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Conformation-Dependent Activation of Type II Adenylyl Cyclase by Protein Kinase C

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Abstract Phorbol ester treatment enhanced the catalytic activity of type II adenylyl cyclase overexpressed in insect cells. In cells coexpressing type II adenylyl cyclase and protein kinase C-α, type II adenylyl cyclase catalytic activity was higher even in the absence of phorbol ester treatment; phorbol ester treatment further and markedly enhanced type II adenylyl cyclase catalytic activity. However, this enhancement, either by phorbol ester treatment or by coexpression of protein kinase C-α, was lost following membrane solubilization with detergents. This attenuation was unaffected by phosphatase inhibitor or salts. In contrast, membrane solubilization did not affect forskolin-stimulated type II adenylyl cyclase catalytic activity. Purified type II adenylyl cyclase was stimulated by forskolin and Gsα, but not by protein kinase C-α. Therefore, a specific mammalian protein kinase C isoenzyme can activate type II adenylyl cyclase, but the mechanism clearly differs from that underlying either Gsα- or forskolin-mediated stimulation. J. Cell. Biochem. 64:492–498. © 1997 Wiley-Liss, Inc.

Key words: adenylylcyclase; protein kinase C; crosstalk; conformation; detergent

A major crosstalk exists between the two signaling pathways, adenylyl cyclase (AC) and protein kinase C (PKC) [Sibley et al., 1987; Houslay 1991]. Over years, scientists have investigated the mechanism of this crosstalk at multiple levels within the two signaling pathways. This crosstalk, however, now seems to be complex because recent molecular cloning studies have revealed that the two enzymes, PKC and AC, are made of multiple subtypes with diverse biochemical properties, and tissue distribution [Ohno et al., 1991; Taussig and Gilman, 1995].

ACII is expressed in the brain, airway smooth muscle cells, and vascular endothelial cells from different sources [Feinstein et al., 1991; Pyne et al., 1994; Manolopoulos et al., 1995]. Biochemically, this isoform is insensitive to calcium/calmodulin, but is stimulated by G$\beta\gamma$ subunits [Taussig and Gilman, 1995]. The fact that ACII integrates signals from G proteins and various receptors, including chemotactic and growth factor receptors [Lustig et al., 1993; Pyne et al., 1994; Tsu et al., 1995], suggests that ACII plays a major role in crosstalk with other signaling pathways. Two independent studies demonstrated that the catalytic activity of ACII expressed in COS cells was increased upon phorbol ester treatment [Yoshimura and Cooper, 1993; Jacobowitz et al., 1993]. The degree of stimulation of this isoform by phorbol ester was greater than that of any other AC isoforms. More recently, phosphorylation and stimulation of ACII by insect PKC was also demonstrated [Jacobowitz and Iyengar, 1994], suggesting that ACII is the major AC isoform contributing to the crosstalk with PKC. How-

Abbreviations used: AC, adenylyl cyclase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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ever, these findings are controversial because mammalian PKC partially purified from the brain did not activate ACII [Lustig et al., 1993], thereby suggesting that PKC does not directly interact with ACII. The reasons for these opposing findings remain unknown.

In this study we attempt to answer two questions. First, can a specific mammalian PKC isoenzyme (PKC-α) potentiate the catalytic activity of ACII? It is known that each cell type expresses a different mixture of PKC isoenzymes, which may have distinct substrate specificity [Sheu et al., 1990; Hsie et al., 1991]. Second, does such potentiation of ACII catalytic activity occur by the same mechanism as those by forskolin and Gsα? Our results showed that ACII was stimulated by PKC-α; however, this stimulation was, unlike that by forskolin and Gsα, conformation-dependent.

METHODS

Overexpression of ACII and PKC-α in Insect Cells

ACII (from Dr. R. Reed, Johns Hopkins University) and PKC-α [Ohno et al., 1987] were overexpressed in High Five insect cells (Invitrogen, CA), as previously described [Kawabe et al., 1994a]. High five cells were infected with recombinant baculovirus and incubated in Grace's medium containing 6% fetal bovine serum at 27°C for 3 days. On the day of harvesting, the cells were incubated in the serum-free medium with or without 100 nM phorbol 12-myristate 13-acetate (PMA) for 30 min at 27°C, and the crude membranes were prepared as previously described [Kawabe et al., 1994a]. These membrane preparations were further mixed with 5 mM Lubrol PX and incubated at 4°C for 1 h, which were used as solubilized membrane preparations without further centrifugation.

