The Journal of Neuroscience, February 15, 1996, 16(4):1317–1323

G_i-Mediated Stimulation of Type II Adenylyl Cyclase Is Augmented by G_q-Coupled Receptor Activation and Phorbol Ester Treatment

Rachel C. Tsu and Yung H. Wong

Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

Synergism between G_s- and G_i- or G_q-dependent signaling pathways has been demonstrated in the stimulation of type II adenylyl cyclase (AC-II). Provision of activated α_s is known to allow numerous G_i-coupled receptors to stimulate AC-II and to potentiate the responses to G_q-coupled receptors. To explore possible interactions between G_i- and G_q-coupled receptors that are independent of α_s, the activity of AC-II was determined after the activation of G_i- and G_q-regulated pathways. Human embryonic kidney 293 cells were transiently cotransfected with cDNAs encoding AC-II and various G-protein-coupled receptors. Agonist-bound G_i-coupled receptors (including the formyl peptide, dopamine-D_2, and δ-opioid receptors) stimulated AC-II activity in the absence of activated α_s, provided that the cells were treated with 100 nM phorbol 12-myristate 13-acetate. Activation of protein kinase C (PKC) thus appears to relieve the requirement for the presence of activated α_s. Stimulation of PKC via G_q-coupled receptors also allowed G_i-coupled receptors to activate AC-II. Coexpression of the m1 muscarinic receptor with the dopamine-D_2 receptor permitted dopamine to stimulate AC-II in the presence of carbachol. The phorbol ester-permissive and α_s-independent stimulation was mediated by G-protein $\beta\gamma$ subunits because it was blocked by the $\beta\gamma$ scavengers α_t and β-adrenergic receptor kinase. These results show that AC-II can efficiently integrate signals generated by G_q- and G_i-coupled receptors via a mechanism that is independent of G_s.

Key words: adenylyl cyclase; cAMP; G-proteins; phorbol ester; protein kinase C; signal transduction

Signal processing is a complex and vital function of the nervous system. Many external signals in the form of neurotransmitters and hormones have to be integrated and processed by the neurons. An example of how intracellular macromolecules convert extracellular signals into a unified message is given by the type II isoform of adenylyl cyclase (AC-II), which is abundant in the brain (Feinstein et al., 1991). AC-II is known to process coincident signals from receptors that are coupled to G_s-, G_i-, or G_q-proteins (Lustig et al., 1993). Like the other adenylyl cyclase isoforms, the type II enzyme can be directly stimulated by the GTP-bound α subunit of G_s (Tang and Gilman, 1992). Activation of AC-II via the G_i pathway is more complex. In the current mechanistic model, activation of a G_i-coupled receptor leads to the release of $\beta\gamma$ subunits from G_i, and the freed $\beta\gamma$ subunits can then stimulate AC-II. However, it was found that the presence of activated $G_{s\alpha}$ is absolutely required for the $\beta\gamma$ subunits to stimulate AC-II (Federman et al., 1992). Thus, when both G_s- and G_i-regulated pathways are simultaneously stimulated, the activity of AC-II is enhanced. Among the various G_i-coupled receptors, the dopamine-D_2, adenosine-A_1, μ- and δ-opioid, melatonin-1c, complement factor C5a, and formyl peptide receptors are able to stimulate the AC-II in the presence of activated $G_{s\alpha}$ (Federman et al., 1992; Tsu et al., 1995a,b; Yung et al., 1995; Chan et al., 1995). Activation of the G_i-coupled pathway alone does not stimulate the type II activity at all. Recent studies suggest that the G_i-proteins can exert an inhibitory influence on the type II enzyme (Chen and Iyengar, 1993).

AC-II can also be activated by external signals that are routed through protein kinase C (PKC). Both phorbol esters (Jacobowitz and Iyengar, 1994) and agonists that act on G_q-coupled receptors (Lustig et al., 1993) are known to stimulate AC-II. These stimulatory effects are attributed to the activation of PKC. It has been shown that type II as well as type VII adenylyl cyclases are activated after phosphorylation by PKC (Jacobowitz et al., 1993; Watson et al., 1995). The phorbol ester-induced stimulation of AC-II is distinct from that seen with α_s and $\beta\gamma$ subunits, and synergistic effects arising from the two pathways have been reported (Lustig et al., 1993). Although acting via different mechanisms, signals generated from G_s- and G_q-coupled receptors nevertheless can converge on the AC-II. In the present investigation, we asked whether AC-II can integrate coincident signals from G_i- and G_q-coupled receptors. Our results indicate that receptors coupled to G_i- and G_q-proteins can stimulate AC-II in a synergistic manner, thereby providing another level of complexity in the regulation of intracellular cAMP.

MATERIALS AND METHODS

Reagents. cDNAs encoding the human *N*-formylmethionyl-leucyl-phenylalanine (fMLP) receptor (in the pCDM8 vector) and the δ-opioid receptor (in the pCDM8 vector) were provided by F. Boulay (LBIO/Laboratoire, Grenoble, France) and Chris Evans (University of California, Los Angeles, CA), respectively. The AC-II cDNA was provided by Randall Reed (The Johns Hopkins University, Baltimore, MD). The β-adrenergic receptor kinase 495–690 ($\beta ARK_{495-690}$) minigene was a gift from Robert Lefkowitz (Duke University Medical Center, Chapel Hill, NC). The origin and construction of other cDNAs have been described previously (Wong et al., 1991, 1992). Pertussis toxin (PTX) was purchased from List Biological Laboratories (Campbell, CA). Human embryonic kidney 293 cells (293 cells hereafter) were obtained from the American Type Culture Collection (CRL-1573; Rockville, MD). [^{3}H]Adenine was purchased from Amersham (Buckinghamshire, UK). Plasmid purification columns were obtained from Qiagen (Hilden, Germany). Staurosporin was purchased from Research Biochemicals (Natick, MA). Cell culture

Received Oct. 12, 1995; revised Nov. 20, 1995; accepted Nov. 28, 1995.

