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Effects of neutral sphingomyelinase on phenylephrine-induced vasoconstriction and Ca^{2+} mobilization in rat aortic smooth muscle

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Abstract

The sphingomyelin pathway is now recognized as an important signal transduction system regulating various cellular functions, in which activation of a neutral sphingomyelinase induced by various extracellular stimulants results in selective degradation of sphingomyelin, yielding bioactive lipid intermediates, ceramides and phosphorylcholine. In the present study, our emphasis has been to examine the effects of exogenous Mg^{2+}-dependent neutral sphingomyelinase, in physiological and pathophysiological magnesium concentrations, on phenylephrine-induced vasomotor tone and on intracellular free Ca^{2+} ($[Ca^{2+}]_i$) mobilization in vitro. Neutral sphingomyelinase (0.001–0.1 U/ml), alone, did not elicit any significant changes in either basal tension or resting levels of $[Ca^{2+}]_i$ in rat aortic smooth muscle; similar results were obtained with phosphorylcholine. However, neutral sphingomyelinase (0.001–0.1 U/ml) and C_2-ceramide or ceramide-1-phosphate, but not phosphorylcholine, attenuated phenylephrine-induced contractions, in isolated rat aortic rings, in a concentration-related manner. The addition of extracellular magnesium in different concentrations (0, 0.3, 1.2, 2.4 mM) modulated the neutral sphingomyelinase-vasorelaxant action in a concentration-dependent manner. Neutral sphingomyelinase-evoked relaxation was only partially endothelium-dependent. Nitric oxide synthase inhibitors, N^G-nitro-L-arginine (L-NNA) and L-N^G-monomethyl-arginine (L-NMMA), an inhibitor of prostanoid synthesis (indomethacin), and pharmacologic amine antagonists, such as atropine, diphenhydramine, cimetidine, propranolol, and methysergide as well as an opiate antagonist, naloxone, all failed to attenuate or interfere with the vasorelaxant responses of neutral sphingomyelinase. Three different inhibitors of protein kinase C (i.e., staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) or bisindolylmaleimide I), when used over a wide concentration range, also failed to interfere with the neutral sphingomyelinase-induced relaxations. Neutral sphingomyelinase inhibited the elevations in $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells caused by phenylephrine. Our results suggest that a Mg^{2+}-dependent sphingomyelin signaling pathway may play an important regulatory role in arterial wall tone.

Keywords: Sphingomyelinase; Smooth muscle, Aortic, rat; Ca^{2+} concentration; Intracellular, free

1. Introduction

Recently, sphingolipids have emerged as new and important putative signal transduction molecules involved in a variety of cellular processes. This is best illustrated in the case of the sphingomyelin pathway. In this pathway, a number of extracellular stimuli, including 1,25-dihydroxyvitamin D_3, tumor necrosis factor-α (TNF-α), endotoxin, interferon, interleukin I, Fax ligands, chemotherapeutic agents, heat and nerve growth factor (Hannun, 1996), cause the activation of sphingomyelinase which hydrolyzes plasma membrane sphingomyelin to produce ceramide and phosphorylcholine (Hannun, 1996; Hannun and Bell, 1993; Mathias and Kolesnick, 1993; Kolesnick, 1994). In turn, ceramide appears to serve as a lipid second messenger to play important roles in a variety of fundamental biological processes, such as cell proliferation, cell differentiation, cell cycle arrest, apoptosis, receptor functions, oncogenesis, immune functions and inflammatory responses (Ballou, 1992; Hannun, 1994, 1996; Hannun and Bell, 1993; Mathias and Kolesnick, 1993; Kolesnick, 1994; Dbaibo, 1997). Ceramide, derived from sphingomyelin, can be

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phosphorylated by ceramide kinase to ceramide-1-phosphate (Dressler and Kolesnick, 1990; Mathias and Kolesnick, 1993), and less than 10% of the ceramide generated is converted to free sphingoid base (Mathias and Kolesnick, 1993). Ceramide, released as a consequence of sphingomyelinase, is now thought to play roles in fundamental processes such as cell proliferation, membrane-receptor functions, oncogenesis, and immune inflammatory responses (Hannun, 1994, 1996; Kolesnick, 1994).

Sphingomyelinases are a group of phospholipases. To date, five types of sphingomyelinases have been identified, and they include the lysosomal Mg^{2+}-independent acidic pH optimal sphingomyelinases, the acidic zinc-dependent neutral sphingomyelinases, the Mg^{2+}-dependent membrane associated neutral pH optimal sphingomyelinases, the neutral Mg^{2+}-independent, and the alkaline sphingomyelinase. These sphingomyelinases differ in tissue distribution, cofactor dependence, mechanism of regulation, and involvement in diverse cellular processes (Liu and Hannun, 1997; Liu et al., 1997). Two of these sphingomyelinases are thought to regulate intracellular levels of ceramide and subsequent ceramide-mediated responses (Liu et al., 1997). Neutral Mg^{2+}-dependent sphingomyelinase is a plasma membrane-bound enzyme that requires magnesium ions (Mg^{2+}) and a neutral pH environment (pH 7.4) (Chatterzee, 1993; Liu et al., 1997). Neutral sphingomyelinase is specific for sphingomyelin and does not hydrolyze other phospholipids (Tamiya-Koizumi et al., 1989; Chatterzee, 1993; Liu et al., 1997).

