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ENDOTHELIN INHIBITS THE ATRIAL NATRIURETIC FACTOR STIMULATED cGMP PRODUCTION BY ACTIVATING THE PROTEIN KINASE C IN RAT AORTIC SMOOTH MUSCLE CELLS*

Rama K. Jaiswal**

Department of Brain and Vascular Research, Cleveland Clinic Research Institute, 9500 Euclid Avenue, Cleveland, OH 44195

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Summary: Preincubation of rat thoracic aortic smooth muscle cells with endothelin inhibits the atrial natriuretic factor (ANF)-induced cGMP accumulation in these cells in a concentration dependent manner. The maximal inhibition of 64% was afforded by $1x10^{-6}$ M endothelin and the half maximal inhibition (IC_{50}) was achieved with $1x10^{-9}$ M endothelin. Endothelin ($1x10^{-6}$ M) also increased the plasma membrane bound protein kinase C (PKC) activity by 4 fold. Hormone-dependent increase in PKC activity was limited to plasma membranes only and some decrease in cytosolic PKC activity was observed. However, phorbol 12-myristate 13-acetate (PMA) ($1x10^{-6}$M) provoked a total loss of cytosolic PKC activity and a net gain in membranous PKC activity indicative of the translocation of the enzyme. Pretreatment of these cells with H-7, a PKC inhibitor, released the endothelin and PMA-mediated attenuation of ANF-stimulated cGMP formation. These results suggest that PKC is involved in the regulation of ANF-induced cGMP accumulation and that the vasoconstrictor activity of endothelin might involve inhibition of the vasorelaxant activity of ANF through the inhibition of cGMP accumulation in smooth muscle cells (SMCs) of the rat aorta.

Atrial natriuretic factor (ANF) is a peptide hormone synthesized in the heart which exerts several potent biological and physiological effects including the relaxation of smooth muscles (1-3). ANF directly activates the membrane-associated form of guanylate cyclase to increase intracellular cGMP (4) and the role of cGMP as a second messenger of the vasorelaxant effect of ANF is very well documented (5). Many physiological and pharmacological functions of ANF including its vasorelaxant effect are antagonized by vasoconstrictor peptides such as angiotensin II (Ang II) and arginine vasopressin (AVP). For example, Ang II down-regulates the ANF receptor in rat vascular SMCs (6) and inhibits the ANF-stimulated cGMP accumulation by stimulating the cGMP phosphodiesterase (7). In an established rat aortic smooth muscle cell line (A10), AVP, which shares many physiological and biochemical properties of Ang II, partially inhibits the ANF-

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** Present address: Room E504, Center for Neurosciences, School of Medicine, 2119 Abington Rd, Case Western Reserve University, Cleveland, OH 44106.

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stimulated cGMP accumulation by a mechanism independent of cGMP phosphodiesterase activation (8). Recently we have demonstrated that one of the regulatory mechanisms of cGMP-mediated ANF signal transduction is through protein kinase C (PKC) which negatively regulates the ANF receptor coupled guanylate cyclase in rat adrenocortical carcinoma cells (9,10).

PKC which has been well recognized in signal transduction induced by many hormones and growth factor (reviewed in 11,12) is also involved in the regulation of smooth muscle contraction (13). Many vasoconstrictor hormones such as Ang II, AVP and bradykinin have been shown to activate and translocate the PKC from cytosol to plasma membrane (14-16) where activated PKC translates the hormonal signal into a physiological response. Endothelin (ET), which is a newly discovered and very potent vasoconstrictor peptide of vascular endothelial origin (17), induces a rapid rise in intracellular inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG) levels derived from phospholipase C mediated hydrolysis of phosphoinositide 4,5, bisphosphate in rat SMCs (18). In view of our previous observations that the activation of PKC by PMA, attenuates the ANF stimulated cGMP accumulation in rat adrenocortical carcinoma cells and that ET induces a rapid rise in DAG levels which mimics the effects of PMA and would be expected to activate the PKC, we investigated in this study whether ET would attenuate the ANF-induced cGMP accumulation by activating the PKC in cultured rat aortic SMCs.

MATERIALS AND METHODS

The synthetic [Arg^{101}-Tyr^{126}]-ANF (26 amino acid rat sequence) used in this study was obtained from Peninsula Laboratory (Belmont, CA). Endothelin-1, Human, was obtained from Bachem (Torrance, CA). $Na^{125}I$ (reductant free) and [$\gamma^{32}P$] ATP were purchased from DuPont-New England Nuclear, (Boston, MA). cGMP radioimmunoassay kit and all other chemicals and cell culture reagents were obtained from Sigma (St. Louis, MO).

