Regulation of type V adenylyl cyclase by PMA-sensitive and -insensitive protein kinase C isoenzymes in intact cells

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Abstract Type V adenylyl cyclase (AC) was stably overexpressed in HEK293 cells (293AC-V). Forskolin-stimulated cAMP accumulation in 293AC-V was 5 times as great as that in control cells. PMA, a protein kinase C (PKC) activator, enhanced cAMP accumulation in 293AC-V cells dose-and time-dependently and this enhancement was abolished by staurosporine. Insulin also enhanced cAMP accumulation in 293AC-V cells. Co-transfection of PKC-ζ, but not PKC-α, potentiated the effects of insulin. These data suggest that type V AC activity is regulated in cells by PKC isoenzymes through different extracellular stimuli.

Key words: Adenylylcyclase; Protein kinase C; Phorbol ester; Insulin; cyclic AMP

1. Introduction

Regulators of adenylyl cyclase (AC) activity are not restricted to G_s and G_i proteins; protein kinase C (PKC), a major mediator in the phosphatidylinositol signal, is another key regulator. Over years, numerous investigators have demonstrated that activation of PKC by phorbol esters alters the intracellular cAMP levels in various tissues and cultured cells. However, the direction and degree of this alteration have been variable among studies [1]. Investigators have tried to explain this variability by the different sites of interaction; PKC may phosphorylate multiple components within the cAMP signaling system, such as receptors, G proteins and AC [2–5].

Although phorbol ester has been used as a classic PKC activator, recent cloning studies have elucidated the presence of a group of PKC isoenzymes, i.e. atypical PKC isoenzymes, that are insensitive to diacylglycerol, and thus to phorbol ester [6]. These isoenzymes are difficult to study because of the lack of specific activators and inhibitors [7,8]. More recent studies have suggested that atypical isoenzymes are activated by extracellular stimuli such as insulin and other growth factors [9–11].

In our previous report using purified enzymes, we have shown that PKC directly phosphorylates and activates type V AC in a PKC isoenzyme-dependent manner [5]. In particular, we have demonstrated that PKC-ζ, an atypical isoenzyme, activates type V AC in vitro. In this study, we have investigated (1) whether the phorbol ester treatment of intact cells recapitulates the activation of type V AC by PKC observed in vitro and (2) whether an extracellular stimulus that potentially activates PKC-ζ also activates type V AC in intact cells.

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Abbreviations: AC, adenylylcyclase; PKC, protein kinase C; IBMX, 3-isobutyl-1-methylxanthine; PMA, phorbol 12-myristate 13-acetate; αPDD, 4α-phorbol 12,13-didecanoate.

2. Materials and methods

2.1. Stable transfection of human embryonic kidney (HEK) cell

HEK 293 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 95% air/5% CO_2 incubator. Cells were transfected with either the type V AC cDNA in pcDNAneo (AC-V) or pcDNAneo alone (control) using the calcium phosphate method. Neomycin-resistant cells were selected in culture medium containing G418 (500 μg/ml).

2.2. Transient transfection of HEK 293 cell

Entire PKC-α or PKC-ζ coding cDNA were constructed in the SRD expression vector [12]. These expression vectors were transfected into 293AC-V cells using the double transfection method as described previously [13]. 1 day after transfection, the cells were subdivided into 12-well plates and incubated for an additional 2 days prior to [^{3}H]adenine labeling.

2.3. [^{3}H]Adenine labeling and cAMP accumulation assay

Cyclic AMP accumulation in intact cells was measured according to the method of Wong et al. [14]. The cells were placed in a serum-free culture medium containing 0.1% bovine serum albumin and [^{3}H]adenine (0.5 μCi/well) for 18 h. The cells were then washed once with a HEPES balanced salt solution (HBSS) (137 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 10 mM D-glucose and 0.2% phenol red) and pretreated with HBSS containing 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) and various reagents for 10 min. Reactions were started by adding 10 μM forskolin and other reagents, and terminated by addition of 12% (w/v) trichloroacetic acid, 0.25 mM ATP and 0.25 mM cAMP after the indicated times. [^{3}H]ATP and [^{3}H]cAMP were separated according to the method of Salomon et al. [15]. cAMP production is expressed as [^{3}H]cAMP/([^{3}H]cAMP + [^{3}H]ATP)×10^3. All assays were performed in duplicate or triplicate, and the values are shown as means ± S.E.M. Student's *t*-test was used for comparisons of groups.

