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Trans-4-methoxy-β-nitrostyrene relaxes rat thoracic aorta through a sGC-dependent pathway

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ABSTRACT

1-Nitro-2-phenylethene (NPe) induces a more potent vasorelaxant effect in rat aorta than its structural analog 1-nitro-2-phenylethane, but mediated through a different mechanism, independent of soluble guanylate cyclase (sGC) stimulation. We hypothesized that introducing an electron donor into the aromatic moiety might stabilize NPe, enhancing its potency and/or interaction with sGC. Therefore, trans-4-methoxy-β-nitrostyrene (T4MN) was synthesized, and mechanisms underlying its vasorelaxant effects were studied in rat aortic ring preparations. In endothelium-intact preparations, T4MN fully relaxed contractions induced by phenylephrine (PHE) with a potency similar to that of its parent drug, NPe. This vasorelaxant effect that was unchanged by endothelium removal, pretreatment with L-NAME, indomethacin, or MDL-12,330 A, but was significantly reduced by tetraethylammonium, 4-aminopyridine, methyl blue, or ODQ. Under Ca^{2+}-free conditions, T4MN did not alter contractions evoked by caffeine, but significantly reduced, in an ODQ-preventable manner, those induced by either PHE or extracellular Ca^{2+} restoration following depletion of intracellular Ca^{2+} stores in thapsigargin-treated aortic preparations. Under the same conditions, T4MN also reduced contractions induced by protein kinase C activator phorbol-12,13-dibutyrate with a potency similar to that evoked by this nitroderivative against PHE-induced contractions. In conclusion, T4MN induces potent vasorelaxation in rat aorta by stimulating the sGC-cGMP pathway through a NO-independent mechanism. Introduction of a methoxy group into the aromatic moiety apparently stabilizes NPe, thereby enhancing its interaction with sGC.

1. Introduction

Nitroderivatives found in higher plants are rare. 1-Nitro-2-phenylethane was the first nitro compound isolated from plants (Gottlieb and Magalhães, 1960), and is responsible for the plant's cinnamon-like scent (Gottlieb, 1972). It is the main constituent of the essential oil of *Aniba canelilla* (H.B.K.) Mez [syn. *Aniba elliptica* A. C. Sm., *Cryptocarya canelilla* Kunth] (Lauraceae), an aromatic plant abundant in the Amazon region, where it is commonly known as “casca preciosa” (precious bark). Previously, we showed that intravenous administration of 1-nitro-2-phenylethane induced two periods of hypotension and bradycardia in either normotensive (de Siqueira et al., 2010) or hypertensive (Interaminense et al., 2011) rats. The first rapid bradycardic and hypotensive phase has a vago-vagal reflex origin while the delayed hypotension was attributed to a direct vasodilatory effect, a finding corroborated by the ability of 1-nitro-2-phenylethane to relax the phenylephrine (PHE)-induced contractions in isolated aortic (de Siqueira et al., 2010; Brito et al., 2013) or superior mesenteric artery (Interaminense et al., 2011, 2013) preparations in a concentration-dependent manner. In rat aortic preparations, vasodilator actions of 1-nitro-2-phenylethane are mediated through stimulation of the soluble guanylate cyclase (sGC)-cGMP pathway independently of endothelial nitric oxide (NO) release, leading to increasing intracellular cGMP levels (Brito et al., 2013).

Conformational analysis of 1-nitro-2-phenylethane showed that this molecule can adopt different conformations with varying dihedral angles between phenyl and nitro groups linked at sp^3 carbon atoms (Vale et al., 2013). We recently showed that 1-nitro-2-phenylethene (NPe, Fig. 1A), a structural analog of 1-nitro-2-phenylethane, formed by substitution of the alkan for the alkene moiety, was nearly 3.5 times

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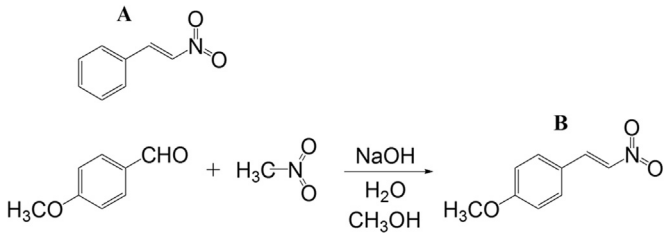

Fig. 1. Chemical structure of 1-nitro-2-phenylethene (or trans-β-nitrostryrene) **(A)** and a general procedure for synthesis of its structural analog, trans-4-methoxy-β-nitrostyrene **(B).**

more potent at relaxing aortic ring preparations than its parent molecule (Arruda-Barbosa et al., 2014). However, the mechanism by which NPe induced vasorelaxation was quite different from that of 1-nitro-2-phenylethane, as it was independent of sGC stimulation (Arruda-Barbosa et al., 2014). Given that interactions between drugs and receptors (or enzymes) are related not only to hydrophobic coupling, but also to polar bonds that require a certain electronic density of specific atoms (Gallé et al., 2013), electronic structure is an important issue, since it represents energy levels of the molecule that can be increased by adding an electron-acceptor (e.g. NO_2). It is well known that methoxy groups exert two opposite effects: inductive (electron withdrawing) and resonance (electron-donating) effects, but the latter effect is more powerful than the former, thereby conferring an electron-donating property upon these groups. Methoxy groups are strong electron donors due to resonance effects that are comparable to those of chloro and other halogen substituents, mainly due to their decreasing oxidation potential (Bessems et al., 1995) and ionization potential (IP) (Diniz et al., 2004).

In the present study, we hypothesized that methoxy substitution in the para-position of the aromatic ring would stabilize NPe, further increasing its interaction with sGC and/or vasorelaxant potency. Thus, using NPe as a lead compound for electronic structural modifications, trans-4-methoxy-β-nitrostyrene (T4MN, Fig. 1B) was synthesized, and mechanisms underlying its vasorelaxant effects were studied in aortic ring preparations.