Cell culture and incubation conditions:

Pure vascular SMCs were isolated from thoracic aorta of 10 week old male Sprague Dawley rats by enzyme the dispersion method of Smith et al (19). The cells were subcultured by 0.2% trypsin and used for experiments at confluent growth between the second and fifth passages. The cells were subcultured in 24 well plates to a density of 10^5cells/well to determine the cGMP production or in 10 cm petri dishes to measure the PKC activity.

Measurement of cGMP

The cells were grown to confluency in 24 well plates and serum-deprived overnight prior to the experiments. The medium was removed and cells were washed two times with serum free M199 containing 0.5 mM isobutylmethylxanthine (IBMX) and the cells were preincubated for 10 min at 37^0 C in the same medium. Unless otherwise noted, 0.5mM IBMX was present throughout subsequent incubation period. After the preincubation period, media was replaced with fresh media (0.9 ml) containing various concentrations of ET. The cells were incubated for 5 min at 37^0C and then 0.1 ml of ANF solution was added and the incubation was continued for another 10 min. The media was aspirated at the end of incubation and 1.0 ml of 1M perchloric acid was added to each well and the cells were scraped and transferred to a plastic tube. After neutralization with 10 M KOH, the mixture was sonicated for 15 sec on ice and the tubes were kept at 4^0C for 30 min. The tubes were centrifuged at 1000 x g for 10 min and the supernatant was removed and used for cGMP determination by RIA after acetylation (20).

Preparation of the Cell Fractions for PKC Estimation

Cells grown in 10 cm petri dishes were washed two times with serum free M199 (10 ml) and stimulated by various agents. The medium was aspirated at the end of the incubation period and the cells were washed once with 20 mM Tris-HCl buffer pH 7.5 containing 5 mM DTT, 2 mM

EDTA, 2 mM EGTA, 0.25 M sucrose, 0.1 mM phenyl methyl sulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin (Buffer A). The cells were scraped in 2.5 ml buffer A and homogenized by sonication (30 sec). Homogenates from two petri dishes for each treatment were pooled. A 50 µl portion of the homogenate was taken for protein determination and the rest was centrifuged at 100,000x g for 35 min at 4°C. The supernatant was used as a cytosolic fraction and the pellet was solubilized by 1.0 ml of Triton X-100 (1% in buffer A) by incubation for 40 min on ice with occasional shaking. The solution was diluted to the original volume of homogenate with buffer A so that the final concentration of Triton X-100 would be 0.2%. The solubilized preparation was centrifuged at 100,000x g for 35 min and the supernatant obtained was used as the solubilized particulate fraction. The cytosolic and particulate fractions were used immediately for the partial purification of PKC by DE 52 chromatography.

Partial Purification of PKC

The crude extracts from the membrane and cytosolic fractions were applied to a 2.0 cm x 0.9 cm DE52 column equiliberated with buffer B (20 mM Tris-HCl, pH 7.5 containing 10 mM DTT, 2 mM EDTA and 2 mM EGTA). Columns were washed with 10 ml of buffer B and the kinase activity was eluted with a linear NaCl gradient (0-0.3M) in buffer B.

PKC Assay

Separate aliquots (100 µl) of the membrane and cytosolic fractions from the columns were assayed for the enzyme activity by measuring the transfer of ^{32}P from [$\gamma^{32}P$]ATP to histone III-S essentially following the method of Granet et al (21).

Other Methods

Protein determinations were done with the Bio Rad protein assay kit using bovine serum albumin as a standard.

RESULTS

As shown in Fig. 1, ANF in the presence of 0.5 mM IBMX stimulates the intracellular levels of cGMP in a time and dose dependent manner in cultured rat aortic SMCs. ANF ($1x10^{-7}$

