Vasorelaxant Action of *N*-*p*-Nitrophenylmaleimide in the Isolated Rat Mesenteric Artery

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The vasorelaxant response of *N*-*p*-nitrophenylmaleimide (4-NO_2-NPM) was evaluated. The mesenteric rings (1–2 mm i.d.) were suspended by cotton thread for isometric tension recordings in a Tyrode's solution at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2, under a resting tension of 0.75 g. 4-NO_2-NPM induced relaxation in mesenteric rings pre-contracted with phenylephrine (Phe; 10 μM, pD_2 = 6.7 ± 0.3) or KCl (80 mM, pD_2 = 3.9 ± 0.2). This effect was significantly attenuated after removal of the vascular endothelium, N^G-nitro L-arginine methyl ester (L-NAME; 100 μM), atropine (1 μM), indomethacin (10 μM), L-NAME + indomethacin or 1*H*-[1,2,3]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; 10 μM). L-Arginine (1 mM) reversed the inhibitory effect of L-NAME. In endothelium-intact preparations pre-incubated with 20 mM KCl, tetraethylammonium bromide (TEA; 1 mM) or glibenclamide (Glib; 10 μM), the vasorelaxant effect was significantly attenuated when compared to controls (endothelium intact). In denuded rings, separate incubation with 20 mM KCl, TEA or Glib did not change the relaxation when compared with that obtained in denuded rings. 4-NO_2-NPM inhibited in a concentration-dependent and non-competitive manner the concentration-response curves induced by $CaCl_2$. In calcium-free medium, the transient contractions induced by Phe (10 μM) or caffeine (20 mM) were inhibited. The relaxant effect induced by 4-NO_2-NPM appeared to be due to endothelial muscarinic receptors activation, NO and prostacyclin release and K_{ATP} and BK_{Ca} (Ca^{2+}-activated K^+ channels) endothelium-dependent activation. Inhibition of the Ca^{2+} influx and inhibition of the Ca^{2+} release from intracellular IP_3- and caffeine-sensitive stores are also involved in the vasorelaxation.

Key words: N-p-Nitrophenylmaleimide, Endothelium-Derived Factors, Mesenteric Rings

Introduction

Cyclic imides, including maleimide, succinimide, glutarimide, phthalimide, naphthalimide, among others, are compounds which generally possess an imide ring and the group –CO–N(R)–CO– as part of the structure. Different and important biological properties have been attributed to these compounds, such as analgesic, antimicrobial, anticonvulsant, antitumour and antispasmodic activities (Cechinel-Filho *et al*., 2003). The attention to these compounds increased since the discovery of phyllanthimide, an alkaloid present in low concentration in the active aerial parts of *Phyllanthus sellowianus* (Tempesta *et al*., 1988). Using this compound as a model some of us synthesized a great number of compounds belonging to different sub-classes of cyclic imides and reported their biological effects in several experimental models (Cechinel-Filho *et al*., 2003).

In a broad pharmacological screening performed in our laboratory with different cyclic imides, we verified that *N*-*p*-nitrophenylmaleimide (4-NO_2-NPM, Fig. 1) produced relaxant action in smooth muscles of isolated rat mesenteric superior rings. Previous studies have shown that this compound exhibits antifungal (Lima *et al*., 1999) and antibacterial (Prado *et al*., 2004) properties. The present study aimed to elucidate the action

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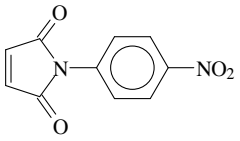

Fig. 1. Chemical structure of *N-p*-nitrophenylmaleimide (4-NO_2-NPM).

mechanism involved in the vasorelaxant effect induced by 4-NO_2-NPM in rat superior mesenteric artery.

Material and Methods

Animals

Male Wistar rats (*Rattus norvegicus*), weighing 200–300 g, were used in all experiments. Experimental protocols and procedures were approved by the Laboratório de Tecnologia Farmacêutica Animal Care and Use Committee. Animals were housed under conditions of controlled temperature [(25 ± 1) °C] and lighting (light on 6:00 a.m.–6:00 p.m.), had access to food and tap water *ad libitum*.