2. Materials and methods

2.1. Synthesis of trans-4-methoxy-β-nitrostyrene

1-((*E*)−2-nitro-vinyl)-(4-methoxy)-benzene or T4MN was synthesized by employing the Claisen-Schmitd procedure (Vogel, 1989; Ford et al., 1994) with *p*-anisaldehyde and nitromethane as substrates (0.1 and 0.12 eq., respectively) (Fig. 1B). The aromatic aldehyde was 'one-pot' converted, with a 96% yield, to the corresponding β-nitrostyrene by treatment with 0.05 eq. of NaOH in methanol and water at 0–10 °C (1:2) (Rosini et al., 1991). The resulting precipitate was filtered and dried under vacuum to yield the desired β-nitrostyrene derivative, T4MN. The *trans* product is preferable to the *cis* form due to a stereoselective reaction that gives a product of low energy. T4MN was crystallized in ethanol as yellow solid-crystals; m.p. 86.6–88.2 °C (86–88 °C; Sigma-Aldrich standard). The final product was identified by NMR (^{1}H and ^{13}C NMR) and FT-IR spectroscopy and compared with properties in the literature (Wang and Wang, 2002). IR ν_{max} (cm^{-1}) 806.25, 1624.06, 1456.26, 2900, 1600, 1550, 1498, 1375; ^{1}H NMR (CD_3SOCD_3, 300 MHz) δ 7.87 (dd, 2 H), 7.52 (dd, 2 H), 7.47 (d, 1 H), 6.87 (d, 1 H), 3.76 (s, 3 H); ^{13}C NMR (CD_3SOCD_3, 75 MHz) δ 162.89, 139.12, 135.09, 131.33, 122.48, 114.87, 55.50.

2.2. Animals

Adult male Wistar rats (280–340 g) were kept under conditions of constant temperature (22 ± 2 °C) with a 12/12 h light/dark cycle and free access to food and water. All animals were maintained in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). All procedures described here were reviewed by and approved by local animal ethics committee (126/14).

2.3. Solutions and drugs

The perfusion medium used was a fresh modified Krebs-Henseleit solution (KHS, pH 7.4) of the following composition (in mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2{\cdot}2H_2O$ 2.5; KH_2PO_4 1.18; $MgSO_4{\cdot}7H_2O$ 1.18; glucose 11. Calcium-free solutions were prepared by omitting $CaCl_2$ from normal KHS. Salts were purchased from Merck (Darmstradt, Germany) and Vetec (Rio de Janeiro, Brazil). PHE hydrochloride, acetylcholine (ACh) chloride, ethylene glycol-bis(b-aminoethyl ether)N,N,N´,N´-tetraaceticacid (EGTA), indomethacin, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), glybenclamide, methylene blue (MB), thapsigargin, phorbol-12,13-dibutyrate (PDB), L-N^G nitroarginine methyl ester (L-NAME), cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL- 12,330 A), caffeine, sodium nitroprusside (SNP) and verapamil were purchased from Sigma. They were first dissolved in distilled water and then with KHS to achieve desired concentration in the bath chamber (except EGTA which was added directly to the Ca^{2+}-free KHS). T4MN and NPe were dissolved in ethanol, brought up to the desired concentrations using KHS, and sonicated just before use. Indomethacin (cyclooxygenase inhibitor), L-NAME (inhibitor of nitric oxide synthase), ODQ (guanylate cyclase inhibitor), MB (guanylate cyclase inhibitor), MDL-12,330 A (adenylate cyclase inhibitor), 4-AP (blocker of voltage-operated K^+ channels), TEA (nonspecific K^+ channel blocker) or glybenclamide (blocker of ATP-sensitive K^+ channels) were applied to the bath 10 min prior to pre-contraction with PHE.

2.4. Tissue preparation and experimental protocols

Rats were killed by stunning followed by rapid exsanguination. For isometric tension recording, the thoracic aorta was removed and placed in cold oxygenated KHS buffer. Cylindrical ring-like segments of this artery (1 mm x 5 mm in length), free of fat and connective tissue, were mounted between two steel hooks in a 5-ml isolated tissue chamber containing gassed (95% O_2 and 5% CO_2) KHS, at 37 °C, under a resting tension of 1 g, which was readjusted every 15 min during a 45-min equilibration period before drug administration. Isometric tension was recorded using an isometric force displacement transducer (Grass Model FTO3, Quincy, MA, USA) connected to an acquisition system (PM-1000; CWE Inc., Akron, OH, USA). Vessels were initially exposed twice to 60 mM KCl to check their functional integrity. After 30 min, rings were contracted with a concentration (0.1 μM) of PHE inducing 50–70% of the contraction induced by KCl. Then ACh (1 μM) was added to assess endothelium integrity. In one series of experiments, endothelium was removed immediately after dissection by gentle rubbing of the aortic lumen with a stainless steel wire. The absence of ACh-induced relaxation was taken as an indicator of successful endothelium removal. Control rings were exposed only to the vehicle used to dissolve T4MN. Mechanisms underlying the vasorelaxant effects of T4MN were studied in aortic preparations contracted with the α_1-adrenergic agonist PHE.

2.4.1. Investigation of the role of the vascular endothelium and potassium channels

Effects of cumulative concentrations of T4MN (0.56–558.1 μM) on sustained contractions evoked by KCl (60 mM) or PHE (1 μM) were studied in either endothelium-intact or endothelium-denuded aortic ring preparations maintained in Ca^{2+}-containing medium. Vasorelaxant effects of T4MN were also studied in preparations primed with PHE

(1 μM) pre-treated with indomethacin (10 μM) or L-NAME (100 μM). As a positive control, vascular effects of the NO donor SNP (0.1 nM-10 μM, n =8) or the vehicle used to dissolve T4MN were studied in PHE-preconstricted aortic preparations with intact endothelium. In the latter preparations, vasorelaxant effects of T4MN were studied after pretreatment with TEA (5 mM), glybenclamide (10 μM) or 4-AP (1 mM) to assess the role of potassium channels.

2.4.2. Investigation of the involvement of cyclic nucleotides

Aortic preparations with intact endothelium were pretreated with MDL-12,330 A (3 μM) to assess the involvement of adenylate cyclase while they were pretreated with ODQ (10 μM) or MB (10 μM) to investigate the role of guanylate cyclase in vasorelaxant effects of T4MN.