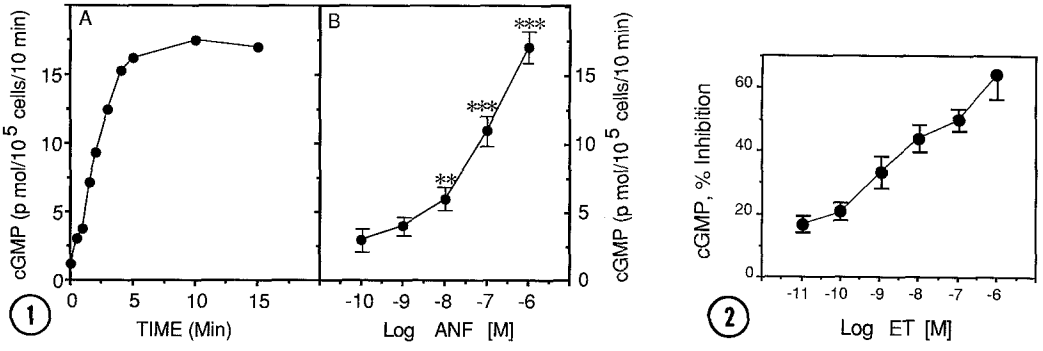

Fig. 1. Effect of ANF on intracellular cGMP formation in cultured rat thoracic aortic smooth muscle cells. [A] Time response curve for the formation of cGMP in response to 0.1 µM ANF and [B] dose response curve of ANF on formation of intracellular cGMP. Confluent cells ($1x10^5$ cells/well) were incubated at 37° C in 1.0 ml M199 containing 0.5 mM IBMX in the presence of various concentrations of ANF. cGMP contents were determined by radioimmunoassay after acetylation as described in "Materials and Methods". Each point is the mean of three determinations; bar indicates SEM statistically different from control: $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$.

Fig. 2. Dose response curve of ET inhibition of 0.1 µM ANF-stimulated cGMP formation in rat aortic smooth muscle cells. cGMP was determined by radioimmunoassay as described in "Material and Methods". Data are mean±SE of three separate experiments performed in duplicate.

M) induced a rapid rise in intracellular cGMP, observed as early as 30 sec and peaking at about 5 min and then remaining unchanged up to 15 min (Fig. 1A). When SMCs were stimulated with ANF at concentrations ranging from 1 x 10^{-6}M to 1 x 10^{-10}M, a concentration dependent increase in intracellular cGMP content was observed (Fig 1B). A highly significant rise in cGMP content ($P < 0.001$) was observed with 1 x 10^{-6}M and 1 x 10^{-7}M ANF. The concentration of ANF needed to induce a half maximal increase of cGMP in these cells was 1x10^{-9}M.

ET (1 x 10^{-6} M) did not have any effect of basal cGMP content in these cells. However, ET inhibited the intracellular cGMP production induced by 1 x 10^{-7}M ANF in a concentration dependent manner (Fig. 2). The maximal inhibition of 64% was achieved with 1 x 10^{-6}M ET and the IC_{50} value for ET (32% inhibition of cGMP accumulation induced by ANF) was 1 x 10^{-9}M. To determine whether ET can activate and translocate the PKC in rat SMCs, PKC activity was measured in both membranous and cytosolic fractions of SMCs that were treated with either 1 x 10^{-6}M ET or PMA for 5 min. PKC was partially purified by DE52 column chromatography; both membranous and cytosolic forms of PKC were eluted between 0.1 - 0.15M NaCl. PKC activity expressed as counts per minute of ^{32}P incorporated into histone per 10 min/100 µl of each fraction is shown in Figure 3. Almost all of the PKC activity was found in the cytosol of control cells, as shown in panel A. However, treatment of these cells with 1x10^{-6}M PMA, a tumor promoting phorbol ester which mimics endogenous diacylglycerol in binding protein kinase C to membrane phospholipids, thereby activating the enzyme (22), resulted in a complete translocation of PKC activity from the cytosol to the plasma membranes (Panel B). Treatment of these cells with 1x10^{-6}M ET for 5 min resulted in a major increase in particulate (membrane bound) PKC activity and at the same time a relatively small loss of cytosolic PKC activity was observed (Fig 3, panel C). Much of the increased PKC activity in particulate fraction can be explained by a shift of the enzyme from cytosol to membrane but a part of the increase in particulate PKC at this time appears unexplainable. This effect was different from that of PMA where PKC activity was completely translocated to the membrane fraction at the expense of cytosolic PKC activity.

In order to test that ET attenuates the ANF-stimulated accumulation of cGMP in rat SMCs by activating the PKC, I tested the ability of H-7, [1-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride), a widely used inhibitor of PKC (9,24) to block the effect of ET on ANF-stimulated cGMP in these cells. As it is observed in Fig. 4, preincubation of SMCs (10 min) with 0.1 mM H-7 before the addition of ET (1x10^{-6}M) completely released the inhibition caused by ET on the ANF-stimulated cGMP levels. ET did not significantly altered the basal levels of cGMP in these cells.

