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Isoform-Specific Sensitization of Adenylyl Cyclase Activity by Prior Activation of Inhibitory Receptors: Role of $\beta\gamma$ Subunits in Transducing Enhanced Activity of the Type VI Isoform

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SUMMARY

Many different types of cells develop increased adenylyl cyclase activity (sensitization) on prior treatment with drugs such as opiates that acutely inhibit the enzyme. We found that human embryonic kidney (HEK) 293 m2 cells, which express the inhibitory m2 muscarinic cholinergic receptor, exhibit a large increase in forskolin-stimulated cAMP synthesis when the cells are preincubated with the muscarinic agonist carbachol for ≥5 min and forskolin stimulation is performed in the presence of the muscarinic antagonist atropine. To determine whether a specific isoform of adenylyl cyclase is susceptible to the adaptation induced by prior activation of inhibitory receptors, cells were transfected with expression vectors encoding adenylyl cyclase types I, II, and VI, representing three major groups of the adenylyl cyclase family. Preincubation of the cells with carbachol for 30 min resulted in a significant increase in prostaglandin E_1-stimulated cAMP accumulation in cells expressing type VI, but not type I or type II, adenylyl cyclase. A similar selective increase in activity from type VI adenylyl cyclase was observed for prior treatment with the D_2 dopamine agonist quinpirole and stimulation of cAMP synthesis with human chorionic gonadotropin in cells transfected with expression vectors coding for the cognate receptors. We next investigated whether $\beta\gamma$ subunits play a role in the sensitization of type VI adenylyl cyclase activity; using expression of α_T to inhibit $\beta\gamma$-mediated effects, we found that the quinpirole-induced sensitization of type VI adenylyl cyclase was abolished. However, $\beta\gamma$ subunits do not seem to directly activate type VI adenylyl cyclase, in contrast with their ability to directly activate the type II enzyme. Therefore, $\beta\gamma$ subunits liberated after activation of inhibitory receptors seem to indirectly cause an increase in activity of type VI adenylyl cyclase. Indirect activation of the type VI enzyme by $\beta\gamma$ subunits is a novel mechanism contributing to the sensitization of adenylyl cyclase.

Drugs or hormones that acutely inhibit the activity of AC often induce an additional phenomenon, namely, an increase in activity of the enzyme (sensitization) that is evident after abrupt withdrawal of the inhibitor (1, 2). Mechanisms of sensitization are not known; however, the more extensively studied adaptation of desensitization, which occurs after activation of AC stimulatory receptors such as β-adrenergic receptors, has been elucidated in some detail (3). Sensitization was originally described at the cellular level in NG108–15 mouse neuroblastoma X rat glioma hybrid cells chronically treated with opiate drugs (4, 5) and was proposed to represent a biochemical correlate of drug withdrawal and addiction (4). Sensitization of AC by prior activation of inhibitory receptors has been documented subsequently in many types of cells and may represent a general cellular adaptive mechanism (1, 2). There has been renewed interest in up-regulation of the AC system in the setting of chronic exposure to drugs of abuse (6). The acute and chronic regulation of AC has been proposed to play a significant role in both the reinforcing and addictive properties of abused drugs such as opiates or cocaine (7). However, the molecular mechanisms involved in AC sensitization remain obscure.

Eight isoforms of mammalian membrane-bound AC have been cloned (8, 9). These eight isoforms can be grouped into three major families based on patterns of regulation and structural relatedness (8, 9). Before characterization of the cloned AC isoforms, the mechanism of the acute inhibition of AC activity by drugs or hormones was uncertain; the relative roles of the α_i or $\beta\gamma$ subunits liberated from the G_i class of G proteins in inhibiting AC were very unclear (10, 11). However, characterization of cloned and expressed isoforms of AC has revealed a complex and distinctive pattern of regulation by α_i and $\beta\gamma$ subunits among the isoforms. For example, the type I isoform is susceptible to inhibition by either α_i or $\beta\gamma$ subunits, whereas members of the type V/VI family are in-

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ABBREVIATIONS: AC, adenylyl cyclase; HEK, human embryonic kidney; PGE_1, prostaglandin E_1; hCG, human chorionic gonadotropin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

hibited by the α_i subunit but not the $\beta\gamma$ subunit. In contrast, types II and IV are relatively unresponsive to inhibition by Gi subunits and indeed are conditionally activated by $\beta\gamma$ subunits liberated from G_i proteins (8, 9). In addition, distinctive patterns of regulation of the major isoforms of AC by protein kinase C and calcium have been found (8, 9). Among the eight isoforms, it seems that no two are regulated in an identical manner (9).

We wondered whether the isoforms of AC have a differential sensitivity to an adaptive increase in activity by prior activation of inhibitory receptors. If a particular isoform or isoforms were susceptible to this adaptation, the known regulatory characteristics of the isoforms (8, 9) might provide insight into the mechanism for induction and/or expression of the sensitization of AC. To address this question, we chose representatives from each of the three major families of cloned mammalian AC. These isoform families, represented by types I, II, and VI, can be distinguished by their unique pattern of regulation by both $\beta\gamma$ subunits and calcium (8, 9).

Initially, we established that sensitization of AC can occur in HEK 293 cells, a model system that has been extensively used to express and characterize the various isoforms of AC (12–14). A cell line expressing the inhibitory m2 muscarinic cholinergic receptor was used in these studies (15). We found that preincubation of the cells with the muscarinic agonist carbachol induced a large increase in cAMP synthesis from endogenous AC(s) expressed in these cells on blockage of the action of carbachol by atropine. Expression of the types I, II, and VI isoforms demonstrated a striking difference in their susceptibility to sensitization by prior activation of an inhibitory receptor. A novel, indirect role for $\beta\gamma$ subunits in mediating sensitization of the type VI isoform of AC is suggested by these results.

