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Nitric oxide donors enhance calcitonin gene-related peptide-induced elevations of cyclic AMP in vascular smooth muscle cells

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Abstract

Vasorelaxant effects of calcitonin gene-related peptide (CGRP) are dependent on endothelium-derived nitric oxide (NO) in some arteries. The mechanism involved is still not clear. In the present study, we used NO donors (sodium nitroprusside (SNP) and 6-(2-hydroxy-1-methyl-2-nitrisohydrazino)-*N*-methyl-1-hyxanamine (NOC-9)), cyclic GMP elevator (brain natriuretic peptide (BNP)) and a selective type III (cyclic GMP-inhibited) phosphodiesterase (PDE) inhibitor 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK&F94120) to investigate involvement of NO, cyclic GMP and type III PDE in CGRP-induced accumulation of cyclic AMP in cultured rat aortic smooth muscle cells. SNP (10 μM), NOC-9 (10 μM) and BNP (1 μM) all increased intracellular cyclic GMP to similar levels (2- to 2.5-fold above basal) and caused significant enhancement of CGRP (10 nM)-induced cyclic AMP accumulation similar to that caused by 10 μM SK&F 94120. The data are therefore consistent with our hypothesis that the mechanism of endothelium-dependent vasorelaxation effect of CGRP involves cyclic GMP-mediated inhibition of type III PDE and subsequent accumulation of cyclic AMP in smooth muscle cells.

Keywords: CGRP (calcitonin gene-related peptide); Nitric oxide (NO); cGMP; Phosphodiesterase inhibitor; cAMP; Smooth muscle cell, vascular

1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide expressed predominantly in the central and peripheral nervous systems (reviewed in Ref. Wimalawansa, 1996). CGRP receptors are widely distributed in the body and CGRP is reported to be the most potent endogenous vasodilator in humans that has been discovered to date. In the cardiovascular system, CGRP is known to regulate inotropy, chronotropy, and vascular tone. One of the major biological effects of CGRP is relaxation of vascular smooth muscle. Intravenous administration of CGRP can cause a dose-dependent decrease of mean arterial blood pressure. According to its potent vasodilator effect, CGRP and its agonists or antagonists have been considered to become therapeutic agents for many kinds of diseases like coronary heart disease, myocardial ischemia, heart failure, renal failure, peripheral vascular disease, hypertension, and septic shock.

Circulating levels of CGRP are elevated in older rats and older humans in comparison with younger ones (Grunditz et al., 1986; Wimalawansa, 1991). The concentration of CGRP in the circulation is also elevated during pregnancy (Stevenson et al., 1986). Furthermore, plasma levels of CGRP are elevated in human patients with septic shock (Joyce et al., 1990). Likewise, in animal models of endotoxin shock, the plasma levels of CGRP are also dramatically elevated after several hours (Wang et al., 1992; Arden et al., 1994). All these results indicate that CGRP may be an important regulator of functions in the body under various physiological and pathological conditions.

The physiological role of CGRP is only partially understood. The mechanism of CGRP causing vasodilation is also not clear. In feline cerebral artery, pig coronary artery, and rat caudal artery, the vasorelaxations induced by CGRP are independent of endothelium (Edvinsson et al., 1985; Shoji et al., 1987; Fiscus et al., 1992). In contrast, the vasorelaxations caused by CGRP in aorta (Brain et al., 1985; Kubota et al., 1985; Grace et al., 1987; Fiscus, 1988; Fiscus et al., 1991; Wang et al., 1991; Gray and Marshall, 1992a,b; Hao et al., 1994) and larger (proximal) coronary

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arteries (Prieto et al., 1991) of the rat as well as cerebral, coronary, gastric and radial arteries of human (Thom et al., 1987) are dependent on endothelium.