DISCUSSION

In the present study I have investigated the effect of ET on ANF induced cGMP accumulation in cultured rat SMCs. ET is a newly discovered potent vasoconstrictor and shares many physiological and biochemical properties of other endogenous vasoconstrictor peptides such as Ang II, and AVP. ANF stimulates cGMP production in aortic SMCs in a time and dose dependent manner indicating that these cells are responsive to the ANF signal. Furthermore, these data demonstrate that ET inhibits ANF-induced cGMP content in these cells in a concentration

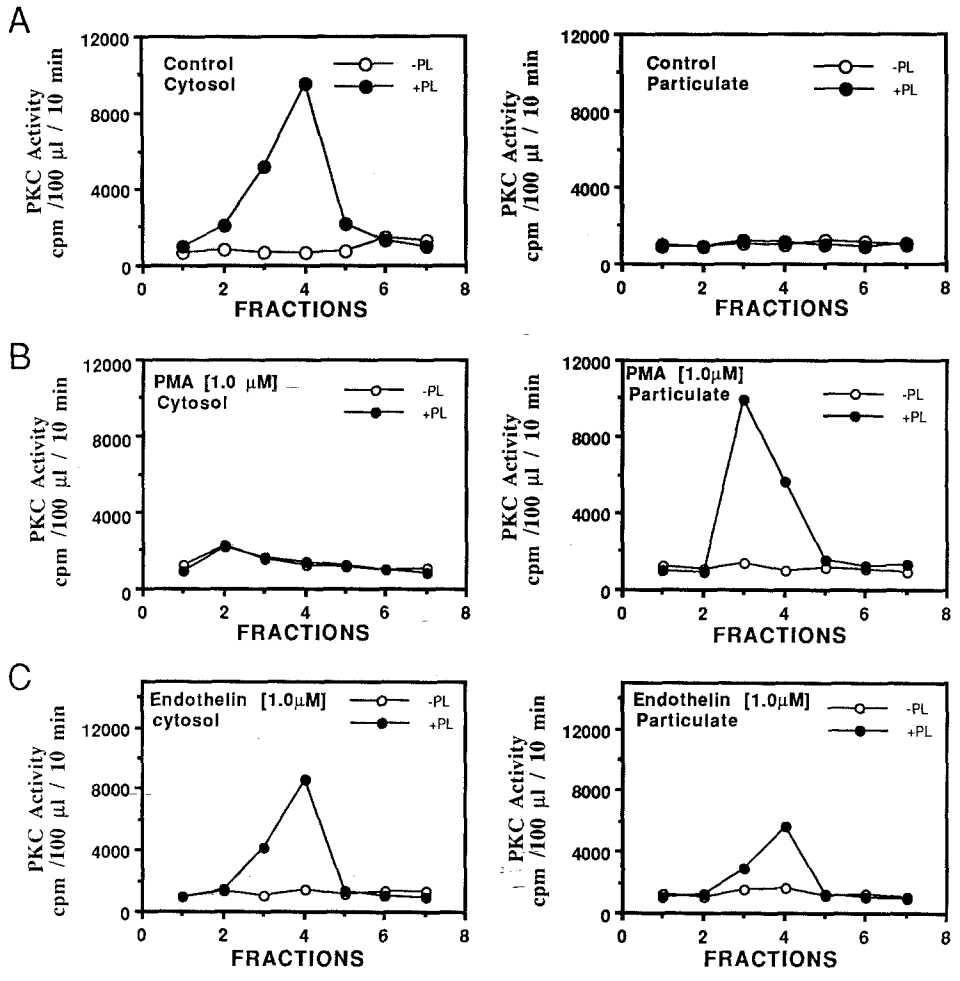

Fig. 3. Distribution of PKC activity in cultured rat aortic smooth muscle cells after treatment with PMA and ET. Cytosolic and membranous fractions were prepared as described in "Materials and Methods" from confluent cultures of aortic smooth muscle cells in a 10 cm petri dish untreated or incubated for 5 min with 1.0 μM PMA or 1.0 μM ET. The cytosol or the detergent solubulized fractions were applied to a DE52 column (2.0 cm x 0.9 cm) . After washing with buffer B the enzyme activity was eluted with a 0-0.3M NaCl gradient in buffer B. Fractions (2.5 ml) were collected and PKC activity was determined in 100 μl aliquots in absence (O) or presence (●) of phosphatidyl serine and diolein. PKC activity is expressed as cpm of ^{32}P incorporated/100 μl sample/10 min.

dependent manner. Inhibition of the ANF-induced cGMP accumulation by ET is not caused by activation of phosphodiesterase activity since all experiments were done in the presence of 0.5 mM IBMX, a potent phosphodiesterase inhibitor.