Synthesis of 4-NO_2-NPM

4-NO_2-NPM was obtained by the reaction of maleic anhydride with 4-nitroaniline in diethyl ether and dehydration of the corresponding maleamic acid by treatment with acetic anhydride/sodium acetate at 70 °C and recrystalization with *n*-hexane (yield 61%), as previously described (Lima *et al*., 1999).

Preparation of isolated rat superior mesenteric artery rings

Rats were killed by stunning and exsanguination. The superior mesenteric artery was removed, cleaned from connective tissue and fat, and sectioned in rings (1–2 mm long), which were suspended by cotton threads in organ baths containing 10 ml of Tyrode's solution (composition in mM: NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0; and glucose, 5.6.), gassed with a mixture of 95% O_2 and 5% CO_2, and maintained at 37 °C for isometric tension recordings. The stabilization period was 1 h under a resting tension of 0.75 g. During this time the solution was changed each 15 min to prevent the accumulation of metabolites. The isometric tension was recorded by a force transducer (Model GM2 Gould, Valley View, OH, USA) coupled to an amplifier-recorder (Gould). Endothelium was removed by gently rubbing the intimal surface of the vessels. The presence of functional endothelium was assessed by the ability of acetylcholine (ACh; 10 μM) to induce more than 80% relaxation of pre-contracted vessels with phenylephrine (Phe; 10 μM), and the absence, less than 10%, of relaxation in the presence of ACh was taken as an evidence that the vessel segments were functionally denuded of endothelium.

Drugs

The following drugs were used: cremophor, atropine sulfate, acetylcholine hydrochloride, indomethacin, N^G-nitro L-arginine methyl ester (L-NAME), L-phenylephrine chloride, L-arginine, ethyleneglycol bis(*β*-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), caffeine, 1*H*-[1,2,3]-oxadiazolo[4,3-*α*]quinoxalin-1-one (ODQ), tetraethylammonium bromide (TEA) and glibenclamide (Glib), all from Sigma. The stock solutions were dissolved in distilled water, except indomethacin that was dissolved in sodium bicarbonate, and ODQ was prepared as stock solution in dimethyl sulfoxide. 4-NO_2-NPM was solubilized in a mixture of distilled water and cremophor, diluted to the desired concentrations with distilled water just before use.

Statistics

Two pharmacological parameters were analyzed: E_{max} (maximal effect generated by the agonist) and pD_2 (–log EC_{50}, where EC_{50} is the half maximal effective concentration). Results are expressed as means ± standard error of the mean (SEM). Student's *t*-test and one-way analysis of variance using Bonferroni or Dunnet test (ANOVA) were used to analyze the data, and results were considered significant when $p < 0.05$.

Effect of 4-NO_2-NPM on mesenteric rings pre-contracted with Phe or KCl

In the first set of experiments, after equilibration, steady tension was evoked by Phe (10 μM) for endothelium-intact and -denuded rings to induce contraction of similar magnitude, and 4-NO_2-NPM was added cumulatively (10^{-9}–10^{-3} M). The ability of 4-NO_2-NPM to attenuate

the 80 mM KCl-induced sustained contraction in the rings was also examined. To investigate the possible mechanism(s) responsible for 4-NO_2-NPM-induced relaxation. The preparations with endothelium were pre-contracted with Phe for 30 min after being pre-incubated with one of the following inhibitors: atropine (1 μM), a non-selective antagonist of the muscarinic receptors, L-NAME (100 μM), an inhibitor of NO synthase (NOS), L-NAME + L-arginine (1 mM), NOS substrate, L-arginine alone, indomethacin (10 μM), an inhibitor of cyclooxygenase (COX), L-NAME + indomethacin, and ODQ (10 μM), separately.