More recently, studies from our laboratory have shown that the membrane-permeable C_2-ceramide can attenuate phenylephrine-induced contractions in isolated rat aortic rings and inhibit elevation in $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells evoked by phenylephrine (Zheng et al., 1999); similar results were obtained with sphingosine (unpublished findings). Collectively, these results suggest that the sphingomyelin pathway may play an important regulatory role in arterial wall tone. Bacterial neutral sphingomyelinase has been shown to activate the sphingomyelin pathway directly (Kim et al., 1991). Exogenously applied bacterial neutral sphingomyelinase (from *Bacillus cereus*) functions at neutral pH (Chatterzee, 1993) and has been considered a useful tool in tissue culture experiments to induce changes in cellular ceramide levels in an attempt to mimic the biological effects of activation of cellular sphingomyelinase (Raines et al., 1993; Linardic and Hannun, 1994). The present study was designed, therefore, to investigate the effects of its key initial enzyme, neutral sphingomyelinase, on contractile regulation of isolated rat thoracic aortic rings and Ca^{2+} mobilization in cultured rat aortic smooth muscle cells. Our studies, herein, suggest that ceramide and ceramide-1-phosphate are generated in response to neutral sphingomyelinase in rat aortic smooth muscle, and these sphingolipids are probably the putative second messengers responsible for neutral sphingomyelinase-induced relaxation.

2. Materials and methods

2.1. Animals, vessels, cell preparations and solutions

The experiments were performed on thoracic aortas obtained from adult Wistar male rats (weighing 200–250 g). The animals were killed by stunning and subsequent decapitation. Aortas were isolated according to previously established methods (Altura and Altura, 1974). The vessels for the ring segments were carefully excised and cleaned, and the tissues cut into approximately 3 to 4 mm lengths. For intact tissue preparations, extreme care was taken to avoid damage of endothelial cells. For denuded arteries, the intima of the vessels was gently rubbed with a wire to rub off endothelial cells (Zhang et al., 1992a), and the tissues were placed in normal Krebs-Ringer bicarbonate solution (NKRB) at room temperature. The composition of the NKRB was (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10, and $NaHCO_3$ 25. When Mg^{2+}-free solution (Mg^{2+}-free NKRB) was utilized, $MgSO_4$ was replaced by an isosmolar amount of NaCl.

The procedure employed to isolate and culture single aortic vascular smooth muscle cells and the use of digital imaging microscopy with the fluorescent indicator, fura-2, has been reported (Zhang et al., 1992b,c). Briefly, aortic vascular smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) mixed 1:1 with Ham's (v/v) nutrient mixture F-12, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.1% lipid mixture and supplemented with 20% fetal bovine serum at 37°C in a humidified atmosphere composed of 95% air–5% CO_2. Morphological examination of confluent cultures revealed vascular smooth muscle cells exhibiting a crisscross pattern, hills-and-valleys, and nodular structures when examined by phase-contrast microscopy (Zhang et al., 1992b,c). Immunohistochemical staining with a monoclonal antibody, recognizing exclusively α-smooth muscle actin, indicated that over 97% of the cultures were pure vascular smooth muscle cells (Zhang et al., 1992b). Cells were maintained in the culture medium through passage 3 and harvested with 1% trypsin-EDTA and then seeded onto 12 mm circular coverslips at an almost confluent density.

2.2. Assessment of vascular reactivity, and denudation of endothelium

The ring segments of rat thoracic aortas were arranged, isometrically, under resting tensions of 1.5–3.0 g, respectively. All tissues were equilibrated, initially, for 2 h in chambers containing 20 ml of NKRB at 37°C, gassed continuously with a 95% O_2–5% CO_2 mixture, and connected to force displacement transducers using Grass Model 7 polygraphs (Grass Model FT 03, Grass instrument, Quincy, MA) before data collection. The loading tensions were adjusted periodically and maintained throughout the

equilibration period. The incubation media were routinely changed every 10–15 min as a precaution against interfering metabolites (Altura and Altura, 1970), and only then were changes in isometric tensions of the vascular smooth muscle preparations recorded. The stable level of tension developed in response to the addition of 60 mM KCl was always measured prior to collection of the data; this was utilized as a reference contractile maximum response, and to validate vascular smooth muscle cell function. To examine the functional viability of an intact endothelium, a concentration–response curve to phenylephrine (10^{-8}–10^{-5} M) was determined for each ring in order to determine the concentration (EC_{50}) producing a half-maximal contractile response to phenylephrine. After the effects of the concentration–response curves were washed out, vascular rings were precontracted with EC_{50} doses of phenylephrine (10^{-6} M), and the presence and absence of endothelium was confirmed by testing for relaxation to acetylcholine (10^{-9}–10^{-7} M), which generally resulted in 90% relaxation in aorta with intact endothelium.

2.3. Experimental procedures for aortic rings

Before phenylephrine EC_{50} concentrations were added, the ring aortic segments (under resting tension) were exposed to NKRB containing various concentrations of sphingomyelinase (0.001–0.1 U/ml), phosphorylcholine (10^{-8}–10^{-4} M), C_2-ceramide (10^{-8}–10^{-4} M) and C_8-ceramide-1-phosphate (10^{-7}–10^{-4} M) for at least 15-min periods for each dose, to determine whether or not the enzyme or sphingolipids had any effects on base-line tension and/or development of spontaneous mechanical activity. After wash out, the rings were re-equilibrated for 15 min in the NKRB solution. In other experiments, EC_{50} concentrations of phenylephrine were added, and when the contraction reached a plateau phase, the neutral sphingomyelinase (0.001–0.1 U/ml) or sphingolipids were added cumulatively to the bath. The results of these experiments are expressed in percentage (%) relaxation of the stable contraction induced by phenylephrine (10^{-6} M) (reference contraction). In some experiments, the extracellular magnesium ions in the NKRB was altered (i.e., to 0, 0.3, 2.4 mM) to determine the role and need of magnesium in the vascular actions of neutral sphingomyelinase. For further clarification of the mechanism of sphingomyelinase-action in a possible role of the endothelium, the rings were exposed to various specific pharmacologic antagonists for 20 min prior to stimulation with neutral sphingomyelinase. To determine whether or not the induced relaxations were affected by endogenous release of prostanoids and nitric oxide from the blood vessels, indomethacin (5×10^{-5} M) and various inhibitors of nitric oxide formation were added, e.g., N^G-nitro-L-arginine (L-NNA) (5×10^{-5} M), and L-N^G-monomethyl-arginine (L-NMMA) (5×10^{-5} M). These specific pharmacologic antagonists were added to the baths for at least 20–30-min periods prior to neutral sphingomyelinase. Other pharmacologic amine antagonists, such as atropine sulfate (10^{-6}–10^{-5} M), diphenhydramine HCl (10^{-5} M), cimetidine HCl (10^{-5} M), propranolol HCl (10^{-5} M), and methysergide maleate (10^{-6}–10^{-5} M) as well as naloxone HCl (10^{-5} M) were also used (added 20 min prior to sphingomyelinase) to determine whether the neutral sphingomyelinase responses were due to release of different types of vasoactive amines (i.e., acetylcholine, histamine, adrenergic amines), and/or opiates. To determine whether or not protein kinase C activation plays any role in neutral sphingomyelinase-induced relaxation, three different inhibitors of protein kinase C, i.e., staurosporine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) or bisindolylmaleimide I, were tested over a wide concentration range; these drugs were also added 15–20 min prior to phenylephrine and before neutral sphingomyelinase addition.

