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Nitric oxide relaxes circular smooth muscle of rat distal colon through RhoA/Rho-kinase independent Ca^{2+} desensitisation

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1 The aim of this study in circular smooth muscle of rat distal colon was to determine whether Ca^{2+} desensitisation, in addition to mechanisms lowering cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), was involved in the relaxation elicited by nitric oxide (NO). Changes in isometric tension and $[Ca^{2+}]_{cyt}$ were recorded simultaneously in fura-2-loaded strips.

2 In methacholine (10^{-5} M)-precontracted preparations, exogenous NO (10^{-4} M), adenosine 5′-triphosphate (ATP; 10^{-3} M) and electrical field stimulation (EFS; 1 ms, 40 V, 4 Hz, 1 min) induced a decrease in smooth muscle tension, which was accompanied by a fall in $[Ca^{2+}]_{cyt}$.

3 The sarcoplasmic/endoplasmic reticulum Ca^{2+} ATP-ase (SERCA) inhibitor thapsigargin (10^{-6} M) did not exert an influence on the decrease in tension produced by exogenous NO, but significantly attenuated the fall in $[Ca^{2+}]_{cyt}$. Both the relaxation and the fall in $[Ca^{2+}]_{cyt}$ to ATP and EFS were unaffected by thapsigargin.

4 Calyculin-A (10^{-6} M), a myosin light chain phosphatase (MLCP) inhibitor, significantly reduced the decrease in tension elicited by exogenous NO, but did not alter the fall in $[Ca^{2+}]_{cyt}$ to exogenous NO. Inactivating RhoA by exoenzyme C3 (2 μg ml^{-1}) or inhibiting Rho-kinase with (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632; 10^{-5} M) had no effect on the decrease of both tension and $[Ca^{2+}]_{cyt}$ generated by exogenous NO.

5 This paper demonstrates that a RhoA/Rho-kinase independent Ca^{2+} desensitisation pathway contributes to the relaxation by NO in circular smooth muscle strips of rat distal colon.
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Abbreviations: ATP, adenosine 5′-triphosphate; AUC, area under the curve; $[Ca^{2+}]_{cyt}$, cytosolic free calcium concentration; cADPR, cyclic ADP-ribose; DMSO, dimethylsulphoxide; EFS, electrical field stimulation; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; Fura-2 AM, fura-2 acetoxymethyl ester; IP_3, inositol triphosphate; MLC_{20}, 20 kDa myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NO, nitric oxide; PKG, protein kinase G; PSS, physiological salt solution; SERCA, sarcoplasmic/endoplasmic reticulum ATP-ase; sGC, soluble guanylate cyclase; Y-27632, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate

Introduction

In gastrointestinal and other smooth muscles, an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is recognised to be an important event in response to contractile agents (for a review see Bolton *et al*., 1999). The major regulatory mechanism of smooth muscle tension is phosphorylation/dephosphorylation of the 20 kDa myosin light chain (MLC_{20}) (Somlyo & Somlyo, 1994). A rise in $[Ca^{2+}]_{cyt}$ initiates contraction by activation of Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) and subsequent phosphorylation of MLC_{20}. Dephosphorylation of MLC_{20} by the Ca^{2+}-independent myosin light chain phosphatase (MLCP) has the opposite effect and produces relaxation.

In recent years, much attention has been focused on regulation of force that is independent of changes in $[Ca^{2+}]_{cyt}$. . This is referred to as 'Ca^{2+} sensitisation' when there is an increase in contractile force for a given concentration of free $[Ca^{2+}]_{cyt}$. The biochemical background is an increase of the Ca^{2+} sensitivity of the contractile apparatus (Somlyo & Somlyo, 2000). At a constant $[Ca^{2+}]_{cyt}$ and MLCK activity, the best-described Ca^{2+}-sensitising mechanism (causing a leftward shift of the $[Ca^{2+}]_{cyt}$–force response curve) involves inhibition of MLCP activity (for a review see Somlyo & Somlyo, 2003). In the literature, there are two well-known pathways for MLCP inhibition: (1) either through the small GTPase RhoA, whereby GTP-bound RhoA translocates to the plasmamembrane and activates Rho-kinase that inhibits MLCP by acting on its regulatory subunit (i.e. MYPT1) or (2) *via* CPI-17, a 17 kDa peptide, whose phosphorylation (on Thr-38) enhances its potency to inhibit the catalytic subunit of MLCP (i.e. PP1c). In addition, other proteins such as protein kinase C, calmodulin-dependent kinase II, integrin-associated kinase, and mitogen-activated protein kinase can also modulate the steady-state level of MLC20 phosphorylation at a constant $[Ca^{2+}]_{cyt}$ (Somlyo & Somlyo, 2003). Under physiological conditions, most contractile agonists that bind to $G\alpha_q$

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protein-coupled receptors produce contraction of smooth muscle by a concerted increase of $[Ca^{2+}]_{cyt}$ and Ca^{2+} sensitisation (Abdel-Latif, 2001).