In the second set of experiments, rings with or without endothelium were obtained with 20 mM KCl, an inhibitor of K^+ efflux, plus Phe, Glib (10 μM), a blocker of the ATP-sensitive K^+ channels (K_{ATP}) or TEA (1 mM), which is at 1 mM a selective blocker of Ca^{2+}-activated K^+ channels (BK_{Ca}). Then, concentration-response curves to 4-NO_2-NPM were obtained. All blocking drugs were added 30 min before the contractions with Phe. The concentration used for each inhibitor of K^+ channels was sufficient to antagonize selectively the channels in arterial smooth muscle (Nelson and Quayle, 1995).

Effect of 4-NO_2-NPM on contractions induced by $CaCl_2$ and Ca^{2+} release from intracellular stores sensitive to Phe and caffeine

To investigate further the mechanism of vasorelaxation induced by 4-NO_2-NPM, concentration-response curves to $CaCl_2$ were constructed using endothelium-denuded rings (Lagaud *et al*., 1999). Briefly, the rings were pre-contracted with KCl (60 mM) to confirm the tissue viability. Tyrode's solution was replaced with hyperpolarizing Tyrode's solution (60 mM KCl) nominally without Ca^{2+} (15 min). Thereafter, concentration-response curves to $CaCl_2$ (10^{-6} to 10^{-2} M) were constructed in the absence or presence of 4-NO_2-NPM (10^{-5}, $3 \cdot 10^{-5}$, 10^{-4}, or $3 \cdot 10^{-4}$ M). To determine whether 4-NO_2-NPM could interfere with Ca^{2+} release from intracellular stores, the denuded rings were pre-contracted with KCl (60 mM), washed and exposed to Ca^{2+}-free Tyrode's solution containing EGTA (1 mM). The rings were then stimulated with Phe (10 μM) or caffeine (20 mM). The contractions of both agonists were obtained in the absence (control) or after incubation with 4-NO_2-NPM ($3 \cdot 10^{-5}$, 10^{-4}, or $3 \cdot 10^{-4}$ M).

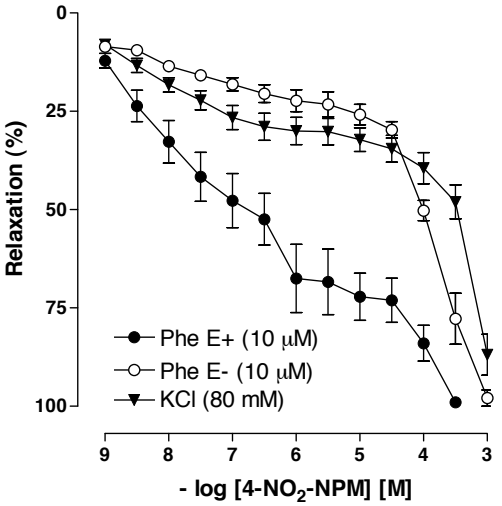

Fig. 2. Effects of increasing concentrations of 4-NO_2-NPM on phenylephrine (Phe, 10 μM)- and KCl (80 mM)-induced contractions in mesenteric rings.

Results

Effect of 4-NO_2-NPM on mesenteric rings pre-contracted with Phe

As showed in the Fig. 2, the 4-NO_2-NPM-induced reductions of Phe-evoked contractions were significantly different in endothelium-denuded preparations ($pD_2 = 3.7 \pm 0.1$) when compared to those in preparations with intact endothelium ($pD_2 = 6.7 \pm 0.3$) suggesting that the vasorelaxant effect attributed to 4-NO_2-NPM may be associated with an endothelium-dependent mechanism (Table I).

Verification of the role of the muscarinic receptors and endothelium-derived factors in the 4-NO_2-NPM-induced vasorelaxant responses

The regulation of vasodilatation by endothelium is determined by three main mediators: NO (Palmer *et al*., 1988), prostacyclin (PGI_2) (Gryglewski *et al*., 1986), and endothelium-derived hyperpolarizing factor (EDHF) (Nakane *et al*., 1991). The formation of NO by NOS can be inhibited by several substituted L-arginine analogues such as L-NAME. In endothelium-intact mesenteric rings pre-treated with L-NAME, an inhibitor of NOS (Moncada and Higgs, 1993), the vasorelaxation induced by the compound was

significantly attenuated (Fig. 3A, Table I). On the other hand, L-arginine or L-arginine + L-NAME had no significant effect on 4-NO_2-NPM-induced relaxation (Table I, Fig. 3C). In the presence of indomethacin, an inhibitor of prostacyclin formation, the vasorelaxation was significantly attenuated. An effect was observed when L-NAME + indomethacin were added together to the organ baths (Fig. 3A, Table I). These results suggested that the endothelium modulated the relaxant responses of 4-NO_2-NPM through the release of NO and of a cyclooxygenase-derived relaxant product.

