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ANGIOTENSIN II AND PHORBOL ESTER ENHANCE ISOPROTERENOL- AND VASOACTIVE INTESTINAL PEPTIDE (VIP)-INDUCED CYCLIC AMP ACCUMULATION IN VASCULAR SMOOTH MUSCLE CELLS

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The importance of Ca^{2+} and cAMP in the regulation of cellular functions has been well demonstrated. We studied the effect of angiotensin II (AII), a potent Ca^{2+}-mobilizing hormone, on cAMP accumulation induced by isoproterenol (ISO) and vasoactive intestinal peptide (VIP) in cultured vascular smooth muscle cells (VSMC). Although the addition of AII alone caused little increase of cAMP, it enhanced ISO- and VIP-induced cAMP accumulations in a dose-dependent manner. This enhancement was mimicked by tumor-promoting phorbol ester but not by Ca^{2+} ionophore. This observation suggested that AII enhanced agonist-induced cAMP accumulation through the activation of protein kinase C in VSMC.

It is well documented that two major groups of humoral agents play important roles in the regulation of vascular smooth muscle contractility. One of them induces the contraction of vascular smooth muscle through the increase of $[Ca]_i$, while the other causes the relaxation through the increase of cAMP (1). It is, thus, possible that these two second messenger systems interact with each other to control the tension of vascular smooth muscle. Based on this hypothesis, effects of Ca^{2+} and of AII on adenylate cyclase activity were investigated in microsomes from vascular smooth muscle (2,3), however, little is known about the interaction of these two systems in intact VSMC.

In previous reports, we showed that cultured VSMC had receptors for AII and β-adrenergic reagents which increase $[Ca^{2+}]_i$ and cAMP, respectively (4,5). Further, our report indicated that the addition of AII elicited

Abbreviations: VSMC, vascular smooth muscle cells; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; AII, angiotensin II; ISO, isoproterenol; VIP, vasoactive intestinal peptide; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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breakdown of phosphotidylinositol in VSMC (5); this cellular event has been postulated to increase both $[Ca^{2+}]_i$ and the formation of diacylglycerol, a presumed physiological activator of PKC (6).

In this communication, we report that AII synergistically enhances the cAMP accumulation induced by ISO or VIP. The possible involvement of PKC activation in this potentiation will be discussed.

Methods

Cultured VSMC were obtained from thoracic aortas of rats by modification of the explant method of Ross (5,6). Age- and sex-matched Wistar Kyoto rats and spontaneously hypertensive rats were the source thoracic aortas. Stock cultures were kept in the modified medium 199 supplemented with 10% fetal bovine serum (4). Cells of the 3rd to 15th passage were used in experiments.

Cyclic AMP determination was performed by modifications of the method described elsewhere (4). VSMC were grown in 6 well cluster plates to reach confluency and were incubated for additional 24-48 h in serum-free medium. Cells were washed with the assay buffer (NaCl 133 mM, KCl 3.6 mM, $CaCl_2$ 1.0 mM, $MgCl_2$ 0.4 mM, D-glucose 16.0 mM, N-2-hydroxethylpiperazine N'-ethane-sulfonic acid 3.0 mM, pH 7.4). VSMC were preincubated at 37°C for 5 min in 1.0 ml assay buffer in the presence or absence of 1.0 mM theophylline as indicated in figure legends. Following the preincubation, cells were incubated for additional 5 min with various test reagents. Preliminary experiments showed that cAMP accumulations induced by VIP and ISO reached maximal values at 5 min. Reagents employed in experiments were VIP, ISO, AII, A23187, and PMA. The reaction was terminated with the addition of ice-cold assay buffer containing 1.0 mM theophylline and cAMP formed was extracted with ice-cold 0.3 N perchloric acid. The extract was neutralized with 3.0 N $KHCO_3$ and kept at -80°C until cAMP was quantified.

cAMP was measured by radioimmunoassay after it was acetylated or succinylated. These procedures increased the sensitivity of radioimmunoassay to the femtomole order (7,8). VSMC in an additional 3 wells of a cluster plate were trypsinized and used in the determination of cell population. The cell population of each dish varied with a standard error of less than 10%.

Because no apparent differences were observed between the responses of VSMC from Wistar Kyoto rats and spontaneously hypertensive rats, results of VSMC from the two strains were combined. Student's t-test was employed for statistical analysis.

ISO, AII, and PMA were from Sigma. VIP was obtained from Peninsula Laboratory. A23187 was from Calbiochem. Cyclic AMP assay kits were purchased from New England Nuclear and Yamasa (Choshi, Japan). Culture medium, supplements, and fetal bovine serum was obtained from Gibco.