Inhibition of ANF induced cGMP accumulation by ET may involve PKC because 1) ET exerts a stimulatory effect on DAG production in rat and bovine vascular SMC (18,25) and therefore would expected to activate PKC and 2) our previous observations indicate that PMA (an activator of PKC) attenuates the ANF stimulated cGMP production in rat adrenocortical carcinoma cells (9,10). To determine whether ET activates PKC in rat aortic smooth muscle cells, the effect

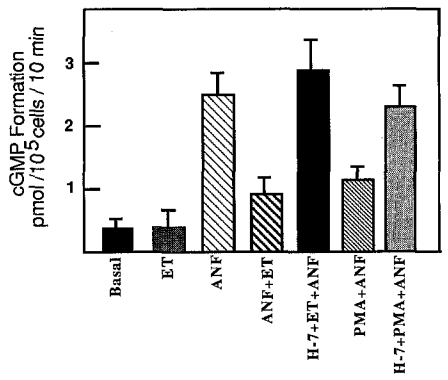

Fig. 4. Effect of PMA, ET and H-7 on the intracellular cGMP formation in rat aortic smooth muscle cells. The conditions of the experiments were identical to those in Fig. 1 except the wells containing H-7 (0.1 mM final) were preincubated for 10 min and then for another 5 min with 1x10-6M ET or PMA in the continuous presence of H-7. The cells were then stimulated with 0.01 μM ANF for another 10 min. Results are shown as the mean % SE (n=3).

of ET on PKC activity and its translocation from cytosol to the membrane was studied. We measured the PKC activity following ET treatment in both cytosolic and plasma membrane (membranous) fractions following partial purification by DE52 chromatography. The results presented here demonstrate that ET causes 4-fold stimulation of the membranous PKC activity over non treated control cells. The increase in membranous PKC activity was not associated with the corresponding decrease in the cytosolic PKC as was expected. Only a part of the increased PKC activity in membrane could be attributed to a translocation of the enzyme from cytosol. In contrast to ET, PMA was shown to increase the PKC activity in plasma membrane and simultaneously decrease the cytosolic PKC activity, suggesting that translocation had taken place. Similar effect of PMA has also been demonstrated in many other cell types (11). Certain hormone-receptor interactions like gonadotropin-releasing hormone, thyrotropin releasing hormone and phenylephrine, an α_1-adrenergic agonist also facilitate the translocation of PKC to the membrane at the expense of cytosolic activity (26-28), while certain other hormones like insulin promote a net increase in membrane PKC activity without translocation (29) and in some cases a net increase in both membrane and cytosolic PKC activity upon hormone stimulation (23). These results suggest that an increase in plasma membrane PKC activity is an important intracellular signal during hormonal stimulation. The differences in PKC activation by ET and PMA deserve at least some kind of explanation. First, it is believed that PKC represents the phorbol ester "receptor" in various tissues (30) and the effect of PMA on the cellular PKC system could be possibly explained by an intercalation of the PMA-PKC complex into the cell membrane. Secondly, ET induced changes in the regulation of PKC system may be more complex than those provoked by PMA. Finally, demonstration of multiple forms of PKC (31), activation of specific isozymic form of PKC by different hormones (15), and substrate specificities (32) further underscore the complexity of PKC system and may ultimately provide insight into variation in PKC activation profiles and subsequent physiological response.

I have also investigated the ability of a PKC inhibitor, H-7, on the ET-mediated inhibition of ANF-induced cGMP production in rat aortic SMCs. One would expect that the presence of H-7 will prevent the inhibition of ANF-induced cGMP accumulation if it caused by ET mediated activation of PKC in these cells. As we expect, preincubation of rat aortic SMCs with $1x10^{-4}$M H-7 completely prevented the inhibition of ANF-induced cGMP production by ET. The relatively higher concentration of H-7 that is required to suppress the effect of ET compared with the concentration needed to inhibit the purified PKC (33) might reflect a decreased ability of these SMCs to incorporate H-7 into the cytosol or a faster metabolic inactivation of H-7 by cytosolic enzymes.

PKC has been shown to be a major stimulus for the contraction of the smooth muscles (13). The direct activation of PKC by PMA induces a slowly developing and sustained contractile response in vascular smooth muscles (34,35). The ET-stimulated increase in PKC may therefore be an important factor in generating long term tonic vasoconstriction and presumable this contraction is facilitated, in part, by the ability of the ET to antagonize the vasorelaxant effect of ANF through the inhibition of the cGMP production in SMC.

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