3. Results and discussion

3.1. Effects of PMA on cAMP accumulation in 293 cells

In cells stably transfected with type V AC (293AC-V), forskolin-stimulated cAMP accumulation was 5 times as great as that in control cells. Treatment of 293AC-V cells with PMA (100 μM), PKC-activating phorbol ester, significantly enhanced forskolin-stimulated cAMP accumulation (Fig. 1). Unstimulated cAMP accumulation in 293AC-V cells, as well as cAMP accumulation in control cells (Fig. 1), was enhanced slightly by the same treatment (data not shown).

3.2. Time course of cAMP accumulation

Forskolin increased cAMP accumulation in 293AC-V cells

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in a time-dependent manner. The accumulation reached a plateau at 20 min after initiating incubation. The time course of cAMP accumulation was similar regardless of the treatment with PMA (Fig. 2).

3.3. PMA dose response of cAMP accumulation

PMA treatment of 293AC-V cells enhanced cAMP accumulation in a dose-dependent manner with an EC_{50} value of 2×10^{-7} M (Fig. 3). αPDD, an inactive phorbol ester analog, had no effects on cAMP accumulation. Maximal cAMP accumulation was achieved with 100 μM PMA. At this concentration, the cAMP levels were increased by 50–100%.

3.4. Effects of staurosporine on PMA-induced cAMP accumulation

Staurosporine, a potent and specific PKC inhibitor, abolished the PMA-induced enhancement of cAMP accumulation in 293AC-V cells (Fig. 4).

The above data indicate that PMA enhanced cAMP accumulation in 293AC-V cells through activation of endogenous, PMA-sensitive PKC. Since type V AC is responsible for most of the catalytic activity in 293AC-V cells (~80%), we conclude that type V AC is stimulated by phorbol ester in intact cells. It was previously shown that phorbol esters stimulate both Ca/calmodulin-sensitive (type I and III) and Ca/calmodulin-insensitive (type II) isoforms [16–18]. Thus, stimulation of AC by phorbol ester may be a more general phenomenon among AC isoforms although the exact mechanisms and degree of this stimulation may vary among them.

We also examined the effects of purified PKC-α on type V AC in membranes prepared from mammalian cells (CMT cells) or insect cells (High Five cells). However, the maximal stimulation of type V AC in such membrane preparations was weaker (10–20%) than that obtained in intact cells (~100%, see Fig. 1) (data not shown). We do not know, however, why PKC could achieve higher stimulation of type V AC in intact cells than in membrane preparations. Another PMA-sensitive PKC isoenzyme, which stimulates type V AC more potently than PKC-α, may exist in intact cells. Alternatively, proteins that facilitate the transportation of PKC to type V AC in the membrane may be involved in intact cells. It has been shown that PKC translocates to the membrane upon stimulation [6]. The presence of proteins has been demonstrated, such as RACK (receptors for activated C-kinase), that recognize an active form of PKC and specify the localization of PKC isoenzymes within the cell [19,20]. These cellular trafficking mechanisms may facilitate the encountering of PKC with type V AC in intact cells, achieving the optimal stoichiometry for the stimulation of type V AC in the membrane. Indeed, when both enzymes, PKC and type V AC, were purified and incubated together, the maximal stimulation of type V AC by PKC increased significantly as shown by us previously [5].

