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Biochemistry

Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2

(epitope tag/recombinant enzyme/Sf9 cells)

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ABSTRACT **Adenylyl cyclase 2 was expressed in Sf9 cells by recombinant baculovirus infection. Phorbol 12-myristate 13-acetate (PMA) treatment of cells expressing adenylyl cyclase 2 (AC2) increased basal activity. This increase was blocked by staurosporine, a protein kinase C inhibitor. PMA treatment increased V_{max} without affecting K_m. Greatest increase in basal activity was seen at physiologically relevant Mg^{2+} concentrations. PMA treatment did not alter sensitivity to guanine nucleotide stimulatory factor (G_s) but enhanced stimulation at all concentrations of activated G_s α subunit tested. AC2 was tagged at the N terminus with an 8-amino acid epitope. Epitope-tagged AC2 was purified to apparent homogeneity in a single step by using an antiepitope antibody-affinity column. The eluate was resolved by SDS/PAGE. Silver staining of the gel showed a 106-kDa band. The purified protein was recognized by antipeptide antibody against a region common to all mammalian adenylyl cyclases. The epitope-tagged enzyme expressed in Sf9 cells was also stimulated by PMA. When cells were labeled with ^{32}P and treated with PMA, a 3-fold increase in ^{32}P incorporation of purified epitope-tagged AC2 was observed. We conclude that activation of protein kinase C results in phosphorylation and stimulation of AC2, a cell-surface G protein effector enzyme. Thus, covalent modification of cell-surface effectors may provide an independent mode for signal transmission through G protein pathways.**

Signal transmission by G protein-coupled signaling systems occurs through a series of protein–protein interactions. Receptors activate heterotrimeric G proteins, which in turn activate effector enzymes. The effector enzymes produce intracellular messengers that elicit cellular responses by regulating protein kinases. With the cloning and characterization of multiple forms of adenylyl cyclases (ref. 1 and the references therein) has come the recognition that different isoforms of adenylyl cyclase can have different signal-recognition capabilities (1). One example is the ability of adenylyl cyclase 2 (AC2) to be stimulated by the activation of protein kinase C (2–4). It had been known for some time that in some cells and tissues cAMP production could be stimulated by the activation of protein kinase C (5–8). Some reports (9, 10) suggested that such regulation may occur at the level of the G protein, whereas others (11, 12) viewed adenylyl cyclase as the target of regulation. Using transient expression in mammalian cells, we (2) and others (3) showed that AC2, but not other isoforms of adenylyl cyclase, was extensively stimulated by activation of protein kinase C. This result suggested that AC2 could be phosphorylated and activated in response to protein kinase C. However, it was not feasible to use transient expression systems to show protein kinase C-mediated phosphorylation of AC2. Hence, we expressed AC2 in Sf9 cells by baculovirus infection. Here we show that AC2 expressed in Sf9 cells is phosphorylated and stimulated by activation of protein kinase C. This demonstration establishes that, in addition to G protein-subunit regulation, covalent modification of an effector is a distinct mechanism by which signals may be transmitted through G protein pathways.

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MATERIALS AND METHODS

Materials. AC-Comm rabbit antiserum was raised against the peptide sequence IGARKPQYDIWGNT common to the C-terminal region of all cloned mammalian adenylyl cyclases. When tested against the recombinant AC2 and adenylyl cyclase 6 expressed in Sf9 cell membranes, the antibody recognized proteins of appropriate sizes in immunoblots. Adsorption of the antibody with the appropriate peptide vitiated recognition of adenylyl cyclase. Detailed characterization of this antibody will be published later. Recombinant Q227L, guanine nucleotide stimulatory factor α subunit (G_s-α) from Juan Codina and Lutz Birnbaumer (Baylor College of Medicine), was synthesized with the terminal deoxynucleotidyltransferase *in vitro* translation system (Promega). Sf9 cells and pVL-1392 were obtained by the Mount Sinai Protein Expression Core Facility from Max Summers. BaculoGold virus was purchased from PharMingen. Sf9 serum-free cell medium was from GIBCO/BRL or Sigma. Dodecyl maltoside and 2-[*N*-morpholino]ethanesulfonic acid (Mes) were from Sigma. Anti-FLAG M2 affinity gel and anti-FLAG antibody were from Kodak-IBI where FLAG is the eight amino acid peptide DYKDDDDK. [α-^{32}P]ATP (25 Ci/mmol; 1 Ci = 37 GBq), and $[^{32}P]H_3PO_4$ were from ICN. Sources of all other materials have been described (2, 13–15).