Purification of ACII, PKC-α, and Gsα

ACII [Feinstein et al., 1991] was purified according to the method originally described by Taussig et al. [1993] and modified by us [Kawabe et al., 1994a]. Recombinant PKC-α was similarly overexpressed in insect cells and purified with a DEAE-anion exchange column and a phosphatidylserine affinity chromatography column, as previously described by us and others [Uchida and Filburn, 1984; Kawabe et al., 1994a]. Recombinant Gsα was overexpressed in insect cells and was purified using the DEAE-MemSep system (Millipore) and hydroxyapatite chromatography [Jones et al., 1993]. Purified Gsα was activated by incubation at 30°C for 30 min in the presence of 100 μM GTPγS and 5 mM $MgCl_2$.

AC Assay

The AC catalytic activity of insect cell membranes (4 μg) was assayed for 15 min at 30°C in a buffer containing 20 mM Hepes (pH 8), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM ATP, 0.1 mM cAMP, 1 mM creatine phosphate, 8 units/ml creatine phosphokinase, 100 μM forskolin, and α-^{32}P-ATP (about 1 μCi/assay tube), unless otherwise specified [Kawabe et al., 1994a]. Protein concentration was measured by the method of Bradford [1976].

PKC Assay

PKC activity was determined by measuring the incorporation of ^{32}P into histone III-S from γ-^{32}P-ATP for 15 min at 25°C [Kawabe et al., 1994a]. The reaction mixture contained 20 mM Tris/HCl (pH 8), 0.05% Lubrol PX, 1 mM dithiothreitol, 50 μg/ml phosphatidylserine, 0.5 mM $CaCl_2$, 100 nM PMA, 1 mg/ml Histone III-S, 10 mM $MgCl_2$, 0.1 mM ATP, and γ-^{32}P-ATP (about 50–100 cpm/pmol). The incorporation of ^{32}P-labeled phosphate into the substrate was assayed with acid precipitation on the nitrocellulose filter (Gibco BRL, MD).

Immunoblotting

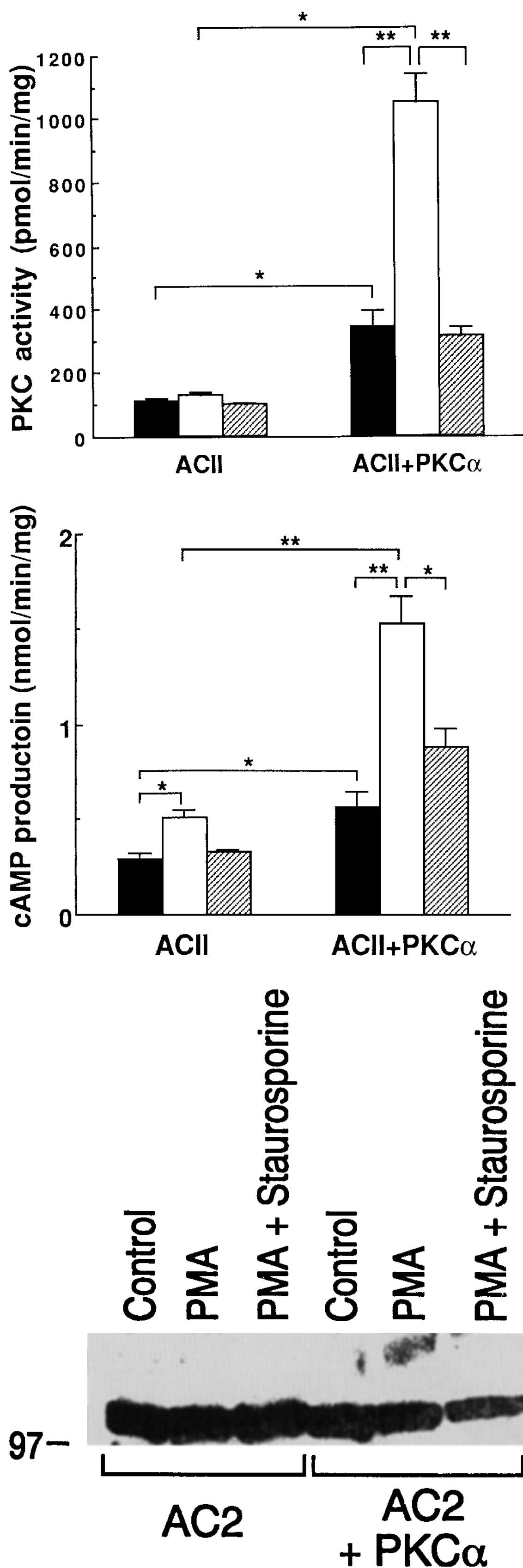
Proteins (5–10 μg) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA). Detection was performed with an anti-ACII antiserum (Santa Cruz Biotech. Inc., CA) using the ECL detection system (Amersham, IL).