Results

Fig. 1 shows that, in the presence of 1.0 mM theophylline, AII augmented VIP-induced cAMP accumulation in a dose-dependent manner. The addition of AII alone had only a small effect on the formation of cAMP. AII increased the

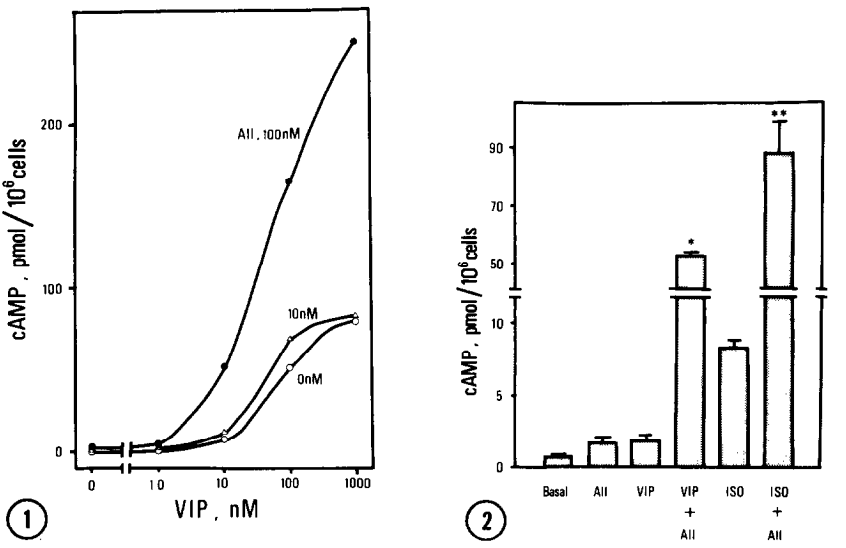

Fig. 1 Effect of various concentrations of AII on VIP-induced cAMP accumulation.
Cultured VSMC were prepared as described in Methods. Following 5 min preincubation, VSMC were further incubated with VIP and/or AII at various concentrations as indicated, for 5 min in the presence of 1.0 mM theophylline. Cyclic AMP was extracted with ice-cold perchloric acid and quantified by radioimmunoassay as described in Methods. Each point indicates mean value of 3 determinations from a representative experiment.

Fig. 2 Effects of AII on VIP- or ISO-induced cAMP accumulation in the absence of theophylline.
Cultured VSMC were prepared as described in Methods. After 5 min preincubation in the assay buffer without theophylline, cells were further incubated for 5 min with test reagents as indicated. Final concentratoins of the test reagents were; AII, 100 nM; ISO, 1.0 μM; and VIP, 1.0 μM. Extraction and measurement of cAMP were performed as described in Methods. Each bar indicates a mean ± SE of 3 determinations from a representative experiment.
$^{*}p<0.01$ compared to VIP only, $^{**}p<0.01$ compared to ISO only.

maximal cAMP accumulation elicited by VIP whereas it did not seem to alter the EC_{50} value. AII similarly enhanced ISO-induced cAMP accumulation (data not shown).

This enhancement of agonist-induced cAMP accumulation by AII was also observed in the absence of the phosphodiesterase inhibitor, theophylline (Fig. 2), suggesting that this phenomenon could occur in in vivo.

VIP alone elicited only a small increase of cAMP in the absence of theophylline (Fig. 2). This was probably due to secondary activation of phosphodiesterase because VIP induced more than ten-fold increases of cAMP in the presence of theophylline (see Fig. 1 and the legend of Fig. 3). On the

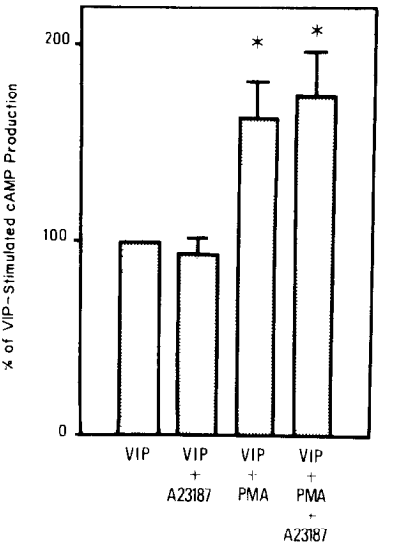

Fig. 3 Effects of A23187 and/or PMA on VIP-induced cAMP accumulation. VSMC grown in 6 well cluster plates were prepared and preincubated with 1.0 mM theophylline as described in Methods. Cells were incubated for 5 min with 1.0 μM VIP alone or with 50 nM A23187 and/or 10 nM PMA as indicated. After the termination of incubation, cAMP was extracted and quantified as described in Methods. Cyclic AMP accumulation was represented as per cent of the VIP-stimulated accumulation (12.0 ± 3.7 pmole/10^6 cells, mean ± SE of 6 experiments). Basal level of cAMP was 1.5 ± 0.3 pmole/10^6 cells (mean ± SE of 6 experiments). Each bar indicates mean ± SE of 4 to 6 experiments. *$p<0.025$ compared to VIP only.

contrary, AII had little effect on cAMP accumulation either in the presence or the absence of theophylline (Fig. 1 and Fig. 2).

Recently, it was indicated that the effects of Ca^{2+}-mobilizing hormones in various cell systems were mimicked by Ca^{2+} ionophore and PKC-activating phorbol esters (9-11). In some cells, these two stimulants were shown to act synergistically (6,9,12). To examine the effects of Ca^{2+} ionophore and of phorbol ester on cAMP accumulation in VSMC, A23187 and/or PMA were without VIP and ISO, elicited little increase of the amount of cellular cAMP (data not shown), however, PMA significantly enhanced VIP-induced cAMP accumulation (Fig. 3). This enhancement was dose-dependent and was observed in the concentrations of PMA which activated PKC (Fig. 4 and see Ref. 12). On the contrary, A23187, either with or without PMA, did not further augment agonist-induced cAMP accumulation (Figs. 3 and Table 1).