Construction of AC2 and FLAG–AC2 Recombinant Baculovirus. AC2 cDNA was excised from pBSII-AC2 and inserted into pVL-1392. Recombinant plasmids were screened for proper orientation by restriction digests. pVL-1392-AC2 was then used to construct pVL-1392-F-AC2. The FLAG epitope DYKDDDDK was incorporated into pVL-1392-AC2 by PCR mutagenesis. The 5′ primer, OJ049 (TACAAGCGGCCGCATGGACTACAAGGACGACGACGATAAGCGGCGGCGCCGCTACC), contained a *Not* I restriction site followed by codons for an initiator methionine, the FLAG epitope, and amino acids 2–6 of AC2. The 3′ primer, OJ048, spanned bp 258–229 of AC2 and included an internal *Nhe* I site. The 215-bp PCR product was subcloned into pVL1392-AC2 from *Not* I to *Nhe* I to yield FLAG-epitope-tagged AC2 (F-AC2). Recombinants were screened by restriction digests, and positive recombinants were verified by sequencing the insert through the integration sites. Recombinant plasmids were individually transfected into Sf9 cells along with BaculoGold virus DNA at the Protein Expression Core Facility.

Abbreviations: AC2, adenylyl cyclase 2; PMA, phorbol 12-myristate 13-acetate; TPO, thyroid peroxidase; F-AC2, FLAG-epitope-tagged AC2; PDD, 4α-phorbol 12,13-didecanoate; G_s, guanine nucleotide stimulatory factor.

Recombinant baculoviruses were purified by two rounds of limiting dilutions and confirmed as recombinant by dot blots. Thyroid peroxidase (TPO)-ΔXS baculovirus (16) was from R. Magnusson (Mount Sinai School of Medicine).

Expression of AC2 and F-AC2 in Sf9 Cells. Sf9 cells were grown in Sf-900 medium (GIBCO) or serum-free insect culture medium 1 (Sigma) at 28°C. Cells were infected with recombinant baculoviruses at a multiplicity of infection of ≈1. Cells were harvested 2–4 days after infection. When appropriate, phorbol 12-myristate 13-acetate (PMA) or 4α-phorbol 12,13-didecanoate (PDD) was added to the culture medium at 1 μM, and cells were incubated for 20–35 min. When staurosporine was included in the treatment, cells were treated with 1 μM staurosporine before PMA addition.

Sf9 cells were lysed by nitrogen cavitation or by homogenization in Dounce homogenizers. After treatments, cells were pelleted at 4°C and washed with lysis buffer containing 20 mM NaHepes (pH 8.0), 4 mM EDTA, 150 mM NaCl, 20 mM $Na_3(SO_4)$, 20 mM NaF, 10 mM β-glycerol phosphate, 2 mM dithiothreitol, and a protease inhibitor mixture of aprotinin at 2 μg/ml, leupeptin at 4 μg/ml, 1 mM 1,10-phenanthroline, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed either by N_2 cavitation at 600 psi (1 psi = 6.9 kPa) for 30 min or by homogenization in a glass Dounce homogenizer (25–35 strokes). Supernatant from a low-speed spin (1000 × g, 10 min) was centrifuged at 100,000 × g for 30–60 min to obtain the membrane fraction. The pellet was resuspended in 10 mM Hepes, pH 8.0/1 mM EDTA/200 mM sucrose/2 mM dithiothreitol/protease inhibitor mixture to a final concentration of 3–5 mg of protein per ml.

Adenylyl Cyclase Assays. Assays were done as described (2, 14) with 2–5 μg of membrane protein for 15 min at 32°C. Basal activities were measured in the presence of 5 mM Mg^{2+}. Forskolin-stimulated activities were measured in the presence of 5 mM Mg^{2+} and 10 μM forskolin.