Endothelium-derived relaxant factor (EDRF, now recognized as nitric oxide (NO)) is involved in the mechanism of CGRP-induced vasorelaxation in rat aorta (Fiscus, 1988; Fiscus et al., 1991; Wang et al., 1991; Gray and Marshall, 1992a,b; Hao et al., 1994). Our previous studies and the studies of Gray and Marshall have shown that CGRP causes endothelium-dependent vasorelaxations in isolated rings of rat abdominal and thoracic aortas that involve elevations of both cyclic AMP and cyclic GMP levels (Fiscus, 1988; Fiscus et al., 1991; Wang et al., 1991; Gray and Marshall, 1992a,b; Hao et al., 1994). We have also shown that the elevations of both cyclic AMP and cyclic GMP levels, like the relaxation, are effectively blocked by inhibitors of EDRF/NO, including hemoglobin, methylene blue, nordihydrograiaretic acid (NDGA), and N_{ω}-nitro-L-arginine (L-NNA) (Fiscus et al., 1991; Hao et al., 1994). An NO donor, nitroglycerin, can substitute for endothelium-derived NO in potentiating the vasorelaxations and cyclic AMP elevations induced by CGRP in rat aortic rings denuded of endothelium (Fiscus et al., 1994). Thus, CGRP appears to have direct vasorelaxant and cyclic AMP-elevating effects in aortic smooth muscle if either endogenous or exogenous NO is present.

We hypothesized that cyclic GMP-inhibited-PDE (type III PDE) was involved in the mechanism of endothelium-dependent vasorelaxation induced by CGRP (Fiscus et al., 1994). As a major form of cyclic AMP-hydrolyzing PDE in rat aortic smooth muscle cells (Lindgren et al., 1991), the type III PDE could rapidly metabolize the newly-synthesized cyclic AMP stimulated by CGRP if the PDE is uninhibited. We have proposed that, when the endothelium is intact, the endothelium-derived NO elevates cyclic GMP levels in smooth muscle cells and inhibits type III PDE, allowing CGRP-induced accumulation of intracellular cyclic AMP and further relaxation (Fiscus et al., 1994). In our previous work, we also found that an intracellular cyclic GMP elevating agent, brain natriuretic peptide (BNP), can uncover and enhance the direct CGRP-induced elevations of cyclic AMP levels and vasorelaxations in rat aortic rings without endothelium (Fiscus et al., 1998), giving support to our hypothesis. However, it is not certain if all of the biochemical interactions leading to these responses occur within the smooth muscle cells of the aortic rings.

In the present study, we investigated the effects of NO, cyclic GMP, and type III PDE on the CGRP-induced cyclic AMP accumulation in a homogenous cultured population of smooth muscle cells isolated from rat thoracic aorta. Our data provide further evidence that cyclic GMP-inhibited PDE may be involved in the vasorelaxant effects of CGRP. Two NO donors, sodium nitroprusside (Murad, 1986) and 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (NOC-9) (Seccia et al., 1996) were used to study the effects of NO. We also used BNP, which is known to elevate cyclic GMP levels in rat aortic smooth muscle cells by activating particulate guanylyl cyclase (Song et al., 1988; Zhou and Fiscus, 1989), a mechanism different from that of NO, and a type III PDE inhibitor, 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK & F94120) (Eckly and Lugnier, 1994). These agents were used to observe the involvement of cyclic GMP and type III PDE in the effects of CGRP on cyclic AMP accumulation in aortic smooth muscle cells.

2. Materials and methods

2.1. Animals

The treatment of the laboratory animals and the experimental protocols of the present study adhered to the guidelines of The Chinese University of Hong Kong and were approved by an Institutional Authority for Laboratory Animal Care. Male Sprague–Dawley rats (240–260 g) were used in our experiments.

2.2. Culture of vascular smooth muscle cells

Vascular smooth muscle cells were isolated from rat thoracic aorta by enzymatic dissociation using standard methods (Beasley et al., 1991). Briefly, three rats were killed by decapitation and exsanguinated. Their thoracic aortas were carefully removed, minimizing exposure to unsterile conditions. After washing five times in sterile Dulbecco's modified Eagle's medium (DMEM) (containing 25 mM HEPES, 100 μg/l gentamicin, 1 mg/ml bovine serum albumin (BSA), pH 7.4), the aortas were removed of the adhering fat and connective tissue in a sterile Biological Safety Cabinet. Then, the aortas were cut open and the endothelium was removed by rubbing the intimal layer with sterile microforceps. The remaining medial layers of the rat aortas were minced and incubated for 1 h at 37°C in DMEM containing 300 U/ml collagenase III, 100 U/ml elastase and 1.0 mg/ml soybean trypsin inhibitor. The digestion medium was removed by 2 min centrifugation at 2000 rpm. After washing one time with phosphate buffered saline (PBS, 1×, pH 7.4), the digested tissue was resuspended in DMEM containing 15% fetal bovine serum (FBS), 200 U/ml penicillin and 200 μg/ml streptomycin. The tissue was triturated 10 times through a 18-gauge needle and sieved through a 150 mesh filter. The cells were washed with PBS three times and plated in 25-cm^2 cell culture flasks containing DMEM, 15% FBS, 200 U/ml penicillin and 200 μg/ml streptomycin. Cells were passaged once every five days by harvesting with trypsin-EDTA and seeded at a 1:5 ratio into 12-well trays. After the first passage, the cells were cultured in DMEM containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were used

between passage 3 and 6 after reaching confluence at 3–5 days. The cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for smooth muscle α-actin, using mouse anti-smooth muscle α-actin antibody (Skalli et al., 1986).