Conversely, smooth muscle relaxation results from a decrease in $[Ca^{2+}]_{cyt}$ and/or a reduced Ca^{2+} sensitivity of the contractile apparatus (i.e. 'Ca^{2+} desensitisation'). Desensitisation to Ca^{2+} can be mediated by protein kinase G (PKG), which is activated by the NO/cGMP pathway. Inhibition of RhoA/Rho-kinase, and thus activation of MLCP, is probably the most important mechanism of Ca^{2+} desensitisation by the cGMP/PKG pathway (Chitaley & Webb, 2002). PKG-mediated phosphorylation of MYPT1 (Surks *et al*., 1999) and of telokin (Wu *et al*., 1998; Walker *et al*., 2001), a 17 kDa acidic peptide whose sequence is identical to the COOH terminus of MLCK, represent alternative routes leading to Ca^{2+} desensitisation. Decreased sensitivity of the inositol triphosphate (IP_3)-receptor, inhibition of IP_3 synthesis and enhanced removal of Ca^{2+} from the cytosol by sarcoplasmic/endoplasmic reticulum ATP-ase (SERCA) have been proposed to mediate cGMP-dependent smooth muscle relaxation by lowering the $[Ca^{2+}]_{cyt}$ (Lincoln *et al*., 2001).

In circular smooth muscle of Wistar–Han rat distal colon, both NO and adenosine 5′-triphosphate (ATP) together with an unknown neurotransmitter are involved as mediators of nonadrenergic noncholinergic relaxation (Van Crombruggen & Lefebvre, 2004). NO was shown to induce relaxation in this smooth muscle preparation in part by activation of soluble guanylate cyclase (sGC) and a rise in cGMP, and in part by direct activation of small conductance Ca^{2+}-dependent K^+ (SK)-channels. The aim of the present study in circular smooth muscle strips of rat distal colon was to investigate the mechanism of the cGMP-mediated part of the NO-induced relaxation in rat distal colon and to assess whether Ca^{2+} desensitisation is involved.

Methods

Tissue preparation

Male Wistar–Han rats (300–440 g; obtained from Janvier, Le Genest St-Isle, France) were killed by decapitation. An approximately 4 cm length of distal colon was removed from 2 cm above the pelvic brim in oral direction, opened along the mesenteric border and pinned mucosa side up in physiological salt solution (PSS) of composition (mM; Salomone *et al*., 1995): NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; glucose, 10; $MgCl_2$, 1.25 and $CaCl_2$, 1.25. After the mucosa and submucosa were dissected away, thin sheets of full-thickness muscle (about 6 mm wide and 10 mm long) were cut along the circular axis and were then stretched and pinned flat. At both ends, the tissue was then clipped with titanium clips so that a flat, rectangular area was situated between both clips. All experimental procedures were approved by and carried out in accordance with the guidelines of the Ethical Committee for laboratory animals from the Faculty of Medicine and Health Sciences at Ghent University.

Measurement of contractile tension and cytosolic calcium concentration

Small clipped sheets of full-thickness rat distal colon muscle were first incubated for 4 to 5 h at room temperature in PSS containing 5×10^{-6} M of the fluorescent calcium indicator fura-2 acetoxymethyl ester (fura-2 AM) and 0.05% cremophor EL. The fura-2 loaded tissues were then transferred to fresh PSS, washed for 5 min in order to remove uncleaved fura-2 AM from the tissue, and *via* the attached titanium clips mounted between two hooks in a 1.5 ml cuvette filled with PSS (composition as above but supplemented with 4×10^{-6} M guanethidine to block effects of activating sympathetic nerves) kept at 37°C and gassed with 95% O_2/5% CO_2. The bottom hook was connected to a stationary support, while the top hook was attached to an isometric force transducer (Grass FT03), the applied tension being adjusted using a micrometer onto which the transducer was mounted. In our study, the flat rectangular area of tissue between the clips was positioned with the luminal side (i.e. circular muscle layer) towards the excitation light beam (cf. infra). Platinum plate electrodes (20×5 mm, distance in between 13 mm) were attached to two opposite corners of the cuvette so that the tissue was mounted in between and electrical field stimulation (EFS) was applied by means of a Grass S88 stimulator. The cuvette was part of a spectrofluorimeter (Quantamaster QM-2000-4, Photon Technology International (PTI)); changes in cytosolic calcium level were measured simultaneously with the contractile tension by alternatively illuminating the circular muscle side with two excitation wavelengths (340 and 380 nm) obtained from a xenon high-pressure lamp coupled to an excitation monochromator; the emitted fluorescence light from the circular muscle surface was collected by a photomultiplier through an emission monochromator set at 510 nm. The fluorescence signals evoked by excitation at 340 and 380 nm (with a frequency of 1 Hz), together with the contractile tension, were recorded on a computer by using the data acquisition hardware (PTI) and the data recording software FeliX™ (PTI).