RESULTS

Co-Expression of PKC-α With ACII

As shown in Figure 1A, the endogenous PKC activity of insect cell membranes overexpressing ACII was relatively low, but was significantly increased by co-expression of PKC-α (~350%) and further potentiated with PMA treatment (~1,000%).

Similarly, the endogenous AC activity was low, but overexpression of ACII increased the activity at least by 10-fold (data not shown). AC activity was measured as cAMP production us-



ing membrane preparations. Treatment of insect cells overexpressing ACII with phorbol ester increased AC catalytic activity (~180%) (Fig. 1B). Coexpression of PKC-α with ACII resulted in a higher ACII catalytic activity even in the absence of PMA treatment (~190%). PMA treatment further and markedly enhanced catalytic activity (~360%). Thus, the effect of PMA on ACII catalytic activity was much greater in cells expressing ACII and PKC-α than in cells expressing ACII alone.

We also examined the effect of staurosporine, a PKC inhibitor [Matsumoto and Sasaki, 1989]. If the increases in ACII catalytic activity upon coexpression of PKC-α PMA treatment were mediated by PKC, these increases should be attenuated in the presence of staurosporine. Indeed, this was the case. AC catalytic activity, as well as PKC activity, in the presence of PKC-α or PMA treatment was attenuated when the cells were treated with staurosporine.

These data suggest that PKC-α isoenzyme stimulated ACII more efficiently than insect PKC. Alternatively, there is insufficient endogenous insect PKC. The fact that coexpression of PKC-α alone increased AC catalytic activity without PMA-stimulation suggests that PKC-α can stimulate ACII in a tonic manner. Coexpres-

Fig. 1. Coexpression of ACII and PKC-α. **A:** Effect of PMA on PKC catalytic activity. **B:** Effect of PMA on AC activity. High five cells from a single plate overexpressing either ACII by itself or with PKC-α were divided into three groups and treated with vehicle control (closed bars), 100 nM PMA (open bars), or 100 nM PMA plus 1 μM staurosporine (striped bars). Staurosporine treatment of the cells was performed for 30 min before PMA treatment. The AC catalytic activity of insect cell membranes (4 μg) was assayed for 15 min at 30°C in a buffer containing 20 mM Hepes (pH 8), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM ATP, 0.1 mM cAMP, 1 mM creatine phosphate, 8 units/ml creatine phosphokinase, 100 μM forskolin, and α-^{32}P-ATP (about 1 μCi/assay tube) [Kawabe et al., 1994a]. PKC activity was determined by measuring the incorporation of ^{32}P into histone III-S from γ-^{32}P-ATP for 15 min at 25°C [Kawabe et al., 1994a]. The reaction mixture contained 20 mM Tris/HCl (pH 8), 0.05% Lubrol PX, 1 mM dithiothreitol, 50 μg/ml phosphatidylserine, 0.5 mM $CaCl_2$, 100 nM PMA, 1 mg/ml Histone III-S, 10 mM $MgCl_2$, 0.1 mM ATP, and γ-^{32}P-ATP (about 50–100 cpm/pmol). The incorporation of ^{32}P-labeled phosphate into the substrate was assayed with acid precipitation on the nitrocellulose filter (Gibco BRL, MD). Means ± SEM are shown from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ differences. **C:** Immunoblot of insect cell membranes overexpressing ACII with/without PKC-α. A representative immunoblot of ACII in the membrane preparations used in the above experiments is shown. A molecular size marker (97 kD) is also shown.