Experimental Procedures

Plasmids. Mammalian expression vectors for AC types I, II, and VI (pCMV-ACI, pCMV-ACII, and pCMV-ACVI)) were kindly provided by Dr. Dermont Cooper (University of Colorado, Denver, Colorado) and have been described previously (16). Expression vectors coding for the lutropin receptor (LHR-pCIS), the D_2 dopamine receptor (D2R-pcDNA-I), a mutationally activated form of the α subunit of G_s (αs-Q227L-pcDNA-I), the α subunit of transducin ($\alpha\tau$-pcDNA-I), and null vector DNA (pcDNA-I) were kindly provided by Dr. Henry Bourne (University of California, San Francisco) and have been described previously (17).

Cell culture. The parental HEK 293 cell was obtained from the American Type Culture Collection (Rockville, MD), and the cell line transfected with and expressing the m2 muscarinic cholinergic receptor, HEK 293 m2, was kindly provided by Dr. Ernest Peralta (Harvard University, Cambridge, MA) and has been described previously (15). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, NY) supplemented with $NaHCO_3$ (3.7 g/liter) and heat-inactivated fetal bovine serum (10%) (HyClone Laboratories, Logan, UT) in a humidified atmosphere of 5% CO_2 at 37 C. Selective pressure was maintained on the HEK 293 m2 line by including geneticin (G-418, 500 μg/ml) (GIBCO-BRL).

Transfection of cells. Cells were seeded 3 days before the start of transfection at 3.0×10^6 cells/100-mm dish and were ~50% confluent at the time of transfection. Transfection was performed according to the DEAE-dextran method (18) in $NaHCO_3$- and serum-free medium containing 0.40 mg/ml DEAE-dextran, 0.10 mM chloroquine, and 20 mM Na-HEPES, pH 7.3. Total plasmid DNA concentration was 25 μg/100-mm dish, with 5 μg of each expression vector coding for a specific gene and sufficient null vector to provide the total amount. Cells were incubated with the plasmid DNA for 4 hr at 37 C, without CO_2, and then washed with balanced salt solution (0.75 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 6 mM glucose, 25 mM Na-HEPES, pH 7.0) and subjected to a 5-min shock with 15% dimethylsulfoxide in the balanced salt solution. After the cell monolayer was washed twice with medium, the cells were incubated in complete growth medium for an additional 20 hr before being seeded for experiments. Cells were seeded at 0.25×10^6/16-mm well and grown for an additional 2 days before treatment.

Drug treatment of cells. Preincubation of cells with inhibitory drug was accomplished by adding carbachol (10–5 M) or the D_2 dopamine agonist quinpirole (5×10^{-7} M) to the complete growth medium as indicated. Preincubations with inhibitory drugs were for 30 min except where indicated. For measurement of cAMP accumulation in intact cells, the medium with or without inhibitory drug was removed by aspiration and replaced with $NaHCO_3$- and serum-free treatment medium containing Na-HEPES (20 mM, pH 7.3) and an agent to stimulate AC activity; either forskolin (5×10^{-5} M), PGE_1 (10^{-6} M), or hCG (5 μg/ml) was used. To assess the withdrawal response when cells were preincubated with inhibitory drug, the muscarinic cholinergic antagonist atropine (10^{-6} M) or the D_2 dopamine antagonist spiperone (5×10^{-7} M) was included for preincubation with carbachol or quinpirole, respectively. Alternatively, to assess the acute inhibitory response in cells not pretreated with drug, carbachol (10^{-5} M) or quinpirole (5×10^{-7} M) was included with the stimulatory agent. Treatment with stimulatory agent was for 2 min at room temperature (forskolin-stimulated cAMP synthesis was linear for at least this time period).

Assay of cAMP. Incubations were terminated by the addition of an equal volume (0.25 ml) of 0.2 N HCl to the wells. Cell counts were made from separate wells. cAMP was measured through the use of radioimmunoassay (19).

Results

HEK 293 m2 cells, transfected to express the m2 muscarinic cholinergic receptor (15), were chosen to study the regulation of AC activity by the muscarinic agonist carbachol in our initial experiments. The parental cell line, HEK 293, has been used extensively to express genes coding for AC as these cells can be transfected efficiently and have relatively low endogenous AC activity (12–14). As reported in the original description of the HEK 293 m2 cell line (15), simultaneous addition of carbachol with an agent that stimulates AC activity resulted in an inhibition of the activity. Carbachol (10^{-5} M) inhibited forskolin- or PGE_1-stimulated cAMP accumulation in the cells by 24.7 ± 4.0 and 61.9 ± 5.7%, respectively (six and five experiments, respectively); these inhibitory responses were blocked by atropine (10^{-6} M). The parental HEK 293 cell possesses endogenous m1 muscarinic cholinergic receptors that are not coupled to the inhibition of AC activity (14, 20). We confirmed that carbachol did not inhibit the stimulation of cAMP accumulation by either forskolin or PGE_1 in this parental cell line not expressing m2 receptors. Therefore, responses mediated by m2 receptors could be determined by comparing the stable transfectant with the parental cell.

