Phorbol Ester and Atrial Natriuretic Peptide Receptor Response on Vascular Smooth Muscle

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At least two types of receptors for natriuretic peptides have been reported: biologically active receptors coupled with guanylate cyclase (atrial natriuretic peptide [ANP]-B receptors) and clearance receptors (ANP-C receptors). To elucidate the role of protein kinase C (PKC) in the regulation of ANP-B receptors, vascular smooth muscle cells in culture were treated with phorbol ester. Incubation with receptor agonists and phorbol ester led to the desensitization of receptor-mediated cyclic guanosine monophosphate (ANP-B receptor response) in rat vascular smooth muscle cells. Although a PKC inhibitor and downregulation of PKC by long-term incubation of cells with phorbol esters blocked the phorbol ester-induced desensitization of the ANP-B receptor response, they did not block the ANP-induced desensitization of the ANP-B receptor response. In addition, when desensitization by phorbol esters was observed, ANP was still capable of desensitization. These observations suggest that the mechanism for regulating ANP-B receptor sensitivity may be both PKC-dependent and PKC-independent and mediated by phorbol esters and ANP, respectively. (Hypertension 1992;19:314–319)

KEY WORDS • atrial natriuretic peptides • protein kinase C • phorbol esters • vascular smooth muscle

Atrial natriuretic peptide (ANP), a peptide hormone released from mammalian atria into the general circulation, exhibits several biological effects such as natriuresis, diuresis, and relaxation of vascular smooth muscle. ANP has been proposed as an active participant in the regulation of blood pressure through receptor-mediated actions in the vasculature. Recent studies on the characterization of ANP receptors have revealed a heterogeneity of ANP receptor-recognition sites in membrane preparations and in cultured cells from various tissues. Three distinct receptor subtypes for natriuretic peptides have been proposed: ANP-A receptor, which is a membrane form of guanylate cyclase; ANP-B receptor, a membrane form of guanylate cyclase that is preferentially activated by brain natriuretic peptide (BNP) rather than ANP; and ANP-C receptor, which is not coupled to guanylate cyclase. In the present study, we defined biologically active ANP receptors that are coupled to guanylate cyclase as ANP-B receptors. In vascular smooth muscle cells (VSMC), ANP-B receptors probably mediate vasorelaxation, and ANP-C receptors are found at a very high concentration (more than 95% of the total number of ANP binding sites).

Cultured VSMC provide a useful model for studying the regulation of ANP receptors. Like many other receptor systems, ANP receptors are susceptible to agonist-induced desensitization. Although several studies have shown homologous and heterologous receptor desensitization of ANP receptors, those investigations focused on desensitization of ANP-C receptors but not of ANP-B receptors.

We recently evaluated ANP-B receptor response by measuring the receptor-mediated formation of cyclic guanosine monophosphate (cGMP) and showed that glucocorticoids affected the ANP-B receptor response. In the present study, we investigated homologous (ANP) and heterologous (phorbol ester) desensitization of the ANP-B receptor response.

Methods

Materials

Type II collagenase, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride (H-7), Nonidet P-40, and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, Mo. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, trypsin EDTA (Versine), and fetal calf serum (FCS) were purchased from GIBCO Laboratories, Grand Island, N.Y. cGMP radioimmunoassay kits were purchased from Gibco Laboratories, Grand Island, N.Y. cGMP radioimmunoassay kits were purchased from Amersham Japan Co., Tokyo. Multiwell pipettes and flasks were purchased from Becton Dickinson and Co., Oxnard, Calif. Rat ANP-(1-28) and rat BNP-(1-45) were purchased from the Peptide Institute, Osaka, Japan.

Cell Culture

VSMC were grown from explants of 14-week-old normotensive Wistar rat renal arteries, with animals
handled as described previously.14-16 Cells were identified as VSMC according to their morphological and growth characteristics as previously reported.14,15 VSMC were grown in DMEM supplemented with 10% FCS. Cells from passages 3 to 5 were used and were subcultured after trypsinization on a weekly basis since cells became confluent in 1 week. Each plate was replenished twice weekly with fresh medium.

