Signaling Mechanisms for the Selective Vasoconstrictor Effect of Norbormide on the Rat Small Arteries

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ABSTRACT

Norbormide (NRB) is a selective vasoconstrictor agent of the rat small vessels. The mechanisms underlying the selective vasoconstrictor effect of NRB are unknown. To investigate whether phospholipase C (PLC) signaling pathway plays a role in NRB-induced vasoconstriction, we performed experiments in NRB-contracted tissues, namely, rat caudal arteries (RCA) and smooth muscle cells derived from rat mesenteric arteries (MVSMCs). An NRB-insensitive vessel, namely rat aorta (RA), served as a control tissue. In RCA and RA we measured either isometric tension or formation of inositol phosphates (IPs), the latter taken as an index of PLC activation. In MVSMCs, we measured intracellular free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) in the presence of external Ca\textsuperscript{2+}, NRB (2–50 \mu M) stimulated IPs formation in RCA but not in RA, and increased [Ca\textsuperscript{2+}]\textsubscript{cyt} in MVSMCs. In the absence of external Ca\textsuperscript{2+}, NRB (50 \mu M) increased IPs formation in RCA but was unable to increase [Ca\textsuperscript{2+}]\textsubscript{cyt} in MVSMCs. In RCA, in the presence of external Ca\textsuperscript{2+}, NRB-induced contraction was inhibited by calphostin C (0.2–1 \mu M), an inhibitor of protein kinase C (PKC), and by SK&F 96365 (30 \mu M), an inhibitor of the store-operated calcium channels, but was poorly affected by verapamil, an L-type calcium channel blocker. However, verapamil was much more effective when external Ca\textsuperscript{2+} was substituted by Sr\textsuperscript{2+}. These results suggest that NRB elicits its tissue and species-selective vasoconstrictor effect by stimulating PLC-PKC pathway and increasing Ca\textsuperscript{2+} influx through both verapamil-sensitive and -insensitive calcium channels. Ca\textsuperscript{2+} release from sarcoplasmic reticulum seems not involved in NRB vasoconstriction.

NORB (Fig. 1) has been proposed to be a selective vasoconstrictor agent for the rat small arteries either in vivo (Roszkowski, 1965) or in vitro (Bova et al., 1996). In rat caudal, renal, and mesenteric artery rings, NRB induces an endothelium-independent contraction that is observed also in single myocytes isolated from the caudal artery, indicating the myogenic nature of the effect (Bova et al., 1996). On the contrary, in rat aorta, as well as in small and large arteries of nonrat species, NRB shows a vasorelaxant effect that can be presumably ascribed to a calcium entry blocker activity (Bova et al., 1996). NRB shows calcium entry blocker properties also in guinea pig heart, where it induces negative inotropic and dromotropic effects, reduces coronary resistances, and inhibits L-type calcium current in ventricular myocytes (Bova et al., 1997). Thus, NRB seems to be a calcium entry blocker agent endowed with selective vasoconstrictor activity for the rat small arteries (Bova et al., 1996).

The contractile effect of NRB is abolished in the absence of extracellular calcium; therefore, it is mainly promoted by calcium entry. Since calcium entry through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange system has been excluded and NRB-induced contraction is affected by very high concentrations (above 100 \mu M) of verapamil or diltiazem (Bova et al., 1996), it is possible that nonvoltage-dependent calcium channels are involved. The type of calcium channels activated by NRB as well as the mechanism(s) underlying this activation is not elucidated. Since it has been shown that some vasoconstrictor agents, which stimulate the PLC signaling pathway, activate calcium channels that are insensitive to calcium entry blockers (Karaki et al., 1997), the goal of the present study was to verify whether also NRB could act by stimulating such a signaling pathway.