Immunoaffinity Purification. To solubilize the adenylyl cyclase, 4–5 mg of Sf9 membrane protein per sample was pelleted for 30 min at 60,000 × g and resuspended in 1 ml of 150 mM NaCl/5 mM EDTA/20 mM Hepes, pH 8.0/20% (vol/vol) glycerol/1 mM EGTA/0.8% dodecyl maltoside (17). The suspension was gently shaken at 4°C for 90 min. Supernatants (60,000 × g, 30 min) were incubated with 15–20 μl of solubilization buffer-preequilibrated anti-FLAG M2 affinity gel (Kodak) for 3 hr at 4°C. The gel was washed two to three times with solubilization buffers containing 0.8%, 0.4%, and 0.05% dodecyl maltoside. F-AC2 was eluted by incubation with 0.1 M glycine, pH 3.0, for 5–10 min in a 90-μl vol. Eluates were immediately neutralized with 10 μl of 1 M NaHepes, pH 8.0, and rapidly frozen on dry ice/acetone.

Electrophoresis and Immunoblotting. These procedures were done as described (15). When needed, densitometry of gels and films was measured with the Hoefer GS-300 scanning densitometer on IBM-PC-running Hoefer GS-350 software. Intensity of band in arbitrary units was estimated from areas under the peaks.

^{32}P Labeling in Sf9 Cells. Sf9 cells were collected at 48 hr after infection and washed with phosphate-free Grace's medium/20 mM Mes, pH 6.2. Cells were incubated in the same medium with 0.5 mCi of $[^{32}P]H_3PO_4$ per ml for 3 hr and treated with PMA or vehicle, as described. After treatment, the cells (7.5×10^8) were washed twice with 40 ml of ice-cold lysis buffer and resuspended in 20 ml of the same buffer. Cells were lysed by homogenization in Dounce homogenizers. Membranes were prepared as described above.

All experiments were repeated thrice with multiple batches of Sf9 cell membranes. Results were qualitatively similar. Typical experiments are shown.

RESULTS

Expression of AC2 in Sf9 cells resulted in a 12-fold increase in basal adenylyl cyclase activity in Sf9 cell membranes as compared with cells infected with baculovirus virus containing the TPO gene (Fig. 1*A*). From 14 independent experiments mean increase in basal activity was 11.1 ± 5.7-fold. Activities in uninfected cell membranes were comparable to those in TPO-baculovirus-infected cells. Treatment of baculovirus-infected cells with PMA for 15 min before lysis resulted in 3-fold enhancement of the expressed AC2 activity in Sf9 membranes (Fig. 1*B*). In 10 experiments, PMA treatment resulted in 2.7 ± 0.5-fold increase in activity in AC2-baculovirus-infected Sf9 membranes, as compared with 1.1 ± 0.4-fold in TPO-baculovirus-infected Sf9 membranes. The inactive phorbol ester PDD did not affect activity. Maximal

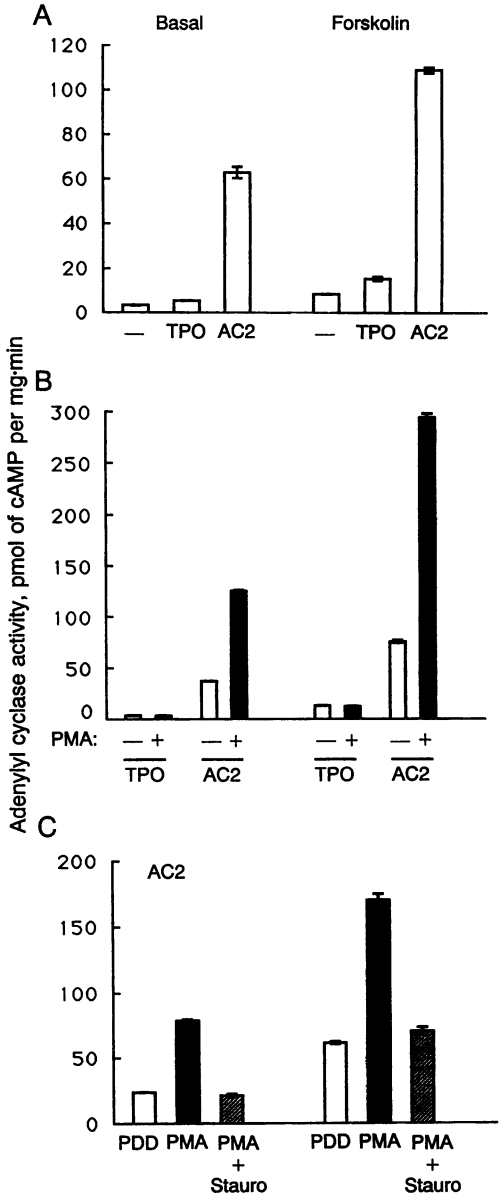

FIG. 1. Expression of AC2 in Sf9 cells. (*A*) Basal and forskolin-stimulated adenylyl cyclase activities were measured in membranes of Sf9 cells without infection (−), infected with TPO-baculovirus (TPO), or infected with AC2 baculovirus (AC2). (*B*) Measurement after treatment without (−) or with (+) PMA before membrane preparation. (*C*) After treatment with the phorbol esters PDD or PMA with or without 1 μM staurosporine (Stauro). All values are means ± SD of triplicate determinations.