This work was supported by Grants RIG92/93.SC05 and HKUST 169/93 M from the University and Grants Committee of Hong Kong. We thank François Boulay for the human fMLP-receptor cDNA, Chris Evans for the δ-opioid receptor, Robert Lefkowitz for the βARK fragment, and Randall Reed for the AC-II.

Correspondence should be addressed to Yung H. Wong at the above address.

reagents were obtained from Life Technologies (Gaithersburg, MD), and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and transfection. The 293 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (v/v), 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere at 37°C in 5% CO_2. COS-7 cells were kept in DMEM containing 10% fetal calf serum. DEAE-dextran-mediated transfection of 293 and COS-7 cells on 12-well plates has been described previously (Tsu et al., 1995a). All cDNAs used in the transfection were purified by Qiagen chromatography.

cAMP accumulation. Twenty-four hours after transfection, 293 cells were labeled with [^{3}H]adenine (1 μCi/ml) in growth medium for 16–20 hr with or without PTX (100 ng/ml). The labeled cells were rinsed once with 2 ml of assay medium (MEM containing 20 mM HEPES, pH 7.4) and incubated at 37°C for 30 min with 1 ml of assay medium containing 1 mM 1-methyl-3-isobutylxanthine and the indicated drugs. Intracellular [^{3}H]cAMP was isolated by sequential chromatography as described previously (Saloman et al., 1974; Wong, 1994). The level of [^{3}H]cAMP was estimated by determining the ratios of [^{3}H]cAMP to total [^{3}H]ATP and [^{3}H]ADP pools as reported previously (Wong et al., 1991). Absolute values for cAMP levels varied between experiments, but variability within any single experiment was generally <10%.

RESULTS

Phorbol 12-myristate 13-acetate allows G_i-coupled receptors to stimulate AC-II in the absence of activated $G_s\alpha$

We and others have shown previously that many G_i-coupled receptors can activate AC-II via the βγ subunits released from G_i (Federman et al., 1992; Tsu et al., 1995a,b; Yung et al., 1995; Chan et al., 1995). For βγ subunits to stimulate AC-II, the GTP-bound α subunit of G_s must also be present (Federman et al., 1992; Taussig et al., 1994). Such a requirement allows the cell to integrate extracellular signals generated through G_s and other G-protein pathways into the intracellular messenger cAMP. The absolute requirement of activated G_s suggests that the G_s-coupled pathway acts as the major circuitry control. Recent reports indicate that the G_q-coupled pathway has stimulatory influence on AC-II (Lustig et al., 1993; Koch et al., 1994). Activated PKC phosphorylates and stimulates AC-II (Jacobwitz and Iyengar, 1994). We therefore examined whether G_i- and G_q-coupled pathways can converge on AC-II by reconstructing all or part of these pathways in transfected 293 cells. The 293 cells were cotransfected with cDNAs encoding AC-II and a G_i-coupled receptor, in the absence or presence of the mutationally activated α_s-Q227L (Masters et al., 1989), and assayed for cAMP accumulation. As reported previously (Tsu et al., 1995a), the G_i-coupled fMLP receptor was unable to stimulate AC-II in the absence of α_s-Q227L (Fig. 1) or AC-II (data not shown). The provision of α_s-Q227L allowed the fMLP receptor to activate the AC-II in an agonist-dependent and PTX-sensitive manner (Fig. 1). Interestingly, previous treatment of the transfected 293 cells with 100 nM of phorbol 12-myristate 13-acetate (PMA) for 10 min allowed fMLP to stimulate AC-II, even in the absence of α_s-Q227L (Fig. 1). In agreement with previous findings (Jacobowitz et al., 1993; Koch et al., 1994), PMA treatment also raised the basal AC-II activity by approximately sixfold. On the contrary, transfected 293 cells that had been treated with the inactive 4-α-PMA (100 nM for 10 min) did not respond to fMLP, nor did they exhibit an elevated basal activity for AC-II (Fig. 1). The fMLP-induced stimulation of AC-II in PMA-treated cells was mediated via G_i-proteins because PTX effectively abolished the response (Fig. 1).

To test whether the permissive effect of PMA on the fMLP receptor is receptor-specific, we repeated the experiments using other G_i-coupled receptors. Both dopamine-D_2 (Federman et al., 1992) and δ-opioid (Tsu et al., 1995b) receptors are known to

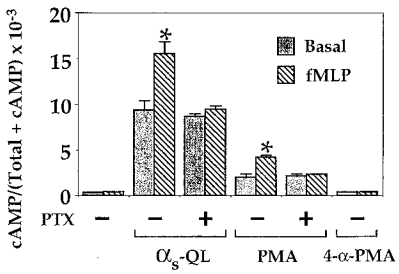

Figure 1. fMLP-induced stimulation of AC-II in the presence of α_s-Q227L or with PMA treatment. The 293 cells were cotransfected with cDNAs encoding the fMLP receptor (0.25 μg/ml) and AC-II (0.25 μg/ml), with or without α_s-Q227L (α_s-*QL*; 0.025 μg/ml). As indicated, transfected cells were exposed to different conditions before assaying for cAMP accumulation in response to fMLP (200 nM). Where indicated, *PTX* (100 ng/ml) was administered to cells during their labeling with [^{3}H]adenine. Cells were treated with either *PMA* (100 nM) or *4-α-PMA* (100 nM) for 10 min before stimulation by fMLP. cAMP accumulation was performed in the presence of 20 mM HEPES and 1 mM 3-isobutyl-1-methylxanthine. Data shown represent the mean ± SD of triplicate determinations in a single experiment; two additional experiments yielded similar results. *Asterisks*, fMLP significantly stimulated cAMP accumulation above the basal value; paired *t* test, $p < 0.05$.