2.4. $[Ca^{2+}]_i$ studies in single cultured vascular smooth muscle cells

Intracellular free Ca ($[Ca^{2+}]_i$) in single vascular smooth muscle cells was measured according to previously established methods (Zhang et al., 1992b,c). Cultured cells were preloaded with 2 μM fura-2/acetoxymethyl (AM) esters in the culture medium without fetal bovine serum for 30 min in a humidified air incubator at 37°C. To improve the loading efficiency, 0.12% pluronic F-127 was used in the loading medium. Following dye-loading, the coverslips, containing fura-2-loaded cells, were washed three times with HEPES buffer solution at room temperature and placed in a tissue chamber on a temperature-controlled stage of a Nikon fluorescence microscope, and subsequently superfused with HEPES buffer solution (pH 7.4, 37°C). The HEPES buffer solution contained (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH. Measurement of $[Ca^{2+}]_i$ was performed using a PTI model 4000 Deltascan, LPS-220 Image Analyzer, Video Scope (Photon Technology International, South Brunswick, NJ). The cultured cells, preloaded with fura-2/AM, were excited, alternately, at 340 and 380 nm. The emission intensity was recorded at 510 nm using a silicon intensified target (SIT) camera.

Fluorescence ratios (R) were obtained by dividing the 340-nm images by the 380-nm images. Then the $[Ca^{2+}]_i$ of single vascular smooth muscle cells was calculated by using the equation:

$$[Ca^{2+}]_i = K_d B[(R - R_{min})/(R_{max} - R)].$$

A K_d of 224 nM was used for the fura-2/Ca^{2+} complex. Calibrating K_d requires making measurements for the completely ion-free and ion-saturated indicator and for the indicator in the presence of known Ca^{2+} concentrations (to determine K_d); this K_d was based on our

previous experiments with these cells (Zhang et al., 1992b, 1997). *B* is the ratio of fluorescence intensity of fura-2 to Ca-bound fura-2, with excitation at 380 nm. Calibration parameters were determined using small volumes of buffered calibrating solutions (pH 7.2, 37°C) containing 3 μM fura-2 pentapotassium salt and various $[Ca^{2+}]$ (Zhang et al., 1992b,c, 1997). The intensity of the recorded images at 340 and 380 nm was corrected by subtracting them from background fluorescence recorded at the corresponding wavelengths. The resulting images were then used to calculate $[Ca^{2+}]_i$ in vascular smooth muscle cells. Calibration showed that our 340:380 ratio fell on the linear portion of the calibration curve. Particular care was taken to minimize photobleaching of the dye. Experiments were carried out in total darkness, and exposure to excitation light was less than 2 s in all experiments.

2.5. Chemicals and reagents

Neutral sphingomyelinase (EC 3.1.4.12), from *B. cereus*, staurosporine, H-7, bisindolylmaleimide I, C_2-ceramide, C_8-ceramide-1-phosphate, phosphorylcholine HCl, and naloxone HCl were all obtained from Sigma (St. Louis, MO). Indomethacin was purchased from Merck (Rahway, NJ); acetylcholine, L-NNA, L-NMMA, propranolol HCl and diphenhydramine HCl were obtained from CALBIOCHEM (La Jolla, CA). Phenylephrine HCl and atropine sulfate were obtained from MANN Res. Lab. (New York, NY). Fura-2 acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR). Cimetidine HCl was obtained from Smith Kline Beecham (Philadelphia, PA). Methysergide maleate was purchased from SANDOZ Pharmaceuticals, (Hanover, NJ). All other chemicals and reagents were obtained from Fisher Scientific (NJ); these were commercial products of the highest grade available.

2.6. Statistical analyses

Where appropriate, results are expressed as means ± S.E.M. Differences bewteen means were analyzed using non-paired *t*-tests or analysis of variance followed by a Newman–Keule test. Statistical significance was assumed when $P < 0.05$.

