

## RESEARCH PAPER

## Vasodilator efficacy of nitric oxide depends on mechanisms of intracellular calcium mobilization in mouse aortic smooth muscle cells

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**Background and purpose:** Reduction of intracellular calcium ( $[Ca^{2+}]_i$ ) in smooth muscle cells (SMCs) is an important mechanism by which nitric oxide (NO) dilates blood vessels. We investigated whether modes of  $Ca^{2+}$  mobilization during SMC contraction influenced NO efficacy.

**Experimental approach:** Isometric contractions by depolarization (high potassium,  $K^+$ ) or  $\alpha$ -adrenoceptor stimulation (phenylephrine), and relaxations by acetylcholine chloride (ACh), diethylamine NONOate (DEANO) and glyceryl trinitrate (GTN) and SMC  $[Ca^{2+}]_i$  (Fura-2) were measured in aortic segments from C57Bl6 mice.

**Key results:** Phenylephrine-constricted segments were more sensitive to endothelium-derived (ACh) or exogenous (DEANO, GTN) NO than segments contracted by high  $K^+$  solutions. The greater sensitivity of phenylephrine-stimulated segments was independent of the amount of pre-contraction, the source of NO or the resting potential of SMCs. It coincided with a significant decrease of  $[Ca^{2+}]_i$ , which was suppressed by sarcoplasmic reticulum (SR)  $Ca^{2+}$  ATPase (SERCA) inhibition, but not by soluble guanylyl cyclase (sGC) inhibition. Relaxation of  $K^+$ -stimulated segments did not parallel a decline of  $[Ca^{2+}]_i$ . However, stimulation (BAY K8644) of L-type  $Ca^{2+}$  influx diminished, while inhibition (nifedipine, 1–100 nM) augmented the relaxing capacity of NO.

**Conclusions and implications:** In mouse aorta, NO induced relaxation via two pathways. One mechanism involved a non-cGMP-dependent stimulation of SERCA, causing  $Ca^{2+}$  re-uptake into the SR and was prominent when intracellular  $Ca^{2+}$  was mobilized. The other involved sGC-stimulated cGMP formation, causing relaxation without changing  $[Ca^{2+}]_i$ , presumably by desensitizing the contractile apparatus. This pathway seems related to L-type  $Ca^{2+}$  influx, and L-type  $Ca^{2+}$  channel blockers increase the vasodilator efficacy of NO.

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**Keywords:** vasodilatation; nitric oxide; intracellular calcium; smooth muscle cell; mouse aorta; pre-contraction; C57Bl6; SERCA; nifedipine

**Abbreviations:**  $[Ca^{2+}]_i$ , cytosolic calcium concentration; CPA, cyclopiazonic acid; DEANO, diethylamine NONOate; eNOS, endothelial nitric oxide synthase; GTN, glyceryltrinitrate;  $IP_3$ , inositol trisphosphate; L-NAME,  $N^G$ -nitro-L-arginine methyl ester; L-NNA,  $N^G$ -nitro-L-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SERCA, sarco-endoplasmic reticulum calcium ATPase; SMC, smooth muscle cell; SR, sarcoplasmic reticulum

## Introduction

Constriction and dilation of blood vessels in response to environmental demands are controlled by changes in the cytosolic  $Ca^{2+}$  levels ( $[Ca^{2+}]_i$ ) in the vascular smooth muscle cell (SMC). In response to mechanical factors or vasoactive agonists, vessels constrict by the increase of SMC  $[Ca^{2+}]_i$  either through  $Ca^{2+}$  influx via  $Ca^{2+}$ -permeable ion channels and/or  $Ca^{2+}$  release from sarcoplasmic reticulum (SR)  $Ca^{2+}$  stores.

Calmodulin interacts with the released  $[Ca^{2+}]_i$  which finally leads to phosphorylation of myosin and subsequent muscle contraction. Dilation, on the other hand, is often initiated in the endothelial cells with the stimulation of  $Ca^{2+}$ -dependent endothelial nitric oxide synthase (eNOS) and release of nitric oxide (NO). The released NO stimulates SMCs to relax either directly and/or indirectly by removal of the elevated  $[Ca^{2+}]_i$ . NO can also cause dilation by decreasing the sensitivity of the contractile apparatus for  $[Ca^{2+}]_i$  (Akata, 2007a,b). Indirect effects of NO involve the activation of soluble guanylyl cyclase (sGC) to produce cGMP, which in turn affects  $Ca^{2+}$  influx and/or efflux; or leads to decreased sensitivity of the contractile apparatus to intracellular  $Ca^{2+}$  via stimulation of myosin phosphatase and the dephosphorylation of myosin

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(Carvajal *et al.*, 2000). Direct effects of NO include inhibition of SMC  $\text{Ca}^{2+}$  release elicited by inositol trisphosphate ( $\text{IP}_3$ ) (Ji *et al.*, 1998), cADP-ribose (Yu *et al.*, 2000) or caffeine (Li *et al.*, 2000); acceleration of  $\text{Ca}^{2+}$  sequestration into intracellular stores via stimulation of the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) (Cohen *et al.*, 1999; Adachi *et al.*, 2004), and activation of  $\text{K}^+$  channels (Bolotina *et al.*, 1994; Quignard *et al.*, 2000).

However, the question remains whether relaxation is dependent on the mechanisms involved in the preceding contraction. The direct and indirect effects of NO on  $[\text{Ca}^{2+}]_i$  in SMCs at least suggest that the efficacy of NO to cause relaxation depends on the ability of the SMC to reduce the elevated  $[\text{Ca}^{2+}]_i$ . This ability differs according to the type of blood vessel (Asano and Nomura, 1999; 2000; Nomura and Asano, 2000), its (patho)physiological condition (atherosclerosis, diabetes, hypertension, etc.) (Lewis *et al.*, 2005; Van Assche *et al.*, 2007) and the type of SMC pre-activation (Plane *et al.*, 1998; Lehen'kyi *et al.*, 2002). As a consequence, the efficacy of NO to cause relaxation is expected to depend upon the specific mechanisms by which  $\text{Ca}^{2+}$  is mobilized during contraction.

In order to investigate whether the mechanisms of  $\text{Ca}^{2+}$  mobilization during the contraction determine the relaxing capacity of NO, contractions of mouse aortic segments were initiated by increasing  $[\text{Ca}^{2+}]_i$  by two widely used and different stimuli. On the one hand, elevated external  $\text{K}^+$  depolarises the SMC and activates  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels. Both processes, i.e. membrane depolarization and activation of L-type  $\text{Ca}^{2+}$  channels, have been described to be involved in blood pressure regulation (Moosmang *et al.*, 2003) and hypertension (Sonkusare *et al.*, 2006). On the other hand,  $\alpha_1$ -adrenoceptor stimulation with phenylephrine causes intracellular  $\text{Ca}^{2+}$  release from SR  $\text{Ca}^{2+}$  stores triggered by  $\text{IP}_3$  receptors (Karaki *et al.*, 1997). Yet, even those contractions are associated with depolarization and opening of L-type  $\text{Ca}^{2+}$  channels (Plane *et al.*, 1998; Quignard *et al.*, 2000; Richards *et al.*, 2001; Akata, 2007a). Following high- $\text{K}^+$  or -phenylephrine contractions, the relaxing capacity of NO was investigated by studying relaxation by endogenous NO evoked by administration of acetylcholine chloride (ACh), or by sources of exogenous NO, that is diethylamine NONOate (DEANO) or glyceryl trinitrate (GTN), and by modulating  $\text{Ca}^{2+}$  influx during depolarization or  $\text{Ca}^{2+}$  release from internal stores during agonist activation.