stimulate AC-II in the presence, but not in the absence, of activated α_s. In 293 cells coexpressing AC-II and either the dopamine-D_2 or the δ-opioid receptor, no agonist-induced stimulation of AC-II was observed (Fig. 2). In contrast, significant stimulation of AC-II activity was obtained in cells treated with 100 nM PMA (Fig. 2). PMA treatment increased the basal AC-II activity by three- to eightfold, and the agonist-induced responses were blocked by PTX (Fig. 2). Hence, it appears that receptors for

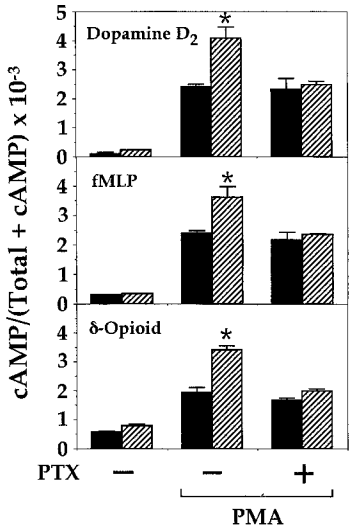

Figure 2. Enhancement of PMA-stimulated AC-II activity by G_i-coupled receptors. The 293 cells were cotransfected with cDNAs (0.25 μg/ml per construct) encoding AC-II and one of three G_i-coupled receptors: *Dopamine D_2*, *fMLP*, or *δ-Opioid*. Transfected cells were labeled with [^{3}H]adenine in the presence or absence of *PTX* (100 ng/ml) and subsequently assayed for cAMP levels in response to dopamine (1 μM), fMLP (200 nM), or DPDPE (100 nM). Basal cAMP levels are indicated by *solid bars*, and agonist-stimulated activities are represented by *hashed bars*. Before drug exposure, some cells were incubated with *PMA* (100 nM) for 10 min. Results shown are the mean ± SD from a representative experiment; two independent experiments gave similar results. *Asterisks*, Agonists to the G_i-coupled receptors significantly enhanced the PMA-stimulated AC-II activity; paired *t* test, $p < 0.05$.

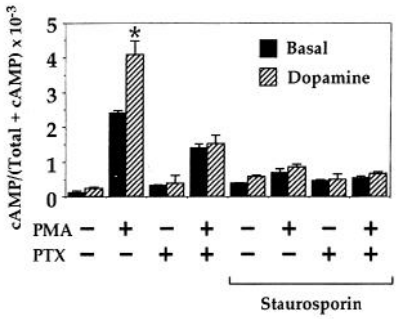

Figure 3. Requirement of PKC activation in G_i-mediated stimulation of AC-II in the absence of activated α_s. The 293 cells were cotransfected with cDNAs encoding the dopamine D_2 receptor (0.25 μg/ml) and AC-II (0.25 μg/ml). Where indicated, transfected cells were treated with *PTX* (100 ng/ml, 16–20 hr), *Staurosporin* (500 nM, 30 min), and/or *PMA* (100 nM, 10 min) in sequential order before the addition of dopamine (1 μM, 30 min) for cAMP assays. Data shown are the mean ± SD of triplicate determinations in a single experiment; two independent experiments yielded similar results. *Asterisks*, Dopamine significantly enhanced the PMA-stimulated AC-II activity; paired t test, $p < 0.05$.

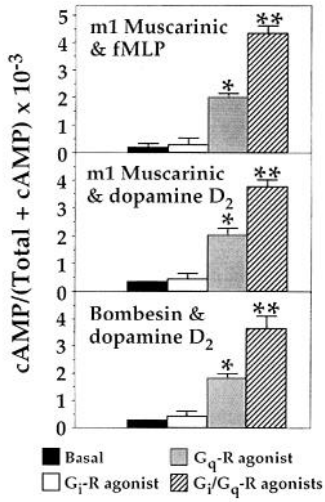

Figure 4. Synergistic effect of G_q- and G_i-coupled receptors on the stimulation of AC-II. The 293 cells were cotransfected with cDNAs (0.25 μg/ml per construct) encoding AC-II, a G_q-coupled receptor (m1 muscarinic or bombesin), and a G_i-coupled receptor (fMLP or dopamine D_2) as indicated. Transfected cells were assayed for cAMP accumulation in the presence of one or more agonists to the coexpressed receptors: fMLP (200 nM), CCh (200 μM), dopamine (1 μM), and bombesin (100 nM). Data shown represent the mean ± SD of triplicate determinations in one experiment; two other experiments yielded similar results. *Asterisks*, Agonists to G_q-coupled receptors significantly stimulated the AC-II activity above basal values; paired t test, $p < 0.05$. *Double asterisks*, Activation of the G_i-coupled receptors significantly potentiated the AC-II responses to agonists acting on the G_q-coupled receptors; paired t test, $p < 0.05$.

a variety of ligands (catecholamines, neuropeptides, or chemotactic factors) can stimulate AC-II via a G_i-linked mechanism that is dependent on PKC activation.

The involvement of PKC was examined further with the kinase inhibitor staurosporin. The 293 cells were cotransfected with cDNAs encoding the dopamine-D_2 receptor and AC-II and then assayed for the synergistic effect of PMA and dopamine on cAMP accumulation. As illustrated in Figure 3, when the transfected cells were pretreated with 500 nM staurosporin for 30 min before the PMA treatment, the ability of dopamine to stimulate AC-II activity was completely abolished. Moreover, the elevated basal activity that normally accompanies PMA treatment was absent in cells pretreated with staurosporin (Fig. 3); staurosporin did not affect the basal activity of AC-II. Collectively, these results indicate that in the absence of GTP-bound α_s, activation of PKC is required for G_i-mediated stimulation of AC-II.