After being mounted in the cuvette, the muscle sheet was equilibrated for 30 min under a resting tension of 0.75 g meanwhile continuously being perfused with PSS at a constant flow rate of 8 ml min^{-1}. Subsequently, the perfusion was stopped and the muscarinic agonist metacholine (10^{-5} M) was injected into the cuvette to induce contraction of the muscle strip. At 5 min after the contraction reached maximum, one of three relaxant stimuli was applied:either a bolus of exogenous nitric oxide (NO; 10^{-4} M) or of ATP (10^{-3} M), or EFS (40 V, 4 Hz, 1 ms for 1 min) *via* the platinum plate electrodes. The influence of thapsigargin, exoenzyme C3, Y-27632, calyculin-A, nicotinamide and tetrodotoxin was tested upon these relaxant stimuli in parallel tissues. These compounds were added into the cuvette after the maximum contraction and left in contact with the tissue for 5 min before the relaxant stimulus was applied. When the solvent was not deionised water, it was also tested for effects *per se*.

At the end of the experiment, the fura-2 Ca^{2+} signal was calibrated. The maximal ratio (R_{max}) was measured in calcium saturating medium by adding ionomycin (2×10^{-5} M) in high KCl solution (i.e. PSS in which the NaCl concentration remained unchanged but in which the KCl concentration was increased to 10^{-1} M), while the minimal ratio (R_{min}) was obtained in calcium-free medium in the presence of EGTA (8×10^{-3} M). The autofluorescence was measured at 340 and at 380 nm by quenching the fura-2 fluorescence with $MnCl_2$ (5×10^{-3} M) and was subtracted from all values. The cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) was calculated according to the equation of Grynkiewicz *et al*. (1985).

Drugs used

Drugs used during the study were: ATP; (Boehringer Mannheim, Germany), calyculin-A (Tocris, Bristol, U.K.), cremophor EL (Sigma, Bornem, Belgium), dimethylsulphoxide (DMSO; Sigma), ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA; Sigma), exoenzyme C3 (from clostridium botulinum, Calbiochem, Leuven, Belgium), fura-2 acetoxy-methylester (fura-2 AM; Calbiochem), guanethidine (Sigma), ionomycin (Sigma), metacholine (Schuchardt, München, Germany), $MnCl_2$ (Sigma), nicotinamide (Sigma), tetrodotoxin (Sigma), thapsigargin (Sigma), (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632; Calbiochem). Drugs were dissolved in deionised water with the exception of calyculin-A and thapsigargin, which were dissolved in dimethylsulphoxide (in the concentration used, this vehicle had no effect on the tissue). A saturated NO solution was prepared from gas (Air Liquide) as described by Kelm & Schrader (1990), by bubbling argon gas and then NO gas through three consecutive in-line connected gas-tight vials, the first two containing KOH solutions, the latter PSS supplemented with 4×10^{-6} M guanethidine.

Data analysis

In the contractility trace, the area under the curve (AUC) of a relaxant response was measured (AUC_A). The AUC of the methacholine-induced contraction was measured for the 30 s period immediately preceeding the application of the relaxant stimulus and the value was normalized for the same duration as the relaxant stimulus (AUC_B). The end of the relaxation was defined as the moment where a clearcut and maintained increase in tension developed. The relaxant response was then expressed as $(1-AUC_A/AUC_B) \times 100\%$. The amplitude of the decrease in $[Ca^{2+}]_{cyt}$ induced by one of the relaxant stimuli applied (in the absence or presence of pharmacological compounds under study) was calculated in a similar way. All results represent means ± s.e.mean (s.e.m.) with *n* referring to tissues obtained from different animals (unless otherwise indicated). Statistical analysis is performed by an unpaired Student's *t*-test, with a *P*-value of 0.05 or less taken to indicate a significant difference.