Materials and Methods

Assay of Inositol Phosphates in Rat Caudal Artery and Aortic Strips

Rat caudal arteries and aortae were dissected, cleaned of adventitia, opened longitudinally to remove the endothelium, and incubated in ASPET Journals on April 29, 2017 jpet.aspetjournals.org Downloaded from
Fig. 1. Structure of norbormide.

bated for 60 min in physiological salt solution (PSS) at 37°C. To evaluate the formation of phosphoinositide metabolites, the strips were labeled with 40 μCi of d-myo-[3H]inositol (specific activity 21 Ci/mmol; NEN, Boston, MA) for 3 h in 3 ml of PSS at 37°C. After this incubation, the arteries were washed for 10 min in fresh PSS containing 5 mM d-myo-inositol and for further 10 min in PSS with 5 mM d-myo-inositol and 10 mM LiCl. Each artery was then transferred in an individual vial and incubated for 10 min at 37°C in PSS with LiCl and the desired concentration of NRB. Thereafter, the arteries were removed from the medium, quickly frozen in liquid nitrogen, and pulverized in a mortar with 0.5 ml HCl 0.1 N. The IPs were extracted by the addition of 2 ml of a mixture containing CHCl₃/CH₃OH/HCl (200:400:5, v/v/v), 0.6 ml of CHCl₃, and 0.6 ml of 0.1 N (Hawkins et al., 1993), and two phases were obtained after a centrifugation at 2500 rpm for 15 min. The upper phase containing the water-soluble [3H]inositol-labeled metabolites was taken for high

The purity of cultures was verified immunocytochemically as previously described (Golovina, 1999). Briefly, mesenteric smooth muscle cells (SMCs) were fixed in ice-cold 95% ethanol and labeled with the nucleic acid stain 4',6'-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) to identify all cells. SMCs were then identified with a monoclonal antibody against SMCs α-actin (Boehringer-Mannheim, Indianapolis, IN); fluorescein isothiocyanate-conjugated goat anti-mouse antibody was used as the secondary stain (Jackson ImmunoResearch Laboratories, West Grove, PA). All 4',6'-diamidino-2-phenylindole-stained cells in the primary cultures cross-reacted with the SMCs α-actin antibody, indicating the purity of the SMCs cultures.

Measurement of [Ca²⁺]ᵮₑₓ. Details of the digital imaging methods used for measuring [Ca²⁺]ᵮₑₓ have been published (Golovina, 1999). Briefly, mesenteric SMCs were grown on coverslips and loaded with 3.3 μM fura-2 AM (TEFLABS, Austin, TX) for 30 min at 22–23°C, under an atmosphere of 5% CO₂, 95% air. The cells were subsequently perfused for 20 to 30 min with a PSS at a rate of 2.0 ml/min (at 35°C) to permit intracellular esterases to cleave intracellular fura-2 AM into active fura-2. Fluorescent images were recorded by a charge-couple device camera (Stanford Photonics, Palo Alto, CA) coupled to an inverted microscope (Carl Zeiss, Thornwood, NY). Imaging acquisition and analysis were performed with a MetaFluor/MetaMorph imaging system (Universal Imaging, West Chester, PA). Video frames containing images of cell fluorescence were digitized at a resolution of 512 horizontal x 480 vertical pixels. To improve the signal-to-noise ratio, eight consecutive video frames were averaged at the video frame rate (30 frames/s). [Ca²⁺]ᵮₑₓ was calculated from the ratio of fura-2 fluorescence excited at 380 and 360 nm (Goldman et al., 1990). In most experiments, four to eight cells in a single field were imaged, and one arbitrarily chosen, peripheral, cytosolic area (10–12 x 10–12 pixels) from each cell was spatially averaged.

Solutions. Standard PSS contained 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.4 mM MgCl₂, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 11.5 mM glucose, and 10 mM HEPES, at pH 7.35, maintained at 37°C. In Ca²⁺-free PSS, CaCl₂ was omitted and 50 μM EGTA was added.

Recording of Mechanical Force in Rat Caudal Artery Rings
Contractions of rat caudal artery rings were recorded as previously described (Bova et al., 1996). Briefly, male Sprague-Dawley rats (250–300 g) were killed by decapitation and the ventral caudal artery was removed. The vessels were cleaned of connective tissue and cut into rings of 2-mm length. The endothelium was removed by gently rubbing the lumen of the rings with a very thin rough-surfaced tungsten wire. The rings were vertically suspended between 100 μm o.d. tungsten wires in organ baths filled with 15 ml of PSS. Tension was recorded on a pen recorder (Ugo Basile, Varese, Italy) via an isometric force displacement transducer (Ugo Basile). Rings were stretched passively to impose a resting tension of 1.5 g that was found to be optimal by testing the response to phenylephrine at different imposed tensions. The rings were allowed to equilibrate for 60 min, and then the responsiveness of each ring was tested by applying a maximally effective concentration of either phenylephrine (10 μM) or KCl (80 mM). To verify the absence of the endothelium, the rings were contracted with 1 mM phenylephrine and then exposed to 2 μM carbamylcholine. The absence of the endothelium was revealed by the lack of carbamylcholine-induced relaxation.