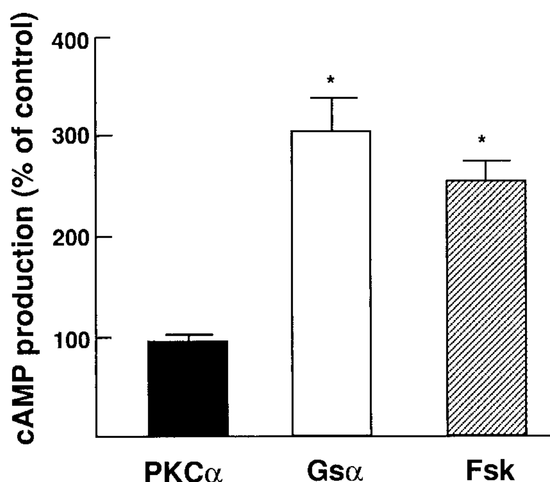


Fig. 2. Effect of purified PKC-α, Gsα, and forskolin on purified ACII. The effects of purified PKC-α (closed bar), purified GTPγS-Gsα (open bar), and forskolin (striped bar) were compared. Purified ACII was stimulated with each stimulator. Data are shown as a percentage of the AC catalytic activity without stimulants. In the absence of stimulants, the catalytic activity was about 0.2 μmol/min/mg. Means ± SEM are shown from three to six independent experiments. *$P < 0.05$ differences from the values without stimulation.

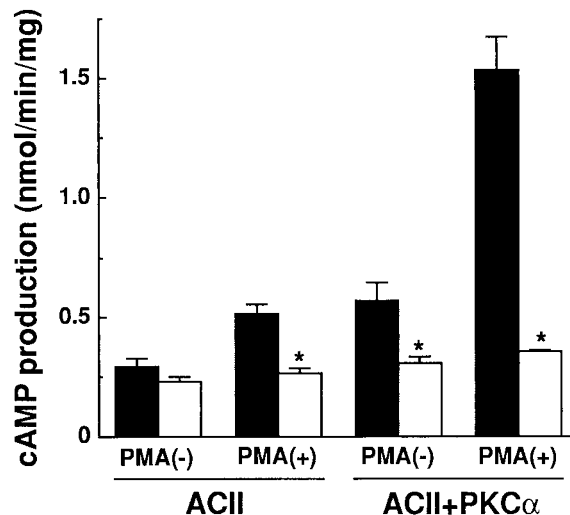


Fig. 3. Effect of Lubrol on AC catalytic activity. The catalytic activity of ACII was measured in crude (closed bars) and solubilized (open bars) membrane preparations. Prior to membrane preparation, cells overexpressing ACII with/without PKC-α were treated with phorbol ester or its vehicle. Means ± SEM are shown from three independent experiments. *$P < 0.05$ differences from the values without Lubrol-treatment.

sion of PKC-α did not alter the expression of ACII as assessed by immunoblotting (Fig. 1C).

Activity of Purified ACII With Purified PKC-α

We next examined the ability of purified PKC-α to stimulate purified ACII as we previously described for ACV [Kawabe et al., 1994b]. Purified ACII (~0.1 μg) was incubated with purified PKC-α (about 300 mU) in a reaction mixture containing 20 mM Tris/HCl (pH 8), 0.1 mM ATP, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 50 μg/ml phosphatidylserine, and 100 nM PMA. After incubation at 25°C for 10 min, cAMP production was initiated by the addition of a buffer containing 40 mM HEPES (pH 8), 1 mM EDTA, 2 mM EGTA, 2 mM creatine phosphate, 16 μg/ml creatine phosphokinase, 0.2 mM cAMP, 0.1 mM ATP, and α-^{32}P-ATP (about 1 μCi/assay tube). In contrast to the above data, the purified PKC-α did not stimulate ACII catalytic activity while GTPγS-Gsα and forskolin stimulated ACII catalytic activity (Fig. 2).

Effect of Solubilization

The above two experiments demonstrated that PKC-α stimulated ACII in intact cells, but not when purified. Thus, we hypothesized that the sensitivity of ACII to PKC is lost during the process of enzyme purification. The first step in purifying ACII is solubilization of membranes with detergent [Taussig et al., 1993], which would change the conformation of this enzyme.