Synergistic effects of G_q- and G_i-coupled receptors on AC-II activity

If activation of PKC is required for the G_i-mediated stimulation of AC-II, then agonists acting on G_q-coupled receptors should be able to mimic the effects of PMA. We transfected 293 cells with cDNAs encoding the AC-II, the m1-muscarinic receptor, and either the fMLP or dopamine-D_2 receptor. Activation of the m1-muscarinic receptor by 200 μM carbachol (CCh) increased cAMP accumulation by approximately eightfold (Fig. 4). The CCh effect has been attributed previously to the phosphorylation of AC-II (Jacobowitz et al., 1993). On the contrary, agonists acting on the fMLP or dopamine-D_2 receptors had little or no effect on the activity of AC-II (Fig. 4). In the presence of 200 μM CCh, both fMLP and dopamine were able to stimulate AC-II activity, and the formation of cAMP was elevated by ~100% compared with that obtained with CCh alone (Fig. 4). Similar results were obtained when we replaced the m1-muscarinic receptor with the bombesin receptor, another G_q-coupled receptor. As shown in Figure 4, simultaneous activation of bombesin and dopamine-D_2 receptors significantly increased cAMP accumulation beyond that seen in the presence of bombesin alone. It is of interest to note that the magnitude of synergism between G_q- and G_i-coupled receptors on the activity of AC-II was similar to that seen with PMA and G_i-coupled receptors. Other G_q-coupled receptors such as those for angiotensin II and cholecystokinin can substitute for the m1-muscarinic and bombesin receptors (our unpublished data).

Stimulation of AC-II by G-protein $\beta\gamma$ subunits

Although not unequivocally proven, it is generally assumed that the G_i-coupled receptors stimulate AC-II via the actions of $\beta\gamma$ subunits (Tang and Gilman, 1991). Stimulation of AC-II by different G-protein $\beta\gamma$ subunits, in the presence of activated $G\alpha_s$, has been well documented (Federman et al., 1992; Taussig et al., 1994; Ueda et al., 1994). In all previous reports, GTP-bound α_s was absolutely required for the $\beta\gamma$ actions on AC-II. It is therefore pertinent to examine whether PMA can replace activated α_s and permit $\beta\gamma$ subunits to stimulate AC-II. As a control, 293 cells were cotransfected with cDNAs encoding the AC-II and α_s-Q227L in the absence or presence of different combinations of $\beta\gamma$ complexes. Coexpression of AC-II and α_s-Q227L with either $\beta_1\gamma_2$ or $\beta_2\gamma_2$ in 293 cells produced cells that exhibit increased basal cAMP accumulation (Fig. 5). In the absence of α_s-Q227L, none of the $\beta\gamma$ complexes stimulated AC-II (data not shown). However, similar studies using $\beta_3\gamma_2$ did not produce cells with elevated cAMP levels (Fig. 5). These results are consistent with previous findings that the former two combinations, but not $\beta_3\gamma_2$, were able to yield functional $\beta\gamma$ complexes (Iñiguez-Lluhi et al., 1992; Pronin and Gautam, 1992; Schmidt et al., 1992; Ueda et al., 1994) that can activate AC-II in the presence of GTP-bound α_s (Tsu et al., 1995a). To bypass the requirement of GTP-bound α_s, 293 cells coexpressing AC-II and $\beta\gamma$ complexes were incubated with 100 nM PMA for 10 min before the cAMP assays. Compared with control (cells transfected with AC-II and vector), the PMA-induced stim-

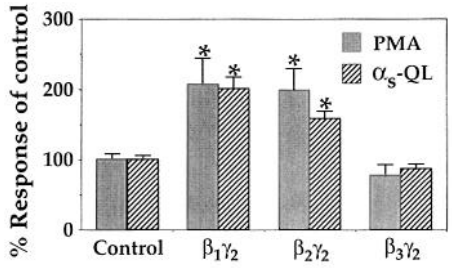

Figure 5. Coexpression of $\beta\gamma$ subunits stimulates AC-II after PMA treatment or in the presence of α_s-Q227L. The 293 cells were cotransfected with the cDNAs encoding AC-II (0.25 μg/ml) and various combinations of β (0.5 μg/ml) and γ (0.25 μg/ml) subunits, with or without α_s-Q227L (α_s-*QL*; 0.01 μg/ml). The cDNAs for the $\beta\gamma$ subunits were replaced by equivalent amounts of the vector pcDNA1 in the controls. Basal cAMP accumulation was determined for cells coexpressing α_s-Q227L. Transfected cells lacking the α_s-Q227L were treated with *PMA* (100 nM for 10 min) before cAMP assays. Results are expressed as a percentage of the cAMP accumulation observed in the *Control* cells. Data shown represent the mean ± SD of triplicate determinations in a single experiment; two other experiments produced similar results. *Asterisks*, Significantly different from the corresponding control; paired *t* test, $p < 0.05$.

ulation of basal cAMP accumulation was significantly higher in cells coexpressing AC-II and either $\beta_1\gamma_2$ or $\beta_2\gamma_2$ (Fig. 5). Again, coexpression of AC-II with $\beta_3\gamma_2$ was without effect. Thus, PMA appeared to allow viable $\beta\gamma$ subunits to stimulate AC-II, which otherwise would require the presence of activated α_s.