Solutions. Standard PSS (PSS-Ca²⁺) contained 125 mM NaCl, 5 mM KCl, 2.7 mM CaCl₂, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose, at pH 7.35, maintained at 37°C and bubbled with 95% O₂, 5% CO₂. In some experiments, CaCl₂ has been replaced with equimolar concentrations of SrCl₂ (PSS-Sr²⁺)
NRB was a kind gift of I.N.D.I.A. S.p.A. (Padova, Italy). Phenylephrine hydrochloride, carbamylcholine chloride, and verapamil were from Sigma. SK&F 96365 was from Biomol Research Laboratories (Plymouth Meeting, PA). Calphostin C was from Calbiochem-Novabiochem Corp. (La Jolla, CA). NRB was dissolved in dimethylformamide. The maximum concentration of dimethylformamide was 0.1% and had no effect on IPs levels or the contractile responses of rat caudal arteries as well as on [Ca²⁺]cyt of rat mesenteric vascular smooth muscle cells. Calphostin C was dissolved in dimethyl sulfoxide. At the maximum concentrations reached in the medium (0.1%), dimethyl sulfoxide had no effect either on resting or stimulated tone of artery rings. Due to the photoactivatable properties of calphostin C, the experiments with it were under fluorescence lighting. All the other drugs were dissolved in twice distilled water.

Data Analysis and Statistics

IPs were measured as cpm/mg of wet weight. Contractile responses of the arteries were measured as milligrams of developed tension. Data are expressed as the means ± S.E.M. Significance was evaluated by Student’s t test for paired or unpaired observations, as appropriate. A P value smaller than 0.05 was considered significant.

Results

Effect of NRB on IPs Levels in Rat Caudal Artery and Aorta. NRB (2–50 μM) stimulated the formation of tritiated IPs in rat caudal arteries prelabeled with D-myoinositol in a concentration-dependent manner, with an increase in [³H]inositol phosphates from 700 ± 160 cpm/mg of wet weight in untreated vessels to 8000 ± 372 cpm/mg of wet weight in arteries treated with 50 μM NRB (Fig. 2).

The activation of IPs production was still observed when the arteries were exposed to the maximal concentration of Ca²⁺-free PSS, with an increase in [³H]inositol phosphates from 211 ± 25 to 1146 ± 16.5 cpm/mg of wet weight (n = 4, P < 0.05). In rat aorta, NRB (2–50 μM) did not modify IPs levels (Fig. 2).

Effect of NRB on Intracellular Ca²⁺ in Rat Mesenteric Artery Vascular Smooth Muscle Cells. The effect of NRB on [Ca²⁺]cyt has been investigated in vascular smooth muscle cells obtained from rat mesenteric arteries preloaded with the fluorescence probe fura-2. NRB, at a concentration of 50 μM, increased [Ca²⁺]cyt from 152 ± 5 nM (n = 48) to 566 ± 117 nM (n = 23) (P < 0.05). This effect was abolished in Ca²⁺-free solution (n = 15). In Fig. 3, a representative experiment shows the effect of NRB in a single cell exposed to NRB first in the presence and then in the absence of extracellular Ca²⁺. We have excluded that tachyphylaxis could be responsible for the lack of effect of NRB because in the presence of external Ca²⁺ superimposable Ca²⁺ responses were elicited by exposing cells (n = 10) twice to NRB and even a single exposure of cells (n = 33) to NRB in the absence of extracellular Ca²⁺ did not evoke Ca²⁺ transient.

Effect of Calphostin C on NRB-Induced Contraction. The effect of the selective protein kinase C (PKC) inhibitor calphostin C (0.2 and 1 μM) on NRB-induced contraction in rat caudal artery rings is shown in Fig. 4. At the highest concentration, calphostin C almost completely inhibited the response to NRB (0.5–25 μM).