2.4.3. Investigation of the involvement of calcium channels

Under Ca^{2+}-free conditions, we investigated whether T4MN inhibits contractions induced by exogenous Ca^{2+} in aortic ring preparations with intact endothelium depolarized by either KCl (60 mM) in the presence of EGTA (50 μM) to activate voltage-operated calcium channels (VOCCs) or by PHE (1 μM) in the presence of verapamil (1 μM) to preferentially activate receptor-operated calcium channels (ROCCs). Thereafter, a cumulative concentration-response curve for Ca^{2+}(0.1–20 mM) was developed. After washing the preparation by changing the bath chamber solution to remove just Ca^{2+} from the medium, preparations were incubated for 5 min with T4MN (55.8 or 167.4 μM). Cumulative concentration-response curve for $CaCl_2$ (0.1–20 mM) were then repeated and compared with that performed in the absence of the nitroderivative.

Another series of experiments was conducted under Ca^{2+}-free conditions to assess whether T4MN interferes with contractions evoked by Ca^{2+} influx through store-operated Ca^{2+} channels (SOCCs) activated by Ca^{2+} store depletion (Putney, 1997). In aortic preparations with intact endothelium initially bathed in Ca^{2+}-free KHS (containing EGTA 100 μM), depletion of intracellular Ca^{2+} stores was achieved through at least three successive exposures to PHE (10 μM) until no detectable contraction was recorded. Each stimulus was followed by washing the preparation with Ca^{2+}-free KHS to remove PHE from the extracellular medium. Thereafter, a control cumulative concentration-response curve for Ca^{2+}(0.1–20 mM) was performed 10 min after the addition to the bath of thapsigargin (1 μM), an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Thastrup et al., 1990). In a separate set of aortic rings with intact endothelium, T4MN (55.8 or 167.4 μM) alone or in the presence of ODQ (10 μM) was added following thapsigargin treatment and its effects on the cumulative concentration-response curve for $CaCl_2$ (0.1–20 mM) were evaluated.

2.4.4. Investigation of the involvement of intracellular calcium

In a first series of experiments, we investigated whether T4MN affects contractions evoked by sarcoplasmic reticulum Ca^{2+} channels activated by inositol triphosphate (IP_3). For this purpose, aortic rings with intact endothelium were first washed with Ca^{2+}-free KHS for 6 min (containing 1 mM EGTA) and a transient contraction was elicited by PHE (1 μM). After washing the tissues with normal KHS, 60 mM KCl was added to refill internal Ca^{2+} stores followed by washing with Ca^{2+}-free solution and addition of T4MN (55.8 or 167.4 μM), 1 min later. After 5 min, the second contraction of PHE (1 μM) was measured in the presence of T4MN alone, or in association with ODQ (10 μM).

In a second series experiments performed under Ca^{2+}-free conditions, we used caffeine, a pharmacological tool to assess whether T4MN interferes with contractions induced by Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors. Aortic rings with intact endothelium were stimulated with 20 mM caffeine to produce a transient contraction at 25 °C. After washing with Ca^{2+}-containing medium, tissues were stimulated with 60 mM KCl to reload Ca^{2+} internal stores. Thereafter, preparations were washed with Ca^{2+}-free KHS and a second transient contraction was elicited by caffeine in the presence of T4MN (167.4 μM), added 5 min prior to caffeine administration.

Finally, inhibitory effects of increasing concentrations (0.56–558.1 μM) of T4MN on the contraction elicited by the PKC activator PDB (1 μM) were studied in aortic rings with intact endothelium bathed in Ca^{2+}-free medium (containing 1 mM EGTA).

2.5. Statistical analysis

Results are expressed as mean ± S.E.M.. IC_{50} values, defined as the concentration (μM) of T4MN (or NPe) required to produce half-maximal reduction of the contractile response, were calculated by interpolation from semi-logarithmic plots, and were expressed as geometric means [95% confidence interval]. Significance ($P < 0.05$) of results was assessed by paired Student's *t*-test, Mann-Whitney *U*-test, and one- or two-way analysis of variance (ANOVA), followed by Dunnett´s multiple comparison test as appropriate.

3. Results

3.1. Effects of T4MN on sustained contractions induced by KCl or PHE in aortic rings. Role of the endothelium

In endothelium-containing aortic ring preparations (n =13), T4MN (0.56–558.1 μM) fully relaxed the sustained contractions induced by 60 mM in a concentration-dependent manner (Figs. 2A, 2C; $P < 0.001$, one-way ANOVA), with an IC_{50} that did not differ significantly from that obtained in preparations without a functional endothelium (n =10) (Table 1; $P > 0.05$, Mann-Whitney *U*-test). In both preparations, vasorelaxant effects of T4MN are reversible upon washout. T4MN (0.56–558.1 μM) also similarly and concentration-dependently relaxed the sustained contractions evoked by PHE (1 μM) in preparations with (n =8) or without (n =12) a functional endothelium (Fig. 2D), with IC_{50} values that were significantly lower than those obtained for relaxing KCl-induced contractions (Table 1; $P < 0.01$, Mann-Whitney *U*-test).

Cumulative addition of vehicle, in the same concentration range used to dissolve T4MN, has no significant effect on either KCl- (Figs. 2B, 2C; n =15) or PHE-induced (Fig. 2D; n =15) contractions ($P > 0.05$, one-way ANOVA). Furthermore, the positive reference drug, SNP, (0.1 nM-10 μM) fully relaxed the sustained contractions induced by PHE (1 μM) in a concentration-dependent manner ($P < 0.001$, one-way ANOVA), with an IC_{50} value of 4.90 (2.96–11.82) nM. Pretreatment of aortic endothelium-intact rings with L-NAME (100 μM, n =5) (Fig. 2D) or indomethacin (10 μM, n =5) (Fig. 3C) did alter the vasorelaxant effects of T4MN against the contractions induced by 1 μM PHE (Table 1; $P > 0.05$, Mann-Whitney *U*-test).