3. Results

3.1. Neutral sphingomyelinase relaxes phenylephrine-induced contractions in rat thoracic aortic rings

Incubation of isolated, resting rat thoracic rings with neutral sphingomyelinase (0.001–0.1 U/ml), alone, did not elicit any significant changes in basal tension (data not shown, $n = 8$). However, treatment of phenylephrine-contracted rat aortic rings with and without endothelium, with neutral sphingomyelinase (0.001–0.1 U/ml), resulted in concentration-dependent relaxation, respectively, during stable contraction evoked by 10^{-6} M phenylephrine (Fig. 1). The relaxation-responses in endothelium-intact rings were of a somewhat greater magnitude ($P < 0.01$) compared to endothelium-denuded rings, indicating that the responses were only partially dependent on intact endothelial cell function (Fig. 2). That the response to neutral

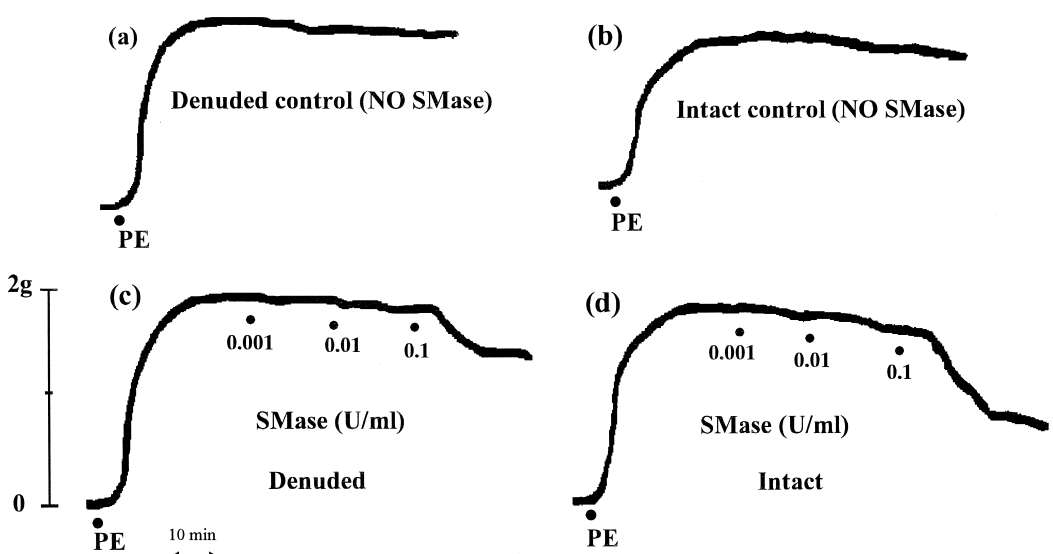

Fig. 1. Typical recording of neutral sphingomyelinase (0.001–0.1 U/ml)-induced relaxations of phenylephrine-contracted (10^{-6} M) aortic rings, in intact and denuded tissue controls. Vertical bar = tension; horizontal bar = time — min. Abbreviations: PE, phenylephrine; N-SMase, neutral sphingomyelinase. (a) Without endothelium control (no neutral sphingomyelinase added), (b) with endothelium control (no neutral sphingomyelinase added), (c) effects of neutral sphingomyelinase (0.001–0.1 U/ml) on denuded rat aortic rings, (d) effects of neutral sphingomyelinase (0.001–0.1 U/ml) on intact rat aortic rings. $n = 6–8$ each.

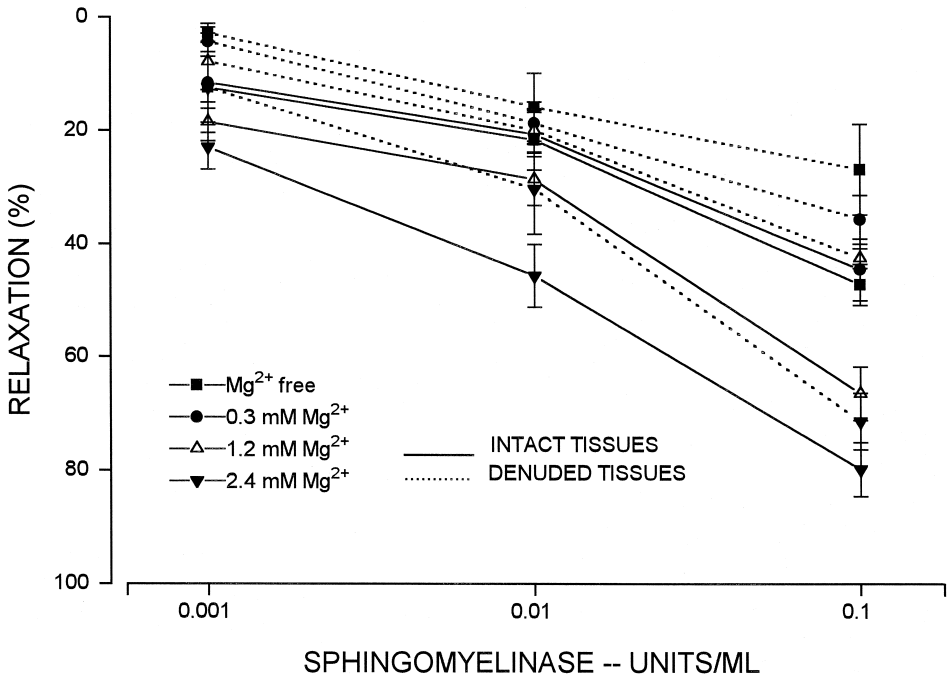

Fig. 2. Effects of neutral sphingomyelinase on phenylephrine (10^{-6} M)-induced contraction in intact and denuded rat aortic rings exposed to different extracellular magnesium concentrations. Except for 0 mM extracellular magnesium, 0.3, 1.2 and 2.4 mM extracellular magnesium were added to [Mg^{2+}]-free NKRB yielding total extracellular magnesium of 0.3, 1.2 and 2.4 mM, respectively. Relaxation data are expressed as percent decrease from precontraction state. Values are shown as means ± S.E., $n = 8$ each.

sphingomyelinase was only slightly attenuated, but not abolished, after de-endothelialization implies that neutral sphingomyelinase exerts most of its relaxant actions directly on the vascular smooth muscle cells.