One of the hallmarks of $\beta\gamma$-mediated signal transduction in mammalian systems is its weaker potency in eliciting the response. Compared with responses mediated by G-protein α subunits, a 10-fold higher concentration of $\beta\gamma$ subunits might be required (Lee et al., 1993). For both fMLP and δ-opioid receptors, the EC_{50} values for agonist-dependent, $\beta\gamma$-mediated stimulation of phospholipase C are indeed 10-fold larger than those for α_i-mediated inhibition of adenylyl cyclase (Tsu et al., 1995b,c). Using the δ-opioid receptor as an example, we examined the dose–response relationship between [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and AC-II stimulation in PMA-treated cells. In 293 cells coexpressing AC-II and the δ-opioid receptor, after PMA treatment, DPDPE stimulated cAMP accumulation in a dose-dependent and saturable manner (Fig. 6). The resultant EC_{50} value for DPDPE was 6 nM. This figure closely resembles that for DPDPE-mediated stimulation of phospholipase C (Tsu et al., 1995b) and is substantially higher than the EC_{50} of DPDPE-induced inhibition of cAMP accumulation. The higher EC_{50} value supports the notion that the PMA-permissive, G_i-linked stimulation of AC-II is mediated via $\beta\gamma$ subunits.

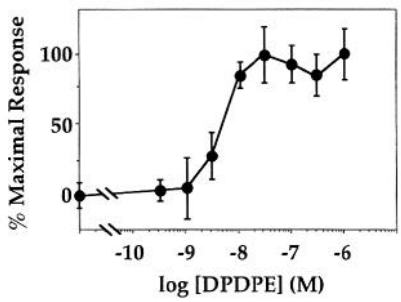

Figure 6. Agonist dose response for G_i-mediated stimulation of AC-II after PMA treatment. The 293 cells were cotransfected with the δ-opioid receptor and AC-II cDNAs as in Figure 2. Transfected cells were treated with PMA (100 nM for 10 min) and subsequently assayed for cAMP accumulation after exposure to varying concentrations of the δ-opioid agonist *DPDPE* (up to 1 μM). Results are expressed as percent maximal stimulation by DPDPE. Data shown represent the mean ± SD of triplicate determinations in a single experiment; two additional experiments yielded similar results.

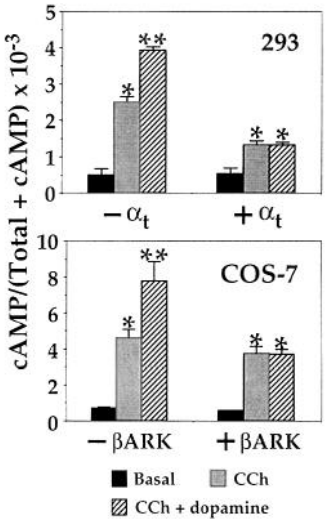

Figure 7. Blockade of the G_i-mediated enhancement of CCh-induced stimulation of AC-II by α_t and βARK. *293* cells were cotransfected with cDNAs (0.25 μg/ml) encoding AC-II, dopamine-D_2, and m1-muscarinic receptors with or without (+ or −, respectively) α_t. *COS-7* cells were cotransfected as with the 293 cells, except the α_t cDNA was replaced with the *βARK* fragment 495–689 (2 μg/ml). Cells were then labeled with [^{3}H]adenine (1 μCi/ml) 1 d before stimulation with CCh (200 μM) in the absence or presence of dopamine (1 μM). Data represent triplicate determinations in a single experiment; two independent experiments yielded similar results. *Asterisks*, Significantly higher than the corresponding basal values; paired *t* test, $p < 0.05$. *Double asterisks*, Dopamine significantly enhanced the CCh response; paired *t* test, $p < 0.05$.

To ascertain that $\beta\gamma$ subunits are involved in the G_i-mediated stimulation of AC-II after PMA treatment, we exploited the ability of transducin (α_t) and βARK to bind $\beta\gamma$ subunits. Both α_t and βARK can block $\beta\gamma$-mediated responses by scavenging free $\beta\gamma$ subunits (Federman et al., 1992; Koch et al., 1994). Cotransfection of 293 cells with AC-II, α_t, dopamine-D_2, and m1-muscarinic receptors produced cells in which dopamine did not potentiate the ability of CCh to stimulate AC-II (Fig. 7). Furthermore, coexpression of α_t partially inhibited the ability of CCh to stimulate cAMP accumulation (Fig. 7). In contrast, dopamine significantly enhanced the CCh response when α_t was omitted in the transfection (Fig. 7). These results are indicative of the full and partial involvement of $\beta\gamma$ subunits in mediating the G_i- and G_q-linked stimulation of AC-II, respectively.

Using a similar approach, we attempted to demonstrate the ability of $\beta ARK_{495-690}$ (a truncated form of βARK that has been shown to bind $\beta\gamma$ subunits tightly) to prevent the stimulation of AC-II by G_i-coupled receptors. In 293 cells, we were unable to demonstrate blockade of G_i-mediated stimulation of AC-II by $\beta ARK_{495-690}$ reproducibly. Nevertheless, coexpression of $\beta ARK_{495-690}$ with AC-II, the dopamine-D_2, and m1-muscarinic receptors in COS-7 cells caused a loss of dopamine-induced enhancement of the CCh-stimulated cAMP accumulation (Fig. 7). These results in COS-7 cells are consistent with the idea that by removing free $\beta\gamma$ subunits, $\beta ARK_{495-690}$ effectively abolished the