To test the effect of preactivation of m2 receptors on AC activity, HEK 293 m2 cells were incubated with carbachol (10^{-5} M) for various times, and the subsequent forskolin-stimulated cAMP accumulation was measured in the presence of the atropine to block the inhibitory action of carbachol. Preincubation of the cells with carbachol rapidly induced an increase in the forskolin-stimulated cAMP accu-

mulation (Fig. 1). The increase in stimulation was near-maximal after 5 min of preincubation with carbachol; an apparent maximum of a 3.5-fold increase in forskolin-stimulated cAMP accumulation was attained after 20 min preincubation with carbachol. A 48-hr preincubation with carbachol did not result in further augmentation in cAMP responsiveness (data not shown). In a series of 12 experiments, a 30-min preincubation of the cells with carbachol resulted in a 4.7 ± 0.4-fold increase in the subsequent stimulation of cAMP accumulation by forskolin in the presence of atropine. In the parental HEK 293 cells with endogenous phospholipase C-coupled m1 receptors but not expressing m2 receptors, carbachol induced a small increase in forskolin-stimulated cAMP accumulation of 0.4 fold (seven experiments). Consequently, the large effect in HEK 293 m2 cells can be attributable to carbachol-induced activation of the m2 rather than m1 receptors. Preincubation of the HEK 293 m2 cells with carbachol also induced an increase in PGE_1-stimulated cAMP accumulation (2.1 ± 0.1-fold increase, 11 experiments).

After this preliminary characterization of the carbachol-induced increase in endogenous AC activity in HEK 293 m2 cells, we investigated whether specific isoforms of AC are susceptible to this adaptive increase in activity by prior activation of inhibitory receptors. Cells were transfected with control vector or a vector coding for a representative of each of the major isoforms of AC, type I, II, or VI. Three assessments were subsequently performed: (i) confirmation that each of the different isoforms of AC was expressed using three different AC stimulatory agents (Fig. 2), (ii) determination of the effect of activation of the m2 receptors with carbachol simultaneous with these stimulators of AC for the different isoforms of AC (Fig. 3), and (iii) determination of the effect of prior activation of m2 receptors with carbachol on the subsequent stimulation of activity in cells expressing the different isoforms of AC (Fig. 4). Fig. 2 presents the expression of activity resulting from treatment of the cells with forskolin, PGE_1, or hCG for cells transfected with vectors coding for ACI, ACII, or ACVI, relative to cells transfected

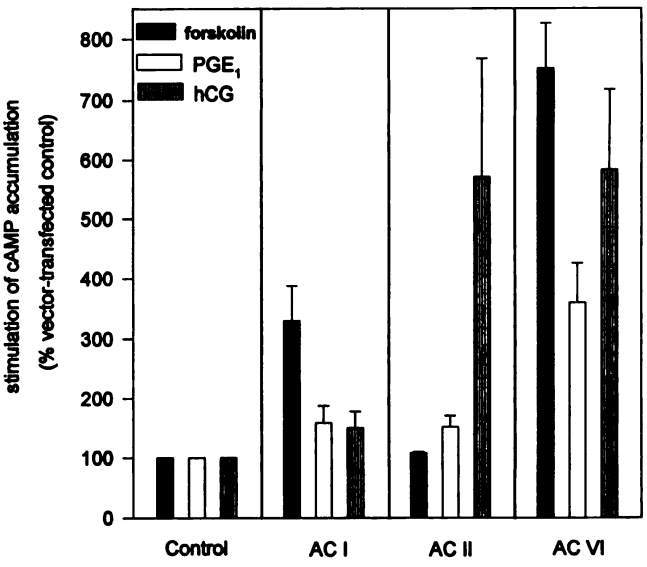

Fig. 2. Expression of adenylyl cyclases types I, II, and VI after transfection of HEK 293 m2. Cells were transfected with control vector or vectors encoding AC types I, II, or VI, as indicated. Three days after the start of transfection, cells were stimulated with forskolin (5×10^{-5} M), PGE_1 (10^{-6} M), or hCG (5 μg/ml) for 2 min. cAMP data were normalized to vector-transfected control cells for each treatment (forskolin, 50.9 ± 8.6; PGE_1, 18.0 ± 3.2; and hCG, 42.2 ± 4.1 pmol cAMP/10^6 cells). Data are mean ± standard error of six (forskolin and PGE_1) or two (hCG) experiments.

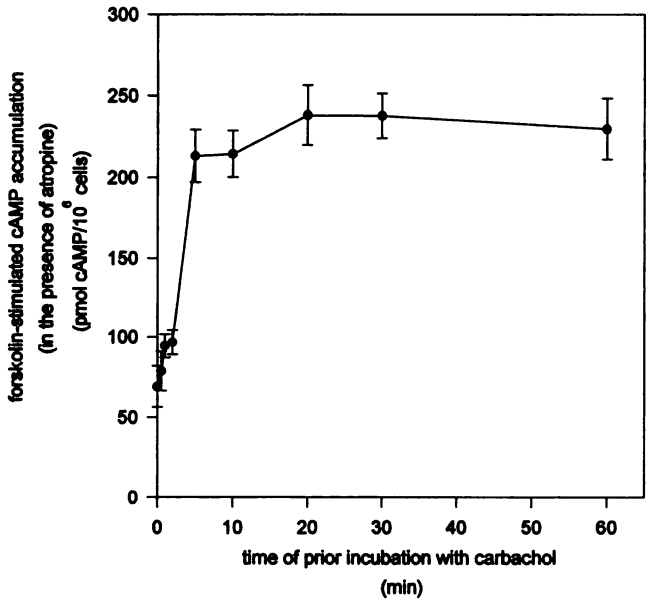

Fig. 1. The effect of preincubation with carbachol on forskolin-stimulated cAMP accumulation in HEK 293 m2. Cells were incubated with or without carbachol (10^{-5} M) for various times as indicated. The medium was removed and replaced with treatment medium containing forskolin (5×10^{-5} M) and atropine (10^{-6} M), and the cells were incubated for 2 min. cAMP data are mean ± standard deviation of replicate culture dishes. This experiment was repeated once with similar results. Atropine had no effect on forskolin stimulation of cAMP accumulation in control cells not incubated with carbachol (data not shown).