3.2. Investigation of the role of potassium channels

Vasorelaxant effects of T4MN (0.56–558.1 μM) against PHE-induced contractions were significantly reduced by TEA (5 mM, n =10) or 4-AP (1 mM, n =8) (Fig. 3A; $P < 0.05$, two-way ANOVA), as evidenced by the significant increase in IC_{50} values (Table 1; $P < 0.05$, Mann-Whitney *U*-test) while they remained unchanged following pre-treatment with glybenclamide (10 μM, n =4) (Fig. 3A; $P > 0.05$, two-way ANOVA and Table 1; $P > 0.05$, Mann-Whitney *U*-test).

3.3. Investigation of the involvement of cyclic nucleotides

Concentration-effect curve of T4MN (0.56–558.1 μM) was significantly shifted to the right following inhibition of sGC by either ODQ (10 μM, n =11) or MB (10 μM, n =11) in aortic preparations pre-contracted with PHE (Fig. 3B, $P < 0.05$ by two-way ANOVA), with IC_{50} values significantly higher than the control value (Table 1; $P < 0.05$,

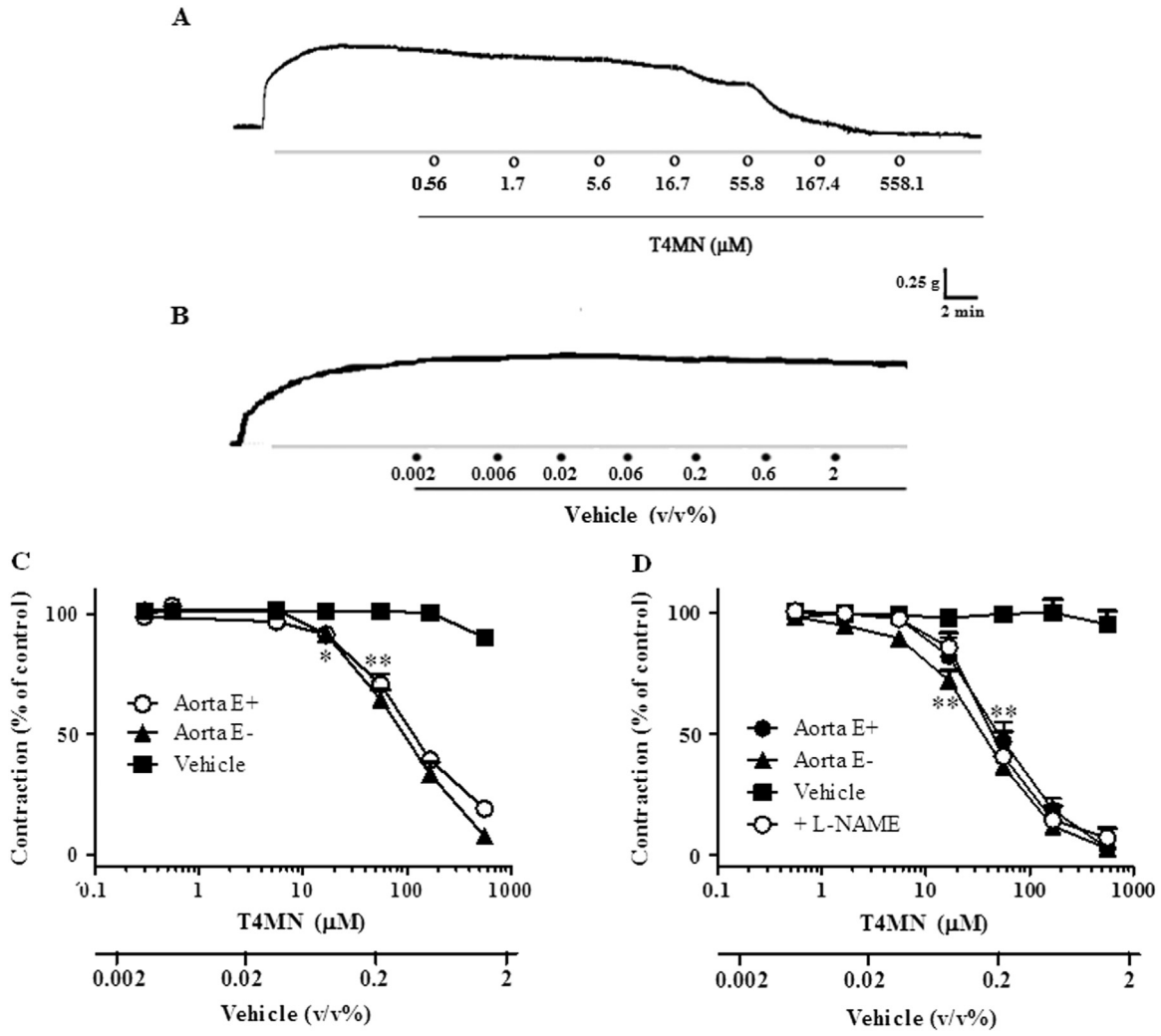

Fig. 2. Typical trace recordings of the effect of trans-4-methoxy-β-nitrostryrene (T4MN, 0.56–558.1 μM) **(A)** and its vehicle **(B)** on sustained contractions induced by 60 mM KCl in rat isolated aortic preparations. Concentration-effect curves for relaxant effects of T4MN (0.56–558.1 μM) in endothelium-intact (E+) and -denuded (E-) aortic rings pre-contracted with KCl (60 mM) **(C)** or phenylephrine (PHE, 1 μM) **(D)**. In panel **D**, note that pretreatment with L-NAME did not alter the vasorelaxant effects of T4MN. In panels **C** and **D**, note that vehicle added cumulatively at the same concentration (% v/v) range used to dissolve T4MN did not change the KCl- and PHE-induced contractions, respectively in preparations with intact endothelium. Results are expressed as means ± S.E.M. (n =10–15 per group). $^{*}P < 0.05$, $^{**}P < 0.001$ by two-way ANOVA followed by Dunnett´s test with respect to vehicle. Calibrations: vertical 0.25 g, horizontal 2 min.

Mann-Whitney *U*-test). In contract, vasodilating activity of T4MN was not affected by the adenylate cyclase inhibitor MDL-12,330 A (3 μM, n =6) (Fig. 3C; p > 0.05, two-way ANOVA).