## Methods

### Aortic segments

The studies were approved by the Ethical Committee of the University of Antwerp, and the investigations conform to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). C57Bl6 mice ( $n = 68$ , food and water *ad libitum*, 12/12 light-dark cycle) were used at the age of 4–5 months. After anaesthesia (sodium pentobarbital, 75 mg·kg<sup>-1</sup>, i.p.), the thoracic aorta was carefully removed, stripped of adherent tissue and dissected systematically. Starting 3 mm from the origin of the left subclavian artery (where the azygos

vein crosses the aorta) down to the diaphragm, the descending thoracic aorta was cut in segments of 2 mm width. Vessels were immersed in Krebs Ringer solution (KR solution, 37°C, 95%  $\text{O}_2$ /5%  $\text{CO}_2$ , pH 7.4) with (in mM): NaCl, 118; KCl, 4.7;  $\text{CaCl}_2$ , 2.5,  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4$ , 1.2;  $\text{NaHCO}_3$ , 25, CaEDTA 0.025; and glucose 11.1. High- $\text{K}^+$  solution was prepared as KR solution, but 44.1 mM NaCl was replaced with KCl. In all experiments, indomethacin (10  $\mu\text{M}$ ) was present to avoid any vasomotor interference caused by prostanoids (Okon *et al.*, 2002).

### Isometric tension measurements

Aortic segments were mounted between two parallel tungsten wire hooks in 10 mL organ baths. Tension was measured isometrically with a Statham UC2 force transducer (Gould, Cleveland, OH, USA) connected to a data acquisition system (Powerlab 8/30, ADInstruments, Spechbach, Germany), and contractile force was reported in mN. Segments were gradually stretched until a stable loading tension of 16 mN was attained (optimal pre-load to attain maximal force development by phenylephrine or high  $\text{K}^+$ ). Endogenous NO release was induced by the cumulative administration of ACh ( $10^{-9}$  to  $10^{-5}$  M). Relaxation experiments with exogenous NO (from DEANO or GTN, both  $10^{-9}$  to  $10^{-5}$  M) or with the membrane-permeable analogue of cGMP (8-Br-cGMP,  $10^{-9}$  to  $10^{-5}$  M) were performed in segments in which NO formation was inhibited with a combination of 300  $\mu\text{M}$   $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester (L-NAME) and 300  $\mu\text{M}$   $\text{N}^{\text{L}}$ -nitro-L-arginine (L-NNA).

### Combined assay of isometric tension and SMC $\text{Ca}^{2+}$

In order to avoid the interference of endothelial  $\text{Ca}^{2+}$  signals with the SMC cytosolic  $\text{Ca}^{2+}$  assay, the endothelium was mechanically removed by rubbing the interior of the segment with braided silk thread. The lack of a raised intracellular  $\text{Ca}^{2+}$  and concomitant relaxation of pre-activated segments, when stimulated with 1  $\mu\text{M}$  ACh, was used as a test for successful removal of endothelial cells. Endothelium-denuded segments were mounted in a wire (40  $\mu\text{m}$ ) myograph (Danish Myotechnology A/S, Aarhus, Denmark) above an inverted microscope (Axiovert 200, Carl Zeiss, Zaventem, Belgium) immersed in Krebs–Ringer solution (37°C) and continuously aerated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (pH 7.4). The segment was loaded *in situ* with 10  $\mu\text{M}$  Fura-2 AM in Krebs–Ringer solution with 0.02% pluronic for 120 min at room temperature. Then, the temperature was increased to 37°C, and the segment was set to its normalized diameter (Mulvany and Halpern, 1976). In short, segments were stretched gradually until the calculated internal diameter reached a value equal to the internal circumference the vessel would have *in vivo*, when fully relaxed and under a transmural pressure of 100 mm Hg. Before starting an experiment, the horizontal and vertical apertures of the microscope photometer were set on the surface of the strip (covering about 10% of the surface area, 40 $\times$  objective). Excitation wavelengths (340 and 380 nm for Fura-2) were delivered at a frequency of 1 Hz with a DeltaRAM Multiwavelength Illuminator (Photon Technology International (PTI), Birmingham, NJ, USA). Emission (dichroic mirror, emission filter PO# OROM9712127, WO#A018983, Omega Optical, Inc.,

Brattleboro, VT, USA) was measured with a microscope photometer (D-104, PTI). The single emission (510 nm) ratio at dual excitation (340 and 380 nm) was used as a relative measure of free  $[Ca^{2+}]_i$  (relative units) and was analysed with Felix software (PTI). At the end of the experiment, 2 mM  $MnCl_2$  was added to the segment to determine background emission values, which were subtracted from the respective emission values during the experiment. Absolute levels of  $[Ca^{2+}]_i$  were not reported due to the uncertainty of the conventional calibration method in intact tissues (Cohen *et al.*, 1999). Contractile force was measured simultaneously and reported in  $mN \cdot mm^{-1}$ .

#### Data analysis

All results are expressed as mean  $\pm$  SEM;  $n$  represents the number of mice, and  $s$  the number of segments. Dose–response curves were fitted with sigmoidal dose–response equations with variable slope, which revealed maximal relaxation responses ( $E_{max}$ ) and the negative logarithm of the concentration resulting in 50% of the maximal contraction or relaxation ( $pEC_{50}$ ) for each vessel segment. Two-way analysis of variance with Bonferroni's *post hoc* test and unpaired Student's *t*-test (GraphPad Prism, version 5, GraphPad Software, San Diego, CA, USA) were used to compare means of the different experimental groups. When different segments of the same aorta underwent the same protocol, the average of the different segments was used for statistical comparison between different animals. A 5% level of significance was selected.

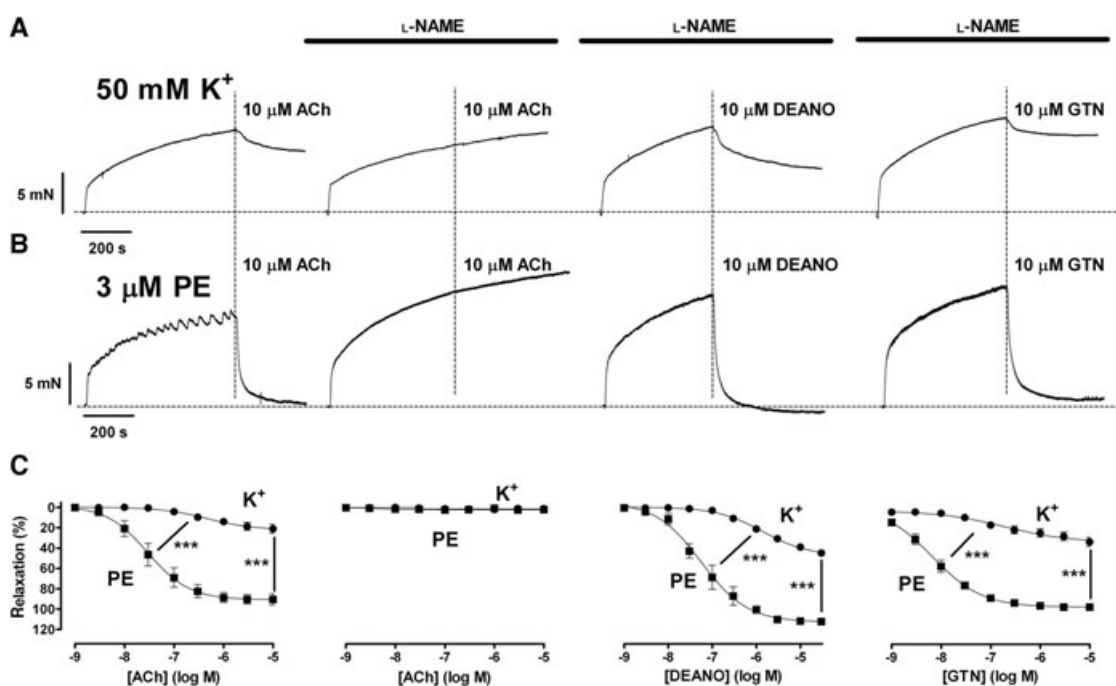
#### Materials

Sodium pentobarbital (Nembutal) was obtained from Sanofi (Brussels, Belgium); indomethacin from Federa (Brussels, Belgium); phenylephrine hydrochloride, ACh, L-NNA, L-NAME, DEANO, cyclopiazonic acid (CPA), nifedipine from Sigma (Bornem, Belgium); Fura 2-AM from Molecular Probes (Invitrogen, Merelbeke, Belgium); GTN from Merck VWR (Leuven, Belgium); verapamil hydrochloride, ( $\pm$ ) BAY K8644, 8-Br-cGMP sodium salt from TOCRIS (Bristol, UK); and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from ASCENT (Weston-Super-Mare, UK).