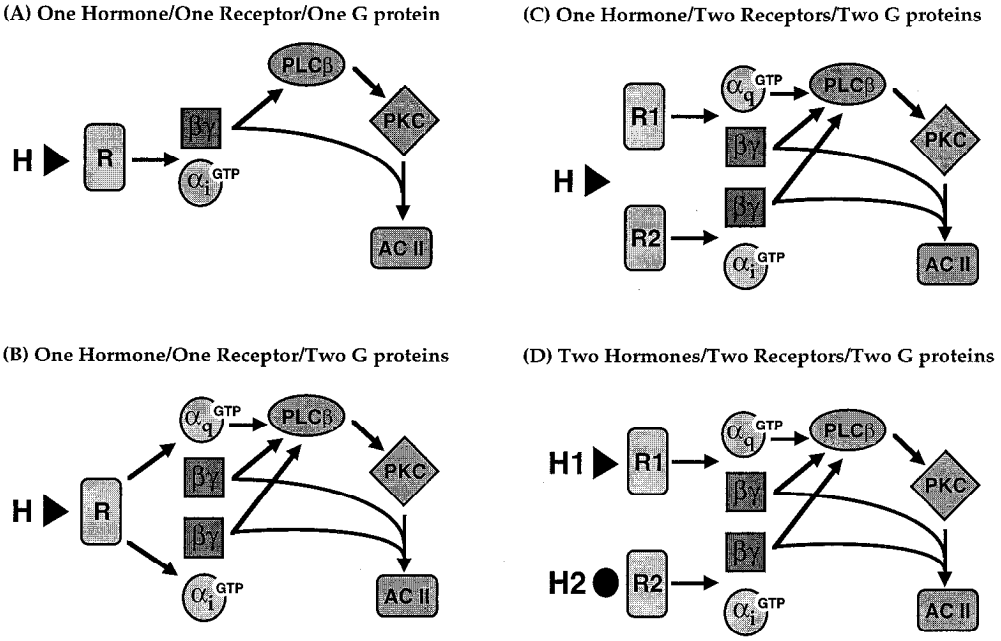

Figure 8. Mechanistic models in which G_i-coupled receptors can activate AC-II in an α_s-independent manner. Four models (*A–D*) for the activation of AC-II by G_i-released $\beta\gamma$ subunits are depicted. *H*, Hormone; *R*, receptor; *PLCβ*, phospholipase Cβ; *PKC*, protein kinase C; *AC II*, type II adenylyl cyclase.

ability of G_i-coupled receptors to stimulate AC-II. The observed differences between 293 and COS-7 cells are presently undefined.

DISCUSSION

Among the multiple isoforms of adenylyl cyclases cloned to date, the type II and IV isoforms are unique for their positive responsiveness to G-protein $\beta\gamma$ subunits. Previous reconstitution (Tang and Gilman, 1991) and transfection (Federman et al., 1992) studies have established that the stimulation of AC-II by $\beta\gamma$ subunits requires the presence of activated α_s. Simultaneous activation of G_s and either G_i or G_q would elevate intracellular cAMP to levels much greater than that seen with any one of these G-proteins alone. Indeed, activation of G_i-*proteins alone does not induce cAMP formation. The AC-II* thus serves as a molecular switch for the integration of coincident signals (Lustig et al., 1993). The present finding that PMA can alleviate the need for activated α_s and allow $\beta\gamma$ subunits to stimulate AC-II indicates that the control for AC-II is complex and multifactorial. Although in this scenario stimulation of AC-II is completely independent of G_s, it should be noted that the level of cAMP accumulation observed is only a small fraction of that attainable by G_s activation (Fig. 1). Thus, even though G_s is not absolutely essential, it remains one of the most potent activators of adenylyl cyclase.

The locus of the PMA action remains to be determined. One of the defined targets for PKC is AC-II itself. Numerous studies have confirmed that AC-II serves as a PKC substrate and that its activity is stimulated after phosphorylation (Jacobwitz et al., 1993; Yoshimura and Cooper, 1993; Jacobwitz and Iyengar, 1994). Some of the G-protein α subunits have also been demonstrated to serve as substrates for PKC. Both α_i (Katada et al., 1985) and α_z (Lounsbury et al., 1991) can be phosphorylated by PKC. However, neither α_s nor the $\beta\gamma$ subunits contain potential phosphorylation sites for PKC. The functional significance of phosphorylating the α subunits of G_i and G_z remains unclear. Because neither α_i nor α_z stimulates AC-II (Taussig et al., 1994), any gain of function after phosphorylation by PKC is unlikely to activate the type II isozyme. In fact, one would expect to see an inhibition of AC-II activity if PKC-mediated phosphorylation activates the α subunits of G_i and G_z. By the same analogy, loss of function of these phosphorylated α subunits might lead to increased cAMP accumulation attributable to removing the inhibitory control. It has been reported that α_{i2} can inhibit AC-II and that this inhibitory effect can be abolished by PKC-mediated phosphorylation (Chen and Iyengar, 1993). It is conceivable that an increase in cAMP production by AC-II could be caused by the removal of inhibitory control by PKC-mediated phosphorylation; but this would only occur if there was constitutive suppression of AC-II activity in the absence of phorbol esters. Moreover, such a mechanism does not explain the stimulatory effects on AC-II by agonists acting on G_i-coupled receptors. The most plausible explanation is that PMA-induced activation of PKC leads to the phosphorylation of AC-II, and this modification somehow allows the $\beta\gamma$-interacting domain of the enzyme to become responsive. Residues 956–982 of AC-II are critical for regulation by $\beta\gamma$ subunits (Chen et al., 1995), whereas the putative PKC-responsive region has been assigned to the C terminus (Levine and Reed, 1995). Although the $\beta\gamma$-interacting and the PKC-phosphorylation sites are far apart in the primary amino acid sequence of AC-II, their topology may be significantly closer in the tertiary structure. Only by analyzing the crystal structure of AC-II may one be able to resolve this problem.