3.4. Investigation of the involvement of calcium channels

In endothelium-intact aortic preparations incubated in Ca^{2+}-free medium in the presence of a high extracellular KCl concentration (60 mM), increasing extracellular concentrations of $CaCl_2$ (0.1–20 mM, n =13) evoked concentration-dependent contractions ($P <$ 0.001, one-way ANOVA), an effect that became significant at 0.3 mM ($P < 0.05$, one-way ANOVA and Dunnett´s test) and attained a maximal magnitude at 10 mM (Fig. 4A). These contractions due to VOCC activation were abolished by T4MN at 167.4 μM (n =5) (Fig. 4A; $P <$ 0.01, two-way ANOVA) but they remained unaffected by T4MN at 55.8 μM ($P > 0.05$; two-way ANOVA, n =6) (data not shown).

In aortic rings incubated in Ca^{2+}-free medium and stimulated with PHE (1 μM) in the presence of verapamil (1 μM) to preferentially activate ROCCs (Fig. 4B), T4MN at 167.4 μM (n =6), but not at 55.8 μM (n =6, data not shown), also abolished the concentration-dependent contractions due to $CaCl_2$ (0.1–20 mM, n =7) (Fig. 4B; $P >$ 0.05, two-way ANOVA).

A third series of experiments was conducted under Ca^{2+}-free conditions to assess whether T4MN interferes with contractions evoked by Ca^{2+} influx through SOCCs activated by Ca^{2+} store depletion. In aortic preparations in which intracellular Ca^{2+}stores were depleted with repeated exposure to PHE followed by thapsigargin, the expected contractions due to Ca^{2+} addition (0.1–20 mM, n =12) were unaltered by 16.7 μM T4MN (n =6, data not shown), but were abolished by T4MN at 55.8 μM (n =10) (Fig. 5; $P < 0.001$, two-way ANOVA). Such an inhibitory effect of T4MN was blunted by 10 μM ODQ (Fig. 5; $P <$ 0.05, two-way ANOVA, n =5).

3.5. Investigation of the involvement of intracellular calcium signaling

Under Ca^{2+}-free conditions, PHE (1 μM) induced a transient contraction which corresponded to 56.01 ± 6.09% (Fig. 6A, n =14) of a reference contraction induced by 60 mM K^+ in Ca^{2+}-containing medium. Pre-exposure of aortic ring preparations to 167.4 μM (n =6), but not to 55.8 μM (n =5, data not shown) T4MN significantly reduced the PHE-induced phasic contraction to 2.52 ± 1.31% (Fig. 6A, $P < 0.05$, one-way ANOVA followed by Dunnett´s test). This inhibitory effect of T4MN was reversed in preparations pretreated with 10 μM ODQ (Fig. 6A, n =7).

Table 1
IC_{50} values for the vasorelaxant effects of *trans*-4-methoxy-β-nitrostyrene in aortic ring preparations pre-contracted with KCl (60 mM) or phenylephrine (1 μM) subjected to various pre-treatments. Values are expressed as geometric mean [95% confidence interval] and number within parentheses indicates the number of preparations for each group.

Contractile agent/Pre-treatments	IC_{50} (μM)	n
Phenylephrine		
Control	57.64 [27.85–87.45]	(08)
Endothelium removal	40.09 [32.31–49.50]	(12)
+ TEA	372.66 [255.17–490.14][a]	(10)
+ 4-AP	500.58 [258.35–742.86][a]	(08)
+ Glybenclamide	98.23 [51.23–144.74]	(04)
+ L-NAME	55.92 [24.22 –87.62]	(05)
+ ODQ	325.50 [192.27–453.20][a]	(11)
+ Methylene blue	188.92 [58.32–319.47][a]	(11)
+ Indomethacin	84.59 [65.79–103.35]	(05)
+ MDL–12,330 A	66.64 [30.47–102.86]	(06)
KCl		
Control	120.10 [89.69–150.47][b]	(13)
Endothelium removal	108.89 [62.56–155.21][b]	(10)

[a] $P < 0.05$, by Mann-Whitney *U*-test with respect to value obtained in endothelium-containing preparations pre-contracted with phenylephrine (control).
[b] $P < 0.01$ by Mann-Whitney *U*-test with respect to the corresponding values in preparations pre-contracted with phenylephrine.

Under similar conditions, caffeine (20 mM, n =7) induced a transient contraction with a magnitude that corresponds to 12.26 ± 3.48% of a reference contraction induced by 60 mM K^+ in Ca^{2+}-containing medium. This contraction remained unchanged by 167.4 μM T4MN (Fig. 6B; $P > 0.05$, paired Student´s *t*-test, n =5).

In aortic rings incubated in Ca^{2+}-free medium, PDB (1 μM) induced long-lasting and sustained contraction corresponding to 115.60 ± 10.30% of the K^+-induced contraction. When the contraction reached a steady state, cumulative addition of increasing concentrations (0.56–558.1 μM) of T4MN (n =9) significantly reduced the PDB-induced contractions in a concentration-dependent manner ($P < 0.001$, one-way ANOVA). The IC_{50} value (75.12 [16.29–133.95] μM) of this effect did not differ from that of the inhibitory effects of T4MN against PHE-induced contractions in aortic pre-parations incubated in Ca^{2+}-containing medium ($P > 0.05$, Mann-Whitney *U*-test).

4. Discussion

This study demonstrated that T4MN has interesting vasorelaxant properties that are independent of endothelium integrity. Although T4MN was shown to be equipotent with its parent drug NPe, the mechanism by which it induced vasorelaxation is quite different. This mechanism seems to involve sGS stimulation, leading to a decrease in intracellular Ca^{2+} levels and activation of outward potassium channels on the plasmalemma, as was reported for 1-nitro-2-phenylethane (Brito et al., 2013).