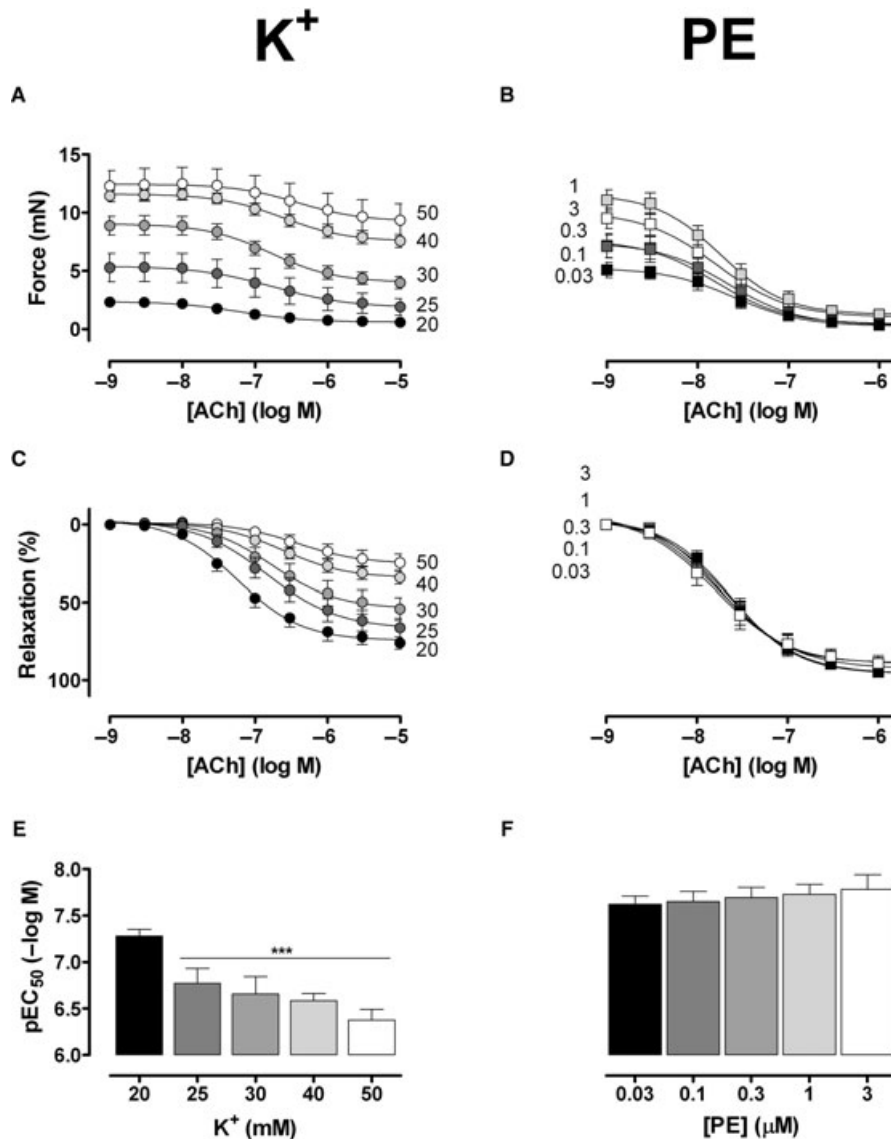
#### Results

##### The relaxing capacity of NO in segments contracted by phenylephrine or high $K^+$

In order to study the relaxing capacity of endogenous NO, the aortic segments were maximally contracted with 50 mM  $K^+$  or 3  $\mu$ M phenylephrine followed by the addition of 10  $\mu$ M ACh (Figure 1). Relaxations to ACh were complete in segments contracted by 3  $\mu$ M phenylephrine, but were significantly attenuated in those contracted with 50 mM  $K^+$ . Moreover, the sensitivity of aortic segments to ACh was significantly lower when the contraction was elicited by high  $K^+$  (Figure 1C). When contractions were reduced by lowering the concentration of  $K^+$  or phenylephrine, the relaxing capacity of NO was still significantly lower in segments contracted with  $K^+$  compared with those contracted with phenylephrine, although both elicited identical force (Figure 2). Similar results were



**Figure 1** Representative examples of contractions elicited by 50 mM  $K^+$  (A) and 3  $\mu$ M phenylephrine (PE) (B) followed by relaxations with 10  $\mu$ M acetylcholine chloride (ACh) in the absence of L-NAME/L-NNA, and with 10  $\mu$ M ACh, 10  $\mu$ M diethylamine NONOate (DEANO) or 10  $\mu$ M glyceryl trinitrate (GTN) in the presence of 300  $\mu$ M L-NAME/L-NNA. (C) Concentration–response relationships for ACh ( $n = 5$ ) in the absence, and ACh ( $n = 3$ ), DEANO ( $n = 7$ ) and GTN ( $n = 7$ ) in the presence of L-NAME/L-NNA of contractions by 50 mM  $K^+$  and 3  $\mu$ M phenylephrine. Results show mean  $\pm$  SEM and were obtained in endothelium-intact segments. \*\*\* $P < 0.001$   $K^+$  versus phenylephrine (one-way analysis of variance).



**Figure 2** Absolute (A,B) and relative (C,D) force of segments stimulated with 20, 25, 30, 40 and 50 mM  $K^+$  (A,C), or 0.03, 0.1, 0.3, 0.3, 3  $\mu$ M phenylephrine (PE) (B,D) followed by addition of acetylcholine chloride (ACh) ( $1 \times 10^{-9}$  to  $1 \times 10^{-5}$  M). (E,F)  $pEC_{50}$  of ACh for contractions induced by increasing  $[K^+]$  (E) or [phenylephrine] (F). Results show mean  $\pm$  SEM,  $n = 6$  endothelium-intact segments; \*\*\* $P < 0.001$  phenylephrine versus  $K^+$ .

**Table 1** Tension induced by 0.3 or 3  $\mu$ M phenylephrine (PE), and by 25 or 50 mM  $K^+$ , and respective  $E_{max}$  and  $pEC_{50}$  of relaxations by DEANO

Contraction constrictor		Tension (mN)	Relaxation $E_{max}$ (%)	$pEC_{50}$ (-logM)
PE ( $\mu$ M)	0.3	9.8 $\pm$ 0.5	103 $\pm$ 6	7.31 $\pm$ 0.15
$K^+$ (mM)	25	10.0 $\pm$ 1.0	91 $\pm$ 6	6.41 $\pm$ 0.10 <sup>b</sup>
PE ( $\mu$ M)	3.0	12.7 $\pm$ 1.4 <sup>a</sup>	104 $\pm$ 2	7.14 $\pm$ 0.17
$K^+$ (mM)	50	17.4 $\pm$ 1.3 <sup>a,b</sup>	54 $\pm$ 3 <sup>a,b</sup>	6.01 $\pm$ 0.08 <sup>a,b</sup>

Results show mean  $\pm$  SEM,  $n = 5-7$  endothelium-intact segments.

<sup>a</sup> $P < 0.001$  high versus low concentration of constrictor.

<sup>b</sup> $P < 0.001$   $K^+$  versus phenylephrine.

DEANO, diethylamine NONOate.

obtained with exogenous NO (10  $\mu$ M DEANO or 10  $\mu$ M GTN) in the presence of NOS inhibitors (Figure 1, Table 1); in that condition, application of 10  $\mu$ M ACh did not evoke relaxation (Figure 1).