The activation of the receptor endothelial M_3 sub-type induces vasorelaxation in the adjacent smooth muscle cells by the release of endothelium-derived relaxant factors (EDRFs) and consequently of participation of the cGMP pathway (Moncada and Higgs, 1993). In the presence of atropine, a non-selective antagonist of the muscarinic receptor, the vasorelaxation induced by 4-NO_2-NPM was significantly affected. ODQ, a selective inhibitor of the guanylyl cyclase enzyme (Garthwaite *et al*., 1995), inhibited the endothelium-dependent vasorelaxant action of 4-NO_2-NPM in the vessel studied (Fig. 3B, Table I). Such finding further confirms the involvement of the NO-cGMP pathway in 4-NO_2-NPM-mediated vasorelaxant responses.

Effect of high concentration of K^+, TEA or Glib in the relaxant effect of 4-NO_2-NPM

In the presence of high concentration of K^+ (20 mM), TEA, a blocker of BK_{Ca} at concentrations ≤ 1 mM, or Glib, a selective antagonist of the ATP-sensitive K^+ channels (K_{ATP}), the relaxations induced by 4-NO_2-NPM were significantly attenuated in endothelium-intact rings (Fig. 4A, Table I). However, with the preparations in endothelium-denuded rings and in the presence of blockers cited above, the concentration-response curves of 4-NO_2-NPM were not significantly affected (Fig. 4B, Table I). These results suggest that the BK_{Ca} and K_{ATP} seem to be involved in relaxation induced by 4-NO_2-NPM in rings of the superior mesenteric artery isolated from rats, and their activation depends on the presence of vascular endothelium.

Verification of the 4-NO_2-NPM effect on K^+-depolarizing solutions (80 mM KCl) tonus in intact rings

4-NO_2-NPM at concentrations ranging from 10^{-9} M to 10^{-3} M inhibited the sustained tonic contraction induced by 80 mM KCl in a concentration-dependent manner (pD_2 = 3.9 ± 0.2). However, this antagonism was less potent when compared with preparations pre-contracted with Phe (pD_2 =

Table I. Comparison of E_{max} and pD_2 values of 4-NO_2-NPM against tonic contractions induced by 10 μM phenylephrine in isolated rat mesenteric rings.

Experimental condition	E_{max} (% relaxation)	pD_2
Endothelium intact	99.1 ± 0.8	6.7 ± 0.3
Endothelium denuded	97.9 ± 2.0	3.7 ± 0.1***
L-NAME (100 μM)	78.9 ± 3.3***	3.6 ± 0.4***
Indomethacin (10 μM)	76.6 ± 3.0	4.7 ± 0.4*
L-NAME + indomethacin	82.0 ± 1.9*	3.8 ± 0.3***
Atropine (1 μM)	79.2 ± 2.3**	5.2 ± 0.3**
ODQ (10 μM)	77.7 ± 4.3*	4.4 ± 0.1***
L-Arginine (1000 μM)	100 ± 0	6.4 ± 0.2
L-NAME + L-arginine	100 ± 0	6.5 ± 0.3
Endothelium intact + KCl (20 mM)	72.6 ± 3.2[a]**	4.8 ± 0.3***
Endothelium denuded + KCl (20 mM)	99.7 ± 1.0	3.8 ± 0.3
Endothelium intact + TEA (1 mM)	77.3 ± 4.1**	4.3 ± 0.2***
Endothelium denuded + TEA (1 mM)	83.9 ± 8.8	3.2 ± 0.1
Endothelium intact + Glib (10 μM)	74.7 ± 4.8***	5.2 ± 0.2**
Endothelium denuded + Glib (10 μM)	95.7 ± 2.6	4.3 ± 0.2

The values are means ± SEM. n = 6 experiments. ANOVA followed by Bonferroni's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. endothelium intact.

