited by ryanodine which suggests the involvement of similar mechanisms as those described in blood vessels. A problem with this conclusion, however, is our additional finding here that a range of K_{Ca} inhibitors had no effect on the ryanodine-sensitive relaxations to both endogenous (neural) or exogenous (SNP) nitrergic stimulation. One

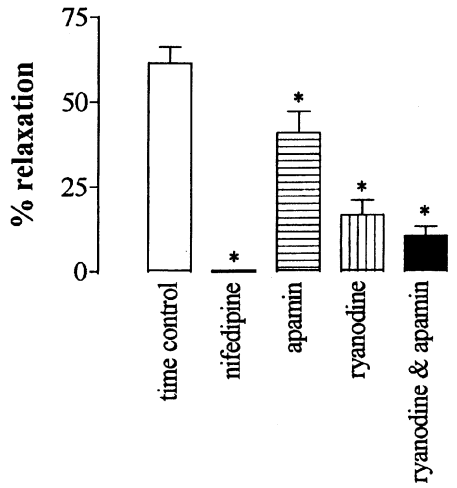

Figure 7 The effect of ryanodine (10 μM) and ion channel inhibitors on relaxations to a single concentration of adenosine 5′ triphosphate (ATP; 30 μM) in carbachol contracted strips of mouse isolated longitudinal gastric fundus in the absence (time control) or presence of either nifedipine (0.3 μM), apamin (0.3 μM), ryanodine (10 μM) or a combination of ryanodine (10 μM) and apamin (0.3 μM). Values (mean ± s.e.mean, $n=4$) are expressed as per cent reversal of the initial contraction to carbachol. (*) denotes a significant ($P<0.05$) difference from time control.

obvious explanation for this apparent lack of effect of K_{Ca} inhibitors in the mouse longitudinal gastric fundus is that NO does not activate K_{Ca} either directly (Bolotina *et al*., 1994) or indirectly (Ward *et al*., 1992; Robertson *et al*., 1993; Archer *et al*., 1994; Yamakage *et al*., 1996) *via* cyclic GMP and ryanodine-sensitive Ca^{2+} release (Porter *et al*., 1998). Instead, the inhibitory effect of ryanodine observed here on nitrergic relaxations may have been due to an action or actions unrelated to inhibition of Ca^{2+} sparks such as inhibition of neurotransmitter release, as demonstrated for sympathetic nerves in the rat vas deferens (Smith & Cunnane, 1996). Also Publicover *et al*. (1993) claimed that ryanodine-sensitive Ca^{2+} release is important in the amplification of NO release by NANC nerve-innervated interstitial cells of Cajal (ICC). If similar mechanisms are present in the mouse longitudinal gastric fundus, then ryanodine could inhibit Ca^{2+}-induced Ca^{2+} release from intracellular pools of the nerve terminals or ICC containing NOS and as such prevent the activation of neuronal or ICC NO synthase (NOS). Such a 'prejunctional' or 'ICC' inhibitory effect of ryanodine on nitrergic neurotransmission, however, appears unlikely in the mouse longitudinal gastric fundus for two reasons. First, ryanodine inhibited relaxation to exogenous NO (i.e. SNP), which presumably only acts postjunctionally on smooth muscle cells. Also, the amplifying effect of ICC on the response to exogenous NO suggested by Publicover *et al*. (1993) was unlikely to have occurred in the mouse longitudinal gastric fundus since relaxations to SNP were unaffected by the NOS inhibitor, L-NOARG. A second reason as to why it is unlikely that ryanodine acted prejunctionally or *via* ICC in our experiments was that caffeine, which releases Ca^{2+} from the SR (for a review; Ehrlich *et al*., 1994), caused ryanodine-sensitive relaxations that were unaffected by L-NOARG and thus does not support a ryanodine-sensitive Ca^{2+} release mechanism in the activation of either nNOS or ICC NOS.