The vasorelaxant effect of T4MN is independent of the integrity of the endothelial layer, as it remained unaltered by removal of the vascular endothelium. Released NO and prostaglandins from endothelial cells are not involved in the vasodilator effect of T4MN, as it remained unchanged following pretreatment with L-NAME and indomethacin, respectively. Since maximal efficacy for the relaxant effect of T4MN was unchanged by endothelium removal, it is reasonable to postulate that its vasodilatory effects have a predominantly myogenic nature. This hypothesis is reinforced by findings that with the same potency, T4MN inhibited contractions evoked by the selective α_1-agonist, PHE, and those produced by PDB, which activates protein kinase C under Ca^{2+}-free conditions.

The actions of cGMP are attributed to activation of protein kinase G (PKG), a serine/threonine kinase composed of an NH_2-terminal domain, a regulatory domain, and a catalytic domain (Hofmann et al., 2006). In

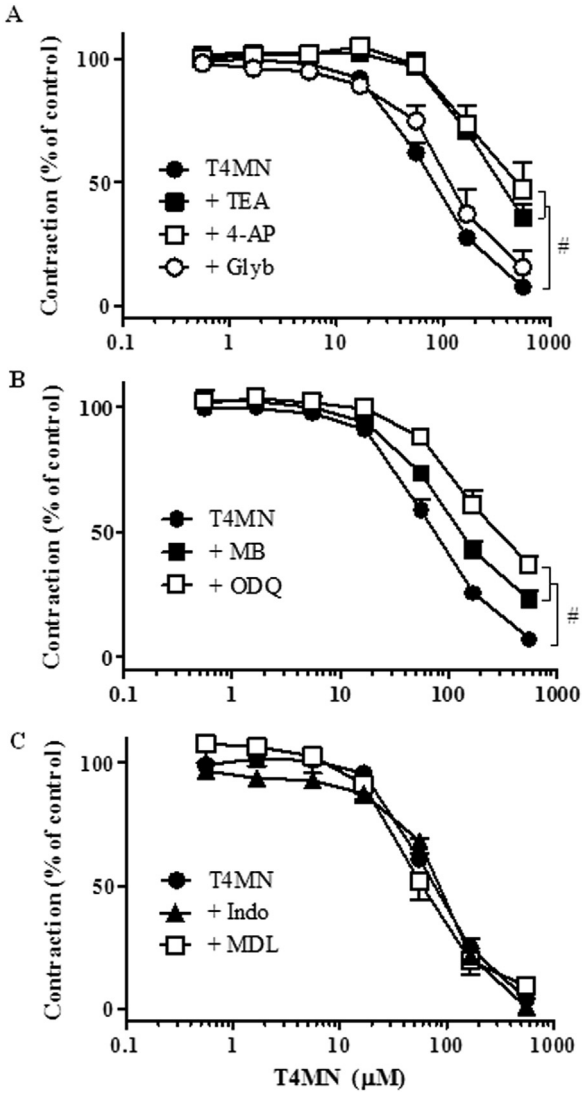

Fig. 3. Effects of pretreatment with tetraethylammonium (TEA, 5 mM), 4-aminopyridine (4-AP, 1 mM), or glybenclamide (Glyb, 10 μM) **(A)**, ODQ (10 μM), methylene blue (MB, 10 μM) **(B)**, indomethacin (Indo, 10 μM), MDL-12,330 A (3 μM) **(C)** on the vasorelaxant effects of trans-4-methoxy-β-nitrostryrene (T4MN, 0.56–558.1 μM) in endothelium-intact aortic preparations pre-contracted with phenylephrine (1 μM). Note that only TEA, 4-AP, MB and ODQ pretreatments showed statistical differences with respect to the control curve (T4MN alone). Results are expressed as means ± S.E.M. (n =4–15 per group). #$P < 0.01$ by two-way ANOVA with respect to the control curve (T4MN alone).

turn, PKG, activated by cGMP, can modulate different target effectors in vascular smooth muscle (VSM) leading to **(i)** decreased intracellular calcium levels that can be achieved through inhibition of calcium release from sarcoplasmic reticulum (IP_3-sensitive Ca^{2+} channels) and/or decreased influx of extracellular calcium (voltage-operated Ca^{2+} and store-operated Ca^{2+} channels), **(ii)** hyperpolarization of VSM membrane potential (outward K^+ channels) and reduction in the sensitivity of the contractile machinery (MLC_{20}). Our data suggest that the vasorelaxant effects of T4MN involve, at least in part, stimulation of the sGC pathway independently from endothelial NO release, leading to increased cGMP levels in aortic rings. This hypothesis is supported by the fact that vasodilator effects of T4MN in aortic preparations were significantly inhibited by both ODQ and MB, two guanylate cyclase inhibitors (Gruetter, 1981; Garthwaite et al., 1995). The present finding contrasts with a previous study showing that the sGC/cGMP pathway is not involved in vasodilatation induced by the parent drug, NPe (Arruda-Barbosa et al., 2014). Cyclic AMP (cAMP), another cyclic nucleotide