2.3. Cyclic GMP accumulations in vascular smooth muscle cells induced by NO donors, BNP or SK&F94120

When the cells reached confluence in 12-well trays, the media were replaced with DMEM containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and either (a) no additions, (b) SNP (1, 10 or 100 μM), (c) NOC-9 (1, 10 or 100 μM), (d) BNP (1 μM) or (e) SK&F94120 (1 or 10 μM). Four minutes later, the media were rapidly removed and cell extracts were obtained for cyclic GMP determination (see cyclic GMP and cyclic AMP measurements below). We selected the time course as 4 min since we experienced that cyclic GMP level could be elevated to maximum in 4–10 min. Cyclic AMP level will reach the maximum in 2–3 min.

2.4. Cyclic AMP elevations caused by CGRP in vascular smooth muscle cells

The intracellular cyclic AMP levels induced by CGRP were determined by incubating the smooth muscle cells with CGRP (10 nM) for 3 min. To determine the interactions of NO donors, BNP and type III PDE inhibitor with CGRP on cyclic AMP levels in the cells, the monolayers of cells were incubated with DMEM containing SNP, NOC-9, BNP or SK&F94120 for 4 min. Then, CGRP was added to the medium and the cells were incubated for another 3 min. The media were removed and cell extracts were obtained for cyclic AMP measurement after these incubations (see below).

2.5. Cyclic GMP and cyclic AMP measurements

Cyclic GMP or cyclic AMP was extracted from the cells by rapid aspiration of the medium and addition of 1.0 ml 0.1 N HCl to each well. The cell fragments were scratched off the bottom of the culture trays and the suspensions were centrifuged at 10,000 rpm and 4°C for 10 min. The supernatants were frozen at −70°C before assay for cyclic GMP or cyclic AMP while the precipitates were used for protein measurement.

The cyclic GMP content of cell extracts was determined by radioimmunoassay using Amersham's cyclic GMP [^{125}I] assay kits with Amerlex-M™ magnetic separation. The cyclic AMP content of cell extracts was measured by radioimmunoassay using Biotrak™ cyclic AMP [^{125}I] assay kits with Amerlex-M™ magnetic separation from Amersham. The protein content of each well was determined by the method of Lowry et al. (1951), using a protein assay kit from Sigma (St. Louis, MO, USA). The cyclic nucleotide contents of the smooth muscle cells were expressed as pmol/mg of protein in each well.

2.6. Chemicals and drugs

Rats were supplied by a colony of Sprague–Dawley rats from the Laboratory Animal Service Center, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong. Synthetic rat CGRP (α-CGRP) was purchased from Bachem (Torrance, CA, USA), Phoenix Pharmaceuticals (Mountain View, CA, USA) and Sigma. Sodium nitroprusside (SNP), HEPES, gentamicin, bovine serum albumin (BSA) and collagenase III were purchased from Sigma. 6-(2-hydroxy-1-methyl-2-nitrisohydrazino)-*N*-methyl-1-hyxanamine (NOC-9) was bought from Calbiochem-Novabiochem (San Diego, CA, USA). Brain natriuretic peptide-32 (rat) was bought from Phoenix Pharmaceuticals. Elastase and soybean trypsin inhibitor were purchased from Worthington Biochemical (Lakewood, NJ, USA). DMEM, FBS, antibiotics (Penicillin–Streptomycin), trypsin-EDTA and PBS were purchased from GIBCO BRL Products, Life Technologies (Gaithersburg, MD, USA). Cyclic AMP and cyclic GMP assay kits were purchased from Amersham Life Science (Buckinghamshire, UK). Protein assay kits were purchased from Sigma. Anti-smooth muscle α-actin was bought from Boehringer Mannheim Biochemical (Mannheim, Germany). Hydrochloride (HCl) was a product of BDH Laboratory Supplies (Poole, England). 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK&F94120) was a kind gift from Dr. Theodore J. Torphy, SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA).