Effect of Ca²⁺ and Sr²⁺ on the Sensitivity of NRB-Induced Contractions to Verapamil. In PSS-Sr²⁺, the responses of rat caudal artery rings to NRB were lower than those obtained in PSS-Ca²⁺ (n = 4) (Fig. 5). The effect of the calcium entry blocker verapamil on cumulative concentration-response curves to NRB in the presence of either external Ca²⁺ or Sr²⁺ is shown in Fig. 6. A very high concentration (500 μM) of verapamil was necessary to obtain 83% inhibition of NRB concentration-response curve in PSS-Ca²⁺ (Fig. 6A). In PSS-Sr²⁺, the same inhibitory effect was obtained by using 1 μM verapamil (Fig. 6B), a concentration that did not affect NRB contraction in PSS-Ca²⁺ (Fig. 6A).

![Fig. 2. Effect of NRB on [³H]inositol phosphate levels in rat caudal artery and aorta. The arteries were exposed to NRB (2–50 μM) for 10 min and assayed for [³H]inositol phosphates as described under Materials and Methods. Results are expressed as cpm/mg/wet weight. Each point is the mean ± S.E. of four experiments.](image)

![Fig. 3. Representative record showing the effect of NRB on [Ca²⁺]cyt in the presence and in the absence of extracellular Ca²⁺ in a single rat mesenteric vascular smooth muscle cell in primary culture. In the presence of extracellular Ca²⁺, 50 μM NRB raised [Ca²⁺]cyt from 153 to 1130 nM. The same concentration of NRB did not affect [Ca²⁺]cyt when the cell was preincubated for 3 min in Ca²⁺-free PSS.](image)
were obtained using diltiazem instead of verapamil (data not shown).

**Effect of SK&F 96365 on NRB-Induced Contraction.**
The effect of the putative store-operated calcium channel inhibitor SK&F 96365 on NRB-induced contraction in rat caudal artery rings is shown in Fig. 7. The contractile responses to NRB (1–25 μM) were almost completely inhibited by 30-min pretreatment with 30 μM SK&F. This inhibitory effect was not modified by 1 μM verapamil (*n* = 4).

**Discussion**
The main conclusion of this study is that the selective vasoconstrictor effect of NRB on the rat small vessels is mediated by stimulation of PLC. This conclusion is supported by the findings that 1) NRB induces increases in IPs levels in rat caudal arteries in a range of concentrations overlapping that inducing vasoconstriction in the same vessel; 2) such an
ular Ca2+ and therefore activates PLC even in the absence of extracellular Ca2+. This has been ascribed to a lack of calcium release from the SR (Sakata and Penner, 1997). IP3 releases Ca2+ from intracellular stores by interacting with specific receptors coupled to the calcium channels in the sarcoplasmic reticulum (SR) (Karaki et al., 1997). A functional consequence of this effect is that vasoconstrictors activating PLC induce a transient contraction of some vessels in the absence of extracellular Ca2+ (Karaki et al., 1997). In rat caudal artery, NRB does not induce contraction in Ca2+-free solution, suggesting that Ca2+ release from SR does not contribute to its vasoconstrictor effect (Bova et al., 1996). A dissociation between PLC activation and lack of contribution of the IP3-SR system to the contractile process has been reported in some vessels for other vasoconstrictor agents activating PLC (Karaki et al., 1997). This has been ascribed either to a lack of calcium release from the SR (Sakata and Karaki, 1992) and/or to the inability by the released Ca2+ to trigger the contractile process (Karaki et al., 1997). Our results show that in rat caudal arteries NRB produces IPs and therefore activates PLC even in the absence of extracellular Ca2+; nevertheless, in isolated primary cultures of vascular smooth muscle cells obtained from rat mesenteric artery, a vessel that is contracted by NRB (Bova et al., 1996), the drug does not induce a calcium transient in the absence of extracellular Ca2+. Therefore, the lack of contraction observed in Ca2+-free solution is unlikely due to a lack of IP3 formation, but rather to a lack of calcium release from the SR.