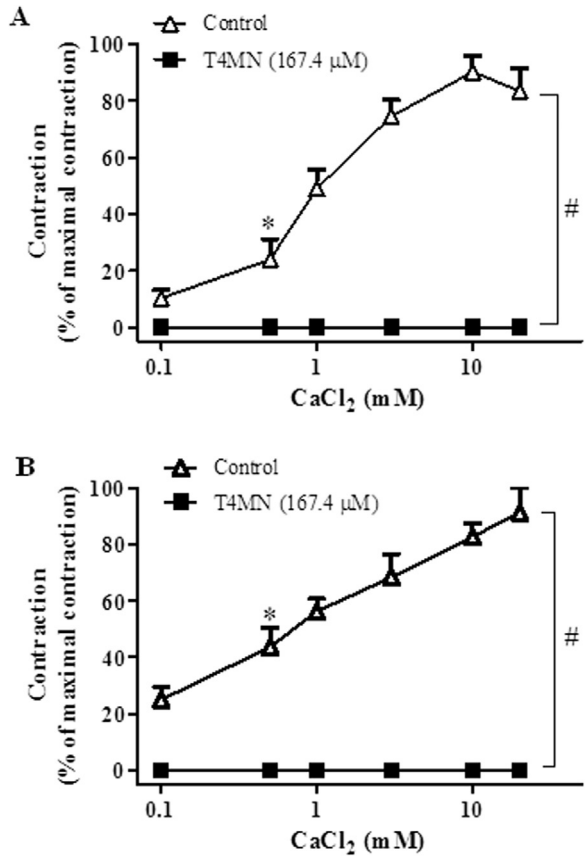

Fig. 4. Inhibitory effects of trans-4-methoxy-β-nitrostryrene (T4MN, 167.4 μM) on the cumulative concentration-effect curve dependent on addition of extracellular Ca^{2+}(0.1–20 mM) in endothelium-intact, KCl-stimulated **(A)** or phenylephrine-stimulated **(B)** aortic ring preparations from rats incubated in Ca^{2+}-free medium. Results are expressed as means ± S.E.M. (n =5–13 per group); *first significant effect for control curve or for curves obtained in presence of T4MN ($P < 0.05$, one-way ANOVA followed by Dunnett´s test); $^{\#}P < 0.01$ by two-way ANOVA, T4MN vs. concentration-contraction curve of Ca^{2+} alone (control).

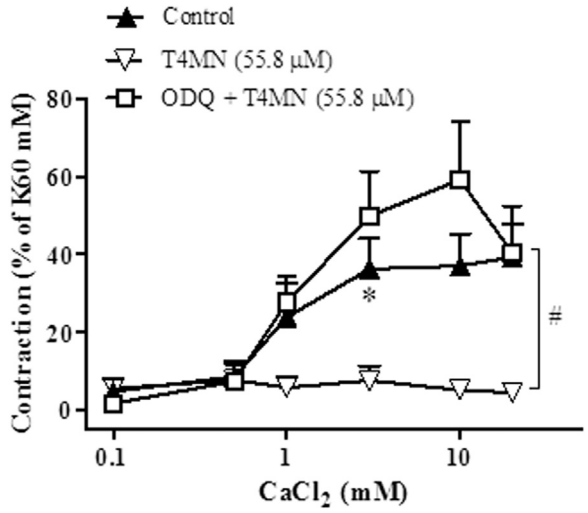

Fig. 5. Inhibitory effects of trans-4-methoxy-β-nitrostryrene (T4MN, 55.8 μM) on the cumulative concentration-effect curve dependent on addition of extracellular Ca^{2+}(0.1–20 mM) in endothelium-intact phenylephrine-depolarized aortic ring preparations pretreated with thapsigargin, and incubated in Ca^{2+}-free medium. Results are expressed as means ± S.E.M. (n =5–12 per group); *first significant effect for control curve ($P < 0.05$, one-way ANOVA followed by Dunnett´s test); $^{\#}P < 0.001$ by two-way ANOVA, T4MN vs. concentration-contraction curve of Ca^{2+} alone (control).

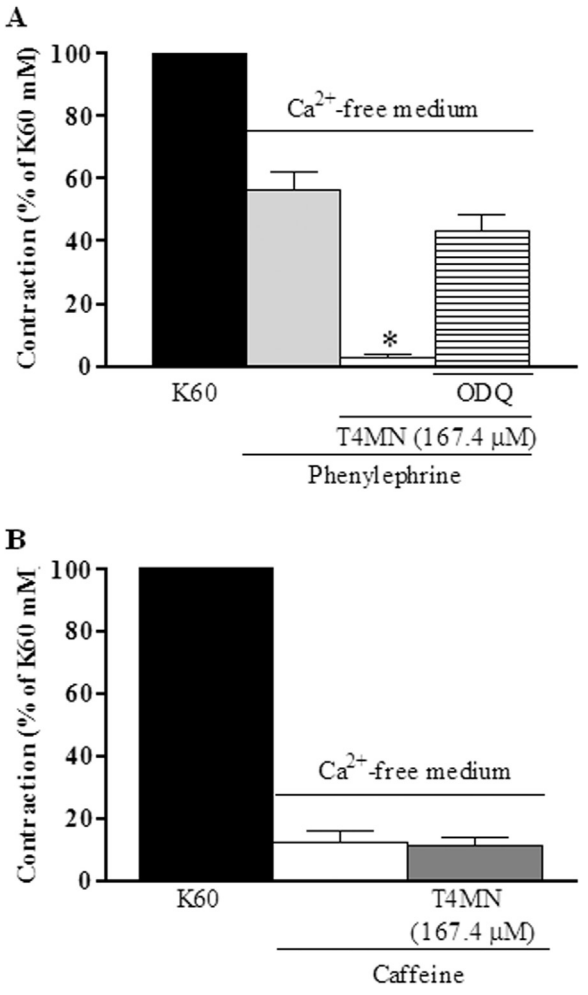

Fig. 6. Inhibitory effects of trans-4-methoxy-β-nitrostryrene (T4MN, 167.4 μM) on contractions evoked by phenylephrine (PHE, 1 μM) **(A)** or caffeine (20 mM) **(B)** in aortic ring preparations with intact endothelium maintained under Ca^{2+}-free conditions. Results are expressed as means ± S.E.M. (n =4–14 per group). $^{*}P < 0.01$ by one-way ANOVA followed by Dunnett´s test with respect to PHE-induced contraction in the absence of T4MN. K60: reference contraction in response to 60 mM K^{+}.

synthesized from intracellular ATP by adenylate cyclase, can also activate PKG in VSM, leading to reduction in intracellular Ca^{2+} and vasorelaxation (Lincoln et al., 1990). Although activation of PKG by cAMP requires nearly a 10-fold-higher cAMP concentration than cGMP concentration, it is unlikely that it contributes to the mediation of T4MN-induced vasorelaxation since this effect was unaltered by the adenylate cyclase inhibitor MDL-12,330 A. A similar profile of action was previously reported for the NO-independent stimulator of sGC, 1-nitro-2-phenylethane (Brito et al., 2013).