2.7. Statistical analysis

The data were analyzed using one-way ANOVA and further analyzed using the Student–Newman–Keuls (S–N–K) test for multiple comparisons between treatment groups. A P value of < 0.05 was used to indicate significant difference between treatment group means. The data are presented as mean values ± the standard error of the mean (SEM). The n values given in the figure legends represent the number of culture wells used for treating with the agents and measuring cyclic nucleotide levels.

3. Results

3.1. Effects of NO donors on cyclic GMP levels in the vascular smooth muscle cells

The NO donors SNP and NOC-9 caused elevations of cyclic GMP in rat aortic vascular smooth muscle cells in

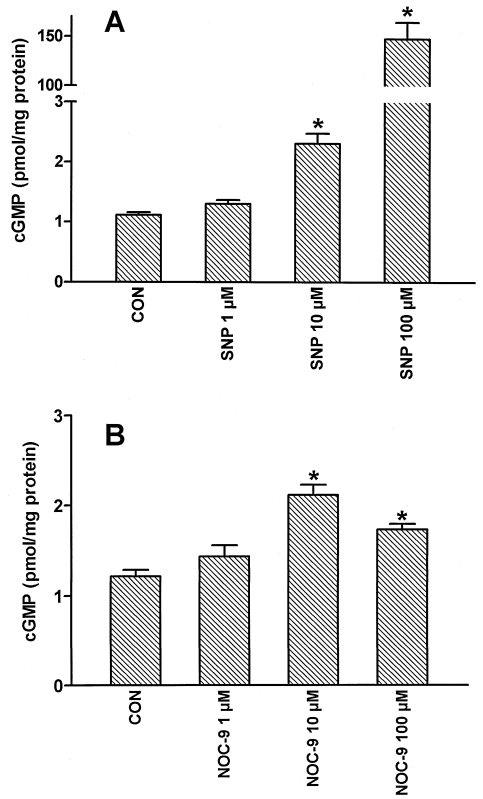

Fig. 1. Effect of nitric oxide donors, sodium nitroprusside (SNP) and 6-(2-hydroxy-1-methyl-2-nitrisohydrazino)-*N*-methyl-1-hyxanamine (NOC-9), on cyclic GMP levels in the cultured rat aortic smooth muscle cells. (A) Vascular smooth muscle cells were incubated for 4 min in DMEM with or without SNP (1, 10 or 100 μM). (B) Vascular smooth muscle cells were incubated for 4 min in DMEM with or without NOC-9 (1 μM, 10 μM or 100 μM). $^*P < 0.05$ compared to the control values. For each treatment group: $n = 6$.

culture (Fig. 1). Four minutes incubation with 100 μM SNP caused about 100-fold elevation of intracellular cyclic GMP from 1.50 ± 0.13 pmol/mg protein to 132.8 ± 8.4 pmol/mg protein, while 10 μM SNP increased the cyclic GMP levels about two-fold from 1.11 ± 0.04 pmol/mg protein to 2.31 ± 0.17 pmol/mg protein. We did not see any significant increase of intracellular cyclic GMP levels when the vascular smooth muscle cells were incubated with 1 μM SNP for 4 min (Fig. 1A).

The NOC-9 also elevated intracellular cyclic GMP levels of vascular smooth muscle cells (Fig. 1B). Incubating the cells with 1 μM NOC-9 for 4 min did not cause any significant elevations of intracellular cyclic GMP levels. NOC-9 with a concentration of 10 μM caused about two-fold elevation of cyclic GMP levels in the vascular smooth muscle cells from 1.22 ± 0.07 pmol/mg protein to 2.12 ± 0.12 pmol/mg protein. However, NOC-9 did not cause any more increase of intracellular cyclic GMP levels when its concentration was increased from 10 μM up to 100 μM. The elevation of cyclic GMP levels caused by 100 μM NOC-9 was from 1.22 ± 0.07 pmol/mg protein to 1.74 ± 0.06 pmol/mg protein.

3.2. Effect of another cyclic GMP elevator, BNP, on cyclic GMP levels in the vascular smooth muscle cells

In contrast to NO donors that elevate cyclic GMP by activating soluble guanylyl cyclase, BNP elevates cyclic GMP by activating particulate guanylyl cyclase in the vascular smooth muscle cells (Song et al., 1988; Zhou and Fiscus, 1989). Incubating the cells 4 min with 1 μM BNP caused the intracellular cyclic GMP levels to increase about three-fold from 1.63 ± 0.17 pmol/mg protein to 4.65 ± 0.21 pmol/mg protein (Fig. 2A).