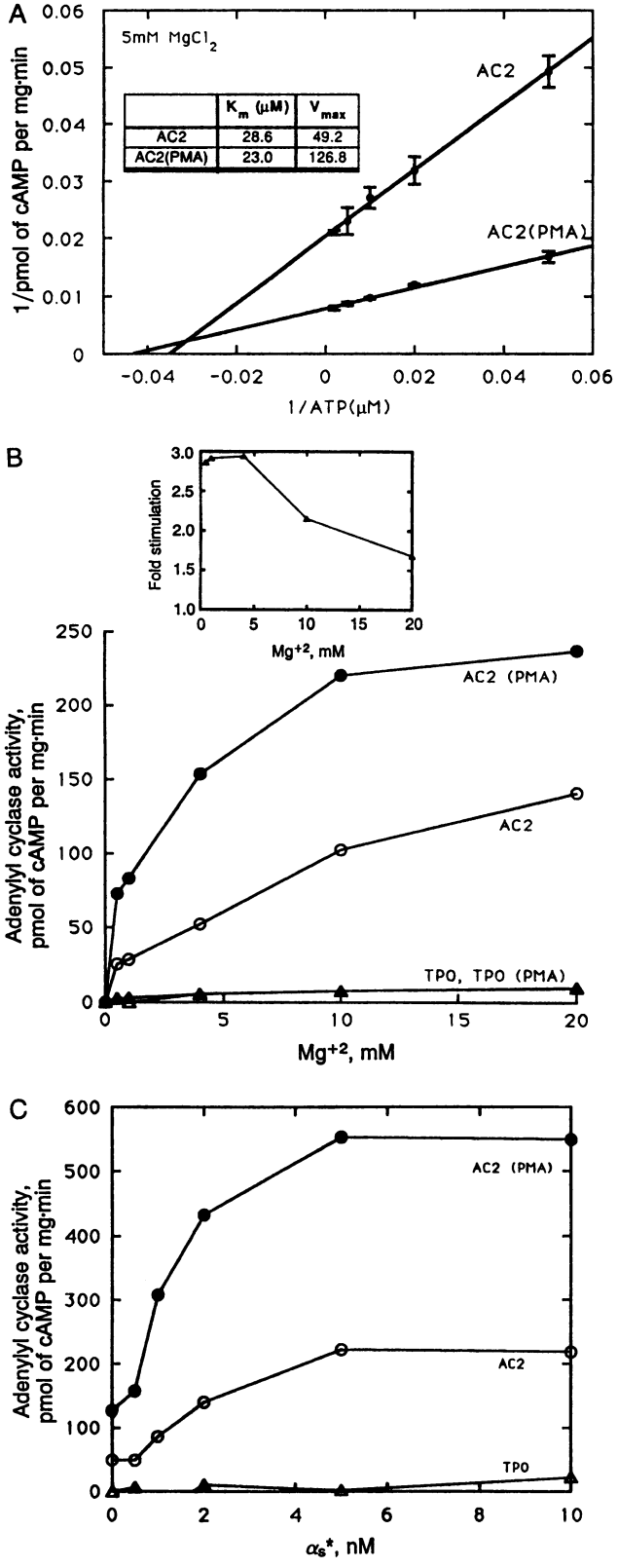

FIG. 2. Effects of PMA treatment on AC2. (*A*) Lineweaver–Burk plot of various ATP concentrations on AC2 activity in control and PMA-treated cells. (*B*) Basal AC2 activity in control (○) and PMA-treated (●) Sf9 cell membranes at various Mg^{2+} concentrations. (*Inset*) Fold stimulation due to PMA treatment as a function of Mg^{2+} concentration. (*C*) Stimulation by various concentrations of rabbit reticulocyte lysate-expressed activated G_s-α (Q227L) of control (○) and PMA-treated (●) AC2. All values are means of triplicate determinations. Coefficients of variance for B and C were <10%.

enhancement was observed at 100 nM PMA (data not shown). We routinely used 1 μM PMA for treatment. Inclusion of staurosporine during the PMA treatment blocked the activity increase (Fig. 1*C*). These data show that AC2 expressed in Sf9 cells can be stimulated by activation of protein kinase C.