Solubilization of membranes with Lubrol did not alter the catalytic activity of ACII under basal or forskolin-stimulated conditions. In contrast, the enhancement of ACII catalytic activity by PMA and/or co-expression of PKC-α was negated by solubilization (Fig. 3). All ACII catalytic activities were decreased to the level prior to PMA treatment in cells not co-expressing PKC-α. The negation of the PMA effect was not specific to Lubrol; other detergents such as dodecyl maltoside similarly abolished the PMA-induced enhancement (data not shown). Thus, our data suggest that the potentiation of ACII catalytic activity induced by PKC depends on the presence of an intact membrane structure.

Effect of Phosphatase Inhibitor and Salts

Lubrol may activate phosphatase that dephosphorylates ACII and negates the PKC effect. Thus, we examined the effect of Lubrol in the presence of okadaic acid, a serine/threonine phosphatase inhibitor. Okadaic acid (5 μM or vehicle) was added to PMA-treated insect cell membranes overexpressing ACII and PKC-α, followed by incubation for 1 h at 4°C during solubilization with Lubrol. However, ACII catalytic activity did not differ from that without

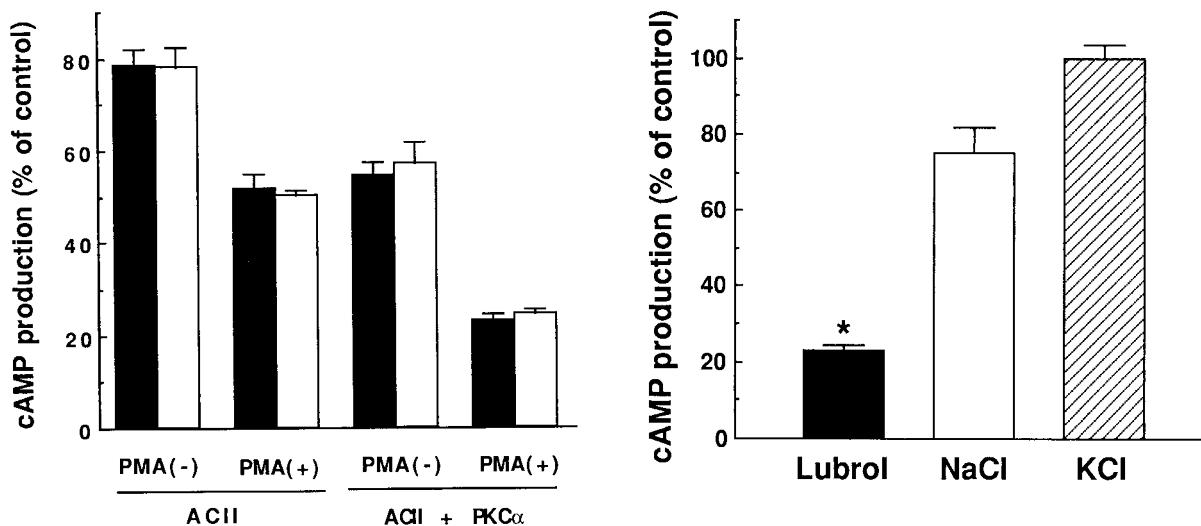


Fig. 4. Effects of phosphatase inhibitors and salts. **A:** Effects of okadaic acid. Inhibition of ACII catalytic activity after Lubrol-solubilization was compared in the presence (open bars) and absence (closed bars) of okadaic acid treatment. Five μM okadaic acid (or vehicle) was added to insect cell membranes overexpressing ACII with/without PKC-α and incubated for 1 h at 4°C before solubilization with Lubrol. Data are shown as percentage of the AC catalytic activity without Lubrol-solubilization. Means ± SEM from three independent experiments are shown. **B:** Effects of salts. Effect of Lubrol-solubilization was compared with that of NaCl and KCl treatment. After phorbol ester treatment of the cells overexpressing ACII and PKC-α, cell membranes were incubated in the presence of 5 mM Lubrol, 2 M NaCl, or 400 mM KCl for 1 h at 4°C, followed by AC assays. Means ± SEM from three independent assays are shown. $^*P < 0.05$ difference.

okadaic acid treatment (Fig. 4A). Thus, it is unlikely that the activation of a phosphatase was responsible for this effect.