3.3. Effect of the type III phosphodiesterase inhibitor SK&F94120 on cyclic GMP levels in the vascular smooth muscle cells

As we expected, the type III PDE inhibitor, SK&F94120, had no effect on intracellular cyclic GMP levels of vascular smooth muscle cells (Fig. 2B). The cyclic GMP levels in the smooth muscle cells incubated 4 min with 1 μM and 10 μM SK&F94120 were 0.99 ± 0.08 pmol/mg protein and 1.30 ± 0.10 pmol/mg protein, re-

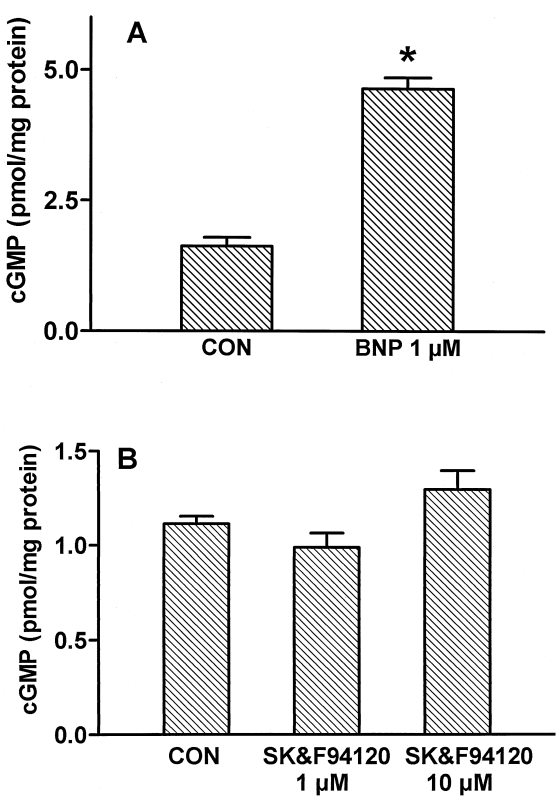

Fig. 2. Effects of brain natriuretic peptide (BNP) and SK&F94120 on cyclic GMP contents of the cultured rat aortic smooth muscle cells. (A) Vascular smooth muscle cells were incubated for 4 min in DMEM with or without BNP (1 μM). (B) Vascular smooth muscle cells were incubated for 4 min in DMEM with or without SK&F94120 (1 or 10 μM). $^*P < 0.05$ compared to the control values. For each treatment group: $n = 6$.

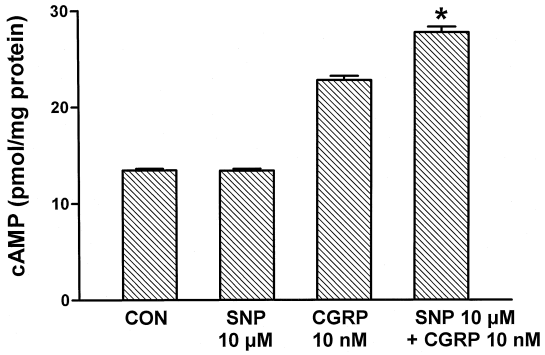

Fig. 3. Effect of sodium nitroprusside (SNP) on CGRP-induced cyclic AMP accumulation in the vascular smooth muscle cells. Vascular smooth muscle cells were incubated for 4 min in DMEM with or without SNP (10 μM). Then 10 nM CGRP was added to some of the wells and the cells were incubated for another 3 min before cyclic AMP determination. $^{*}P < 0.05$ compared to the cyclic AMP levels in the cells treated by CGRP by itself. For each treatment group: $n = 6$.

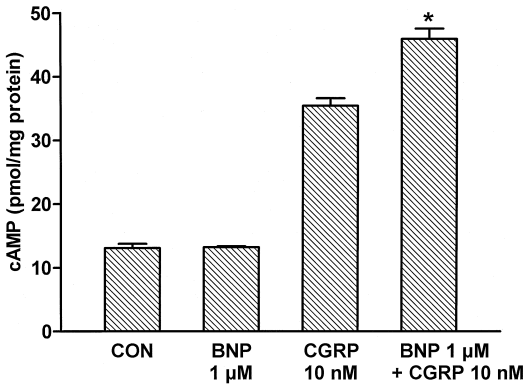

Fig. 5. Effects of BNP on CGRP-induced cyclic AMP accumulation in the vascular smooth muscle cells. Vascular smooth muscle cells were incubated for 4 min in DMEM with or without BNP (1 μM). Then 10 nM CGRP was added to some of the wells and the cells are incubated for another 3 min before cyclic AMP determination. $^{*}P < 0.05$ compared to the cyclic AMP levels in the cells treated by CGRP by itself. For each treatment group: $n = 6$.