Results

General observations

As depicted in Figure 1, all rat distal colon circular smooth muscle preparations showed small spontaneous changes of $[Ca^{2+}]_{cyt}$ and tension superimposed on the baseline $[Ca^{2+}]_{cyt}$ and the basal tension, respectively. Based on the mean responses obtained in a single tissue from all rats studied, the mean resting $[Ca^{2+}]_{cyt}$ was established to be 115.4 ± 5.6 nM ($n = 38$). Addition of the muscarinic acetylcholine receptor agonist methacholine (10^{-5} M) to the circular muscle strips induced a quick increase in tension followed by phasic activity. The mean $[Ca^{2+}]_{cyt}$ in the 5 min period following the initial peak tension after administration of methacholine (10^{-5} M) was calculated to be 408.7 ± 15.9 nM ($n = 38$).

Addition of exogenous NO (10^{-4} M) or ATP (10^{-3} M) to methacholine-precontracted circular smooth muscle strips produced pronounced relaxations that were accompanied by marked decreases in $[Ca^{2+}]_{cyt}$ (Figure 2a, b). Similar changes were also observed upon EFS (40 V, 4 Hz, 1 ms for 1 min)

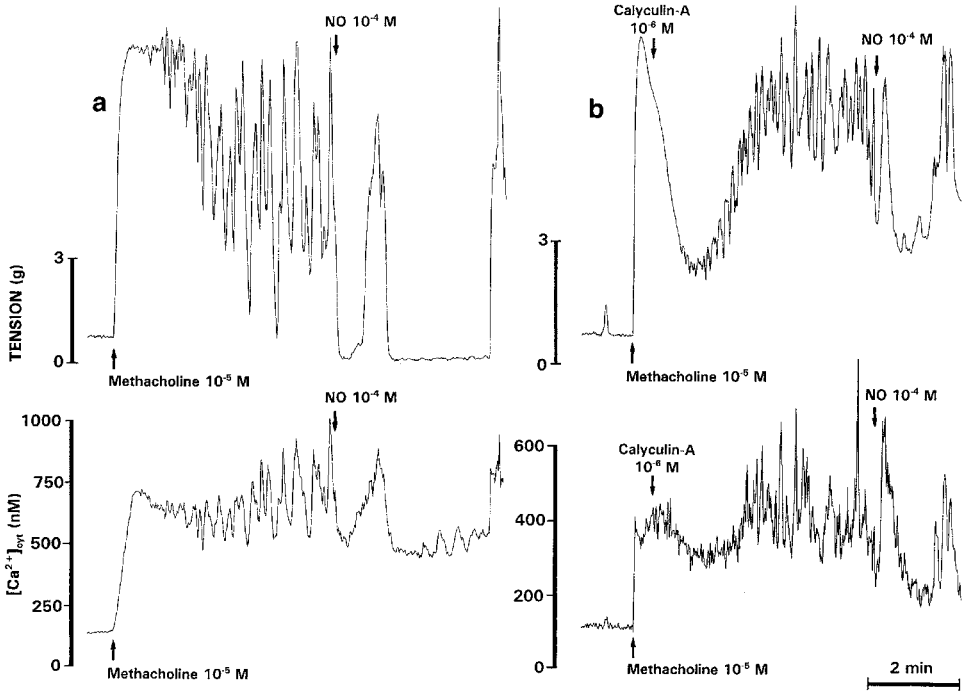

Figure 1 Representative tracings illustrating the decrease in $[Ca^{2+}]_{cyt}$ (bottom part) and the decrease in tension (upper part) induced by addition of exogenous NO (10^{-4} M) to methacholine (10^{-5} M)-precontracted circular smooth muscle strips of rat distal colon in the absence (a) and in the presence (b) of calyculin-A (10^{-6} M).