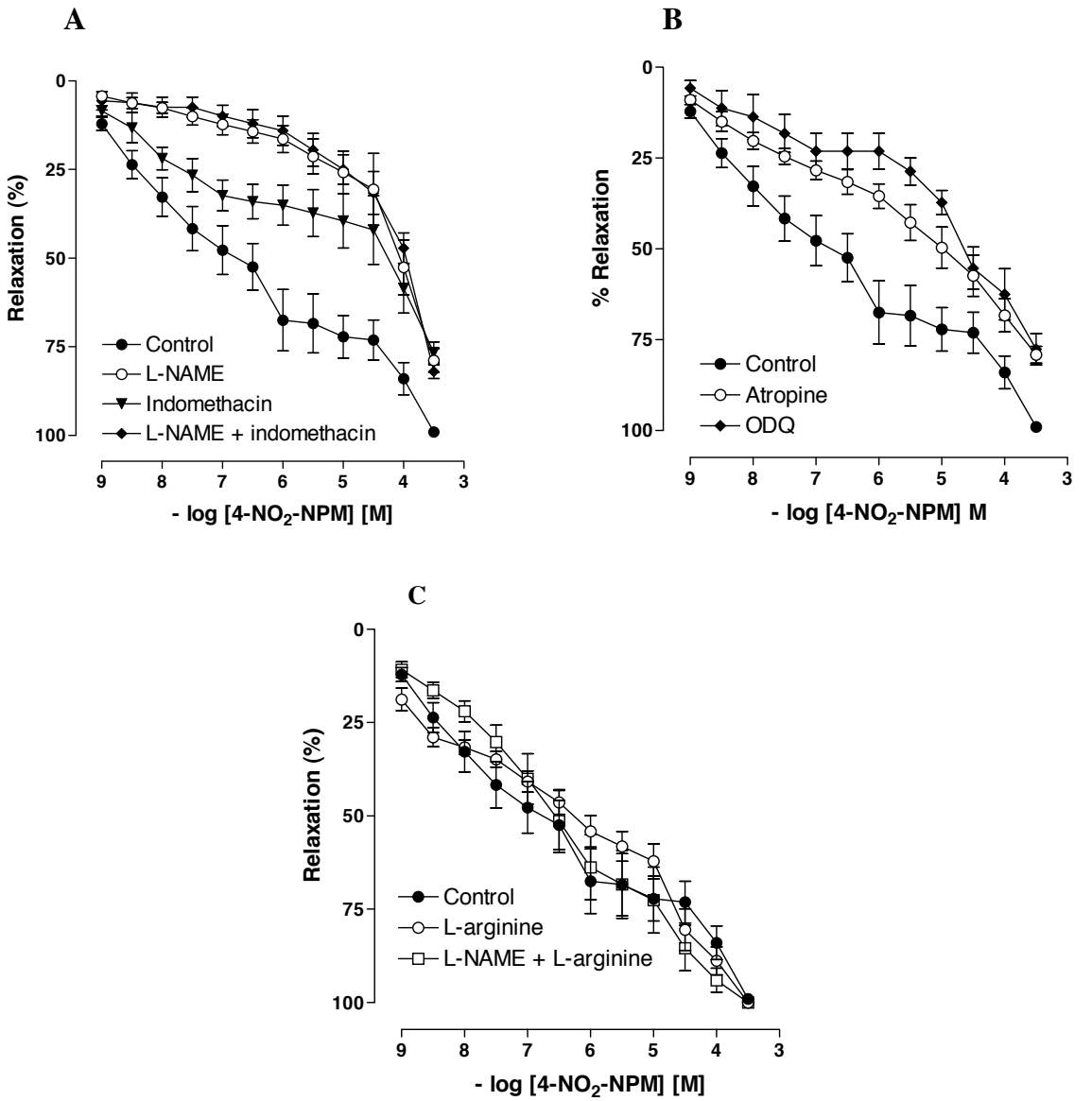

Fig. 3. Relaxation responses induced by increasing concentrations of 4-NO_2-NPM in intact rat mesenteric superior rings endothelium contracted with phenylephrine: (A) in the presence of L-NAME (100 μM), indomethacin (10 μM), L-NAME + indomethacin; (B) in the presence of atropine (1 μM), ODQ (10 μM); (C) in the presence of L-arginine (1 mM), L-NAME + L-arginine.