**Measurement of Cyclic GMP**

After incubation with or without phorbol esters, cells were washed three times with 2 ml DMEM and then stimulated for 10 minutes with different concentrations of ANP dissolved in DMEM with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Rapid absorption and the addition of 1.5 ml ice-cold 65% ethanol stopped the reaction. After evaporation by a centrifugal evaporator, the dry residue was dissolved in an assay buffer. cGMP levels were determined by radioimmunnoassay using the Amersham cGMP radioimmunoassay kit as previously described.13

**Membrane Guanylate Cyclase Activity**

After PMA or vehicle treatment for 4 hours, 175-cm² culture flasks on ice were washed twice with 5 ml cold N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)-buffered saline; were scraped into 3 ml of a cold mixture of 20 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol; and were homogenized by passage 10 times through a 22-gauge needle. After centrifugation for 15 minutes at 5,000g and washing with the same buffer, membrane proteins were solubilized by incubation on ice for 30 minutes in a solution containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM dithiothreitol. After centrifugation for 15 minutes at 5,000g, supernatant fluids were adjusted to equal protein concentrations and incubated for 10 minutes with or without 1 µM ANP. Incubation was continued for 10 minutes at 37°C after diluting 100 µl of each sample into a solution containing a final concentration of 20 mM HEPES, pH 7.4, with 1 mM guanosine triphosphate, 1 mM MnCl₂, and 1 mM IBMX in a total reaction volume of 250 µl. The reaction was terminated with 750 µl of 50 mM sodium acetate, pH 4.0, and then the solution was boiled for 3 minutes. cGMP was assayed using the Amersham radioimmunoassay kit. Protein was measured by the Coomassie blue method of Bradford.17

**Cell Fractionation and Assay of Protein Kinase C**

VSMC detached from culture dishes by incubation with DMEM were washed twice with an ice-cold assay buffer [50 mM Tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5), buffer containing 2 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.25 mM sucrose, 10 mM 2-mercaptoethanol, 100 µg/ml leupeptin, and 40 µg/ml phenylmethylsulfonyl fluoride] and were sonicated with three 10-second bursts. The homogenates were centrifuged at 100,000g for 60 minutes at 4°C to separate the cytosolic and particulate fractions. The cytosolic fraction was kept on ice with Nonidet P-40 added to a final concentration of 1%. The pellet was resuspended in assay buffer containing 1% Nonidet P-40, was stirred on ice for 1 hour, and then was centrifuged at 100,000g for 30 minutes. Protein kinase C activity was measured by a modification of the method previously reported using the Amersham protein kinase C (PKC) assay system. In brief, a sample of the reaction mixture (50 mM Tris/HCl [pH 7.5], 3 mM calcium acetate, 2 mol% l-α-phosphatidyl-l-serine, 6 µg/ml PMA, 225 µM substrate peptide, 7.5 mM dithiothreitol, and 0.05% wt/vol sodium azide) was mixed with magnesium [γ³²P]ATP and incubated at 25°C for 15 minutes. An acidic reaction-quenching reagent was added to stop the reaction. Phosphorylated peptide was separated on binding paper. After the paper was washed, the extent of phosphorylation was detected by scintillation counting. PKC assay was linear for 15 minutes. PKC activity was determined by subtracting the initial rate of protein kinase activity (in the absence of activators) from the initial rate of protein kinase activity in the presence of phosphatidylserine, calcium acetate, and PMA.

**Statistical Methods**

Statistical analysis was performed by analysis of variance and Scheffe's modified t test. Values of p<0.05 were considered to be significant.

**Results**

**Effect of PMA Pretreatment on Atrial Natriuretic Peptide–Mediated or Brain Natriuretic Peptide–Mediated Cyclic GMP Formation by Vascular Smooth Muscle Cells**