The results obtained using calphostin C, a selective inhibitor of PKC (Kobayashi et al., 1989), indicate that PKC plays a key role in the selective vasoconstrictor effect of NRB. Calphostin C inhibits PKC-dependent vasoconstriction without affecting contractile responses induced by stimuli that do not require PKC activation (Shimamoto et al., 1993). In vascular smooth muscle, activation of PKC produces contraction through mechanisms that are not fully elucidated (Sharma and Bhalla, 1988). PKC increases phosphorylation of specific proteins during sustained contractions (Rasmussen et al., 1987); activates the extracellular signal-regulated protein kinase mitogen-activated protein kinases (Showasser et al., 1998); and opens calcium channels in smooth muscle cells, thereby increasing calcium influx (Fish et al., 1988; Vivaudou et al., 1988; Oike et al., 1993; Karaki et al., 1997). Because NRB contractile effect is completely dependent on the presence of extracellular Ca2+ (Bova et al., 1996), it can be reasoned that the main role of PKC in NRB contraction is to open calcium channels and promote calcium entrance in vascular smooth muscle cells.

Vascular smooth muscle cells express several types of calcium channels. Among these, L-type calcium channels (L-TCCs), nonselective cation channels (NSCCs), and store-operated channels (SOCs) can be activated during the contractile response evoked by vasoconstrictor agents (Karaki et al., 1997). These channels show different functional characteristics: L-TCCs are very sensitive to calcium entry blockers, whereas NSCCs and SOCCs are not (Karaki et al., 1997; Broad et al., 1999); the selectivity for Ca2+ is very high for SOCCs, whereas L-TCCs and NSCCs can be permeated by other cations, namely, Sr2+ (Uvelius et al., 1974; Ebeigbe and Aloamaka, 1985; Parekh and Penner, 1997; Broad et al., 1999); SOCCs can be selectively inhibited by agents like SK&F 96365 (Zhang et al., 1999). On the basis of this knowledge, we used Sr2+ and SK&F 96365 to evaluate the role of L-TCCs, NSCCs, and SOCCs in NRB-induced contraction. In vascular smooth muscle, Sr2+ can substitute Ca2+ in the excitation-contraction process; therefore, vasoconstrictor agents can evoke contractions when Ca2+ is substituted by Sr2+ in the extracellular medium (Ebeigbe and Aloamaka, 1985). In PSS-Sr2+ NRB induces a contractile effect that is very sensitive to calcium entry blockers, indicating that, among the Sr2+-permeable calcium channels, L-TCCs, but not NSCCs, which are insensitive to calcium entry blockers, are activated by the drug. Our results show that in PSS-Ca2+ NRB vasoconstriction is almost completely abolished by the SOCC inhibitor SK&F 96365, indicating these channels as the main calcium influx pathway activated by the drug. A key role of SOCCs has been recently shown also for endothelin-1-mediated vasoconstriction; in rat aorta SK&F 96365 completely relaxed endothelin-1-induced contractions that were not affected by nifedipine (Iwamura et al., 1999; Zhang et al., 1999). Activation of SOCCs is a consequence of intracellular calcium stores depletion (Parekh and Penner, 1997), which in the case of vasoconstrictor agents that activate PLC is mediated by IP3. In the case of NRB, it is difficult to call upon this stimulus, since NRB does not release calcium from the SR either in the whole arterial tissue (Bova et al., 1996) or in single cells (Fig. 3). Since NRB contraction is strongly dependent on PKC, a PKC-dependent activation of SOCCs can be reasonably hypothesized. Such a regulatory mechanism has not been reported in vascular smooth muscle, but it has recently been demonstrated in skeletal muscle (Vazquez et al., 1998).

The PLC/PKC pathway is a signaling mechanism shared by most vasoconstrictor agonists acting by binding to a receptor (Karaki et al., 1997), and several isoforms of either PLC or PKC have been identified in vascular smooth muscle.
Norbormide Selective Vasoconstriction and Phospholipase C


References


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NORBORMIDE

PLC

DAG

Ne Ca2+ release

PKC

Opening of Calcium Channels (Ca2+ = Sr2+)

Verapamil-sensitive

Opening of Calcium Channels (Ca2+ >> Sr2+)

SK&F 96355-sensitive

Fig. 8. Proposed mechanism of action underlying the selective vasoconstrictor effect of NRB on the rat small vessels. See text for description.