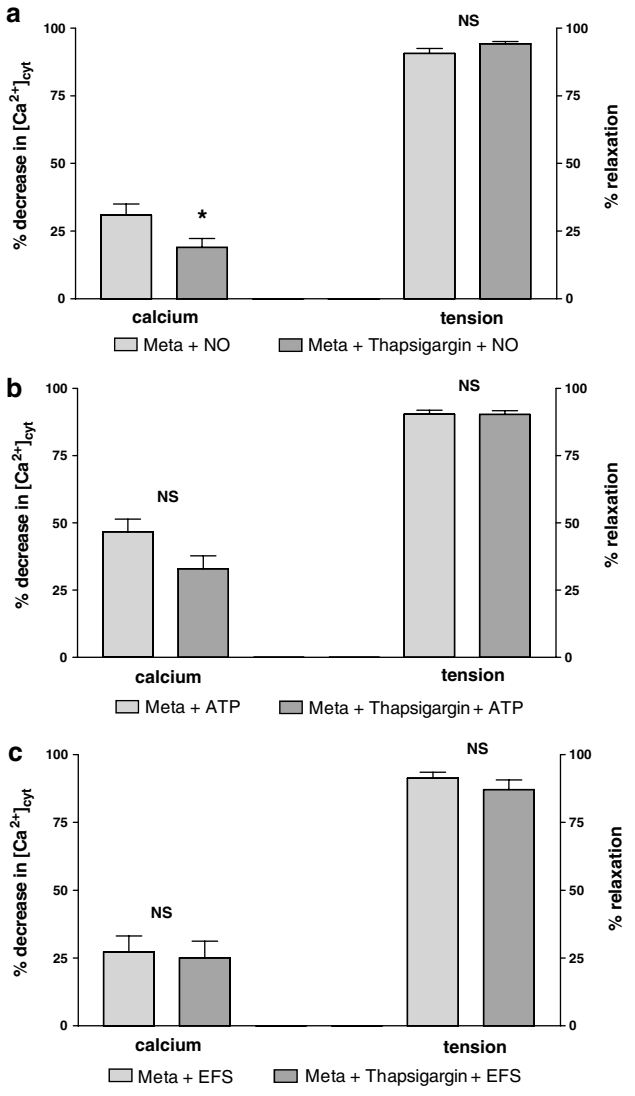

Figure 2 Effect of exogenous NO (10^{-4} M; a), ATP (10^{-3} M; b) and EFS (40 V, 4 Hz, 1 ms for 1 min; c) upon the $[Ca^{2+}]_{cyt}$ and upon the tension of methacholine (10^{-5} M)-precontracted circular smooth muscle preparations of rat distal colon. The effect of thapsigargin (10^{-6} M) upon these relaxant stimuli is also depicted (a–c). Results represent means ± s.e.m. of $n = 6$–7. *$P < 0.05$: significantly different from the response in the absence of thapsigargin (unpaired t-test). NS = nonsignificant.

(Figure 2c). Tetrodotoxin (3×10^{-6} M) completely abolished the responses to EFS ($n = 3$).

Effect of thapsigargin on relaxations induced by NO, ATP and EFS

Thapsigargin (10^{-6} M) had no effect *per se* on the methacholine-induced tension and $[Ca^{2+}]_{cyt}$ increase. Thapsigargin (10^{-6} M) given 5 min before applying exogenous NO (10^{-4} M) did not change the relaxation produced by this agent but it did reduce the decrease in $[Ca^{2+}]_{cyt}$ by about 40% (Figure 2a). In an additional series, we investigated whether application of thapsigargin (10^{-6} M) 5 min before addition of methacholine (10^{-5} M) had a similar result upon the relaxation induced by exogenous NO (10^{-4} M). Addition of thapsigargin (10^{-6} M) induced a very modest increase in basal tension and in basal $[Ca^{2+}]_{cyt}$ only in some tissues. Neither the decrease in tension nor the fall in $[Ca^{2+}]_{cyt}$ in response to exogenous NO (10^{-4} M) was different between the thapsigargin-pretreated strip and the control strip ($n = 6$). Increasing the incubation time of thapsigargin to 30 min yielded similar results.

For the relaxations induced by ATP (10^{-3} M) and EFS (40 V; 4 Hz, 1 ms for 1 min), thapsigargin (10^{-6} M), given 5 min before the relaxant stimulus, did not exert an inhibitory influence on the decrease in tension or on the decrease in $[Ca^{2+}]_{cyt}$ induced by these relaxant stimuli (Figure 2b, c).

Influence of exoenzyme C3, Y-27632, calyculin-A and nicotinamide on relaxations induced by NO

To determine whether Ca^{2+} desensitisation processes were involved in the relaxation induced by exogenous NO (10^{-4} M), exoenzyme C3 or Y-27632 or calyculin-A were tested in a similar protocol as described above (thus given 5 min before exogenous NO). Calyculin-A (10^{-6} M), a widely used inhibitor of MLCP, initially suppressed tension of the methacholine-precontracted strips, which then recovered to a level not significantly different from the control situation; it did not influence the methacholine-induced increase of $[Ca^{2+}]_{cyt}$ (Figure 1b). When exogenous NO (10^{-4} M) was then applied in the presence of calyculin-A (10^{-6} M), the reduction in tension elicited by exogenous NO (10^{-4} M) was significantly smaller than in the absence of calyculin-A (Figure 1b): the values obtained were, respectively, 39.1 ± 5.1% and 92.0 ± 2.1% ($n = 6$; Figure 3a; $P < 0.001$). However, addition of calyculin-A did not alter the decrease in $[Ca^{2+}]_{cyt}$ elicited by exogenous NO (10^{-4} M) ($n = 6$; Figure 3a).