6.7 ± 0.3) (Fig. 2). It is well known that KCl induces smooth muscle contraction through activation of voltage-dependent calcium channels and subsequent release of calcium from the sarcoplasmic reticulum (Gurney, 1994), whereas Phe-induced vasoconstriction is mediated by the stimulation of G-protein coupled to α-adrenoceptors (Pérez-Vizcaino *et al.*, 1998). In both cases, the major resulting effect is an increase in the intracellular calcium concentration through calcium entry. We therefore suggest that the residual vasorelaxant effect observed after NOS inhibition and endothelium removal is due to an NO-independent mechanism.

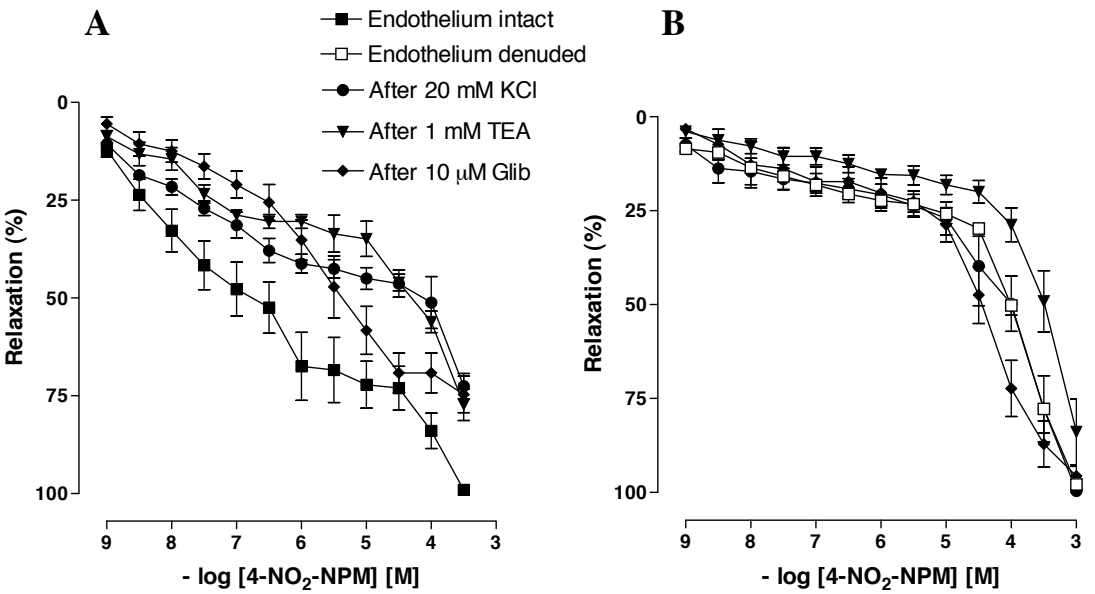

Fig. 4. Relaxation responses induced by increasing concentrations of 4-NO_2-NPM in superior mesenteric rings pre-contracted with phenylephrine after addition of KCl (20 mM), TEA (1 mM) or Glib (10 μM) (A) with endothelium and (B) without endothelium.