We determined whether the activation of protein kinase C was an independent pathway for stimulation of AC2. Other stimulators such as G_s-α and forskolin activate adenylyl cyclase by increasing the V_{max} of the enzyme. We tested whether this also occurred with PMA treatment by comparing basal adenylyl cyclase activities at various concentrations of ATP. Lineweaver–Burk transformation of the data from this experiment shows that PMA treatment increases the V_{max} of the enzyme without affecting the K_m for ATP (Fig. 2*A*), an effect mechanistically similar to that of other stimulators. Two different experiments gave a mean 3-fold increase in the V_{max} and a 25% decrease in the K_m. The greatest extent of sensitization by PMA treatment (≈3-fold) was observed at low Mg^{2+}. Increasing the Mg^{2+} concentration increased the basal activity of untreated AC2 with smaller increases for the PMA-treated AC2, resulting in decreased sensitization by PMA at high Mg^{2+} concentrations (Fig. 2*B*). Stimulation of AC2 by activated (Gln-227 → Leu) G_s-α was enhanced by PMA treatment at all G_s-α concentrations tested, but sensitivity to G_s-α was unaltered (Fig. 2*C*).

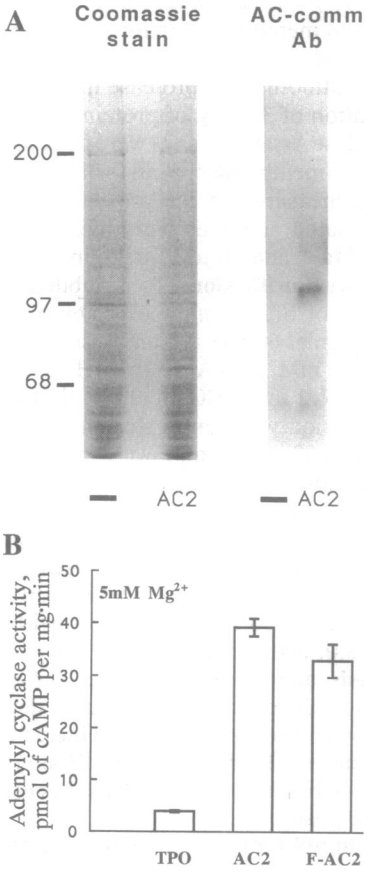

FIG. 3. Identification of AC2 expressed in Sf9 cells. (*A*) (*Left*) Coomassie blue-stained pattern of membranes from TPO control- (−) and AC2-expressing cells. Positions of the molecular markers in kDa are indicated. (*Right*) Immunoblot of TPO- (−) and AC2-expressing membranes with AC-Comm, an antipeptide antibody to a region common to all mammalian adenylyl cyclases. Antiserum was used at a 1:1000 dilution. Detailed immunoblotting procedures have been published (13, 15). (*B*) Basal adenylyl cyclase activity in membranes of cells infected with TPO baculovirus (−), AC2 baculovirus, or F-AC2 baculovirus. All values are means ± SDs of triplicate determinations.

Similarly, PMA treatment enhanced the activity seen with activated α_s and $\beta\gamma$ subunits (data not shown). These data indicate that PMA treatment enhances basal activity by an increase in V_{max} of AC2 and that the increased activity is seen independently of G_s-α action.

The data in Figs. 1 and 2 could imply that adenylyl cyclase was phosphorylated in response to activation of protein kinase C. To determine whether this occurred, we attempted to isolate the expressed AC2. Though adenylyl cyclase activity substantially increases in AC2-expressing Sf9 cells, AC2 is not present at a level detectable by Coomassie staining of membrane proteins (Fig. 3*A Left*). However, immunoblotting with AC-comm, an antipeptide antibody to a region common to all mammalian adenylyl cyclases, indicated that an exogenous adenylyl cyclase was being expressed (Fig. 3*A*, *Right*). Thus recombinant baculovirus infection of Sf9 cells probably did not produce sufficient amounts of AC2 to allow characterization of its phosphorylation state without extensive purification. The use of conventional methods to purify adenylyl cyclase, such as forskolin-affinity chromatography, could have resulted in copurification of endogenous adenylyl cyclases of Sf9 cells along with the expressed AC2 and could have confounded interpretation of results of the phosphorylation experiments. Hence, we tagged AC2 at the N terminus with FLAG, an eight-amino acid peptide (DYKDDDDK). The F-AC2 can be purified by immunoaffinity chromatography on anti-FLAG antibody agarose, which is commercially available. Expression of F-AC2 resulted in adenylyl cyclase activities similar to that seen with AC2 (Fig. 3*B*).