In order to further examine whether enzymatic action of sphingomyelinase is essential for the action of sphingomyelinase on vasomotor tone, we also examined effects of heat-inactivated sphingomyelinase on rat aortic rings. Heat-inactivated neutral sphingomyelinase (56°C for 30 min) had no effect on vasomotor tone or tension in either quiescent or phenylephrine-precontracted rat aortic arteries ($n = 3$, data not shown), indicating that intact enzymatic action of neutral sphingomyelinase is required for its vasorelaxant actions.

3.2. Effects of extracellular magnesium-concentration on neutral sphingomyelinase relaxant action

The bacterial sphingomyelinase (*B. cereus*) is known to be activated by magnesium (Jungner et al., 1997). As shown in Fig. 2, the addition of extracellular magnesium ([Mg^{2+}]$_o$) in different concentrations (i.e., 0, 0.3, 1.2, 2.4 mM) affected the neutral sphingomyelinase (0.001–0.1 U/ml) vasorelaxant action in a concentration-dependent manner. In the presence of 1.2 and 2.4 mM [Mg^{2+}]$_o$, the vasorelaxant effects of neutral sphingomyelinase were significantly enhanced in comparison to low extracellular magnesium, with neutral sphingomyelinase present ($P < 0.05$). This suggests that extracellular magnesium, at low concentrations, is required to achieve active association of the enzyme with its substrate; 1.2 mM [Mg^{2+}]$_o$ is enough, however, to achieve a reaction. But, 2.4 mM [Mg^{2+}]$_o$ is needed to fully activate the enzyme in rat aortic smooth muscle (Fig. 2). A comparison of intact and denuded aortic rings, in different extracellular magnesium, indicate that the intact tissues clearly yield significantly ($P < 0.05$) greater relaxations at both 1.2 and 2.4 mM extracellular magnesium (Fig. 2). Hence, extracellular magnesium at the concentrations of 1.2 and 2.4 mM can produce near-maximal and maximal neutral sphingomyelinase vasorelaxant action, respectively, in phenylephrine-induced contractions. In contrast to the diverse neutral sphingomyelinase relaxation effects, seen with different concentrations of extracellular magnesium on phenylephrine precontraction, in intact aortae, control experiments using the same concentrations of extracellular magnesium, performed without neutral sphingomyelinase ($n = 4$, data not shown), demonstrate that addition of 1.2 and 2.4 mM extracellular magnesium to phenylephrine-induced contractions produced only about 10–15% relaxation, thus much less than the 30–70% relaxation induced in the presence of neutral sphingomyelinase.

3.3. Effects of nitric oxide inhibitors, indomethacin and pharmacologic antagonists on neutral sphingomyelinase-induced vasorelaxation

As normal endothelial cells can release several vasoactive substances, such as nitric oxide and prostacyclin, and

endogenous amines can also be released from the arterial wall, we determined whether or not neutral sphingomyelinase-induced relaxation was due to release of any these substances. In the presence of two different nitric oxide synthase inhibitors [i.e., L-NNA (5×10^{-5} M), L-NMMA (5×10^{-5} M)], or the cyclooxygenase inhibitor, indomethacin (5×10^{-5} M), the vasorelaxations in response to neutral sphingomyelinase were not significantly attenuated, ($n = 8$–10 each, $P > 0.05$) (Fig. 3). These results suggest that the partial endothelium-dependent relaxations to neutral sphingomyelinase are not dependent on either a L-NNA-sensitive or L-NMMA-sensitive nitric oxide release from endothelium, or cyclooxygenase-sensitive prostacyclin generation. Thus, there may exist another possible vasodilator(s) that is synthesized and released from endothelium in response to neutral sphingomyelinase. Although not shown, a wide variety of amine pharmacologic antagonists (i.e., atropine, diphenhydramine, cimetidine, propranolol, methysergide) and an opiate antagonist (naloxane) all failed to attenuate or interfere with the neutral sphingomyelinase-induced relaxations ($n = 6$–8 each, $P > 0.05$).

3.4. Effects of protein kinase C inhibitors, staurosporine, H-7, and bisindolylmaleimide I on neutral sphingomyelinase-induced vasorelaxation

The purpose of these experiments was to determine whether or not the neutral sphingomyelinase-induced relaxation is dependent on activation of protein kinase C. Various protein kinase C inhibitors were added 15 min before and after treatment of phenylephrine-contracted, denuded aortic rings, using staurosporine (5×10^{-8}–5×10^{-6} M), H-7 (5×10^{-8}–5×10^{-6} M) or bisindolylmaleimide I (5×10^{-7}–5×10^{-6} M), respectively. We found that all of these agents, which collectively act on all active sites of protein kinase C, did not significantly attenuate the neutral sphingomyelinase-induced relaxations ($n = 6$ each, $P > 0.05$), but had a tendency to augment the relaxation (Fig. 3). These results suggest that a protein kinase C pathway cannot explain neutral sphingomyelinase's relaxant action on phenylephrine-induced vasoconstriction.

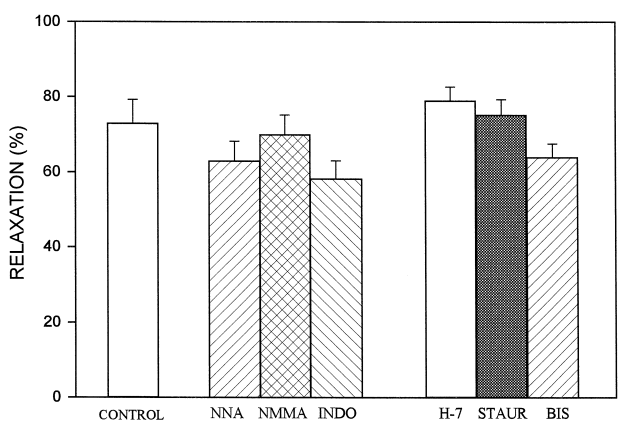

Fig. 3. Lack of effect of nitric oxide synthesis inhibitors (L-NNA, L-NMMA), indomethacin or protein kinase C inhibitors on neutral sphingomyelinase (0.1 U/ml)-induced relaxation on intact rat aortic ring segments. Each bar gives the mean ± S.E. Concentration of nitric oxide inhibitors and indomethacin used was 5×10^{-5} M; protein kinase C inhibitors were used here at 5×10^{-6} M. Data are expressed as percent decrease from precontraction induced by phenylephrine. $P > 0.05$ vs. neutral sphingomyelinase control rings. $n = 8$ each. Abbreviations: STAUR, staurosporine; BIS, Bisindolylmaleimide I; NNA, L-NNA; NMMA, L-NMMA, INDO, indomethacin.