Exoenzyme C3 (2 μg ml^{-1}; inactivator of RhoA) and Y-27632 (10^{-5} M; a specific Rho-kinase inhibitor) had no significant effect upon the methacholine-induced contractile activity nor on the methacholine-induced $[Ca^{2+}]_{cyt}$ increase. Neither exoenzyme C3 (2 μg ml^{-1}) nor Y-27632 (10^{-5} M) influenced the effect of exogenous NO (10^{-4} M) upon tension and $[Ca^{2+}]_{cyt}$ ($n = 4$; Figure 3b, c).

In a final series of experiments, we tested the effect of nicotinamide (10^{-2} M; an inhibitor of ADP-ribosyl cyclase). Having no effect *per se* on methacholine-induced tension and on $[Ca^{2+}]_{cyt}$, nicotinamide (10^{-2} M) also did not change the effect of exogenous NO (10^{-4} M): both the decrease in tension (91.0 ± 2.5%; $n = 4$) and in $[Ca^{2+}]_{cyt}$ (33.4 ± 7.9%; $n = 4$) were not different from the control situation (respectively 92.5 ± 1.3% for tension and 31.4 ± 3.2% for $[Ca^{2+}]_{cyt}$; $n = 4$).

Discussion

The present study reports for the first time on a Ca^{2+} desensitising effect of NO in the colon, more specifically the circular muscle layer of the rat distal colon. Indeed, while thapsigargin did not exert an influence on the decrease in tension produced by exogenous NO, it significantly attenuated the fall in $[Ca^{2+}]_{cyt}$ to NO; on the other hand, calyculin-A, an MLCP inhibitor, significantly reduced the decrease in tension elicited by exogenous NO, but did not alter the fall in $[Ca^{2+}]_{cyt}$ to exogenous NO.

Smooth muscle relaxation can either result from a decrease in $[Ca^{2+}]_{cyt}$ and/or a decrease in myofilament Ca^{2+} sensitivity.

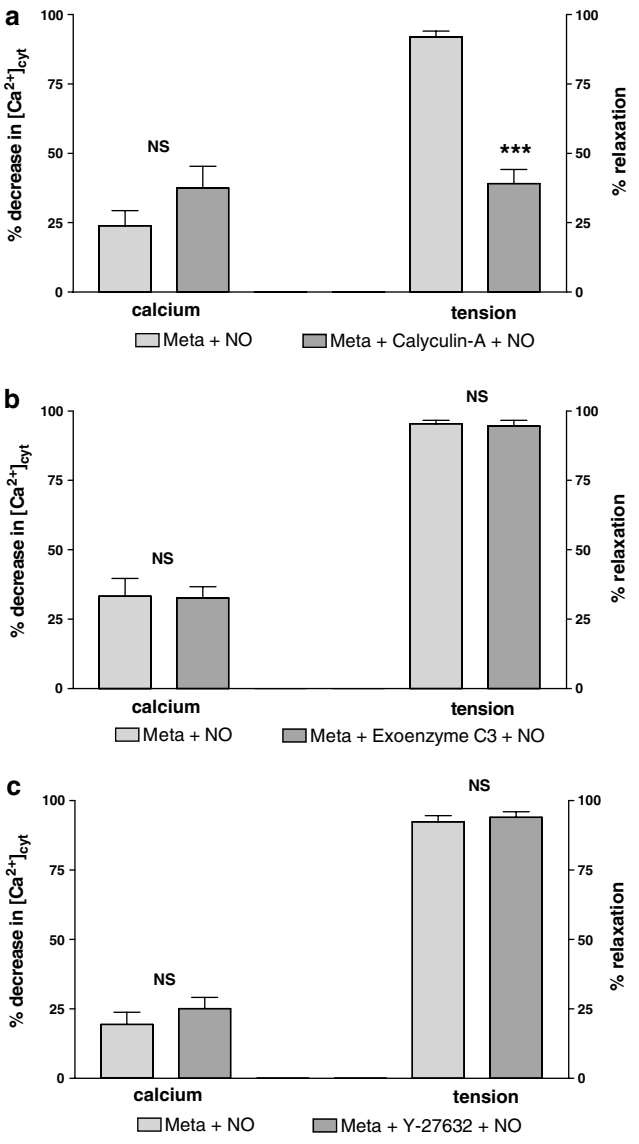

Figure 3 Influence of calyculin-A (10^{-6} M; a), exoenzyme C3 ($2\,\mu g\,ml^{-1}$; b) and Y-27632 (10^{-5} M; c) upon the decrease in $[Ca^{2+}]_{cyt}$ and the decrease in tension induced by addition of exogenous NO (10^{-4} M) to methacholine (10^{-5} M)-precontracted circular smooth muscle strips of rat distal colon. Values are means ± s.e.m. of $n = 4$–6. ***$P < 0.001$: significantly different from the response in the absence of calyculin-A (unpaired *t*-test). NS = nonsignificant.