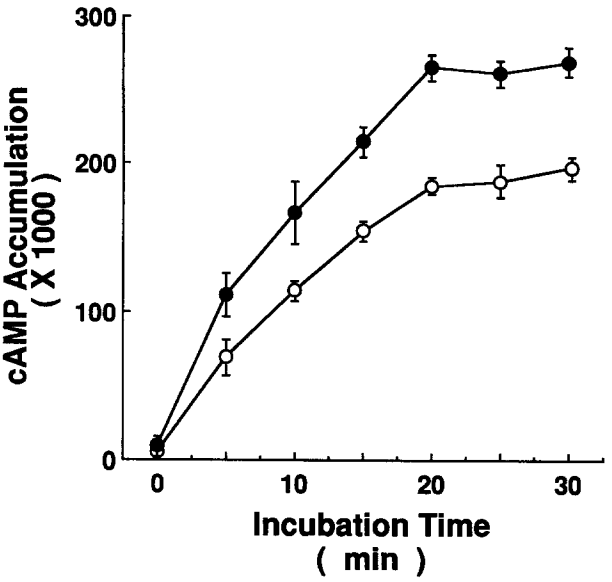

Fig. 2. Time course of cAMP accumulation in 293AC-V cells stimulated by PMA. 293AC-V cells were pre-treated with 100 nM PMA (closed circles) or Me_2SO (open circles) for 10 min, followed by incubation in the presence of 10 μM forskolin for the indicated time. Cyclic AMP accumulation was determined as described in section 2.

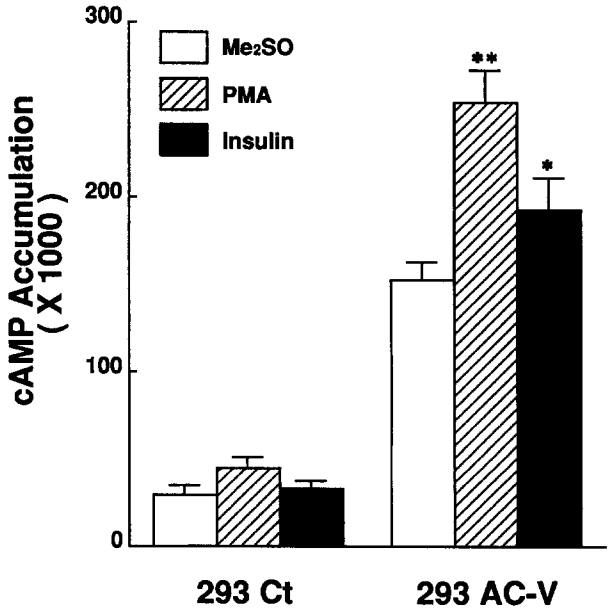

Fig. 1. Effects of phorbol ester on 293AC-V and control cells. 293AC-V (293 AC-V) and control (293 Ct) cells were treated with Me_2SO (vehicle) (open bars), 100 nM PMA (shaded bars), or 100 nM insulin (closed bars) for 10 min, followed by stimulation with 10 μM forskolin for 30 min. Cyclic AMP accumulation was determined as described in section 2. $^*P < 0.05$; $^{**}P < 0.01$ vs. Me_2SO (n = 7–12).

3.5. Effects of insulin on cAMP accumulation in 293 cells

We also examined the effects of insulin on cAMP accumulation in 293AC-V cells (Fig. 1). Insulin enhanced forskolin-stimulated cAMP accumulation although the degree of enhancement was lower (~20%) than that by PMA (50–100%).