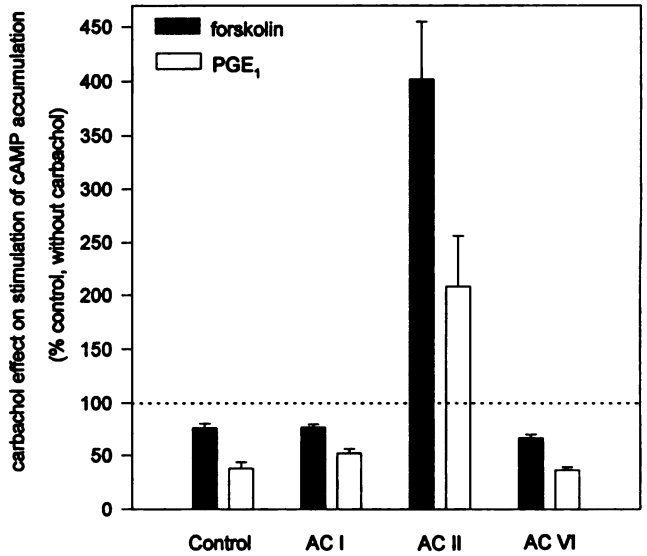

Fig. 3. The effect of simultaneous incubation with carbachol on forskolin- or PGE_1-stimulated cAMP accumulation in HEK 293 m2 cells. Cells were transfected with control vector or vectors encoding AC types I, II, or VI, as indicated. Three days later, cells were stimulated with forskolin (5×10^{-5} M) or PGE_1 (10^{-6} M) in the absence or presence or carbachol (10^{-5} M) for 2 min. cAMP data were normalized to values determined with stimulation in the absence of added carbachol. Data are mean ± standard error of six experiments.

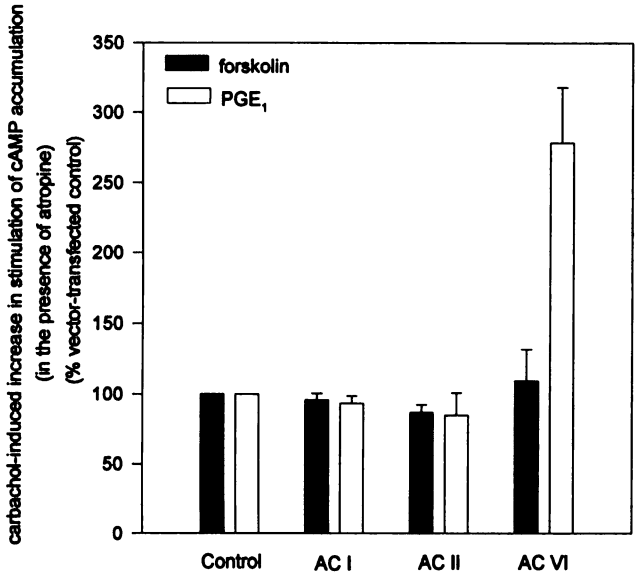

Fig. 4. The effect of preincubation with carbachol on forskolin- and PGE_1-stimulated cAMP accumulation in HEK 293 m2 cells transfected to express ACI, ACII, or ACVI. Cells were transfected with control vector or vectors encoding AC types I, II, or VI, as indicated. Three days later, cells were incubated with or without carbachol (10^{-5} M) for 30 min, then treated with forskolin (5×10^{-5} M) or PGE_1 (10^{-6} M) in the presence of atropine (10^{-6} M) for 2 min. cAMP data were normalized to vector-transfected control cells by calculating the difference in cAMP concentrations from cells incubated with carbachol or not and expressing the differences from cells transfected with exogenous AC relative to the differences from vector-transfected control cells. Data are means ± standard error of six experiments. Preincubation of the cells with carbachol resulted in a significant ($p < 0.01$, paired t test) increase in PGE_1-stimulated cAMP accumulation attributable to ACVI but not to ACI or ACII.

with control vector. There was substantial variation in the expression of the three isoforms of AC, depending on the stimulatory agent used. Such variation is consistent with previously reported studies of expression of AC isoforms in these cells (see Discussion). Nevertheless, there was significant expression of activity from each of the three isoforms of AC with respect to at least one stimulatory agent, indicating that each of the isoforms was being expressed.

An assessment of the ability of carbachol to acutely inhibit the stimulation of cAMP accumulation after transfection and expression of the three isoforms of AC was also performed (Fig. 3). After transfecting the cells with vectors coding for ACI or ACVI, simultaneous incubation of the cells with carbachol and stimulatory agent resulted in the expected carbachol-mediated inhibition of forskolin- or PGE_1-stimulated cAMP accumulation. However, in cells transfected with vector coding for ACII, carbachol did not inhibit but rather potentiated the stimulation of cAMP accumulation in response to either forskolin or PGE_1. This result is consistent with previous data showing that activation of other "inhibitory" receptors coupled to G_i can enhance the stimulation of AC activity in HEK 293 cells expressing ACII (17, 21, 22).