In circular muscle of rat distal colon, exogenous NO induced a relaxation accompanied by a decrease in $[Ca^{2+}]_{cyt}$. Some reports (see e.g. Cohen *et al*., 1999) describe the activation of SERCA (i.e. refilling the endoplasmic reticulum as intracellular Ca^{2+} store) as a possible mechanism by which inhibitory mediators (in case NO) produce a decrease in $[Ca^{2+}]_{cyt}$ and thus elicit relaxation. We therefore investigated the influence of thapsigargin, a specific inhibitor of SERCA, on the effects of NO. In contrast to what has been found in other smooth muscle tissues such as rat gastric fundus (Van Geldre & Lefebvre, 2004) and guinea-pig tracheal smooth muscle (Ito *et al*., 2002), thapsigargin did not induce a consistent increase in tension in circular smooth muscle of rat distal colon when given in basal conditions; in those tissues where an increase in tension was observed, it was modest as was the concomitant rise in $[Ca^{2+}]_{cyt}$. The observation that thapsigargin was without effect on resting tension and $[Ca^{2+}]_{cyt}$ was also described previously in guinea-pig uterine smooth muscle (Coleman *et al*., 2000). When thapsigargin was administered to methacholine-precontracted circular smooth muscle strips of rat distal colon, this SERCA inhibitor significantly attenuated the decrease in $[Ca^{2+}]_{cyt}$ induced by exogenous NO, but exerted no influence on the decrease in smooth muscle tension in response to exogenous NO. This indicates that the decrease in $[Ca^{2+}]_{cyt}$ by NO is at least partially due to activation of SERCA. But the observation that the reduced decrease in $[Ca^{2+}]_{cyt}$ by NO in the presence of thapsigargin did not lead to a reduction of NO-induced relaxation clearly points to an involvement of Ca^{2+} desensitisation mechanisms in the relaxation to NO. We also investigated the effect of thapsigargin when incubated before the application of methacholine. Following this protocol, we could not establish the same results as mentioned above: thapsigargin had no effect on the decrease in $[Ca^{2+}]_{cyt}$ and on the fall in smooth muscle tension in response to exogenous NO. At the moment, we cannot provide a suitable explanation for this discrepancy in the action of thapsigargin. Since inhibition of SERCA did not fully inhibit the decrease in $[Ca^{2+}]_{cyt}$ elicited by NO, other mechanisms must be involved. Therefore, another series of experiments was performed to determine whether inhibition of the cyclic ADP-ribose (cADPR)-mediated Ca^{2+} signalling was involved in the $[Ca^{2+}]_{cyt}$ lowering effect of NO in the experimental strips. Indeed, cADPR can induce an increase in $[Ca^{2+}]_{cyt}$ in response to muscarinic receptor agonists (Ge *et al*., 2003; White *et al*., 2003), while NO has been demonstrated to prevent this cADPR-mediated rise in $[Ca^{2+}]_{cyt}$ by inhibiting ADP-ribosyl cyclase activity (Yu *et al*., 2000). Incubation with nicotinamide, an ADP-ribosyl cyclase inhibitor, however failed to affect the decrease in $[Ca^{2+}]_{cyt}$ induced by NO, hence rejecting this possibility. Other means by which NO might reduce $[Ca^{2+}]_{cyt}$ are enhanced Na^+/Ca^{2+} exchange (Furukawa *et al*., 1991), activation of the sarcolemmal Ca^{2+}-pumping ATP-ase (Imai *et al*., 1990) or a reduction in Ca^{2+} influx *via* hyperpolarisation of the cell membrane (Bolotina *et al*., 1994), but these possible mechanisms of action were not investigated.