3.6. Effects of coexpression of PKC on PMA, insulin-stimulated cAMP accumulation

In order to examine the contribution of specific PKC isoenzymes to the enhancement of cAMP accumulation in 293AC-V cells, we transiently overexpressed PKC-α, a conventional isoenzyme, and PKC-ζ, an atypical isoenzyme [6]. We have chosen a transient PKC expression system since

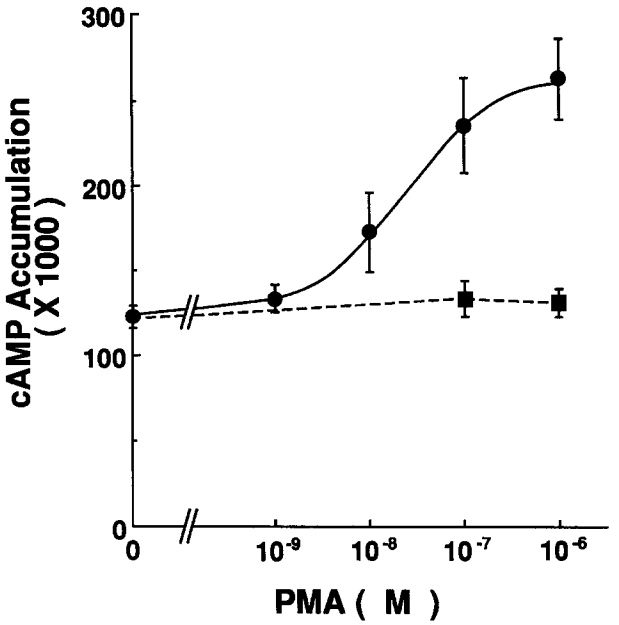

Fig. 3. Dose-dependent stimulation of type V AC by phorbol esters. 293AC-V cells were treated with either αPDD, an inactive phorbol ester (squares), or PMA (circles) for 10 min, followed by incubation in the presence of 10 μM forskolin for 30 min. Cyclic AMP accumulation was determined as described in section 2.

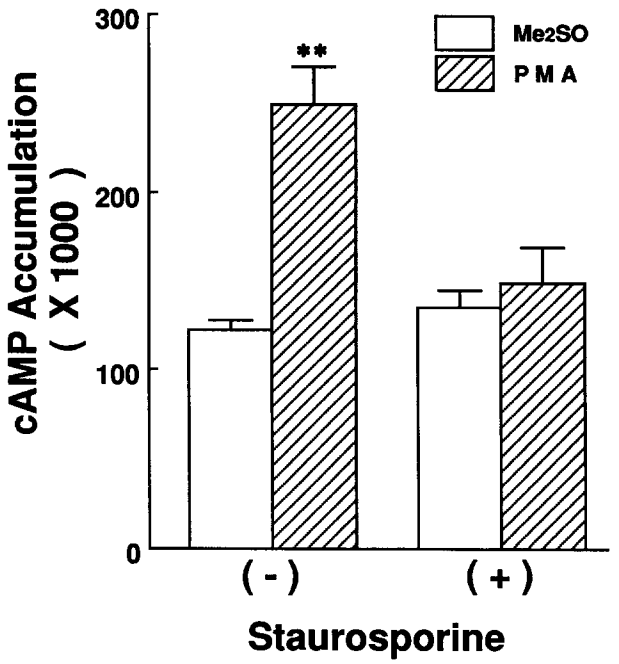

Fig. 4. Effects of staurosporine on the PMA-mediated enhancement of cAMP accumulation. 293AC-V cells were pre-incubated with 100 nM staurosporine (+) or its vehicle, Me_2SO (−) for 30 min, followed by treatment with 100 nM PMA or Me_2SO for 10 min. The cells were then incubated in the presence of 10 μM forskolin. Cyclic AMP accumulation was determined as described in section 2. $^{**}P < 0.01$ vs. Me_2SO ($n = 4$).

stable co-expression of PKC may modify the expression of AC in the cell.

Overexpression of these PKC isoenzymes by itself did not alter cAMP accumulation in 293AC-V cells (control cells, 152.5 ± 10.0; cells overexpressing PKC-α, 162.0 ± 15.5; PKC-ζ, 164.1 ± 10.1, n = 6–13, P = N.S.). PMA treatment of these cells enhanced cAMP accumulation by 60–80% (Fig. 5). However, the degree of enhancement did not differ among these cells, including those overexpressing PKC-α, a PMA-sensitive isoenzyme. The amount of endogenous PMA-sensitive PKC isoenzymes in 293 cells may already be functionally saturated to stimulate type V AC activity. Alternatively, PKC-α may not be involved in cross-talk between the cAMP signal and the diacylglycerol signal in 293 cells.