Results indicated that the different relaxing capacity of NO for high  $K^+$ - or phenylephrine-induced contractions was not dependent on the source of NO, but was dependent on the agonist causing contraction. Hence, all following experiments

were performed in segments in which endogenous NO release was inhibited with L-NAME/L-NNA, using the NO donor DEANO.

The vasodilator efficacy of NO was dependent on the amount of pre-contraction for  $K^+$ , but not for phenylephrine.

#### Relaxing capacity of NO at depolarized membrane potentials

In order to test whether relaxations induced by NO were affected by the membrane potential of SMCs, segments were contracted by  $3 \mu\text{M}$  phenylephrine in the presence of  $50 \text{ mM}$   $K^+$ , but the depolarization-dependent  $Ca^{2+}$  influx and concomitant contractions were prevented by inhibition of L-type  $Ca^{2+}$  channels with  $1 \mu\text{M}$  nifedipine. As shown in Figure 3B, nifedipine completely prevented force development by  $50 \text{ mM}$   $K^+$ , and inhibited the slow phase of force development by phenylephrine by 70%, from  $12.5 \pm 0.8 \text{ mN}$  to  $3.80 \pm 1.1 \text{ mN}$  ( $n = 4$ ). However, maximal relaxation and  $pEC_{50}$  of DEANO were not affected by  $50 \text{ mM}$   $K^+$  plus nifedipine (Figure 3C). These results indicated that the resting membrane potential of the SMC did not determine the relaxing capacity of NO.

#### Relaxing capacity of NO with activation of L-type $Ca^{2+}$ influx

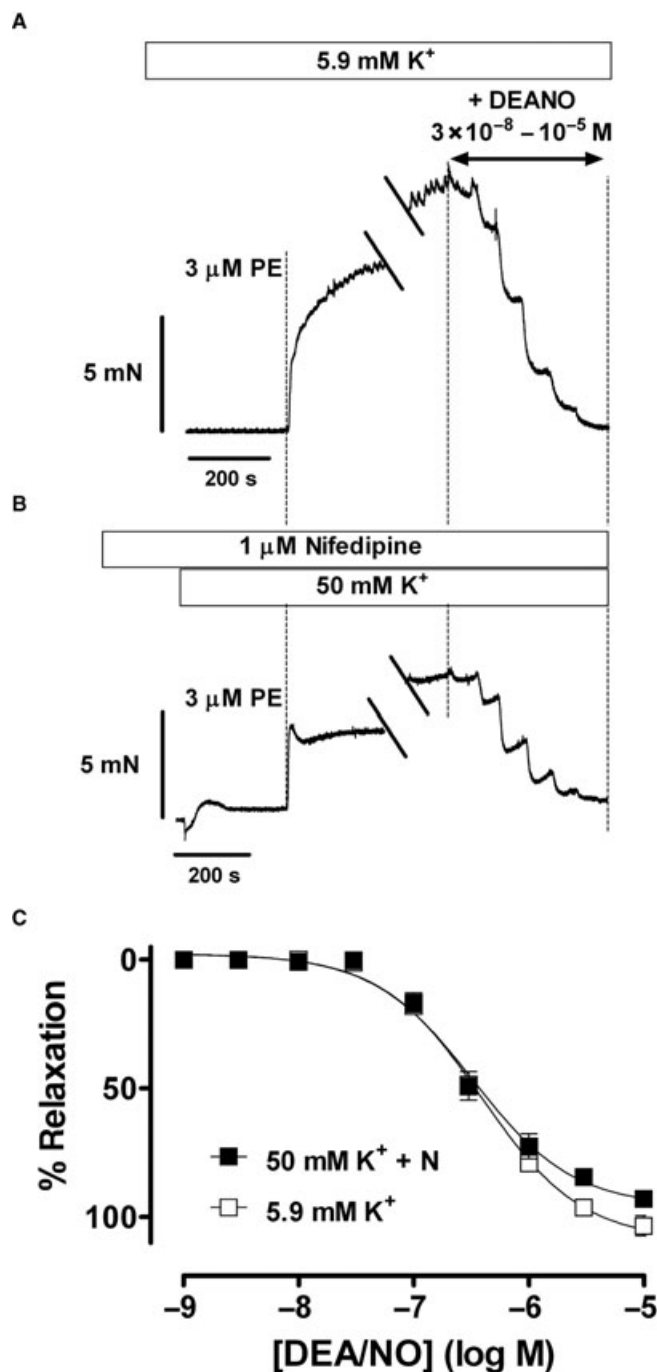
To increase  $Ca^{2+}$  influx during contraction by  $3 \mu\text{M}$  phenylephrine or moderate depolarization with  $20 \text{ mM}$   $K^+$ , segments were incubated with  $30 \text{ nM}$  BAY K8644, an activator of L-type  $Ca^{2+}$  channels. BAY K8644 slightly raised basal tension and significantly increased the contraction by  $20 \text{ mM}$   $K^+$ , without affecting the phenylephrine contraction (Figure 4A,B). Incubation with BAY K8644 shifted the dose-response curves of DEANO significantly to the right in both situations (Figure 4C,D), and this shift could be reversed by nifedipine (data not shown).

#### Relaxing capacity of NO with inhibition of L-type $Ca^{2+}$ influx

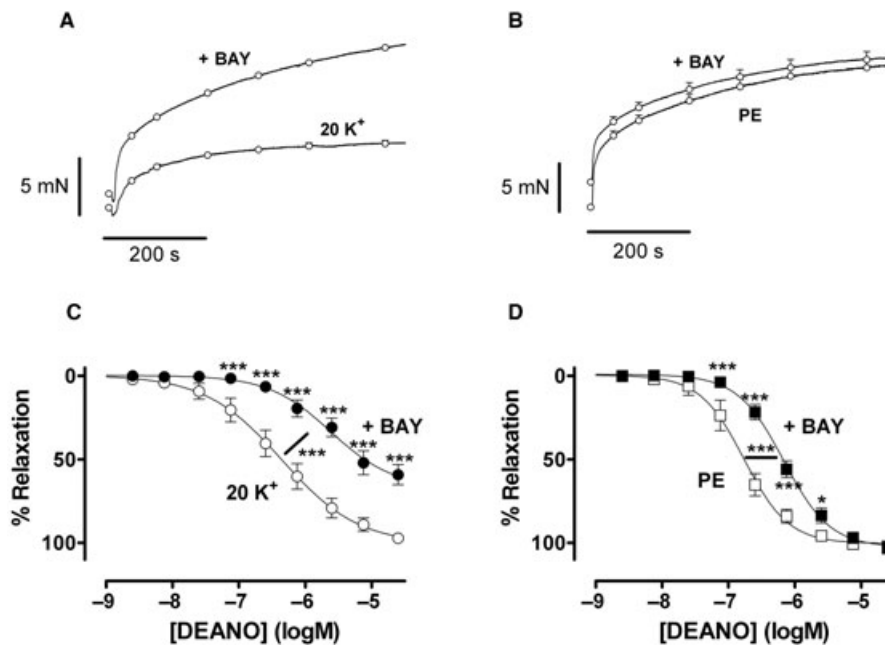
In the next series of experiments, relaxation of contractions by high  $K^+$  and phenylephrine was measured following inhibition of  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels with nifedipine (Figure 5). Nifedipine ( $1\text{--}30 \text{ nM}$ ) caused dose-dependent inhibition of the contraction by high  $K^+$ , but augmented the relaxing capacity of DEANO (Figure 5A,C,E). Similar results were obtained with another L-type  $Ca^{2+}$  channel inhibitor verapamil ( $10\text{--}300 \text{ nM}$ ,  $n = 3$ ). Although nifedipine (Figure 5B) and verapamil (data not shown) also attenuated contractions elicited with  $3 \mu\text{M}$  phenylephrine, they did not affect relaxation induced by DEANO (Figure 5B,D,F). Similar results were obtained with another NO donor GTN ( $n = 3$ ) (data not shown).