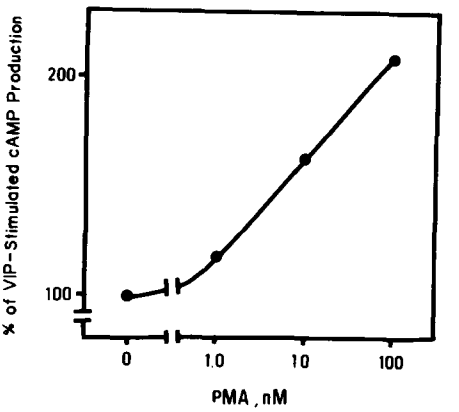

Fig. 4 Effects of various concentrations of PMA on VIP-induced cAMP accumulation.
VSMC grown in 6 well cluster plates were prepared and preincubated with 1.0 mM theophylline as described in Methods. One μM VIP alone or with PMA at various concentrations as indicated were added to cell cultures. After the incubation was terminated, cAMP was extracted and quantified as described in Methods. Cyclic AMP accumulation was represented as per cent of VIP-stimulated cAMP accumulation (12.0 ± 3.7 pmole/10^6 cells, mean ± SE of 6 experiments). Each point indicates mean of 2 to 6 experiments.

Discussion

Our results clearly show that AII, a potent Ca^{2+}-mobilizing hormone, synergistically enhanced VIP- and ISO-induced cAMP accumulation in VSMC. This enhancement was also observed in the absence of phosphodiesterase inhibitor,

Table 1. Effect of A23187 on cAMP accumulation induced by ISO and PMA

Addition	cAMP pmole/10^6 cells
basal	1.17 ± 0.03
ISO	54.0 ± 4.4
ISO + PMA	91.9 ± 2.3
ISO + PMA + A23187 1.0 nM	90.7 ± 1.7
ISO + PMA + A23187 10 nM	89.5 ± 2.7
ISO + PMA + A23187 100 nM	59.8 ± 4.2

Cells were prepared and incubated in the presence of 1.0 mM theophylline as indicated in Methods. The concentrations of reagents added were; ISO, 1.0 μM; PMA, 10 nM. Each value indicates a mean ± SE of 3 determinations from a representative experiment.

theophylline, thus AII-mediated augmentation of cAMP accumulation probably occurs in in vivo.

In the study on the membrane preparation from vascular smooth muscle, Anand-Srivastava observed that AII decreased agonist-stimulated adenylate cyclase activity (3). Our results differed substantially from this observation. This descrepancy may be due to the difference of experimental conditions; in our experiments, we used intact VSMC in which the effects of AII were mediated by the integrated second messenger systems such as Ca^{2+}-calmodulin dependent protein kinase and PKC (6,12). This integration of second messenger systems may be damaged in the process of membrane preparation.

This work also indicates that, in VSMC, PMA mimicked the action of AII to enhance agonist-induced cAMP accumulation. In contrast, A23187 did not enhance cAMP accumulation induced by VIP or ISO. A23187 at higher concentrations rather antagonized the effect of PMA on ISO-induced cAMP accumulation (Table 1). This is consistent with the observation by Piascik et al., which showed that high concentrations of Ca^{2+} decreased adenylate cyclase activity in vascular smooth muscle microsomes (2). As AII elicited sharp increase followed by immediate return of $[Ca^{2+}]_i$ to basal levels and prolonged breakdown of phosphoinositides in VSMC (5), the observed enhancement of agonist-induced cAMP accumulation by AII at 5 min was probably mediated by PKC activation induced by diacylglycerol.

A recent observation indicated that PKC-activating phorbol esters enhanced agonist-induced cAMP accumulation in pinealocytes (13) and in S49 lymphoma cells (14). Thus, this modulation of cAMP metabolism by phorbol esters may be a common mechanism, at least, in some cell systems.

We do not know what process(es) of cAMP metabolism are modulated by AII and PMA. In the recent report on a lymphoma cell line, Bell et al. suggested that the interaction of GTP-binding stimulatory unit and catalytic unit of adenylate cyclase was facilitated by PKC-activating phorbol esters (14). Further investigations are needed to find whether the same mechanism regulates

adenylate cyclase activity in VSMC. Our results indicate that synergistic enhancement of cAMP accumulation by AII is mediated by the stimulation of cAMP production rather than by the inhibition of cAMP degradation, because AII enhanced agonist-induced cAMP accumulation in the presence of 1.0 mM (Fig. 1) or 10 mM (data not shown) theophylline which inhibited phosphodiesterase activity.

In summary, it appears that, in VSMC, AII synergistically enhanced ISO- and VIP-induced cAMP accumulations which were mimicked by PMA but not by A23187. The mechanisms of this enhancement are under investigation.

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