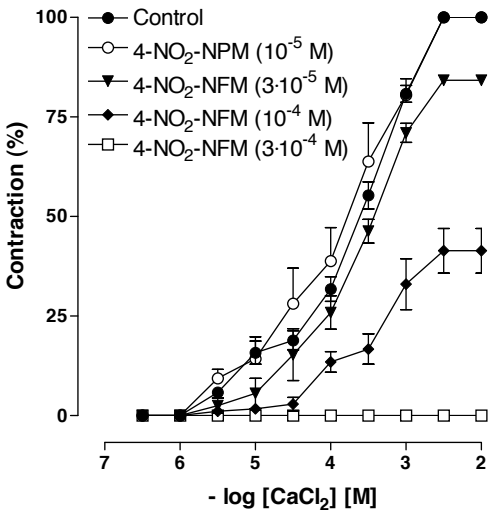

Fig. 5. Effects of increasing concentrations of 4-NO_2-NPM (10^{-5}, $3 \cdot 10^{-5}$, 10^{-4}, and $3 \cdot 10^{-4}$ M) on cumulative concentration-response curves to $CaCl_2$ in depolarizing medium nominally without Ca^{2+} in superior mesenteric rings.

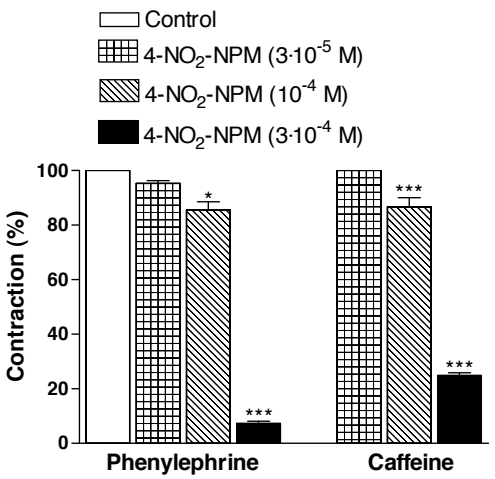

Fig. 6. Effects of increasing concentrations of 4-NO_2-NPM ($3 \cdot 10^{-5}$, 10^{-4}, and $3 \cdot 10^{-4}$ M) on phenylephrine (10 μM)- and caffeine (20 mM)-induced transient contractions, in Ca^{2+}-free media, in superior mesenteric rings.

Effect of 4-NO_2-NPM on contractions induced by $CaCl_2$ and Ca^{2+} release from intracellular stores sensitive to Phe and caffeine

The above-mentioned observation suggests that 4-NO_2-NPM induces a vasodilator effect, at least in part by inhibition of these channels. As can be seen by Fig. 5, the mean cumulative concentration-response curves for $CaCl_2$ alone and in the presence of different concentrations of 4-NO_2-NPM produced a non-parallel and concentration-dependent rightward shift of the concentration-response curves for $CaCl_2$, significantly reducing the maximal response. These results suggest that the inhibitory effect of influx of Ca^{2+} induced by 4-NO_2-NPM in rings of the superior mesenteric artery isolated from rats can be attributed in part to a Ca_v (voltage-operated calcium channels) blockade of the membrane of the vascular smooth muscle.

Since 4-NO_2-NPM relaxed the mesenteric superior aortic rings pre-contracted with Phe, it could be suggested that the compound blocks the Ca^{2+} influx through interference with both voltage- and receptor-operated channels. As can be observed in Fig. 6, the compound significantly inhibited the transient contractions induced by Phe like the transient contractions induced by caffeine. Thus, it seems unlikely that the vascular effects of 4-NO_2-NPM involve a reduction of Ca^{2+} release from intracellular stores sensitive to Phe and caffeine. This result does not rule out the possibility that 4-NO_2-NPM interferes with the sarcoendoplasmatic reticulum Ca^{2+}-ATPases. Further studies should be performed to clarify this point.

In conclusion, the present study demonstrated that 4-NO_2-NPM produced a endothelium-dependent and -independent vasorelaxation in superior mesenteric artery rings. Endothelium-dependent relaxation appeared to be due to endothelial muscarinic receptors activation, NO and PGI_2 release, and activation of K_{ATP} and BK_{Ca}, however, these channels are only activated in the presence of vascular endothelium. Endothelium-independent relaxation is due to inhibition of the Ca^{2+} influx and inhibition of calcium release from intracellular IP_3- and caffeine-sensitive stores.

Acknowledgements

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