#### NO and $Ca^{2+}$ uptake by SERCA

When SERCA was inhibited with  $10 \mu\text{M}$  CPA, force development by  $50 \text{ mM}$   $K^+$  decreased from  $12.9 \pm 0.6 \text{ mN}$  to  $9.5 \pm 0.3 \text{ mN}$  ( $-25\%$ ,  $n = 7$ ), whereas force developed by phenylephrine was not affected (from  $10.1 \pm 1.6 \text{ mN}$  to  $11.4 \pm 1.2 \text{ mN}$ ;  $+9\%$ ,  $n = 6$ ). Relaxation of the high  $K^+$  contraction by DEANO was not affected by CPA ( $pEC_{50}$   $6.30 \pm 0.07$  and  $6.23$



**Figure 3** (A) Representative examples of contractions elicited by  $3 \mu\text{M}$  phenylephrine (PE) at normal resting potential ( $5.9 \text{ mM}$   $K^+$ ), and (B) following depolarization of the membrane potential with  $50 \text{ mM}$   $K^+$ . Contraction by  $50 \text{ mM}$   $K^+$  was inhibited by pre-incubation with  $1 \mu\text{M}$  nifedipine (N). When contractions reached a stable level (breaks in tracings), this was followed by the addition of increasing concentrations of diethylamine NONOate (DEANO). (C) Concentration-response relationships for DEANO of contractions by  $3 \mu\text{M}$  phenylephrine at normal resting potential ( $5.9 \text{ mM}$   $K^+$ ) and depolarized potentials ( $50 \text{ mM}$   $K^+ + \text{N}$ ). Relaxation by DEANO amounted to  $107 \pm 4\%$  with  $pEC_{50}$  of  $6.45 \pm 0.02$  at normal resting potential, whereas at depolarized potentials the maximal relaxation was  $98 \pm 2\%$  with  $pEC_{50}$  of  $6.51 \pm 0.10$  ( $n = 4$ , endothelium-intact segments).



**Figure 4** Mean levels of contraction evoked by 20 mM  $K^+$  (A) ( $n = 8$ ) and 3  $\mu$ M phenylephrine (PE) (B) ( $n = 7$ ) in the absence and presence of 30 nM BAY K8644 (+Bay). Relaxation by diethylamine NONOate (DEANO) in segments constricted by 20 mM  $K^+$  (C) and 3  $\mu$ M phenylephrine (D) in the absence and presence of 30 nM BAY K8644. All segments were endothelium intact. \* $P < 0.05$ , \*\*\* $P < 0.001$  BAY K8644 versus control, repeated measurements analysis of variance.

$\pm 0.14$ , respectively,  $P > 0.05$ ), but was significantly shifted to the right ( $pEC_{50}$  from  $7.01 \pm 0.06$  to  $6.54 \pm 0.17$ ,  $P < 0.05$ ,  $n = 6$ ) for the phenylephrine contraction without significant change in the amplitude of relaxation ( $E_{max}$  of  $110 \pm 4$  and  $104 \pm 3\%$ , respectively). These observations suggest that SERCA-mediated re-uptake of  $Ca^{2+}$  into the SR is involved in the NO-mediated relaxation of contractions elicited by phenylephrine, but probably not involved when contractions are induced by high  $K^+$ .

#### NO-induced decrease of intracellular $Ca^{2+}$

Stimulated  $Ca^{2+}$  uptake to the SR by NO was further studied in experiments in which  $[Ca^{2+}]_i$  was measured in segments contracted with 50 mM  $K^+$  or 3  $\mu$ M phenylephrine, followed by relaxation with 10  $\mu$ M DEANO in the absence or presence of CPA to inhibit SERCA-mediated  $Ca^{2+}$  uptake. For contractions induced by phenylephrine, relaxation by DEANO was accompanied by a significant (about 70%) decrease of  $Ca^{2+}$  mobilized during contraction. NO-induced relaxation was not accompanied by a  $Ca^{2+}$  decrease when segments were contracted by 50 mM  $K^+$  or when segments were contracted by 3  $\mu$ M phenylephrine in the presence of CPA (Figure 6).

#### cGMP-mediated relaxations of contractions by phenylephrine and high $K^+$

Relaxation induced by the membrane-permeable analogue of cGMP (8-Br-cGMP) was compared with responses to DEANO. Maximal relaxations by DEANO and 8-Br-cGMP of contractions induced by 50 mM  $K^+$  or 3  $\mu$ M phenylephrine were not significantly different (Table 2). Segments contracted with phenylephrine were significantly more sensitive to DEANO

**Table 2**  $E_{max}$  (%) and  $pEC_{50}$  for relaxations by DEANO and 8-Br-cGMP of contractions induced by 50 mM  $K^+$  and 3  $\mu$ M phenylephrine (PE)

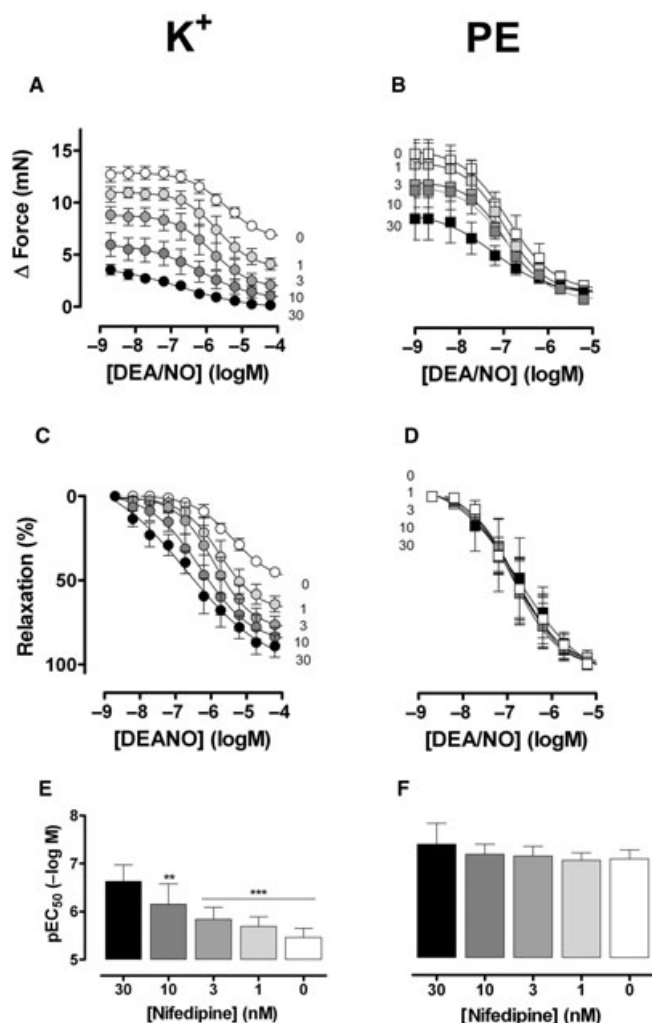
		DEANO	8-Br-cGMP
$E_{max}$ (%)	$K^+$	$54 \pm 3$	$47 \pm 13$
	PE	$105 \pm 2^{***}$	$102 \pm 6^{***}$
$pEC_{50}$ (-logM)	$K^+$	$6.04 \pm 0.08$	$4.36 \pm 0.29$
	PE	$6.68 \pm 0.16^{***}$	$4.65 \pm 0.08$

Results show mean  $\pm$  SEM,  $n = 5$  endothelium-intact segments. \*\*\* $P < 0.001$  phenylephrine versus  $K^+$ . DEANO, diethylamine NONOate.

than segments contracted with  $K^+$  ( $\Delta pEC_{50} = 1.7 - \log M$ ), but the difference in sensitivity was far less pronounced for 8-Br-cGMP ( $\Delta pEC_{50} = 0.29 - \log M$ ). These results indicate that the difference in the relaxing capacity of NO for  $K^+$  or phenylephrine contractions is mainly situated at the level of direct effects of NO and not at the level of NO-mediated increase of cGMP or the subsequent steps.