spectively, while the control level of cyclic GMP was 1.11 ± 0.04 pmol/mg protein. There was no statistically significant difference between the control levels of cyclic GMP and those in the cells following SK&F94120 treatments.

3.4. Effects of NO donors on CGRP-induced elevation of cyclic AMP in the vascular smooth muscle cells

The NO donor SNP synergistically increased the CGRP-induced elevations of cyclic AMP in the vascular smooth muscle cells (Fig. 3). At 10 μM concentration, SNP caused a statistically significant enhancement of CGRP-induced cyclic AMP production in the cells. SNP by itself did not cause any change in the levels of cyclic AMP in the vascular smooth muscle cells.

The NO donor NOC-9 (10 μM) also significantly enhanced the CGRP (10 nM)-induced increase in cyclic AMP levels, from 22.7 ± 0.4 pmol/mg protein to 27.8 ± 0.7 pmol/mg protein (Fig. 4). NOC-9 by itself did not cause any statistically significant changes of intracellular cyclic AMP levels.

3.5. Effect of BNP on CGRP-induced cyclic AMP production in vascular smooth muscle cells

As another agent which elevates intracellular cyclic GMP, BNP also synergistically increased the CGRP-induced cyclic AMP elevation in the vascular smooth muscle cells (Fig. 5). BNP (1 μM) caused the levels of cyclic AMP in the cells treated by 10 nM CGRP to be significantly elevated from 34.9 ± 0.8 pmol/mg protein to 45.9 ± 1.6 pmol/mg protein. BNP by itself did not cause any change of intracellular cyclic AMP levels in vascular smooth muscle cells.

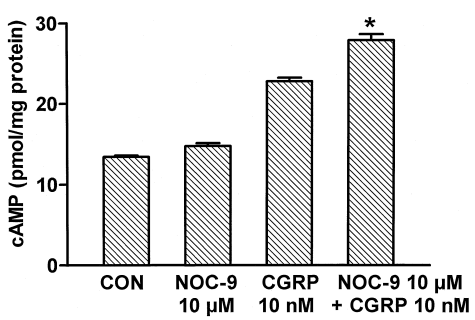

Fig. 4. Effects of 6-(2-hydroxy-1-methyl-2-nitrisohydrazino)-*N*-methyl-1-hyxanamine (NOC-9) on CGRP-induced cyclic AMP accumulation in the vascular smooth muscle cells. Vascular smooth muscle cells were incubated for 4 min in DMEM with or without NOC-9 (10 μM). Then 10 nM CGRP was added to some of the wells and the cells were incubated for another 3 min before cyclic AMP determination. $^{*}P < 0.05$ compared to the cyclic AMP levels in the cells treated by CGRP by itself. For each treatment group: $n = 6$.

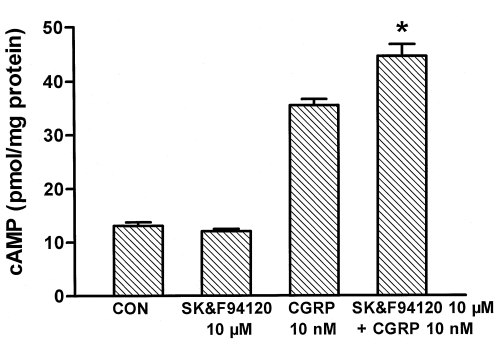

Fig. 6. Effects of SK&F94120 on CGRP-induced cyclic AMP accumulation in the vascular smooth muscle cells. Vascular smooth muscle cells were incubated for 4 min in DMEM with or without SK&F94120 (10 μM). Then 10 nM CGRP was added to some of the wells and the cells are incubated for another 3 min before cyclic AMP determination. $^{*}P < 0.05$ compared to the cyclic AMP levels in the cells treated by CGRP by itself. For each treatment group: $n = 6$.