Table 1
Sphingomyelinase attenuates secondary phase elevation in $[Ca^{2+}]_i$ induced by phenylephrine in rat aortic smooth muscle cells
The values are presented as means ± S.E.M. At least 15–20 cells each were utilized. Abbreviations: SMase, neutral sphingomyelinase; PE, phenylephrine.

Treated with	$[Ca^{2+}]_i$ (nM)
Control	92.2 ± 8.0
SMase (0.1 U/ml) alone	99.3 ± 6.3
PE (10^{-6} M) alone	142.1 ± 7.2[a]
PE + SMase	109.7 ± 5.4[b]

[a] $P < 0.01$, significantly different from control; the $[Ca^{2+}]_i$ was measured 15 min after neutral sphingomyelinase was added to the chamber.
[b] $P < 0.01$, significantly different from phenylephrine alone. Phenylephrine-induced $[Ca^{2+}]_i$ value, alone, was measured 20 min after its addition to the organ bath.

3.5. Effects of neutral sphingomyelinase on $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells

Since Ca^{2+} plays a critical role in the regulation of smooth muscle tone, we examined the effect of neutral sphingomyelinase on phenylephrine-induced changes in $[Ca^{2+}]_i$. Table 1 summarizes the results. Addition of 10^{-6} M phenylephrine to cultured rat aortic smooth muscle cells caused a significant, rapid spike-like rise in $[Ca^{2+}]_i$ from a mean resting level of 92.2 ± 8.0 nM to about 180 ± 6.8 nM followed by a smaller, secondary tonic Ca^{2+} plateau to about 142.1 ± 7.2 nM ($P < 0.01$) (Fig. 4A). Addition of 2 mM EGTA to a Ca^{2+}-free NKRB medium, 20 min prior to phenylephrine, resulted in a complete abolition of the secondary rise in $[Ca^{2+}]_i$ (Fig. 4B). Exposure of cultured rat aortic smooth muscle cells to neutral sphingomyelinase (0.1 U/ml), alone, did not change the resting levels of $[Ca^{2+}]_i$, but it markedly inhibited phenylephrine-induced secondary phase rise of $[Ca^{2+}]_i$ (Fig. 4C). However, after addition of neutral sphingomyelinase, the phenylephrine-induced secondary plateau-like elevation of $[Ca^{2+}]_i$ was clearly inhibited (from 142.1 ± 7.2 to 109.7 ± 5.4 nM, $P < 0.01$) (Fig. 4D). Although not shown, lower concentrations of neutral sphingomyelinase (e.g., 0.001–0.01 U/ml) resulted in concentration-dependent inhibition of phenyl-