The capacity of prior activation of m2 receptors with carbachol to induce an increase in activity of a specific isoform of AC was tested after transfection of the cells with vectors coding for ACI, ACII, or ACVI (Fig. 4). Three days after transfection, the cells were incubated with carbachol for 30 min before treatment with either forskolin or PGE_1 in the presence of atropine to block the response to carbachol. The differences in cAMP concentrations resulting from preincubation with carbachol were calculated, and the differences from cells transfected with exogenous AC were normalized to those from vector-transfected control cells to subtract the carbachol-induced increases resulting from endogenous AC activity expressed in the cells. The results of these experiments revealed a striking difference among the three isoforms of AC. Prior treatment of the cells with carbachol resulted in a significant increase in PGE_1-stimulated activity of ACVI but not of ACI or ACII. Also, the carbachol-mediated augmentation in activity of ACVI was evident for PGE_1 stimulation but not for forskolin stimulation. Expression of ACVI in parental HEK 293 cells did not result in a further increase in carbachol-induced activity, indicating the importance of the m2 receptors (data not shown).

Because activation of inhibitory receptors liberates $\beta\gamma$ subunits from G_i proteins and these $\beta\gamma$ subunits can modify the activity of a variety of effectors (23–25), we wondered whether $\beta\gamma$ subunits released on activation of the m2 receptors could contribute of the augmented activity of ACVI. The $\beta\gamma$ subunits have been shown to directly enhance the $G_{s\alpha}$ activation of ACII (11, 22, 26, 27), and $\beta\gamma$ subunits liberated from G_i proteins in response to activation of several different inhibitory receptors (α_2-adrenergic, D_2 dopamine, and A_1 adenosine) have been shown to enhance the $G_{s\alpha}$ conditional activation of ACII expressed in HEK 293 cells (17, 21, 22). The involvement of $\beta\gamma$ subunits in intact cells was demonstrated by the fact that expression of the α subunit of transducin ($\alpha\tau$) attenuated the capacity of drugs acting through G_i to enhance ACII activity, presumably because expressed $\alpha\tau$ would be expected to bind free $\beta\gamma$ subunits (17). Therefore, we used this ability of $\alpha\tau$ to sequester free $\beta\gamma$ subunits to test the potential role of $\beta\gamma$ subunits on the sensitization of ACVI. Preliminary experiments indicated that expression of $\alpha\tau$ could significantly attenuate the increase in forskolin stimulation of endogenous AC resulting from preincubation of HEK 293 m2 cells with carbachol (data not shown). Therefore, we pursued the question of a possible role for $\beta\gamma$ subunits in mediating the marked increase in activity of ACVI caused by prior activation of an inhibitory receptor.

To answer this question, we adopted the approach of cotransfecting the cells with vectors coding for the requisite components to measure the effects of expression of a stimulatory receptor, an inhibitory receptor, $\alpha\tau$, and ACVI in the same population of transfected cells. To ensure that these experiments worked as expected, a number of control experiments were done in parallel. We confirmed that a G_i-coupled receptor could enhance the $G_{s\alpha}$ activation of ACII and that this activation was attenuated by expression of $\alpha\tau$ as has been found previously (17). HEK 293 m2 cells were cotransfected with expression vectors coding for the inhibitory D_2 dopamine receptor, the mutationally activated α subunit of G_s, and ACII, and with or without an expression vector coding for $\alpha\tau$. The results of one of these control experiments are given in Table 1. Incubation of the transfected cells with the D_2 agonist quinpirole resulted in augmented cAMP accumulation in these cells; this quinpirole-induced increase in cAMP accumulation was dependent on cotransfection with the vector coding for ACII (data not shown). These results confirm previous observations (17). Expression of $\alpha\tau$ completely abolished the increased cAMP accumulation caused by quinpirole. This control experiment indicated that there

TABLE 1

The effect of the α subunit of transducin (αT) on quinpirole stimulation of ACII in HEK 293m2 cells

Cells were transfected with vectors coding for the genes for ACII (pCMV-ACII), the D_2 dopamine receptor (D2R-pcDNA-I), and the constitutively activated α subunit of G_s (αs-Q227L-pcDNA-I) and with or without the vector coding for αT (αT-pcDNA-I) or control vector (pcDNA-I), as indicated. Three days after the start of transfection, cells were treated or not treated with quinpirole (5×10^{-7} M) for 2 min. cAMP data are mean ± standard data of replicate culture dishes. This representative experiment was repeated seven times with similar results. Expression of αT prevented the quinpirole stimulation of ACII.

Vector	[cAMP]	
	pcDNA-I	αT-pcDNA-I
	pmol/10⁶ cells	
Basal	145.0 ± 5.6	62.5 ± 1.7
Quinpirole stimulated	266.3 ± 12.2	69.3 ± 21.6

was sufficient expression of αT to markedly attenuate or abolish the βγ stimulation of ACII, as has been found previously (17). In cells transfected with the vector coding ACVI rather than ACII, there was no effect of quinpirole treatment alone to increase the intracellular cAMP concentration (data not shown), confirming previous evidence that ACVI is not susceptible to direct βγ-mediated enhancement of $G_{s\alpha}$-conditional activation (28).