Given that G_i- and G_q-regulated pathways can converge on AC-II, several interesting situations, as depicted in Figure 8, may arise. A single receptor that can activate both G_i and G_q should be able to stimulate the activity of AC-II in a partially PTX-sensitive manner. For instance, the thrombin receptor is capable of coupling to both G_i- and G_q-proteins (Hung et al., 1992), and receptor activation may lead to the activation of PKC, thereby allowing $\beta\gamma$ subunits released from G_i to stimulate AC-II further (Fig. 8*B*). The α_2-adrenergic receptor is another receptor that is known to possess the ability to interact with both G_i- and G_q-proteins (Conklin et al., 1992). The G_q-dependent pathway for the activation of PKC can be circumvented by the $\beta\gamma$-sensitive phospholipase C β isoforms (Camps et al., 1992; Smrcka and Sternweis, 1993). Colocaliza-

tion of phospholipase C β_2 or β_3 with AC-II should therefore allow receptors that are exclusively G_i-coupled to stimulate the formation of cAMP (Fig. 8*A*). However, because the activation of phospholipase C by $\beta\gamma$ subunits is much less efficient than that by α_q/α_{11}, stimulation of AC-II via this mechanism is unlikely to produce large increases in intracellular cAMP. In neutrophils, the fMLP receptor is predominantly coupled to G_i-proteins (Gierschik et al., 1989), and after binding to fMLP the formation of inositol phosphates is increased and is accompanied by a slight elevation in cAMP levels (Simchowitz et al., 1980). A single hormone or neurotransmitter may bind to two distinct receptor subtypes that are coupled to G_i and G_q, respectively (Fig. 8*C*). The muscarinic, serotonergic, and α-adrenergic-receptor systems contain multiple receptor subtypes that are coupled to either G_i- or G_q-proteins. Reports on the stimulation of cAMP accumulation by these neurotransmitter receptors are abundant. Finally, the ability of $\beta\gamma$ subunits to stimulate AC-II in the presence of activated PKC provides a mechanism by which two disparate receptor systems can integrate their signals in the form of intracellular cAMP (Fig. 8*D*). As demonstrated in Figure 4, simultaneous activation of two different receptor systems (e.g., dopaminergic and muscarinic) can be detected by a cell because of their synergistic actions on AC-II. We predict that other pairs of G_q- and G_i-coupled receptors may have the ability to activate AC-II in a synergistic manner. For example, endogenous cholecystokinin has been reported to enhance the analgesic potentials of opioids in the CNS (Noble et al., 1993). Because the cholecystokinin receptors are coupled to Gq/G_{11}-proteins in the regulation of phospholipase C, and because opioid peptides have been shown to stimulate cAMP production (Cruciani et al., 1993), it is conceivable that AC-II may act as the point of signal convergence in neurons in which both receptors are colocalized with AC-II.

Three lines of evidence strongly suggest the involvement of $\beta\gamma$ subunits in mediating the synergistic effects of phorbol esters and G_i-coupled receptors on AC-II. First, coexpression of functional $\beta\gamma$ complexes and AC-II produced cells with greater responsiveness to PMA. Second, in line with most $\beta\gamma$-mediated responses, high concentrations of agonists acting on the G_i-coupled receptors are required for the PMA-permissive stimulation of AC-II. Third, the synergistic effect can be abrogated by the two known scavengers of $\beta\gamma$ subunits (α_t and βARK). It is noteworthy that the blockade by βARK could not be demonstrated reproducibly in 293 cells. The $\beta ARK_{495-690}$ fragment contains the pleckstrin homology domain, which is implicated in the binding of $\beta\gamma$ subunits (Touhara et al., 1995). $\beta ARK_{495-690}$ also harbors a putative site (T542; four residues upstream of the putative $\beta\gamma$-binding domain) for phosphorylation by PKC, and recent studies have shown that phorbol esters can indeed alter the function of βARK (Chuang et al., 1995). Perhaps PKC-mediated phosphorylation of βARK can eliminate its ability to bind $\beta\gamma$ subunits and that in 293 cells the phosphorylation state of βARK can be better maintained.

In summary, the present study provides evidence that the activity of AC-II can be modulated by signals acting via G_i- and G_q-proteins. In this case, the involvement of G_s is not essential for the stimulation of AC-II. Signal integration by cells or neurons that express AC-II is both complex and versatile. To understand fully how a neurotransmitter or hormone alters the cAMP levels in the target cells, one has to appreciate the cellular complement of G-proteins and effectors as well as the state of the target cells when the stimulus is being received. Preconditioning by PKC-mediated phosphorylation of AC-II can dictate whether a signal from a G_i-coupled receptor will be transformed into a rise in intracellular cAMP. We predict that many G_i- and G_q-coupled receptors can and do produce synergistic effects on AC-II in neuronal tissues.

REFERENCES

Camps M, Carozzi A, Scheer A, Park PJ, Giershik P (1992) Isozyme-selective stimulation of phospholipase C-β2 by G protein $\beta\gamma$ subunits. Nature 360:683–686.

Chan JSC, Chiu TT, Wong YH (1995) Activation of type II adenylyl cyclase by the cloned μ-opioid receptor: coupling to multiple G proteins. J Neurochem 65:2682–2689.

Chen J, Iyengar R (1993) Inhibition of cloned adenylyl cyclases by mutant-activated $G_{i\alpha}$ and specific suppression of type 2 adenylyl cyclase inhibition by phorbol ester treatment. J Biol Chem 268:12253–12256.

Chen J, DeDivo M, Dingus J, Harry A, Li J, Sui J, Carty DJ, Blank JL, Exton JH, Stoffel RH, Inglese J, Lefkowitz RJ, Logothetis DE, Hildebrandt JD, Iyengar R (1995) A region of adenylyl cyclase 2 critical for regulation by G protein $\beta\gamma$ subunits. Science 268:1166–1169.

Chuang TT, LeVine III H, De Blasi A (1995) Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. J Biol Chem 270:18660–18665.

Cruciani RA, Dvorkin B, Morris SA, Crain SM, Makman MH (1993) Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. Proc Natl Acad Sci USA 90:3019–3023.

Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through G_i-protein $\beta\gamma$ subunits. Nature 356:159–161.

Feinstein PG, Schrader KA, Bakalyar HA, Tang W-J, Krupinski J, Gilman AG, Reed RR (1991) Molecular cloning and characterization of a Ca^{2+}/calmodulin-insensitive adenylyl cyclase from rat brain. Proc Natl Acad Sci USA 88:10173–10177.

Gierschik P, Sidiropoulos D, Jakobs KH (1989) Two distinct G_i-proteins mediate formyl peptide receptor signal transduction in human leukemia (HL-60) cells. J Biol Chem 264:21470–21473.