Lubrol may disrupt the interaction of ACII with a putative, regulatory protein that is required to maintain the PKC-mediated activation of ACII. We examined the effect of treatment with high salt concentration, a condition that might similarly dissociate the protein from ACII. Cells coexpressing ACII and PKC-α were treated with phorbol ester. The membranes were then incubated in the presence of 5 mM Lubrol, 2 M NaCl or 400 mM KCl for 1 h at 4°C prior to AC assays. ACII catalytic activity was compared with that in the absence of these treatments (Fig. 4B). Lubrol markedly decreased the activity while KCl had no effects. NaCl decreased the activity to a small degree, but did so even in the absence of PMA treatment. Thus, it is unlikely, at least under the conditions examined in our study, that a putative, regulatory protein(s) was responsible for this effect.

DISCUSSION

Our data indicate that a specific mammalian PKC isoenzyme (PKC-α) activated ACII. This may be important because it was not clear which PKC isoenzyme(s) was capable of stimulating ACII in the past studies. The activation of ACII by PKC-α occurred in two steps in our study. First, coexpression of PKC-α with ACII increased ACII catalytic activity, which suggests that tonic activation of ACII by PKC-α occurred. Importantly, the presence of such tonic regulation has been suggested previously [Bushfield et al., 1991], however, it has been difficult to prove this hypothesis in the absence of molecular tools. Second, this activation was further and markedly potentiated by stimulation of PKC-α with PMA. Thus, PKC-α, a classic PKC isoenzyme [Nishizuka, 1992], was capable of activating ACII.

More important in the present study, the potentiation by PKC-α required the presence of an intact membrane structure. When the structure was disrupted by detergent solubilization or by purification of the enzyme, this effect was lost. It is interesting that this loss was specific to the potentiation by PKC; purified ACII continued to be stimulated by forskolin and Gsα. Thus, the mechanism of activation of PKC was different from that of either Gsα or forskolin. The past conflicting findings [Jacobowitz and Iyengar, 1994; Lustig et al., 1993] might have resulted from the usage of detergent. Our finding may be useful for the investigators who will

study the regulation of adenylyl cyclase; this property may also be used as a tool to determine the catalytic activity of AC induced by PKC.

The different effects of PKC on solubilized or purified ACII vs. membrane-bound ACII clearly contrast with its effects on type V AC (ACV). As we previously demonstrated, ACV is potently stimulated by PKC-α in the purified state although poorly in the membrane. Gsα and forskolin also stimulate purified ACV [Kawabe et al., 1994a]. Therefore, the molecular mechanisms underlying regulation by PKC appear to differ between the two AC isoforms. In contrast, the mechanisms for Gsα- and forskolin-stimulation appear similar in this regard. Conformational changes may be less important for Gsα- and forskolin-stimulation; a recent study showed that a chimeric mutant AC consisting of the cytosolic catalytic domains (ACI/ACII) retained Gsα- and forskolin-stimulation [Yan et al., 1996]. Thus, the cytoplasmic domains may be enough for the regulation by Gsα and forskolin. Nevertheless, these studies indicate that all of them (PKC, Gsα, and forskolin) stimulate both ACII and ACV.

The differences may stem from the amino acid sequence within the transmembrane domains, which is poorly conserved among the various isoforms. These domains determine the membrane topology of AC; disruption of the membrane structure with detergent might differently impact the two isoforms. In contrast, the amino acid sequence in the cytoplasmic catalytic domains, including clusters of putative PKC-phosphorylation sites, is relatively well conserved. Currently, however, we do not know the exact site(s) that is phosphorylated by PKC in these isoforms. The functional sites of phosphorylation by PKC isoenzymes both in vivo and in vitro, as well as the mechanism of catalytic activation by phosphorylation, need to be addressed in future studies. Membrane-perturbing agents, such as ethanol or general anesthetics [Deitrich et al., 1989], may also modify the cAMP signaling differently among the various isoforms. Indeed, a previous study showed that this was the case [Yoshimura and Tabakoff, 1995]. The membrane domain of AC may also function as regulatory domain within the molecule. Taken together, our data suggest that the regulation of AC by PKC is not only isoform-dependent but also depends on the physical state of the enzyme as well.

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