Incubation of aortic segments with 10  $\mu$ M ODQ, an inhibitor of sGC, completely abolished relaxation to DEANO in  $K^+$ -constricted segments, and shifted relaxation curves for contractions by phenylephrine to the right (Figure 7). However, for phenylephrine, more than 70% ( $76 \pm 4\%$ ) of the maximum DEANO response could still be attained in a cGMP-independent manner.

As shown in Figure 8, sGC inhibition by ODQ did not affect the decrease of cytosolic  $Ca^{2+}$  or the concomitant relaxation upon addition of DEANO, suggesting that  $Ca^{2+}$  uptake into the SR was directly stimulated by exogenous NO and not dependent on sGC stimulation by NO. Finally, in the presence of ODQ (10  $\mu$ M) and CPA (10  $\mu$ M), relaxation of the



**Figure 5** Absolute (A,B) and relative (C,D) force of segments stimulated with 50 mM  $\text{K}^+$  (A,C) or 3  $\mu\text{M}$  phenylephrine (PE) (B,D) followed by addition of diethylamine NONOate (DEANO) in the absence (C: open symbols) and presence of increasing concentrations of nifedipine (1, 3, 10, 30 and 100 nM). (E)  $\text{pEC}_{50}$  of DEANO for contractions induced by 50 mM  $\text{K}^+$  or 3  $\mu\text{M}$  phenylephrine in the absence (C) and presence of increasing nifedipine concentrations (1, 3, 10, 30 and 100 nM). Results show mean  $\pm$  SEM,  $n = 4$  endothelium-intact segments. \*\* $p < 0.05$  and \*\*\* $p < 0.001$  phenylephrine versus  $\text{K}^+$ .

phenylephrine-induced contraction by NO was also completely absent (data not shown).

## Discussion

### *The agonist causing contraction determines the vasodilator efficacy of NO*

In the present study, vasodilatation evoked by endogenous (ACh) or exogenous (DEANO, GTN) NO was severely attenuated when mouse aortic segments were pre-activated with high  $\text{K}^+$  rather than with phenylephrine. A further difference was that the vasodilator capacity of NO was equal for different concentrations (and thus different levels of contraction) of phenylephrine, whereas for  $\text{K}^+$  it was strongly dependent on

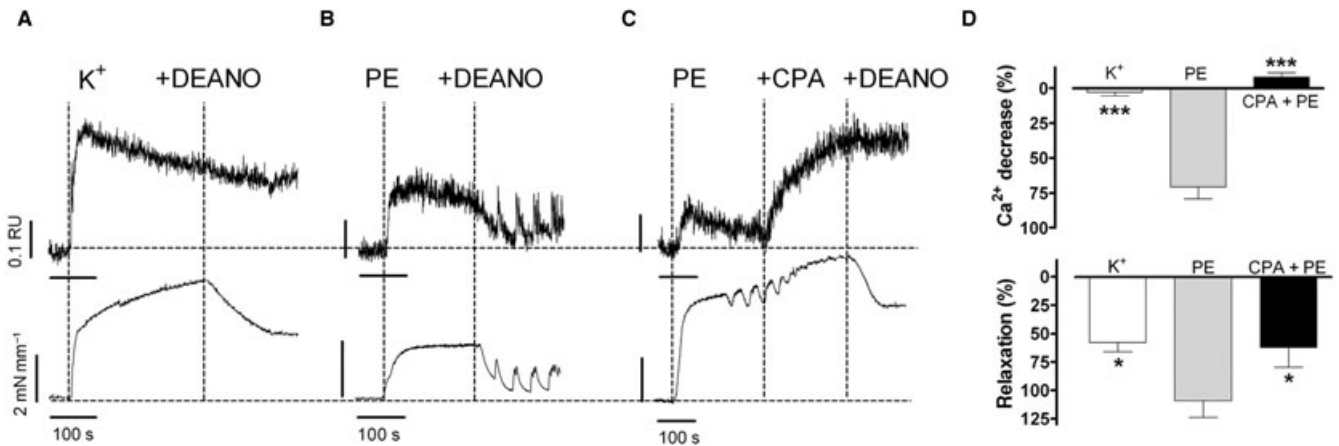
the concentration used to evoke contraction. Moreover, when  $\text{K}^+$  and phenylephrine (Figures 2 and 4; Table 1) evoked identical contraction levels, the vasodilator capacity of NO remained different, suggesting that the mechanisms of  $\text{Ca}^{2+}$  mobilization during contraction determine the vasodilator efficacy of NO.

Because elevation of external  $\text{K}^+$  causes depolarization of the membrane potential of endothelial cells and SMCs, one could argue that the attenuated relaxation of endogenous NO at high  $\text{K}^+$  in comparison with phenylephrine is caused by diminished NO release by the depolarized endothelium and/or by lower NO efficiency in depolarized SMCs. However, the difference was also seen using sources of exogenous NO in the presence of NOS inhibitors. Furthermore, SMC depolarization as the factor underlying the low efficacy of NO was also ruled out. This is because the capacity of NO to reverse phenylephrine-induced contractions was identical in SMCs at normal resting membrane potentials and in depolarized SMCs, at least when, in the latter condition,  $\text{Ca}^{2+}$  influx via L-type  $\text{Ca}^{2+}$  channels was prevented with nifedipine (see also Scarborough and Carrier, 1984). That the relaxing capacity of NO for phenylephrine contractions was not attenuated at elevated external  $\text{K}^+$  further indicates that direct activation of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels by NO, as observed in rabbit aorta (Bolotina *et al.*, 1994; Quignard *et al.*, 2000), is not the factor underlying the greater efficacy of NO in phenylephrine-constricted segments.

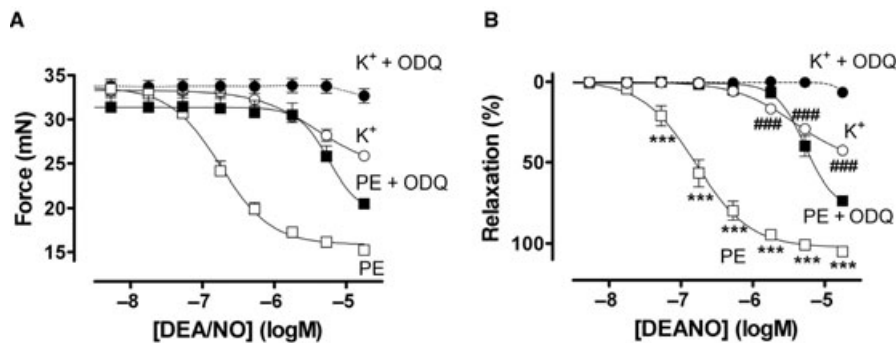
The different ACh-induced relaxation profile of  $\text{K}^+$ - or phenylephrine-constricted segments cannot be due to altered release of NO in both conditions, because exogenous NO also showed a low efficacy in  $\text{K}^+$ -constricted segments. Furthermore, it is important to note that relaxation by ACh in the mouse aorta was absent with NOS inhibition, thus confirming that ACh-induced relaxation is exclusively mediated by NO, without participation of endothelium-derived hyperpolarizing factor (EDHF) or prostacyclin as previously observed by Crauwels *et al.* (2000; 2003). Therefore, the low efficacy of ACh in relaxing  $\text{K}^+$ -constricted rings cannot be due to inhibition of an EDHF-induced relaxation as observed in human small arteries (Buus *et al.*, 2006). Also, altered prostanoid release (Okon *et al.*, 2002) at depolarized potentials (Thapaliya *et al.*, 2000) could be excluded as the underlying factor, because all experiments were performed in the presence of 10  $\mu\text{M}$  indomethacin. As a consequence, the greater efficacy of NO to dilate phenylephrine- compared with high  $\text{K}^+$ -constricted aortic segments can only be attributed to different mechanisms of contraction.