Mironneau *et al*. (1996) reported that low concentrations (1 mM) of caffeine increased ryanodine-sensitive Ca^{2+} spark frequency and evoked K_{Ca}-dependent outward currents in rat portal vein myocytes. Also, in the present study, we found that similar low concentrations of caffeine caused relaxations which were markedly inhibited by ryanodine with the remaining (~50%) response being abolished by the non-selective phosphodiesterase inhibitor IBMX (Selemidis & Cocks, unpublished observations). Therefore, even though K_{Ca} do not appear to be involved in nitrergic relaxations or exogenous NO, our finding with caffeine suggests that ryanodine-sensitive Ca^{2+} release from smooth muscle SR can evoke relaxation of the mouse longitudinal gastric fundus and supports our proposal that ryanodine-sensitive relaxation to NO in this tissue involves Ca^{2+} release from the SR of smooth muscle cells, possibly as Ca^{2+} sparks.

Since part of the NO-mediated relaxations to intramural nerve stimulation and exogenous NO application were blocked by nifedipine, it is probable that hyperpolarization-evoked closure of nifedipine-sensitive L-type VOCCs was involved in each response. Although K^+ channels do not appear to be involved in the relaxation to NO, the nifedipine-sensitive component of this response may be explained by an action of NO, perhaps *via* cyclic GMP or protein kinase G (PKG), to decrease chloride channel activity (Zhang *et al*., 1998), evoke hyperpolarization and thus close L-type VOCCs. The additive inhibitory effects of nifedipine and ryanodine on relaxations to both endogenous and exogenous NO indicate that Ca^{2+} release from the SR had no effect on either membrane potential or the activity of L-type VOCCs. These findings, however, are in direct contrast to protease-activated receptor (PAR; Cocks *et al*., 1999) – and ATP (this study) – induced mechanisms of relaxation in the mouse longitudinal gastric fundus, which although blocked by ryanodine, were also both inhibited by apamin and nifedipine suggesting a role for SK_{Ca}. The inhibition by ryanodine of PAR- and ATP-mediated relaxations in the gastric fundus was unlikely to have been due to a direct effect on SK_{Ca} channels because in the guinea-pig *taenia coli*, ryanodine had no effect on similar apamin-sensitive relaxation to either PAR activation (Cocks *et al*., 1999) or ATP (Selemidis & Cocks, unpublished observations). Therefore, ryanodine-sensitive Ca^{2+} release appears to be able to evoke relaxation of the mouse longitudinal gastric fundus *via* K_{Ca}- and VOCC-dependent mechanisms, but only in response to specific stimuli, like PAR activators and ATP.

Such differential effects of ryanodine on NO, ATP- and PAR-mediated relaxations in the mouse longitudinal gastric fundus has important implications regarding the activation of Ca^{2+} release from the SR not only by different relaxing agonists but by constrictor agents as well. For example, PAR and purinergic receptors (e.g. P_{2Y} receptors) are G protein-coupled to phospholipase C *via* G_q/G_{11} (Burnstock, 1995; Brass *et al*., 1996; Molino *et al*., 1997; Verrall *et al*., 1997). Thus, activation of both types of receptor leads to an increase in IP_3 which is known to cause Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores. However, if IP_3-induced Ca^{2+} release was involved in PAR and purinergic responses in the mouse longitudinal gastric fundus, then it would have most likely activated further Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores *via* a Ca^{2+}-induced Ca^{2+} release mechanism (Boittin *et al*., 1998) since both responses are blocked by ryanodine. By contrast, the contractions to carbachol and U46619 – due to activation of muscarinic and thromboxane A_2 receptors respectively, both most likely G-protein coupled to IP_3 (Thierauch *et al*., 1994; Eglen *et al*., 1996) – were not inhibited by ryanodine which suggests no role for ryanodine-sensitive Ca^{2+} stores.

In conclusion, relaxation of the mouse longitudinal gastric fundus to NO released endogenously by enteric inhibitory nerves and exogenously by SNP involves cyclic GMP-dependent and ryanodine-sensitive mechanisms, although subsequent activation of K_{Ca} is unlikely to be involved. We propose that NO is able to increase Ca^{2+} release from SR Ca^{2+} stores, perhaps in the form of Ca^{2+} sparks, which then activate other Ca^{2+}-sensitive processes to cause smooth muscle relaxation.

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