Calcium influx from extracellular space through calcium channels is known to play a crucial role in VSM contraction. It is known that contraction evoked by high KCl in VSM is due to membrane depolarization leading to Ca^{2+} influx through VOCCs (Somlyo and Somlyo, 1968). On the other hand, activation of α_1-adrenoceptors by PHE lead to a biphasic response that is characterized by an initial phase of contraction elicited by IP_3-induced intracellular Ca^{2+} release from sarcoplasmic reticulum followed by a second tonic phase that results from Ca^{2+} influx through ROCCs (Ehrlich and Watras, 1988). In the present study, T4MN inhibited electromechanically mediated, KCl-induced contractions and, in aortic preparations depolarized with high KCl in Ca^{2+}-free medium, it also reduced or even abolished $CaCl_2$-induced contractions that are due to an increase in Ca^{2+} influx through VOCCs. T4MN also interfered with pharmacomechanical coupling. This hypothesis is supported by the ability of T4MN to reduce PHE-

induced contractions in Ca^{2+}-containing medium and to inhibit $CaCl_2$-induced contractions in aortic preparations stimulated with PHE under Ca^{2+}-free conditions in the presence of verapamil to remove the indirect influence of VOCC-mediated Ca^{2+} influx. Thus, T4MN was able to interfere with contractile events elicited by Ca^{2+} entry through VOCCs and ROCCs. It is known that SOCCs are activated by depletion of Ca^{2+} stores within the sarcoplasmic reticulum, allowing a capacitative Ca^{2+} influx into the cytosol and a sustained contraction (Clapham et al., 2001). Our results revealed that when aortic preparations were submitted to simultaneous depletion of Ca^{2+} stores by PHE and inhibition of SERCA by thapsigargin, the sustained contraction induced by the addition of Ca^{2+} was abolished by T4MN, an effect that was prevented by pretreatment with ODQ.

In the present study, T4MN-induced relaxation was blunted by pharmacological blockade of K^+ channels with TEA and 4-AP, but not by glybenclamide. This suggests that opening of K^+ channels in the plasmalemma may contribute to vasodilatation evoked by T4MN. Such a mode of action is consistent with the ability of T4MN to stimulate sGC and with the finding of higher potency of T4MN on pharmacomechanical coupling than on electromechanically mediated KCl-induced contractions. On the other hand, T4MN was unable to change the transient contraction evoked by caffeine. However, it inhibited the phasic contraction induced by PHE in aortic preparations incubated in Ca^{2+}-free solution, an effect reversed by pretreatment with ODQ. These findings suggest that T4MN effectively inhibits Ca^{2+} mobilization from intracellular stores caused by IP_3 while it was inert against contractions induced by Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors (Karaki and Weiss, 1997). It is noteworthy that while T4MN was able to relax aortic preparations pre-contracted with PHE at lower concentrations (IC_{50} =57.64 μM), its IP_3 signaling blocking effect was evident only at higher concentrations (167.43 μM). This may suggest that T4MN inhibits contractions of VSM at a signaling pathway downstream from the receptor activation.

In concert of all the aforementioned results, T4MN appears to induce vasorelaxant effects through sGC stimulation independently from endothelial NO release. Conventional stimulators such YC-1 and BAY 41–2271 increased cGMP levels in the absence of NO by inducing a conformational change of sGC probably due to their binding to an allosteric site in proximity to the heme group (Zhao et al., 1998; Stasch et al., 2001; Koglin et al., 2003). This stimulatory effect depends on the presence of a reduced prosthetic heme (i.e., heme iron in the ferrous state) of sGC since it crucially decreased after heme removal or oxidation by ODQ (Stasch et al., 2001; Evgenov et al., 2006). By contrast, heme-independent sGC activators such as BAY 58–2667 bind preferentially to the NO-insensitive oxidized (i.e., heme iron in the ferric state) or heme-free forms of sGC. Based upon the present findings with L-NAME and ODQ, T4MN may likely be characterized as an sGC stimulator rather than activator, as was previously proposed for 1-nitro-2-phenylethane (Brito et al., 2013). The sGC-cGMP signal transduction pathway plays an important role in regulation of pulmonary vascular tone and resistance in pulmonary arterial hypertension. Considering that Riociguat (BAY 63–2521), a novel oral NO-independent stimulator of sGC, is the only treatment currently available to treat two of the five forms of pulmonary arterial hypertension (Makowski et al., 2015), T4MN and relative derivatives could be promising candidates not only as antihypertensive drugs, but also for treatment of pulmonary arterial hypertension, heart failure, thrombosis, and erectile dysfunction among other diseases (Evgenov et al., 2006; Stasch and Evgenov, 2013).

We hypothesized that introducing an electron donor into the aromatic moiety would stabilize NPe and further increase its vasorelaxant potency and/or its interaction with sGC. The methoxy group was chosen as the electron donor because of its lower IP, which is the energy required to remove an electron from the highest occupied molecular orbital (HOMO). With this consideration, the frontier orbital energy is the important electronic parameter of molecular structure. The HOMO and the lowest unoccupied molecular orbital (LUMO) are related to nucleophilicity and electrophilicity, respectively. In a recent molecular modeling study using density functional theory (Oliveira et al., 2016), Oliveira and coworkers calculated the HOMO-LUMO energy gap and IP for NPe and T4MN. Compared to NPe, these authors found that **(i)** T4MN displayed a lower IP, which is suggestive of a higher capacity for stabilization of nitrogen-free radicals, and **(ii)** the gap energies of frontier orbitals showed that the transition from HOMO to LUMO is easier for T4MN. Thus, based on these parameters, it can be concluded that the electronic structure of these two nitroderivatives must be different. Therefore, it seems that introduction of a methoxy group into the aromatic moiety stabilizes NPe, increasing its interaction with sGC without changing its vasorelaxant potency.

5. Conclusions

In conclusion, our findings indicate that the mechanism underlying the vasorelaxant effects of T4MN is distinctly different from that of its parent drug, NPe. T4MN-induced vasorelaxation appears mediated by a sGC pathway leading to increased intracellular cGMP, which in turn activates PKG. Such an activation mediates the opening of potassium channels and reduction of intracellular Ca^{2+} by partial inhibition of Ca^{2+} influx through either VOCC or intracellular Ca^{2+} stores. It seems that introduction of a methoxy group into the aromatic moiety stabilizes NPe, thereby enhancing its interaction with sGC.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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