As shown in Figure 1, ANP stimulated the formation of cGMP by guanylate cyclase in intact VSMC. Incubation of VSMC for 4 hours in the presence of PMA (1 µM) led to a suppression of ANP-mediated formation of cGMP by intact cells. The dose–response curve for ANP in PMA-treated cells for 4 hours showed a suppression of the maximum response, generally ranging from 42% to 59% below that of the untreated cells. BNP-induced cGMP formation was compared with ANP-induced cGMP formation in intact VSMC. The maximal response curve for BNP in PMA-treated cells for 4 hours showed a suppression that was almost equal to that observed for ANP. The dose–response curve for ANP in PMA-treated cells for 24 hours showed almost no suppression (Figure 2). Incubation of cells with 1 µM PMA for various periods of time before ANP stimulation of cGMP led to a detectable effect at 2 hours with a maximum effect at 4 hours, whereas incubation of cells with 1 µM ANP for various periods of time before ANP stimulation of cGMP led to a detectable effect at 30 minutes with a maximum effect at 4 hours (Figure 3). The time course response to ANP showed that PMA treatment for 4 hours decreased the ANP-mediated formation of cGMP for 10 minutes after stimulation. Cells treated with different concentrations of PMA exhibited a dose-related decrease in cGMP formation induced by ANP (Figure 4).

**Guanylate Cyclase Activity in Vascular Smooth Muscle Cells**

Particulate guanylate cyclase activity of VSMC was 2.3±0.2 pmol/min/mg in vehicle-treated cells and 2.1±0.2 pmol/min/mg in cells treated with 1 µM PMA for 4 hours. After ANP (1 µM) treatment for 10 minutes, particulate guanylate cyclase activity was 6.3±0.6 pmol/min/mg in vehicle-treated cells and 4.8±0.2 pmol/min/mg in PMA-
ANP : -log(M) 
BNP : -log(M)

FIGURE 1. Line graphs show effect of phorbol 12-myristate 13-acetate (PMA) on the dose–response curve for atrial natriuretic peptide (ANP)–mediated and brain natriuretic peptide (BNP)–mediated formation of cyclic guanosine monophosphate (cyclic GMP) by vascular smooth muscle cells from renal arteries of Wistar rats. After incubation for 4 hours with 1 μM PMA (●) or vehicle (○), cells were stimulated with the indicated doses of ANP or BNP for 10 minutes in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Data (mean±SD) are for one experiment representative of three independent experiments sampled in triplicate at each dose point. *Significantly different from control; p<0.05.

treated cells (Figure 5A). PMA did not affect the basal guanylate cyclase and suppressed ANP (1 μM)-activated guanylate cyclase activity (2.3±0.3 pmol/min/mg).

Desensitization of Atrial Natriuretic Peptide-B Receptors Induced by Atrial Natriuretic Peptide Under Conditions in Which Heterologous (PMA-Induced) Desensitization Occurred

Incubation of cells for 2 hours with PMA suppressed the ANP-induced formation of cGMP to 62.7±2.7% of control values. Under these conditions, an additional 1 hour of incubation with 1 μM ANP further desensitized the subsequent ANP-mediated cGMP response (Figure 6), leading to a response that was 55.3±2.9% of that observed in cells treated with PMA alone. This extent of desensitization of the cGMP response was comparable to that of ANP-induced desensitization of cGMP formation (57% of control) in cells that were not pre-treated with PMA (Figure 5). ANP receptor–mediated cGMP formation in cells treated with both ANP and PMA was statistically different from that in cells treated with either ANP or PMA (Figure 6).

Effect of the Kinase C Inhibitor H-7 on PMA-Induced Desensitization of Atrial Natriuretic Peptide-Mediated Cyclic GMP Formation

The inhibitory effect of PMA on ANP-induced formation of cGMP in VSMC was reduced significantly by H-7. However, preincubation of the cells with 100 μM H-7 for 30 minutes followed by an additional hour of incubation with ANP did not block the desensitization of the subsequent ANP-mediated cGMP response (Figure 7).

Effect of Downregulation of Protein Kinase C on the PMA-Induced and Atrial Natriuretic Peptide-Induced Desensitization of Atrial Natriuretic Peptide-B Receptors

Incubation of cells with 1 μM PMA caused a significant increase in specific PKC activity in the particulate fraction as early as 1 hour after the addition of phorbol ester to the medium (from 2.5±1.0 to 15.1±1.5 pmol/min/10^6 cells). PKC activity was then desensitized in a time-dependent manner. The PKC activity after 1 μM PMA treatment for 4 hours was 2.52±1.2 pmol/min/10^6 cells in the cytosolic fraction, and 7.5±1.2 pmol/min/10^6 cells in the particulate fraction (Figure 8A).