Membranes containing F-AC2 were extracted with detergent, and the extract was passed over anti-FLAG antibody agarose. The gel was washed, and the bound protein was eluted at pH 3.0. When analyzed on SDS/polyacrylamide gels and silver-stained, the eluate migrated as a single 106-kDa band (Fig. 4 *Left*). In immunoblot analysis, this protein band was recognized by AC-Comm, the antipeptide antiserum against the common region of adenylyl cyclase (Fig. 4 *Middle*) and by the anti-FLAG antibody (Fig. 4 *Right*). Evidently F-AC2 can be purified to apparent homogeneity by single-step immunoaffinity chromatography.

We used immunoaffinity purification to determine whether treatment of the F-AC2-baculovirus-infected Sf9 cells with PMA resulted in phosphorylation of the F-AC2 and in enhanced adenylyl cyclase activity. For this purpose cells were

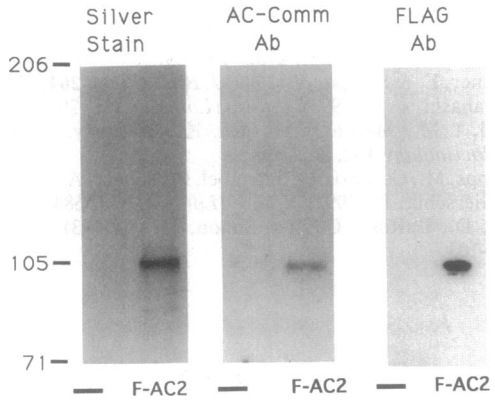

FIG. 4. Characterization of the purified F-AC2 expressed in Sf9 cells. Membranes from TPO baculovirus (−) and F-AC2 were extracted with dodecyl maltoside and passed over the anti-FLAG affinity agarose. The agarose was washed, and samples were eluted. Thirty-microliter aliquots were resolved on SDS/polyacrylamide gels. (*Left*) Silver-stained profile. (*Middle*) Immunoblot with AC-Comm. (*Right*) Immunoblot with FLAG antibody (FLAG-Ab). The anti-FLAG antibody, a mouse monoclonal antibody against the epitope tagged onto AC2, was used at 30 μg/ml for immunoblotting. Molecular markers are in kDa.

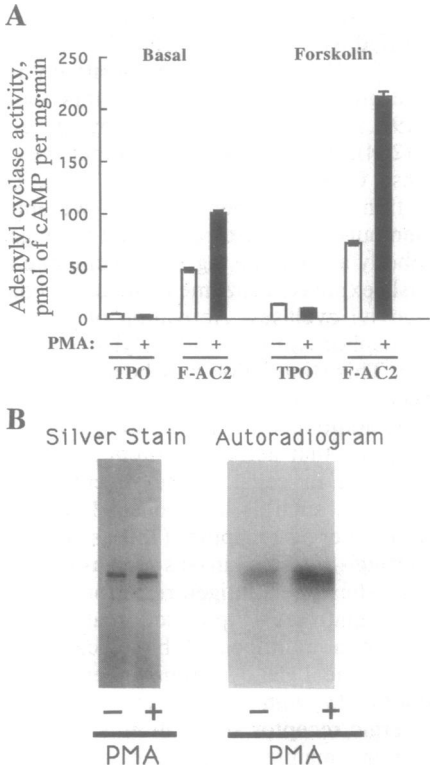

FIG. 5. Activation and phosphorylation of F-AC2 by PMA treatment. Cells infected with TPO baculovirus or F-AC2 baculovirus were labeled with ^{32}P (0.5 mCi/ml) for 3 hr and then treated with 1 μM PMA for 30 min, after which membranes were prepared. (*A*) Basal adenylyl cyclase activity in membranes from TPO- or F-AC2-infected cells treated without (−) or with (+) PMA. Values are mean ± SDs of triplicate determinations. (*B*) Membranes from cells infected with F-AC2 baculovirus and treated without (−) and with (+) 1 μM PMA were extracted, and F-AC2 was purified on an antiepitope antibody-affinity column. Thirty-microliter aliquots of F-AC2 from untreated and PMA-treated cells were resolved on 6% SDS/polyacrylamide gels. Two identical gels were simultaneously run. One was silver stained (*Left*); the other was dried and autoradiographed for 9 days (*Right*).