Insulin enhanced cAMP accumulation in 293AC-V by ~15% (Fig. 1). In clear contrast to the above results, when the cells were transfected with PKC-ζ, the insulin-mediated enhancement of cAMP accumulation was further potentiated (~30%) (Fig. 5). Cells overexpressing PKC-α did not show any further enhancement, indicating that the effect of insulin on cAMP accumulation was unique to the expression of PKC-ζ.

In contrast to the conventional PKC isoenzymes that are activated by Ca and diacylglycerol, PKC-ζ is activated by arachidonic acid and phosphatidylinositol 3,4,5-triphosphate (PIP3) [9,10], which are products of insulin or other growth factor receptor stimulation [21,22]. Indeed, a recent study has shown that PKC-ζ is involved in the physiological functions of insulin in vivo [11]. Stimulation of these receptors initiates a cascade of phosphorylation reaction within the cell, promoting cellular growth and differentiation [23]. It is interesting to note that investigators have demonstrated that insulin also modulates the cAMP signal [24,25]. Our study suggests that insulin regulates the cAMP signal at the level of AC through activating PKC-ζ. A more recent study has demonstrated that epidermal growth factor enhances cAMP production [26] and that this enhancement is unique to the type V isoform [27].

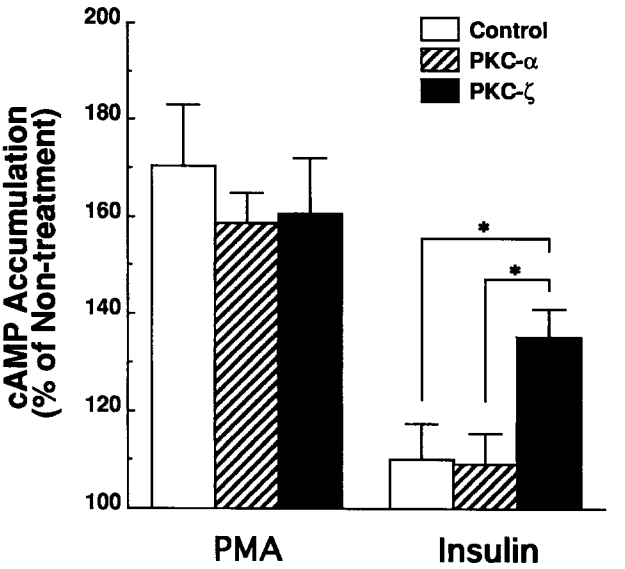

Fig. 5. Transient expression of PKC isoenzymes and its effect on 293AC-V cells. 293AC-V cells were transiently transfected with control plasmid (open bars), PKC-α (shaded bars), and PKC-ζ (closed bars). These cells were pre-incubated with 100 nM PMA (PMA) or 100 nM insulin (Insulin) for 10 min, followed by incubation in the presence of 10 μM forskolin. Cyclic AMP accumulation was determined as described in section 2. The data are shown as % increases from the cAMP accumulation in non-treated cells. $^{*}P < 0.05$ (n = 6–12).

We have previously shown that PKC-ζ stimulates type V AC more potently than PKC-α [5]. We do not know, however, whether the stimulation of AC by insulin is unique to the type V isoform.

In summary, the catalytic activity of type V AC increased in response to PMA and insulin stimulation. In particular, the effect of insulin was augmented by transfecting PKC-ζ. These data suggest that type V AC is regulated by both PMA-sensitive and-insensitive PKC isoenzymes in intact cells, coordinating the activities of these two signal transduction pathways at the level of this enzyme.

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