Incubation of cells with 1 μM PDBu for 24 hours significantly decreased PKC activity from 46.0±3.2 to 6.5±1.0 pmol/min/10^6 cells in the cytosolic fraction, and from 9.0±1.0 to 2.5±0.8 pmol/min/10^6 cells in the particulate fraction (Figure 8B). Under these conditions, the ANP-mediated formation of cGMP was 89% of that in cells not treated with PDBu (data not shown). Cells were incubated with 1 μM PDBu for 24 hours, then washed free of PDBu and reincubated with the indicated concentrations of PMA for 2 hours (Figure 9A). Under these conditions, the inhibitory effect of PMA on the ANP-mediated formation of cGMP was nearly lost. In comparison, in PKC-downregulated cells incubated with the indicated concentrations of ANP for
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1 hour, an agonist concentration-dependent desensitization of subsequent ANP-induced formation of cGMP was observed in the same fashion and to the same extent as that seen in untreated cells (Figure 9B). Thus, it appears that ANP-induced downregulation of ANP-B receptors is independent of PKC.

Discussion

It has been reported that exposure of VSMC to PKC-activating phorbol esters or to a receptor agonist results in desensitization. However, previous studies focused on the desensitization of the ANP-C receptor using the $^{125}$I-ANP binding assay. Few studies have dealt with desensitization of the ANP-B receptor. A lack of correlation between the receptor-binding affinity of ANP and its potency for stimulating cGMP has been reported. The present study has shown that not only ANP-C receptors but also ANP receptors that are coupled to guanylate cyclase (ANP-B receptors) become desensitized. This result is not consistent with a previous study, which reported that ANP-B receptors are not desensitized. These different results could be due to differences in VSMC from renal arteries and those from thoracic aortas.

Although the kinase C inhibitor H-7 suppressed the enzymatic activity of PKC as well as PMA-induced desensitization of the ANP receptor-mediated response in VSMC, it did not influence ANP receptor desensitization (Figure 6). Furthermore, treatment of VSMC with PKC-activating phorbol ester for 24 hours produced a loss of 90% in detectable PKC activity and led to a significant attenuation of the inhibition of ANP receptor function by phorbol ester. In contrast, PDBu-treated cells were able to undergo ANP-induced desensitization (homologous desensitization) of the cGMP response and receptor down-regulation with a time course and concentration dependence similar to those observed in untreated cells. Thus, these findings support the notion that PKC is involved in the observed actions of phorbol ester but does not play an important role in receptor agonist-induced desensitization (homologous desensitization). These results and the

**Figure 3.** Line graph plots time course for the effect of atrial natriuretic peptide (ANP) or phorbol 12-myristate 13-acetate (PMA) treatment on ANP-mediated cyclic guanosine monophosphate (cyclic GMP) formation by vascular smooth muscle cells. Cells were preincubated for the indicated periods of time with 1 μM ANP (▲), 1 μM PMA (●), or vehicle (○), prepared as described in "Methods." The formation of cyclic GMP by subsequent 1 μM ANP for 10 minutes was measured by radioimmunoassay in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Each point represents mean±SD of three determinations. *Significantly different from control; p<0.05.

**Figure 4.** Line graph shows phorbol 12-myristate 13-acetate (PMA) dose-dependency for 1 μM atrial natriuretic peptide (ANP)-mediated formation of cyclic guanosine monophosphate (cyclic GMP). Vascular smooth muscle cells were incubated for 4 hours with the indicated concentrations of PMA (●) or vehicle (○), prepared as described in "Methods" and stimulated with 1 μM ANP for 10 minutes, in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Results are mean±SD of triplicate determinations at each dose point. *Significantly different from control; p<0.05.