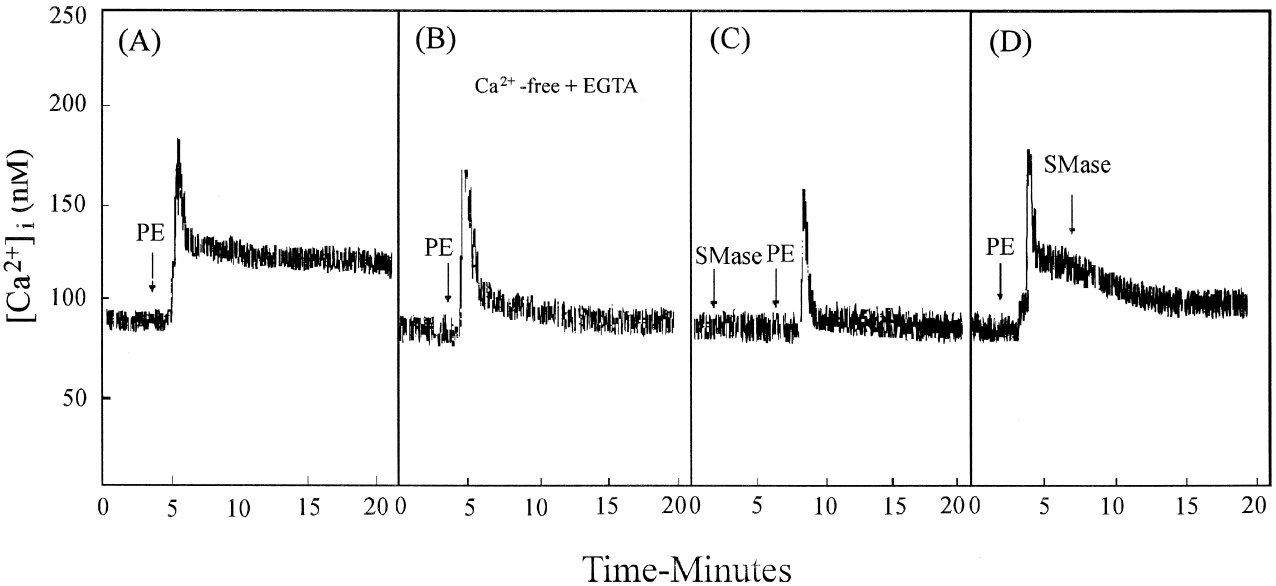

Fig. 4. Effects of neutral sphingomyelinase (0.1 U/ml) on phenylephrine-induced rises in $[Ca^{2+}]_i$ in single aortic smooth muscle cells. Effects of phenylephrine alone on $[Ca^{2+}]_i$ in single aortic smooth muscle cells (A). Phenylephrine-induced rises in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} with 2 mM EGTA added (B). Effects of neutral sphingomyelinase addition before (C) and after (D) phenylephrine (10^{-6} M) on rises in $[Ca^{2+}]_i$ in single aortic smooth muscle cells. Traces shown are representative examples of typical responses of single cells from at least 10 similar separate experiments in various assay conditions. $n = 15$. Phenylephrine (10^{-6} M); neutral sphingomyelinase (0.1 U/ml). Abbreviations: PE, phenylephrine; SMase, neutral sphingomyelinase.

ephrine-induced elevations of $[Ca^{2+}]_i$, thus paralleling the concentration-dependent neutral sphingomyelinase-induced relaxations.

3.6. C_2-ceramide and C_8-ceramide-1-phosphate attenuated relaxations after phenylephrine stimulation

Previously, we demonstrated that C_2-ceramide causes a concentration-dependent relaxation of aortic smooth muscle contractions induced by phenylephrine (Zheng et al., 1999), similar to those demonstrated above (e.g., Fig. 1) for neutral sphingomyelinase. Since neutral sphingomyelinase, acting on sphingomyelin, gives rise to both ceramides and phosphorylcholine, and ceramide can be phosphorylated to ceramide-1-phosphate, via endogenous ceramide kinase, it was important to determine whether either phosphorylcholine or ceramide-1-phosphate can mimic the actions of neutral sphingomyelinase and C_2-ceramide. As shown in Fig. 5 and Table 2, both putative second messengers (i.e., phosphorylcholine and ceramide-1-phosphate) did not produce any change in basal tension, but ceramide-1-phosphate caused a marked relaxation of phenylephrine-induced contractions; phosphorylcholine did not elicit any relaxation ($n = 4$ each, $P < 0.05$). The presence of an intact endothelium appears to contribute, in a small way, to both C_2-ceramide and ceramide-1-phosphate-induced vasodilation. Moreover, in contracted aortic rings, C_2-ceramide and C_8-ceramide-1-phosphate at (10^{-5} M) resulted in 57% and 70% (in 1.2 mM $[Mg^{2+}]_o$) relaxation in the endothelium-intact rings, and 39% and 45% relaxations in the endothelium-denuded rings, respectively (Table 2). Interestingly, when a comparison is made between relaxation in intact and denuded aortic rings, it appears that neutral sphingomyelinase (0.1 U/ml), C_2-ceramide (10^{-5} M) and C_8-ceramide-1-phosphate (10^{-5} M) induce similar types of relaxations in 1.2 mM extracellular magnesium (Fig. 2, Table 2). Although this does not, in itself, represent positive proof, it does suggest the the

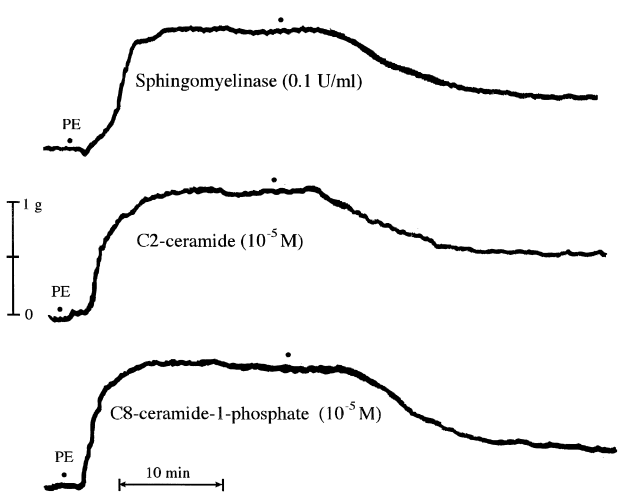

Fig. 5. Comparative effects of neutral sphingomyelinase, C_2-ceramide and C_8-ceramide-1-phosphate on phenylephrine (10^{-6} M)-induced contraction in denuded rat aortic rings. Neutral sphingomyelinase (0.1 U/ml); C_2-ceramide (10^{-5} M), and C_8-ceramide-1-phosphate (10^{-5} M) were added to baths, in 1.2 mM $[Mg^{2+}]_o$, $n = 8$.

Table 2
C_2-ceramide and C_8-ceramide-1-phosphate attenuate phenylephrine-induced contraction in 1.2 mM extracellular magnesium in rat aortic smooth muscle
All values are means ± S.E.M. expressed as percentage of the maximal tension developed to phenylephrine as control. 4–6 tissues were utilized. Abbreviations: Cer, C_2-ceramide; Cer-1-P, C_8-ceramide-1-phosphate.

Agonist	Reduction (%)	
	With endothelium	Without endothelium
Cer (10^{-5} M)	57.9 ± 4.0[a]	39.4 ± 2.4[b]
Cer-1-P (10^{-5} M)	70.5 ± 7.2[a]	45.8 ± 3.2[a]

[a] $P < 0.01$, significantly different from phenylephrine alone control.
[b] $P < 0.05$, significantly different from phenylephrine alone control.

relaxant effect of magnesium-dependent neutral sphingomyelinase on phenylephrine-induced contractions in rat aortic smooth muscle may indeed occur via production of ceramide and ceramide-1-phosphate generation.