To test whether expression of αT could attenuate the increase in ACVI activity resulting from prior activation of an inhibitory receptor, HEK 293 m2 cells were transfected with expression vectors coding for the inhibitory D_2 dopamine receptor and a gonadotropin receptor coupled to the stimulation of AC, with or without vectors coding for ACVI or αT. In these experiments, quinpirole (5×10^{-7} M) inhibited hCG-stimulated cAMP accumulation by 64.8 ± 4.3% and 87.8 ± 2.0% in cells transfected without or with the vector coding for ACVI, respectively (four experiments). Quinpirole-mediated inhibition of cAMP accumulation was blocked by 10^{-6} M spiperone; transfection with the vector coding for αT had no effect on quinpirole-mediated inhibition of cAMP accumulation (data not shown). Each of four groups of transfected cells (with or without ACVI, each with or without αT) was incubated with quinpirole for 30 min and then treated for 2 min with hCG in the presence of the D_2 antagonist spiperone to block the immediate effects of quinpirole. The results of a representative experiment, repeated four times, are shown in Fig. 5. Prior treatment of the cells with quinpirole had no significant effect on hCG-stimulated cAMP accumulation from endogenous ACs. However, transfection of the cells with the vector coding for ACVI resulted in a large increase in hCG-stimulated cAMP accumulation. The activity in cells transfected with ACVI was increased an additional 2-fold after preincubation of the cells with quinpirole. In cells transfected with a vector coding for αT, this quinpirole-induced increase in ACVI activity did not occur, suggesting a role for βγ in sensitization of ACVI.

Discussion

Preactivation of m2 muscarinic cholinergic receptors with carbachol rapidly induced a marked increase in cAMP synthesis from endogenously expressed AC in HEK 293 m2 cells when the action of carbachol was rapidly terminated with atropine. Consequently, HEK 293 cells can be added to the list of many different types of cells in which sensitization of

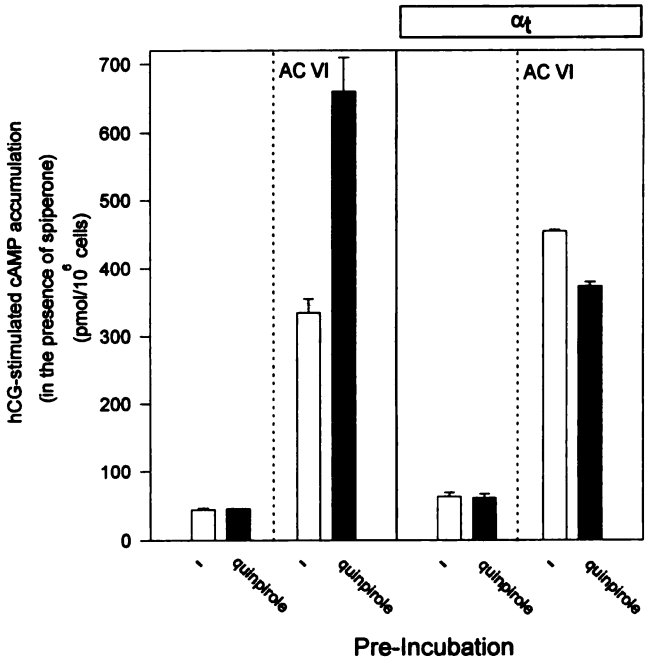

Fig. 5. The effect of the α subunit of transducin (αT) on the quinpirole-induced increase in hCG-stimulated cAMP accumulation attributable to ACVI in HEK 293 m2 cells. Cells were transfected with vectors coding for the genes for the lutropin receptor and the D_2 dopamine receptor and with or without vectors coding for ACVI or αT, as indicated. Three days later, cells were incubated with or without quinpirole (5×10^{-7} M) for 30 min and then treated with hCG (5 μg/ml) in the presence of the dopamine receptor antagonist spiperone (10^{-6} M) for 2 min. cAMP data are mean ± standard deviation of replicate culture dishes. This experiment was repeated four times with similar results.

AC by prior activation of inhibitory receptors has been described (2). A short term incubation of HEK 293 m2 cells with carbachol seemed to cause a maximal increase in forskolin-stimulated cAMP synthesis within 20 min.

Sensitization of AC by prior activation of inhibitory receptors was originally described for the case of treatment of cells with opiate drugs for many hours (4, 5), but the phenomenon is now recognized to be induced by both short (minutes) and long term treatments with a variety of drugs that activate inhibitory receptors (1, 2). The signal transduction mechanism responsible for the induction of sensitization of AC is unknown, although it does not seem to involve the decrease in intracellular cAMP concentration resulting from inhibition of AC activity (29). Also, the molecular alterations responsible for the sensitization of adenylyl cyclase activity are unknown. Expression of enhanced activity is not confined to a particular stimulatory agent or receptor (2). Numerous reports have documented changes in the quantities of G protein α or β subunits after treatment of cells, tissues, or animals with inhibitory drugs for many hours or days, although the results have not been consistent (2). In the current experiments, we focused on rapid sensitization of AC, where changes in protein expression are likely to be unimportant in any case.