**Figure 5.** Line graphs show effect of phorbol 12-myristate 13-acetate (PMA) on rat arterial particulate guanylate cyclase. Panel A: Vascular smooth muscle cells were pretreated with (●) or without (○) 1 μM PMA for 4 hours and were incubated as described under "Methods" in the presence of various concentrations of atrial natriuretic peptide (ANP). Panel B: Vascular smooth muscle cells were pretreated with different concentrations of PMA for 4 hours and were incubated with (●) or without (○) 1 μM ANP as described in "Methods." Control, no PMA. Data presented are mean±SD of quadruplicate determinations; *p<0.05.
lack of effect of H-7 on basal cGMP levels suggest that basal PKC activity does not regulate cGMP formation. A dichotomy of the mechanisms for desensitization induced by these two classes of agents has been suggested for the β-adrenergic receptor adenylate cyclase system in rat glioma C6 cells using a similar approach. However, we cannot exclude the remote possibility that residual PKC activity may play a role in ANP-induced desensitization.

The mechanism of phorbol ester-induced desensitization of the ANP-B receptor is not clear. It has been shown that phorbol esters induce desensitization of adenylate cyclase activity to β-adrenergic agonists by PKC-mediated phosphorylation of the β-adrenergic receptor.

ANP-B receptors contain guanylate cyclase in the receptor molecule; it is unlikely that the activation of PKC by phorbol ester phosphorylates the ANP receptor to uncouple it from membrane-bound guanylate cyclase, leading to desensitization of the receptor response. Another possible explanation is that PKC alters the intrinsic enzymatic activity of the guanylate cyclase moiety. This is also unlikely since PKC did not change the basal guanylate cyclase activity in isolated membrane preparations. Similarly, the possibility that PKC may affect cGMP phosphodiesterase activity seems unlikely because the study of cGMP formation was performed in the presence of IBMX, a phosphodiesterase inhibitor. Therefore, the probable explanation is that PKC decreases the ANP-B receptor number.

We have shown in the present study that in VSMC, the maximal response of BNP-induced cGMP formation and its suppression by PMA was almost the same as that induced by ANP. It has been reported that in the ANP-A-receptor, cGMP formation by BNP was equal to that by ANP and that in the ANP-B-receptor as a subtype of biologically active ANP receptors, BNP more potently stimulated cGMP formation than did ANP; however, this study compared porcine BNP with ANP as an agonist, and there are species differences among porcine, human, and rat BNP in primary structure.

Reevaluation of subtypes of biologically active ANP receptors (ANP-A and ANP-B receptors) should be done using ANP, BNP, and cells of the same species.

In summary, we found that the exposure of rat VSMC to phorbol esters led to the desensitization of the ANP-mediated cGMP response. This observation resembled desensitization induced by preincubating the cells with ANP, except that the phorbol ester process was heterologous. It was evident, however, that phorbol ester-mediated suppression of receptor function is PKC-dependent, whereas ANP-induced desensitization is PKC-independent. Evidence supporting such divergent mechanisms of ANP receptor regulation includes the following: 1) time courses that differ; 2) desensitization...
of the ANP-B receptor response by phorbol ester observed in cells preincubated with ANP; 3) protein kinase inhibitor diminished the effect of phorbol esters without effect on ANP-induced desensitization; and 4) although long-term phorbol ester treatment caused a major loss in the ability of phorbol ester to desensitize ANP-mediated biochemical response, PKC-depleted cells showed unaltered ANP-mediated receptor desensitization.

In conclusion, alterations in ANP-B receptor sensitivity could be mediated by both PKC-dependent and PKC-independent mechanisms, triggered by phorbol esters and ANP, respectively.

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References

FIGURE 9. Line graphs show desensitization of the atrial natriuretic peptide (ANP)-mediated cyclic guanosine monophosphate (cyclic GMP) response induced by phorbol 12-myristate 13-acetate (PMA) or by ANP. Vascular smooth muscle cells were incubated with (A, e) and without (A, o) 1 μM phorbol 12, 13-dibutyrate for 24 hours and prepared for cyclic GMP measurements as described in "Methods." Panel A: Various concentrations of PMA were added for another 2 hours before ANP (1 μM) was added to induce the formation of cyclic GMP. Panel B: Cells were treated with various concentrations of ANP for 1 hour, washed, then stimulated with 1 μM ANP. Data are mean±SD of determinations performed in triplicate. *Significantly different from control; p<0.05.
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