4. Discussion

It is well-known that the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a key role in regulating the functions of every cell type. Smooth muscle cells contract in response to a $[Ca^{2+}]_i$ rise and relax as the $[Ca^{2+}]_i$ decreases (Missiaen et al., 1993). It has been shown that, in phenylephrine-induced contraction of vascular smooth muscle, the primary event is a breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane to produce two intracellular messengers: myo-inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (Exton, 1985). IP_3 causes some release of intracellular Ca^{2+} from intracellular stores, producing a rapid rise in cytosolic Ca^{2+} (Lee and Severson, 1994), but it is short-lived due to the limited size of the internal Ca^{2+} stores. The internal release of Ca^{2+} is responsible for the initial phasic component of contraction, which is succeeded by a slower tonic component dependent on influx of extracellular Ca^{2+} and involving increased Ca^{2+} permeability of the plasma membrane (Exton, 1985).

It has also been suggested that activation of protein kinase C may be involved in the contractile response to phenylephrine (Walsh et al., 1994). In the present study, after addition of neutral sphingomyelinase, phenylephrine-induced vasoconstriction and the rise of $[Ca^{2+}]_i$ were significantly inhibited. As shown in Fig. 4, chelation of extracellular Ca^{2+} with 2 mM EGTA (in the absence of $[Ca^{2+}]_o$) markedly reduced the phenylephrine-stimulated secondary phase rise of $[Ca^{2+}]_i$, indicating diminution of influx of extracellular Ca^{2+}. Treatment with neutral sphingomyelinase (0.1 U/ml), before or after phenylephrine was added, did not affect the initial increase in $[Ca^{2+}]_i$ or the peak value (Fig. 4C,D), but did cause a rapid decline in the secondary phase of $[Ca^{2+}]_i$ in a manner distinguishable from that of control cells cultured for the same period of time (Fig. 4C,D). The phenylephrine-induced $[Ca^{2+}]_i$ increase under such conditions is due to its release from internal stores by IP_3 (Exton, 1985). The $[Ca^{2+}]_i$ profiles obtained, herein, in the presence of neutral sphingomyelinase were nearly the same as those observed in Ca^{2+}-depleted cells in the presence of EGTA (Fig. 4B). Thus, these results lead us to hypothesize that neutral sphingomyelinase blocked phenylephrine-stimulated Ca^{2+} influx from the extracellular source. Moreover, protein kinase C inhibitors, staurosporine, H-7 or bisindolylmaleimide I, which collectively act on all active sites of protein kinase C isozymes, did not significantly attenuate the neutral sphingomyelinase-induced relaxations, indicating that this neutral sphingomyelinase's relaxant action on phenylephrine-induced vasoconstriction is not mediated through protein kinase C.

It has been demonstrated that treatment of HL-60 and U937 cells with bacterial sphingomyelinase from *S. aureus* to hydrolyze outer leaflet sphingomyelin, in both cell types, hydrolyzes approximately 60% or more of the total cellular sphingomyelin (Linardic and Hannun, 1994). It has also been reported that generation of ceramide via use of exogenous sphingomyelinase results in time- and concentration-dependent formation of ceramide-1-phosphate in HL-60 cells. Ceramide can serve as precursor to ceramide-1-phosphate in vitro, via activation of ceramide kinase (Dressler and Kolesnick, 1990; Mathias and Kolesnick, 1993). Moreover, ceramide-1-phosphate can be generated in aortic smooth muscle cells (Morrill et al., 1988). A recent study also showed that cultured vascular smooth muscle cells contain basal ceramide levels that can be increased by incubation with exogenous neutral sphingomyelinase (0.1 U/ml), ranging from a 12-fold rise at 5 min, to 17-fold rise at 30 min (Jones et al., 1997). Another recent study indicates that ceramide levels in vascular smooth muscle cells are clearly Mg^{2+}-dependent (Morrill et al., 1998). Our present findings demonstrate that an optimum concentration of extracellular magnesium is required for neutral sphingomyelinase relaxation in aortic smooth muscle.

More recently, studies from our laboratory have demonstrated that cell-permeable ceramide analogs, i.e., C_2-ceramide, can attenuate phenylephrine-induced contraction in isolated rat aortic rings, as well as inhibit the secondary phase rise in $[Ca^{2+}]_i$ induced by phenylephrine in cultured rat aortic smooth muscle cells (Zheng et al., 1999). In the present studies, both C_2-ceramide and ceramide-1-phosphate caused a relaxation similar to that induced by neutral sphingomyelinase (Fig. 5). The ceramide-1-phosphate relaxation, however, was greater than that induced by C_2-ceramide and consistent with that induced by neutral sphingomyelinase in 1.2 mM extracellular magnesium. It is, thus, possible that this event could occur via activity of a Mg^{2+}-dependent ceramide kinase to promote ceramide phosphorylation. Based on the evidence, herein, the ability

of neutral sphingomyelinase, C_2-ceramide and C_8-ceramide-1-phosphate, but not phosphorylcholine, to inhibit phenylephrine-induced vasoconstriction in an identical manner, coupled with the inhibition of the accompanying secondary rise of $[Ca^{2+}]_i$ evoked by the agonist, suggests that it is more than likely that the neutral sphingomyelinase vasorelaxant action is caused by the accumulation and activation of ceramide as well as a phosphorylation of ceramide to form ceramide-1-phosphate .

In conclusion, our experiments demonstrate that neutral sphingomyelinase, the key initial enzyme of the sphingomyelin pathway is Mg^{2+}-dependent in aortic smooth muscle, and can inhibit phenylephrine-induced contraction and phenylephrine-evoked elevation in $[Ca^{2+}]_i$ in rat aortic smooth muscle, suggesting that a Mg^{2+}-dependent sphingomyelin signaling pathway may play an important regulatory role in arterial wall tone. Lastly, these new experiments provide evidence that both ceramide and ceramide-1-phosphate may be putative second messengers in the neutral sphingomyelinase–sphingomyelin signaling pathway in arterial smooth muscle.

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