Hung DT, Wong YH, Vu T-KH, Coughlin SR (1992) The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. J Biol Chem 267:20831–20834.

Iñiguez-Lluhi JA, Simon MI, Robishaw JD, Gilman AG (1992) G protein $\beta\gamma$ subunits synthesized in Sf9 cells: functional characterization and the significance of prenylation of γ. J Biol Chem 267:23409–23417.

Jacobwitz O, Iyengar R (1994) Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. Proc Natl Acad Sci USA 91:10630–10634.

Jacobwitz O, Chen J, Premont RT, Iyengar R (1993) Stimulation of specific types of G_s-stimulated adenylyl cyclases by phorbol ester treatment. J Biol Chem 268:3829–3832.

Katada T, Gilman AG, Watanabe Y, Bauer S, Jakobs KH (1985) Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. Eur J Biochem 151:431–437.

Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates $G_{\beta\gamma}$-mediated signaling. J Biol Chem 269:6193–6197.

Lee SB, Shin SH, Hepler JR, Gilman AG, Rhee SG (1993) Activation of phospholipase C-β2 mutants by G protein α_q and $\beta\gamma$ subunits. J Biol Chem 268:25952–25957.

Levine LR, Reed RR (1995) Identification of functional domains of adenylyl cyclase using *in vivo* chimeras. J Biol Chem 270:7573–7579.

Lounsbury KM, Casey PJ, Brass LF, Manning DR (1991) Phosphorylation of G_z in human platelets: selectivity and site of modification. J Biol Chem 266:22051–22056.

Lustig KD, Conklin BR, Herzmark P, Taussig R, Bourne HR (1993) Type II adenylyl cyclase integrates coincident signals from G_s, G_i, and G_q. J Biol Chem 268:13900–13905.

Masters S, Miller RT, Chi MH, Chang F-H, Beiderman B, Lopez NG, Bourne HR (1989) Mutations in the GTP-binding site of $G_{s\alpha}$ alter stimulations of adenylyl cyclase. J Biol Chem 264:15467–15474.

Noble F, Derrien M, Roques BP (1993) Modulation of opioid antinociception by CCK at the supraspinal level: evidence of regulatory mechanisms between CCK and enkephalin systems in the control of pain. Br J Pharmacol 109:1064–1070.

Pronin AN, Gautam N (1992) Interaction between G-protein β and γ subunit types is selective. Proc Natl Acad Sci USA 89:6220–6224.

Saloman Y, Londos C, Rodbell M (1974) A highly sensitive adenylate cyclase assay. Anal Biochem 58:541–548.

Schmidt CJ, Thomas TC, Levine MA, Neer EJ (1992) Specificity of G protein β and γ subunit interactions. J Biol Chem 267:13807–13810.

Simchowitz L, Fischbein LC, Spilberg I, Atkinson JP (1980) Induction of a transient elevation in intracellular levels of adenosine-3′,5′-cyclic monophosphate by chemotactic factors: an early event in human neutrophil activation. J Immunol 124:1482–1491.

Smrcka AV, Sternweis PC (1993) Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C β by G protein α and $\beta\gamma$ subunits. J Biol Chem 268:9667–9674.

Tang W-J, Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science 254:1500–1503.

Tang W-J, Gilman AG (1992) Adenylyl cyclases. Cell 70:869–872.

Touhara K, Koch WJ, Hawes BE, Lefkowitz RJ (1995) Mutational analysis of the pleckstrin homology domain of the β-adrenergic receptor kinase: differential effects on $G\beta\gamma$ and phosphatidylinositol 4,5-bisphosphate binding. J Biol Chem 270:17000–17005.

Taussig R, Iñiguez-Lluhi JA, Gilman AG (1993) Inhibition of adenylyl cyclase by $G_{i\alpha}$. Science 261:218–221.

Taussig R, Tang W-J, Hepler JR, Gilman AG (1994) Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. J Biol Chem 269:6093–6100.

Tsu RC, Allen RA, Wong YH (1995a) Stimulation of type II adenylyl cyclase by formyl peptide and C5a chemoattractant receptors. Mol Pharmacol 47:835–841.

Tsu RC, Chan JSC, Wong YH (1995b) Regulation of multiple effectors by the cloned δ-opioid receptor: stimulation of phospholipase C and type II adenylyl cyclase. J Neurochem 64:2700–2707.

Tsu RC, Lai HWL, Allen RA, Wong YH (1995c) Differential coupling of the formyl peptide receptor to adenylate cyclase and phospholipase C by the pertussis toxin-insensitive G_z protein. Biochem J 309:331–339.

Ueda N, Iñiguez-Lluhi JA, Lee E, Smrcka AV, Robishaw JD, Gilman AG (1994) G protein bg subunits: simplified purification and properties of novel isoforms. J Biol Chem 269:4388–4395.

Watson PA, Krupinski J, Kempinski AM, Frankenfield CD (1994) Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. J Biol Chem 269:28893–28898.

Wong YH (1994) G_i assays in transfected cells. Methods Enzymol 238:81–94.

Wong YH, Conklin BR, Bourne HR (1992) G_z-mediated hormonal inhibition of cyclic AMP accumulation. Science 255:339–342.

Wong YH, Federman A, Pace AM, Zachary I, Evans T, Pouysségur J, Bourne HR (1991) Mutant α subunits of G_{i2} inhibit cyclic AMP accumulation. Nature 351:63–65.

Yoshimura M, Cooper DMF (1993) Type-specific stimulation of adenylyl cyclase by protein kinase C. J Biol Chem 268:4604–4607.

Yung LY, Tsim ST, Wong YH (1995) Stimulation of cAMP accumulation by the cloned *Xenopus* melatonin receptor through G_i and G_z proteins. FEBS Lett 372:99–102.