Desensitization of the AC system has been extensively studied and has long been recognized to be an important biological adaptation resulting from activation of receptors (3). Sensitization of AC is now receiving increased interest due to potentially important consequences of this adaptive response. Chronic treatment of rats with morphine has been

reported to result in increases in AC activity in specific brain regions during withdrawal (6). Other alterations were reported to be associated with increases in AC activity, such as increased phosphorylation of several proteins, including the transcription factor cAMP response element binding protein, induction of the proto-oncogenes c-*fos* and c-*jun*, and increased expression of tyrosine hydroxylase (6). Thus, the up-regulated cAMP system has been suggested to play a role in the alteration of gene expression that may contribute to the reinforcing and addictive properties of abused drugs (7). We have found direct evidence that small increments in cAMP that occur during withdrawal from an inhibitory drug can cause increased phosphorylation of cAMP response element binding protein and induction of the c-*fos* gene in the NG108–15 cell line.[1]

We initially considered the idea that the carbachol-induced increase in forskolin-stimulated cAMP synthesis in HEK 293 m2 cells might be mediated through post-translational phosphorylation by protein kinase C or another kinase. Phorbol esters have been variously reported to attenuate or enhance the activity of AC, depending on the cell type, accompanying stimulus, or time of treatment (30–33). Recently, the idea that a differential effect of phorbol esters to enhance AC activity might be due to different forms of the enzyme was tested through expression of the cloned isoforms of AC. Phorbol esters increased the activity of ACII expressed in the parental HEK 293 cells (16, 34, 35), and the increase in activity has recently been associated with phosphorylation of this isoform (36). The phorbol ester-induced increase of activity of ACII is evident both in intact cells (16, 35) and in permeabilized cells (34). We compared the effects of preincubation of HEK 293 m2 cells with carbachol or phorbol ester by subsequently measuring cAMP responses in intact and in permeabilized cells, with or without transfection with an expression vector coding for ACII. We found that the carbachol-induced sensitization of AC was distinguished from the ability of phorbol ester to enhance the activity of exogenously expressed AC type II in terms of both the magnitude of the effect (much greater for carbachol) and the stability of the effect after cell lysis (preserved in case of phorbol ester pretreatment, whereas the effect of carbachol pretreatment is lost in the broken cells) (data not shown). Consequently, the carbachol-induced increase in forskolin-stimulated cAMP synthesis is likely not attributable to a mechanism similar to the protein kinase C-induced phosphorylation of the type II isoform.

The eight known isoforms of mammalian AC have been reported to be differentially regulated not only by protein kinase C but also by calcium and $\beta\gamma$ subunits of G proteins (8, 9). Because the isoforms of AC are differentially regulated, a specific isoform may be susceptible to an increase in activity caused by prior activation of inhibitory receptors. We chose to study representative isoforms from each of the three major families of mammalian AC, types I, II, and VI. Each of these types expressed in HEK 293 cells has been shown to have a distinct pattern of regulation by calcium or $\beta\gamma$ subunits (8, 9), although more than one type may be positively regulated by protein kinase C (34, 35). Although the HEK 293 cell has been considered a favorable model system with which to study the expression of exogenous AC because of the relatively low activity from endogenous AC (12–14), this cell has been reported to express mRNA for several isoforms, including types I, II, III, and VI and a likely type IX (37–40).

In the absence of any knowledge concerning the relative expression of the these isoforms at the protein level, it is difficult to come to any definite conclusions about mechanisms of sensitization in the wild-type cells. Consequently, we turned to transfecting these cells with representatives from the major isoform families of AC. Transfection of the HEK 293 m2 cells with expression vectors coding for AC types I, II, and VI resulted in significant variation in apparent levels of expression, depending on the agent used to stimulate enzyme activity (Fig. 2). Although a similar, direct comparison does not seem to have been performed in previous studies of these isoforms, in general, our results are consistent with those reported previously. For example, hormone/$G_{s\alpha}$ activation of ACI has been reported to be very low in magnitude (41–43). Also, the forskolin stimulation of ACII has been reported to be relatively small (16, 34, 44). In contrast, cotransfection of HEK 293 cells with a vector coding for the lutropin receptor and treatment of the cells with gonadotropin hormone resulted in a large increase in activity from ACII and ACVI (45), similar to our findings.

The ability of carbachol to acutely inhibit the stimulation of cAMP accumulation was preserved after expression of ACI and ACVI but not in the case of ACII (Fig. 3). Carbachol actually enhanced the stimulation of cAMP accumulation by both forskolin and PGE_1 in cells expressing ACII. This result is consistent with the documented ability of $\beta\gamma$ subunits released from activated G_i to enhance the activity of ACII (17, 21, 22).

In HEK 293 m2 cells expressing ACI, ACII, or ACVI, prior activation of the m2 muscarinic cholinergic receptor with carbachol revealed a strikingly difference among the isoforms with respect to the sensitization phenomenon. Preincubation with carbachol caused an increase in activity above that from vector-transfected control cells only in cells expressing ACVI (Fig. 4). The increase in activity from ACVI induced by carbachol seemed to be confined to hormone stimulation (PGE_1 or hCG, Figs. 3 and 5), not forskolin stimulation of cAMP accumulation. Although the reason for this difference is unknown, we speculate that there was no further increase in the stimulation by forskolin because the background increase in endogenous AC activity stimulated by forskolin was very large (Fig. 1).

In preliminary experiments, we obtained evidence that expression of $\alpha\tau$ could attenuate the carbachol-induced increase in forskolin stimulation of endogenous ACs in these cells. Because expression of $\alpha\tau$ attenuates or prevents the $G_{s\alpha}$-conditional enhancement of ACII by $\beta\gamma$ subunits liberated from activated G_i (17), these results suggested the possible involvement of the type II isoform in the carbachol-induced effect. However, the carbachol-induced sensitization of AC activity is induced by prior activation of the receptor and is evident only after muscarinic receptor inactivation by atropine, whereas the enhancement of the type II isoform has been observed in the presence of ligand-activated inhibitory receptors (17, 21, 22). Therefore, we considered the possibility that $\beta\gamma$ subunits might mediate the carbachol-induced sensitization of AC by having an indirect effect on enzyme activity, in contrast to the apparent direct activation of the type II isoform by $\beta\gamma$ subunits (11, 26, 27).