labeled with ^{32}P for 3 hr before PMA treatment. In cells expressing F-AC2, PMA treatment caused a 2-fold increase in basal activity (Fig. 5*A*). In three independent experiments PMA treatment resulted in a mean 2.0 ± 0.4 enhancement of basal activity. Aliquots of the membranes were extracted, and F-AC2 from control- and PMA-treated cell membranes were individually purified by immunoaffinity chromatography. The eluates from the FLAG-antibody agarose were resolved on SDS/polyacrylamide gels. A single band of 106 kDa was seen (Fig. 5*B Left*). When equivalent amounts of proteins were resolved on SDS/polyacrylamide gels, autoradiographic analysis indicated that treatment of F-AC2-expressing cells with PMA caused up to 3-fold increases in the ^{32}P incorporated into F-AC2 (Fig. 5*B Right*). From three independent experiments, ^{32}P incorporation increased 2.4 ± 0.9-fold. Data show that stimulation of protein kinase C results in activation and phosphorylation of AC2.

DISCUSSION

Upon binding agonists, G protein-coupled receptors interact with and activate heterotrimeric G proteins. The G protein subunits, in turn, activate effectors, stimulating production of second messengers. In this system, transmembrane signal transduction occurs by sequential protein–protein interactions. In addition to linear signaling, certain effector isoforms

can receive signals from other pathways (1), as exemplified by AC2. Purified AC2 can be stimulated by α_s, as well as $\beta\gamma$, subunit (17, 18), allowing AC2 to be positively regulated by both G_s- and guanine nucleotide inhibitory factor-coupled receptors (19). AC2 is also activated by protein kinase C stimulation (2–4). It was not known whether the activation of protein kinase C would phosphorylate AC2. To establish phosphorylation of AC2 unequivocally, we epitope-tagged AC2 and then purified the expressed epitope-tagged enzyme with an antibody against the tag. This technique isolated the heterologously expressed enzyme without any contaminating Sf9 cell adenylyl cyclases. Activation of protein kinase C enhanced phosphorylation of adenylyl cyclase and stimulated activity. This stimulation may be an independent input pathway because it reflects an increase in V_{max} of basal activity and is additive with stimulation by G_s-α.

Many receptors that activate protein kinase C stimulate cAMP production in cells. These receptors include G_q-coupled receptors, such as angiotensin II (20), muscarinic (21), and α_1-adrenergic receptors (22), as well as receptors that signal through tyrosine kinases such as the nerve growth factor (23) and the B-cell antigen receptors (24). The added capacity to stimulate cAMP production allows these receptors to produce a multifaceted biological response. For instance, in the pineal gland, norepinepherine stimulation of cAMP production through α_1-adrenergic receptors in concert with β-adrenergic receptor stimulation is believed to play a major role in the maintenance of circadian rhythms (25). Additionally angiotensin II stimulation of steroidogenesis in adrenal cells is mediated by a protein kinase C-dependent increase in intracellular cAMP (20, 26). Thus phosphorylation of AC2 or other members of the type 2 family could have physiological effects in many tissues.

These data and our previous observations showing AC2 as the major target for protein kinase regulation (2), along with the demonstration of different responses of adenylyl cyclase types to G protein $\beta\gamma$ subunits (18) and the different responses of isoforms of phospholipase C β to G protein α and $\beta\gamma$ subunits (27, 28), establish that within G protein signaling systems, the effector is a locus at which information from different receptors is received and processed. This processing of information is often, although not always, superimposed upon the primary linear response, which is the production of intracellular messengers by the effector in response to activation by its specific G protein α subunit. Such superimposition allows the primary receptor signal to be modulated by signals from other receptors, permitting the signal transduction system to temporally integrate multiple signals and produce intracellular messengers at levels reflective of the multiplicity of signals received. Thus, cell-surface signaling systems should be viewed as information-processing networks, where information can enter and exit at multiple points. Within G protein signaling systems the molecular identity of the individual components will determine the specificity of the interconnections and the capability of the system to process information.

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