Gastrointestinal smooth muscle relaxation as a result of Ca^{2+} desensitisation was already suggested by Himpens *et al*. (1989) in the guinea-pig ileum. In permeabilised rabbit ileum, Wu *et al*. (1996) also observed relaxations at a constant $[Ca^{2+}]_{cyt}$ that resulted from activation of MLCP with a subsequent reduction in MLC_{20} phosphorylation. Since the degree of MLC_{20} phosphorylation controls actin–myosin interaction, an increase in the activity of MLCP will lead to a rightward shift of the $[Ca^{2+}]_{cyt}$–force response curve; this in turn reflects a reduction of the apparent Ca^{2+} sensitivity of the contractile apparatus (i.e. Ca^{2+} desensitisation). Calyculin-A, a well-known inhibitor of MLCP (Mitsui *et al*., 1994; Gibson *et al*., 2003), was used to assess the involvement of MLCP in the NO-induced Ca^{2+} desensitisation in our experimental preparations. Our findings that calyculin-A did not alter the decrease in $[Ca^{2+}]_{cyt}$ elicited by exogenous NO, but that the reduction in tension in response to NO was significantly smaller than in the absence of calyculin-A, confirmed that MLCP activation is involved in the NO-induced Ca^{2+} desensitisation. A well-known pathway for NO to activate MLCP goes through inhibition of RhoA/Rho-kinase; we

employed both exoenzyme C3 and Y-27632 to elucidate its involvement. Exoenzyme C3 is a bacterial ADP-ribose transferase that ADP-ribosylates the Asn-41 residue of RhoA, thereby inactivating RhoA and inhibiting Ca^{2+} sensitisation (Fujihara *et al*., 1997); on the other hand, Y-27632 acts more downstream by virtue of its highly selective inhibition of Rho-kinase (Uehata *et al*., 1997). Neither exoenzyme C3 nor Y-27632 antagonised the Ca^{2+} desensitising effect of NO since the effect of NO upon tension and $[Ca^{2+}]_{cyt}$ was left unaltered in their presence. This thus implies that NO does not mediate its Ca^{2+} desensitising effect through the RhoA/Rho-kinase pathway. In contrast, with regard to contractile responses in the rat distal colon, recent findings by Takeuchi *et al*. (2004) demonstrate that in rat distal colon longitudinal smooth muscle the RhoA/Rho-kinase pathway mediates Ca^{2+} sensitization induced by activation of muscarinic receptors. In the literature, several other pathways have been mentioned through which the cGMP/PKG pathway can lead to Ca^{2+} desensitisation: PKG-mediated phosphorylation of telokin (Wu *et al*., 1998), a 17 kDa acidic peptide whose sequence is identical to the COOH terminus of MLCK, and of the regulatory subunit of MLCP itself (i.e. MYPT1) (Surks *et al*., 1999) represent alternative routes that can also lead to Ca^{2+} desensitisation in our preparation.

In addition to NO, also ATP is an established inhibitory neurotransmitter in circular smooth muscle of rat distal colon (Van Crombruggen & Lefebvre, 2004). ATP induced a decrease in tension and a concomitant decrease in $[Ca^{2+}]_{cyt}$, none of which were influenced by thapsigargin indicating that activation of SERCA is not involved in the decrease in $[Ca^{2+}]_{cyt}$ by ATP. This might seem surprising as ATP was shown to induce NO release in rat distal colon; however, NO released by 10^{-3} M ATP acted directly on membrane small conductance Ca^{2+}-dependent K^+ channels without generation of cGMP (Van Crombruggen & Lefebvre, 2004). This might explain why thapsigargin did not influence the effect of ATP. As both nitrergic and purinergic neurotransmitters are involved in EFS-induced relaxation (Van Crombruggen & Lefebvre, 2004), an action of thapsigargin on the nitrergic component of the EFS-induced relaxation might be expected. But thapsigargin did not influence the EFS-induced decrease in $[Ca^{2+}]_{cyt}$. This might be related to the complex interaction between ATP and NO during inhibitory neurotransmission and/or to the redundancy of ATP-mediated mechanisms when NO-mediated mechanisms are influenced.

In conclusion, the relaxation to exogenous NO in circular smooth muscle of rat distal colon was demonstrated to be dependent on Ca^{2+} desensitisation since a reduction of the NO-induced decrease in $[Ca^{2+}]_{cyt}$, established by the SERCA-inhibitor thapsigargin, did not affect the amplitude of the decrease in tension induced by NO; this was further corroborated by the finding that the MLCP-inhibitor calyculin-A reduced the NO-induced decrease in tension without changing the fall in $[Ca^{2+}]_{cyt}$ to NO. As inhibition of the RhoA/Rho-kinase pathway with exoenzyme C3 and Y-27632 did not affect this NO-induced Ca^{2+} desensitisation, alternative routes – yet to be elucidated – must be involved in this process.

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