3.6. Effect of SK&F94120 on CGRP-induced cyclic AMP elevations in vascular smooth muscle cells

SK&F94120, a specific type III PDE inhibitor, acted synergistically with CGRP to elevate cyclic AMP levels in the vascular smooth muscle cells (Fig. 6). The cyclic AMP levels in the cells treated by 10 μM SK&F94120 plus 10 nM CGRP was 44.5 ± 2.2 pmol/mg protein, while the cyclic AMP level in the cells treated by CGRP alone was 35.4 ± 1.1 pmol/mg protein. Similar to the NO donors and BNP, SK&F94120 by itself did not cause any changes on intracellular cyclic AMP levels compared with the control.

4. Discussion

Our data show that NO donors can synergistically enhance the CGRP-induced elevations of cyclic AMP levels, in addition to increasing cyclic GMP levels, in cultured vascular smooth muscle cells of rat aorta. BNP, an agent which elevates intracellular cyclic GMP by a different pathway from that of NO, also enhances the CGRP-induced elevations of cyclic AMP levels in vascular smooth muscle cells. Furthermore, SK&F94120, a specific type III PDE inhibitor, has similar effects on increasing the cyclic AMP elevations induced by CGRP.

One common characteristic of NO and BNP is that they both increase intracellular cyclic GMP levels. NO acts on the heme-moiety of soluble guanylyl cyclase and increase the biosynthesis of cyclic GMP inside cells (Murad, 1986; Fiscus, 1988). BNP acts on particulate guanylyl cyclase and also increases biosynthesis of cyclic GMP in vascular smooth muscle cells (Song et al., 1988; Zhou and Fiscus, 1989). In the present study, we have used NO donors and BNP in concentrations that would give similar elevations of cyclic GMP levels to determine if this leads to enhancement of CGRP-induced elevations of cyclic AMP levels. SNP and NOC-9 at 10 μM caused 2.08- and 1.73-fold elevations of intracellular cyclic GMP levels, respectively, while BNP at 1 μM caused 2.85-fold elevation of cyclic GMP levels. Relevantly, these concentrations of NO donors and BNP also synergistically enhanced CGRP-induced cyclic AMP elevations in the cultured vascular smooth muscle cells. Considering these results, we suggest that cyclic GMP is likely involved in the mechanism of enhancing CGRP-induced cyclic AMP elevations in aortic smooth muscle cells.

A potentially important function of the cyclic GMP in these cells may be to inhibit cyclic GMP-inhibited phosphodiesterase (type III PDE) (Maurice and Haslam, 1990; Lindgren et al., 1991; Jang et al., 1993; Eckly and Lugnier, 1994). Maurice and Haslam (1990) and Jang et al. (1993) had previously shown that a NO donor or atrial natriuretic peptide could enhance isoproterenol-induced elevation of cyclic AMP levels in aortic smooth muscle cells. Thus, the type III PDE may be another important part of the mechanism of regulating accumulation of intracellular cyclic AMP. The augmentation of CGRP-induced intracellular cyclic AMP elevations caused by the type III PDE inhibitor SK&F94120 in the present study further suggests the involvement of type III PDE in the mechanism leading to accumulation of cyclic AMP in these cells.

The CGRP is reported to be the most potent vasodilator in humans currently known (Wimalawansa, 1996). However, the cellular mechanism of vasorelaxation caused by CGRP is not clear. CGRP can cause endothelium-dependent relaxation in some arteries, including rat aorta and many human arteries (Brain et al., 1985; Kubota et al., 1985; Grace et al., 1987; Thom et al., 1987). This endothelium-dependent vasorelaxant effect of CGRP is thought to be mediated by endothelium-derived NO (Fiscus, 1988; Fiscus et al., 1991; Wang et al., 1991; Gray and Marshall, 1992a,b; Hao et al., 1994). In the NO-mediated endothelium-dependent vasorelaxation caused by CGRP, a unique signal transduction pathway is involved. CGRP causes endothelium-dependent vasorelaxations in isolated rings of rat abdominal and thoracic aortas that involves elevations of both cyclic AMP and cyclic GMP levels in arterial tissues (Fiscus, 1988; Fiscus et al., 1991; Wang et al., 1991; Gray and Marshall, 1992a,b; Hao et al., 1994). It has been shown that NO mediates this unique dual signal transduction pathway in rat aorta (Fiscus et al., 1994). However, this mechanism has not been investigated in cultured smooth muscle cells.