### *Mechanisms of contractions by high $\text{K}^+$ or phenylephrine determine the NO-mediated relaxation*

NO-evoked relaxation is mediated by cGMP-dependent and by cGMP-independent pathways (Bolotina *et al.*, 1994; Cohen *et al.*, 1999; Nimmegeers *et al.*, 2007). The cGMP-dependent mechanisms involve reduction in  $[\text{Ca}^{2+}]_i$  and/or a decrease in the sensitivity of the contractile system to  $\text{Ca}^{2+}$  (Carvajal *et al.*, 2000). In the present study, relaxation of constrictions initiated by L-type  $\text{Ca}^{2+}$  channel activation at high  $\text{K}^+$  had similar amplitudes when induced by NO or by the membrane-permeable analogue of cGMP. These effects were abolished by



**Figure 6** Representative examples of Ca<sup>2+</sup> signals (upper tracings) and force signals (bottom tracings) for contractions by (A) 50 mM K<sup>+</sup>, (B) 3 μM phenylephrine (PE) and (C) 3 μM phenylephrine after SERCA inhibition with 10 μM cyclopiazonic acid (CPA) and following addition of 10 μM diethylamine NONOate (DEANO). (D) Summary of A–C showing % decrease of Ca<sup>2+</sup> and % relaxation following addition of 10 μM DEANO (mean ± SEM, *n* = 4 endothelium-denuded segments). \**P* < 0.05; \*\*\**P* < 0.001 versus phenylephrine.



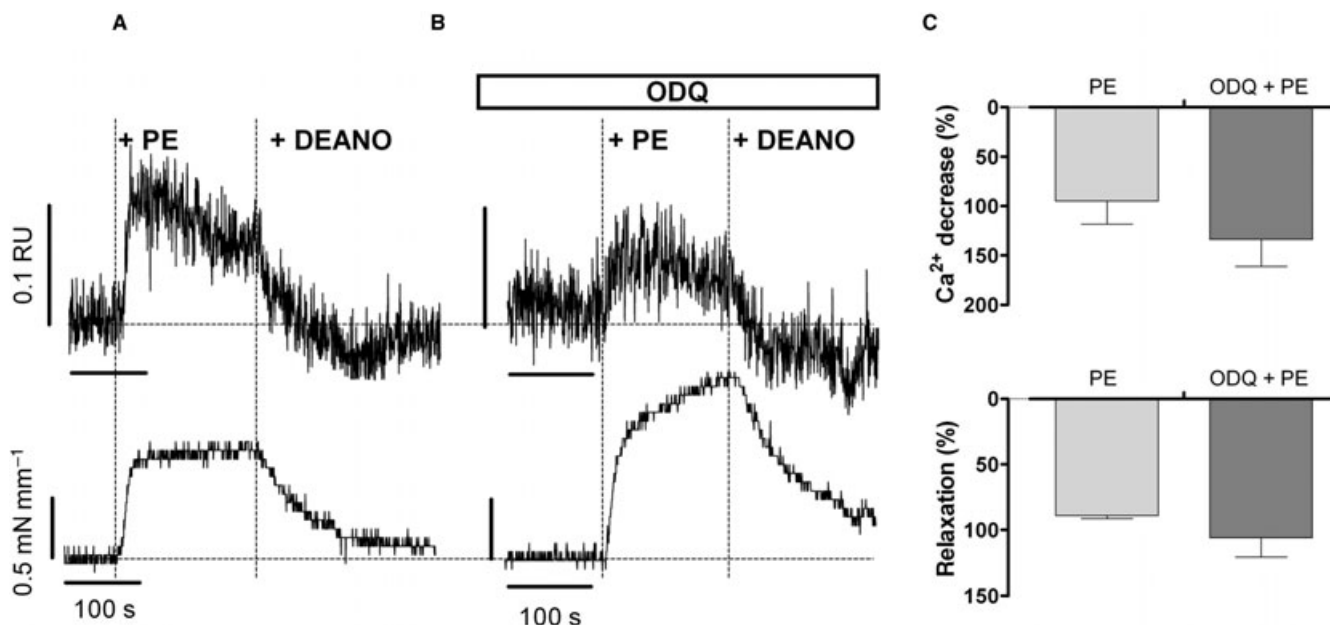
**Figure 7** Relaxation expressed as absolute force (A) or % relaxation (B) by diethylamine NONOate (DEANO) of contractions elicited by 50 mM K<sup>+</sup> and 3 μM phenylephrine (PE) in the absence and presence of 10 μM ODQ. Results were obtained in endothelium-intact segments and show mean ± SEM of the average of three segments per mouse (*n* = 4). \*\*\**P* < 0.001 versus phenylephrine + ODQ, ###*P* < 0.001 versus K<sup>+</sup> + ODQ.

the sGC inhibitor ODQ, were not influenced by the SERCA inhibitor CPA and were not associated with a decrease of cytosolic Ca<sup>2+</sup>. These results indicated that NO-induced relaxation of high K<sup>+</sup>-constricted segments occurred mainly through activation of sGC and subsequent cGMP-mediated effects, which were independent of [Ca<sup>2+</sup>]<sub>i</sub> levels. However, when the initial [Ca<sup>2+</sup>]<sub>i</sub> was recruited from inside the SMC, as with phenylephrine, the NO-induced relaxation was paralleled by a time-matched decrease of [Ca<sup>2+</sup>]<sub>i</sub>, which was about 70% of the [Ca<sup>2+</sup>]<sub>i</sub> mobilized during the preceding contraction. The NO-induced [Ca<sup>2+</sup>]<sub>i</sub> decrease was cGMP independent, as it was not affected by ODQ and it was accompanied by 70% or more of the relaxation response observed in the absence of ODQ. Both the [Ca<sup>2+</sup>]<sub>i</sub> decline and the relaxation were CPA sensitive, suggesting an important role for Ca<sup>2+</sup> uptake into the SR by SERCA (Cohen *et al.*, 1999; Adachi *et al.*, 2004). Therefore, these results indicated that NO-induced relaxation of phenylephrine-mediated constrictions occurred via two pathways: about 70% of the maximum response occurred via a cGMP-independent reduction of [Ca<sup>2+</sup>]<sub>i</sub>, and 30% of the response occurred via NO-stimulated cGMP formation, without reduction of [Ca<sup>2+</sup>]<sub>i</sub>, possibly involving

desensitization. Inhibition of both pathways (ODQ plus CPA) abolished NO-induced relaxations completely. The unique ability of NO to directly stimulate [Ca<sup>2+</sup>]<sub>i</sub> decrease when segments were contracted with phenylephrine is suggested to be at the basis of the greater efficacy of NO to dilate phenylephrine- compared with high K<sup>+</sup>-constricted aortic segments.

A decrease of SMC [Ca<sup>2+</sup>]<sub>i</sub> by NO has been observed in different vascular beds of different species (Chen and Rivers, 2001; Ohi *et al.*, 2001; Buus *et al.*, 2006), but has never been associated with the specific intracellular Ca<sup>2+</sup> pools mobilized during contraction. Although high K<sup>+</sup> and the α-adrenoceptor agonists phenylephrine or noradrenaline elicit comparable sustained vasoconstriction, they mobilize intracellular Ca<sup>2+</sup> via different mechanisms (Karaki *et al.*, 1997). Vasoconstriction by high K<sup>+</sup> could be fully attributed to the depolarization-induced opening of L-type Ca<sup>2+</sup> channel, whereas the relationship between intracellular Ca<sup>2+</sup> and contraction seemed to be more complicated for phenylephrine. Phenylephrine induces Ca<sup>2+</sup> release from internal storage sites, increases [Ca<sup>2+</sup>]<sub>i</sub> in the sub-plasmalemmal region (where Ca<sup>2+</sup>-dependent mechanisms such as ion channels, ion pumps and