[1] J. M. Thomas and B. B. Hoffman, unpublished observations.

Because the stimulation of exogenous ACVI by the endogenous PGE_1 receptor was sensitized by prior activation of m2 receptors in HEK 293 m2 cells, we investigated whether expression of $\alpha\tau$ would block sensitization of ACVI. We also cotransfected both stimulatory and inhibitory receptors in these experiments; this cotransfection approach would eliminate background effects in cells not incorporating plasmid DNA. In an important control experiment, we confirmed that expression of $\alpha\tau$ prevented the $G_{s\alpha}$-conditional enhancement of ACII activity by an "inhibitory" receptor, the D_2 dopamine receptor, as has been demonstrated previously (17). This provided the basis for asking whether $\alpha\tau$ prevented sensitization of type VI AC. In cells transfected with vectors coding for the D_2 receptor and a stimulatory lutropin receptor, prior activation of the D_2 receptor with quinpirole and subsequent withdrawal of the agonist resulted in a significant increase in hCG-stimulated cAMP accumulation in cells expressing ACVI (Fig. 5). In cells expressing $\alpha\tau$, this sensitization of ACVI did not occur. These results suggest that $\beta\gamma$ subunits liberated from G_i on activation of an inhibitory receptor are responsible for the increase in activity of the type VI isoform of AC. Because the activity of ACVI was not potentiated in a test for an effect of $\beta\gamma$ on $G_{s\alpha}$-conditional stimulation, unlike the activity of ACII (Table 1), the actions transduced by $\beta\gamma$ on ACVI are likely to be indirect. We suggest that $\beta\gamma$ subunits liberated from G_i on activation of inhibitory receptors can regulate some other effector, which in turn has a positive effect of the activity of ACVI. However, the connection of these results in cells overexpressing ACVI and the mechanism of sensitization found in the cells in Fig. 1 remain speculative.

The $\beta\gamma$ subunits have been shown to regulate several different effectors. Other than AC, these effectors include K^+ channels, phospholipase Cβ, phospholipase A_2, β-adrenergic and muscarinic receptor kinases, mitogen-activated protein kinase, and pheromone-induced mating in yeast (for reviews, see Refs. 24 and 25). One of these effectors could be involved in the indirect regulation of ACVI by $\beta\gamma$ subunits. Further investigation will be required to determine the potential contribution to sensitization of ACVI by any of these signaling molecules regulated by $\beta\gamma$ subunits.

One possible pathway resulting in an increased activity of the type VI isoform of AC could involve Ca^{2+} channels. Type VI AC has been shown to be inhibited by low micromolar concentrations of Ca^{2+} (41, 46). Both ionophore-induced increases in intracellular Ca^{2+} (47, 48) and capacitative Ca^{2+} entry (48, 49) have been shown to inhibit the activity of ACVI expressed in HEK 293 cells. Receptors that were functionally identified on the basis of their ability to inhibit AC have additional effects in many cells, including the inhibition of Ca^{2+} channel activity (50, 51). Plausibly, activation of an inhibitory receptor could lower intracellular Ca^{2+} concentration via the inhibition of channel activity, and this decrease in Ca^{2+} concentration could result in increased activity of the type VI isoform of AC. However, we think this possibility is unlikely. First, the HEK 293 cells have been reported to have negligible voltage-dependent Ca^{2+} channel activity (52). Second, experiments in our laboratory have indicated that neither Ca^{2+} channel antagonists nor nominally Ca^{2+}-free medium could mimic the effect of carbachol or quinpirole to induce an increase in activity of ACVI (data not shown). Therefore, Ca^{2+} channels are unlikely to be involved in sensitization of ACVI.

We embarked on this study as a first step in defining the regulation of specific isoforms of AC by prior activation of inhibitory receptors. For the model system in this study, we used available inhibitory receptor systems and confined the analysis to short term activation of the receptors. Other effects on the isoforms of AC could result from activation of other inhibitory receptors or from prolonged receptor activation. Recently, mRNA for the type VIII isoform of AC was reported to be increased in brain tissue of rats chronically treated with morphine, but only mRNA for ACVIII was studied (53). Because in at least some brain tissue there may be poor correlation between isoform-specific mRNA and activity of AC (54), it is difficult to draw any comparison between the results of our study and this report of an effect of an inhibitory drug on the mRNA for the type VIII isoform. In view of the multiplicity of signal transduction mechanisms and differential G protein coupling of inhibitory receptors (55, 56), different drugs could have different effect on the isoforms of AC in different cells or tissues.

Expression of distinctly regulated isoforms of AC in HEK 293 cells has provided a model system to address the question of whether a specific isoform is susceptible to an increase in activity by prior activation of inhibitory receptors. The data indicate that the type VI isoform is susceptible to an inhibitor-induced increase in activity and suggest that a novel, indirect mechanism involving $\beta\gamma$ subunits is responsible for mediating sensitization of the type VI isoform of AC. This indirect regulation of ACVI is distinctly different from the previously characterized, direct but conditional activation of ACII by $\beta\gamma$ subunits. The mechanism for this novel action of $\beta\gamma$ awaits further investigation. Sensitization of AC activity is a complicated adaptation as is the antithetical, extensively studied question of desensitization of AC activity by stimulatory receptors.

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