Methylene blue, an agent which inhibits soluble guanylyl cyclase, can block not only the cyclic GMP elevations but also the cyclic AMP elevations and vasorelaxations caused by CGRP in rat thoracic aorta with endothelium (Fiscus et al., 1991). BNP, an agent elevating intracellular cyclic GMP by activating particulate guanylate cyclase, can uncover and enhance CGRP-induced elevations of cyclic AMP and vasorelaxant effects in rat aortic rings without endothelium (Fiscus et al., 1998). Thus, cyclic GMP appears to play an important role in the cyclic AMP-elevating effects of CGRP. A potential pathway of cyclic GMP causing elevation of cyclic AMP levels is the inhibition of cyclic GMP-inhibited-PDE isozyme (also called type III PDE or amrinone/milrinone-inhibited-PDE, one of the 'low-K_m' cyclic AMP-hydrolyzing PDEs) (Fiscus et al., 1994). Rat aorta has been shown to possess high levels of this enzyme (Lindgren et al., 1991). This enzyme is inhibited by 50% in the presence of cyclic GMP at 0.30 μM (Lindgren et al., 1991), suggesting that cyclic GMP, if elevated to sufficiently high levels within smooth muscle cells, could potentially inhibit this PDE and thus lead to accumulation of cyclic AMP. Selective inhibitors of the cyclic GMP-inhibited-PDE, including SK&F94120 (Eckly and Lugnier, 1994), OPC 3911, CI-930 and milrinone (Lindgren et al., 1991), have been shown to cause significant relaxations of isolated rings of rat aorta, suggesting that this enzyme is of major importance in metabo-

lizing the specific pool of cyclic AMP responsible for mediating aortic smooth muscle vasorelaxations.

The data of our present study give further evidence supporting the hypothesis that cyclic GMP and type III PDE are involved in the mechanism of endothelium/NO mediated vasodilator effect of CGRP. NO donors, BNP and SK&F94120 did not cause significant increases of the basal levels of cyclic AMP in the vascular smooth muscle cells, but acted synergistically with CGRP to elevate the intracellular cyclic AMP levels. This may be because the basal levels of cyclic AMP are below the threshold to accumulate under these conditions. However, when CGRP is present, the intracellular levels of cAMP are elevated to a certain extent that cannot be metabolized in a short time and thus will accumulate when type III PDE is inhibited. This accumulation of cyclic AMP caused by CGRP in the presence of elevated cyclic GMP levels may have special importance for the mechanism of CGRP-induced NO-dependent vasodilations in arteries with intact endothelium as well as in situations when exogenous NO has been given, as for example, during therapy with nitrovasodilators, such as nitroglycerin and sodium nitroprusside.

Because CGRP and NO are recognized mediators of endotoxin shock (Joyce et al., 1990; Wang et al., 1992; Arden et al., 1994; Wimalawansa, 1996), this synergistic interaction may be of special relevance to the pathogenesis of endotoxin shock. Furthermore, the synergistic effect of BNP and CGRP on increasing intracellular cyclic AMP levels of vascular smooth muscle cells may also be especially important in the vascular regulation of cerebral arteries, which are reported to be innervated by both BNP- and CGRP-containing paravascular nerves (Saper et al., 1990; Wimalawansa, 1996). Also, congestive heart failure is associated with elevated circulating levels of both BNP (Mukoyama et al., 1991) and CGRP (Wimalawansa, 1996) and a synergistic interaction between these two neuropeptides in vascular smooth muscle cells could be an important response in congestive heart failure.

In conclusion, the present study has shown that NO donors can increase intracellular cyclic GMP levels and then augment the CGRP-induced cyclic AMP elevations in cultured rat aortic vascular smooth muscle cells. BNP, another agent which increases intracellular cyclic GMP levels independent of NO in aorta (Zhou and Fiscus, 1989), also enhances the CGRP-induced cyclic AMP elevations in the cultured vascular smooth muscle cells. These results suggest that cyclic GMP, presumably by inhibiting type III PDE, is an important mediator of the endothelium-dependent effects of CGRP in arteries. Further support for this idea is given by the data of the present study showing that a type III PDE inhibitor, SK&F94120, also increases the cyclic AMP elevation induced by CGRP similar to NO donors and BNP. Thus, NO released from the endothelium or provided as a therapeutic agent likely enhances the direct cyclic AMP and vasodilator effects of CGRP via elevation of intracellular cyclic GMP levels, inhibition of cyclic GMP-inhibited-PDE (type III PDE) and subsequent accumulation of cyclic AMP in the vascular smooth muscle cells.

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