**Figure 8** Representative example ( $n = 4$ ) of  $\text{Ca}^{2+}$  signals (upper panels) and force (lower panels) induced by  $3 \mu\text{M}$  phenylephrine (PE) in the absence (A) and presence (B) of  $10 \mu\text{M}$  ODQ followed by application of  $10 \mu\text{M}$  diethylamine NONOate (DEANO). In two segments, ODQ raised baseline  $\text{Ca}^{2+}$  and tone, but DEANO evoked a similar decline of phenylephrine-induced  $\text{Ca}^{2+}$  and tension. (C) Summary of % decrease of  $\text{Ca}^{2+}$  and % relaxation following addition of  $10 \mu\text{M}$  DEANO. Results show mean  $\pm$  SEM,  $n = 4$  endothelium-denuded segments.

enzymes are located), opens receptor- or store-operated  $\text{Ca}^{2+}$ -permeable cation channels (or directly L-type  $\text{Ca}^{2+}$  channels) and finally increases  $[\text{Ca}^{2+}]_i$  around the contractile elements (Karaki *et al.*, 1997; Akata, 2007a,b). As L-type  $\text{Ca}^{2+}$  influx is much more important for high  $\text{K}^+$ -induced than for phenylephrine-elicited contractions, depolarization-dependent L-type  $\text{Ca}^{2+}$  influx might be the compromising factor in determining the relaxing capacity of NO in constricted mouse aortic segments.

#### L-type $\text{Ca}^{2+}$ influx compromises the vasodilator efficacy of NO

Voltage-dependent L-type  $\text{Ca}^{2+}$  influx is a major  $\text{Ca}^{2+}$  influx pathway in vascular SMCs (Karaki *et al.*, 1997; Berkels *et al.*, 2003; Godfraind, 2005) and also in mouse thoracic aortic segments, because in this preparation nifedipine ( $1 \mu\text{M}$ ) inhibited contractions by high  $\text{K}^+$  completely and by phenylephrine partly. Application of the L-type  $\text{Ca}^{2+}$  channel blockers nifedipine and verapamil not only directly reduced  $\text{Ca}^{2+}$  influx and the concomitant contraction, but, at least for contractions by high  $\text{K}^+$ , also caused a dose-dependent leftward shift of the NO-induced relaxation curves. Indeed, the vasodilator capacity of NO declined when the influx of  $\text{Ca}^{2+}$  via L-type  $\text{Ca}^{2+}$  channels became more prominent as with elevation of external  $\text{K}^+$  or as with application of an activator of L-type  $\text{Ca}^{2+}$  influx (BAY K8644). Vice versa, the vasodilator capacity of NO increased when the influx of  $\text{Ca}^{2+}$  was reduced by lowering external  $\text{K}^+$  or by inhibition of L-type  $\text{Ca}^{2+}$  channels with nifedipine or verapamil. As a result, the vasodilator capacity of NO was strongly dependent on the concentration of external  $\text{K}^+$  used to evoke depolarization and contraction, which might explain the dependence of the vasodilator capacity of NO on the level of pre-contraction by high  $\text{K}^+$ , but not by phenylephrine.

The present study shows that segments contracted by increase of SMC  $[\text{Ca}^{2+}]_i$  via release of  $\text{Ca}^{2+}$  from intracellular stores (phenylephrine) relax better and at lower NO concentrations than segments contracted via L-type  $\text{Ca}^{2+}$  influx (high  $\text{K}^+$ ). Nevertheless, L-type  $\text{Ca}^{2+}$  channels were also involved in the phenylephrine-induced contraction, but inhibition of these channels with nifedipine or verapamil did not affect the vasodilator capacity of NO. Relaxation was, however, compromised by stimulating L-type  $\text{Ca}^{2+}$  influx in phenylephrine-constricted segments with BAY K8644, again pointing to the compromising effect of L-type  $\text{Ca}^{2+}$  influx on the relaxing capacity of NO.

#### NO-sensitive $\text{Ca}^{2+}$ pools

Because NO and  $\text{Ca}^{2+}$  are both non-homogeneously distributed in the SMC (van Breemen *et al.*, 1995; Rembold and Chen, 1998; Asano and Nomura, 2000; Nomura and Asano, 2000), the interaction between NO and  $\text{Ca}^{2+}$  pools may vary according to the mechanisms by which  $\text{Ca}^{2+}$  was increased during contraction. SMC  $\text{Ca}^{2+}$  pools have been described to display different intracellular localization with important consequences for the relationship between intracellular  $\text{Ca}^{2+}$  increase and interaction with the contractile filaments (Karaki *et al.*, 1997). Other complicating events include the large variation in the capacity of  $\text{Ca}^{2+}$  domains to store  $\text{Ca}^{2+}$  (Asano and Nomura, 2000; Nomura and Asano, 2000); the specific intracellular localization of SERCA,  $\text{IP}_3$  and ryanodine receptors (Golovina and Blaustein, 1997; Janiak *et al.*, 2001; Wamhoff *et al.*, 2002); the different sensitivity of the stores to inhibition by CPA (Rembold and Chen, 1998; Blaustein *et al.*, 2002); and finally, the specific sensitivity of the  $\text{Ca}^{2+}$  pools to NO (Ji *et al.*, 1998; Li *et al.*, 2000; Yu *et al.*, 2000). Therefore, to predict the vasodilator capacity of NO in a specific vascular

bed, it is essential to investigate the mechanisms of  $\text{Ca}^{2+}$  mobilization during the preceding contraction. For example, in rat tail arteries (Lehen'kyi *et al.*, 2002; 2005; Soloviev *et al.*, 2004), NO caused a greater decrease in  $[\text{Ca}^{2+}]_i$  when segments were contracted with high  $\text{K}^+$  compared with phenylephrine, whereas the present study described the reverse situation in mouse aorta. Species and vascular bed differences may account for such differences, but according to the present study, it could be directly related to the different mechanisms by which high  $\text{K}^+$  or phenylephrine mobilize contractile SMC  $[\text{Ca}^{2+}]_i$  in rat tail artery or in mouse aortic SMCs. It has at least been shown that bovine tail artery has about 60% less SR than does rat aorta (Ashida *et al.*, 1988).

## Conclusions

According to the present study in mouse aorta, the different relaxing capacity of NO for SMC contractions initiated by depolarization (high  $\text{K}^+$ ) or by  $\alpha_1$  adrenoceptor stimulation (PE) is not dependent on the source of NO (endogenous or exogenous), but depends mainly on the pathways of  $[\text{Ca}^{2+}]_i$  mobilization during contraction. In mouse aorta, NO induces relaxation via at least two pathways. One pathway involves sGC stimulation by NO with the subsequent elevation of cGMP inducing relaxation without detectable changes of  $[\text{Ca}^{2+}]_i$ . This pathway seems to be related to L-type  $\text{Ca}^{2+}$  influx, and the relaxation is most probably due to desensitization of the contractile apparatus to  $\text{Ca}^{2+}$  (Carvajal *et al.*, 2000). The other pathway involves the decrease of SMC  $[\text{Ca}^{2+}]_i$  via a non-cGMP-dependent stimulation of SERCA. The concomitant re-uptake of  $\text{Ca}^{2+}$  into the SR seems most prominent when the  $\text{Ca}^{2+}$  mobilized during contraction originated from the ER. The latter mechanism seems to determine the vasodilator capacity of NO in the mouse aorta. As a consequence, interventions which reduce or increase L-type  $\text{Ca}^{2+}$  influx during contraction will influence the relaxing capacity of NO. Whether these observations are relevant *in vivo* require further investigation. However, our observations may form an alternative explanation for the improved relaxation of atherosclerotic aortic segments in mice treated with the dihydropyridine  $\text{Ca}^{2+}$  channel blocker nifedipine (Berkels *et al.*, 2003; Kyselovic *et al.*, 2005), that was initially attributed to enhanced bioavailability of endothelium-derived NO by the authors. Finally, it would be interesting to test whether L-type  $\text{Ca}^{2+}$  channel blockers also sensitize resistance arteries to the vasodilator effect of NO.

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## Conflicts of interest

None.

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