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Regulation of Adenylyl Cyclase Type VI Activity During Desensitization of the A2a Adenosine Receptor-Mediated Cyclic AMP Response: Role for Protein Phosphatase 2A

YIJUANG CHERN, JIN-YI CHIOU, HSING-LIN LAI, and MING-HSIEN TSAI

Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China (Y.C., H.-L.L., M.-H.T.), and National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Taipei, Taiwan, Republic of China (J.-Y.C.)

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SUMMARY

We reported earlier that inhibition of adenylyl cyclase activity is a mechanism involved in desensitization of the A2a adenosine receptor-mediated cAMP response (A2a desensitization) in rat pheochromocytoma PC-12 cells. Here, we investigated the molecular mechanism that modulates adenylyl cyclase activity during A2a desensitization. Reversible inhibition of forskolin-evoked adenylyl cyclase activity in desensitized cells occurred after incubation with an A2a-selective adenosine agonist (CGS21680). However, when okadaic acid (a relatively protein phosphatase 2A-specific phosphatase inhibitor) was added after agonist removal, adenylyl cyclase activity did not recover. Okadaic acid caused significant dose-dependent inhibition of adenylyl cyclase activity in intact PC-12 cells. Prolonged exposure of okadaic acid-treated PC-12 cells to adenosine agonists did not evoke further inhibition, suggesting that the inhibition of adenylyl cyclase activity during A2a desensitization may operate through a pathway that overlaps with the increased phosphorylation caused by okadaic acid. Inclusion of calcium in the adenylyl cyclase assay significantly inhibited cyclase activity, indicating that PC-12 cells contain Ca^{2+}-inhibitable type VI adenylyl cyclase (AC6). This was confirmed by polymerase chain reaction-based detection of AC6 cDNA. Furthermore, incubation of PC-12 cell membrane fractions with purified protein phosphatase 2A or coexpression of protein phosphatase 2A with AC6 in COS-1 cells significantly increased AC6 activity. To reduce the possible influence of $G_{s\alpha}$ protein, we substituted guanosine-5′-*O*-(2-thio)diphosphate and $MnCl_2$ for GTP and $MgCl_2$, respectively, in some cyclase assays and found that the suppression of AC6 during A2a desensitization and okadaic acid treatment remained largely unchanged. Taken together, these data suggest that phosphorylation of AC6 might account for the inhibition of adenylyl cyclase activity during A2a desensitization in PC-12 cells.

Adenosine modulates a wide variety of physiological responses through specific receptors (1). At least four distinct adenosine receptor subtypes (A1, A2a, A2b, and A3), which associate with adenylyl cyclase to regulate intracellular cAMP content in response to extracellular adenosine, have been reported (2, 3). As with many other G protein-coupled receptors, prolonged activation of the A2a receptor significantly inhibits the response of the cells to subsequent stimulation (A2a desensitization). In rat pheochromocytoma PC-12 cells, we previously found no significant change in the number of binding sites or their affinity for adenosine agonists during A2a desensitization. Instead, inhibition of adenylyl cyclase, down-regulation of $G_{s\alpha}$ protein levels, and activation of phosphodiesterase apparently contribute to A2a desensitization (4).

Genes of at least eight distinct mammalian adenylyl cyclases, which can be further divided into five subfamilies, have been reported. The specific tissue distribution of these different adenylyl cyclases supports biochemical evidence for distinct modes of regulation of cAMP levels (5, 6). These enzymes can all be activated by the α subunit of G_s proteins. In addition, some adenylyl cyclases (types I, III, VI, and VIII) can be modulated by Ca^{2+} (and calmodulin) in a cooperative feedback manner (7). Recently, phosphorylation of adenylyl cyclases has also been suggested to regulate their activity (8, 9). Nevertheless, each adenylyl cyclase type may undergo a distinct mode of regulation by phosphorylation (9).

Regulation of protein phosphorylation is well recognized as one of the most important mechanisms for controlling vari-

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ABBREVIATIONS: PP2, protein phosphatase 2; PP1, protein phosphatase 1; PCR, polymerase chain reaction; OKA, okadaic acid; AC2, -4, -5, and -6, adenylyl cyclase types II, IV, V, and VI, respectively; 8-PT, 8-phenyltheophylline; PKA, cAMP-dependent kinase; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate.

ous biological activities. Both kinases and phosphatases are required to maintain the proper level of phosphorylation of a wide variety of cellular proteins. Two major classes of mammalian serine/threonine protein phosphatases (type 1 and type 2) have been characterized on the basis of their specificity for phosphoprotein substrates and their sensitivity to two thermostable protein inhibitors (10). The PP2s can be further divided into three subgroups, PP2A, PP2B, and PP2C, which are distinct from one another on the basis of their different divalent cation requirements. To explore the potential physiological roles of these protein phosphatases, several protein phosphatase inhibitors (including OKA) have been developed and made commercially available. OKA is a potent and relatively PP2A-specific phosphatase inhibitor that inhibits both PP2A (K_i = 0.2 nM) and PP1 (K_i = 20 nM) (11). Most importantly, this polyether derivative of a C_{38} fatty acid readily permeates cell membranes. Therefore, OKA has been widely used to investigate the role of protein phosphatases in various types of cellular regulation in intact cells.

In this report, we use an A2a-selective agonist, PP2A, and a relatively PP2A-specific inhibitor (OKA) to address the question of whether protein phosphorylation plays a role in regulating adenylyl cyclase activity during A2a desensitization and, if so, which types of adenylyl cyclase are involved. Our data strongly, although indirectly, support the hypothesis that protein phosphorylation of AC6 may account for the inhibition of adenylyl cyclase activity during prolonged exposure of PC-12 cells to adenosine agonists.

Experimental Procedures

Materials. Adenosine agonists and antagonists were obtained from Research Biochemicals (Natick, MA). cAMP and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). OKA was from Boehringer Mannheim Biochemica (Mannheim, Germany). Purified human PP2A was from Upstate Biotechnology (Lake Placid, NY).

Cell culture. PC-12 cells (CRL 1721) were originally obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Gibco) plus 10% horse serum (Gibco), in an incubation chamber gassed with 10% CO_2/90% air at 37°. COS-1 cells were also from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, in 5% CO_2/90% air.

PCR. DNA amplification was carried out in a solution containing 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 1 μg of the desired primers, 0.2 mM levels of each deoxynucleoside triphosphate, DNA template, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), in 50 μl of reaction solution. The reaction proceeded for 40 PCR cycles (94° for 1 min, 55° for 1 min, and 72° for 4 min). The primers for AC5 and AC6 were as follows: 5′-CGGAAAGARGAGAAGGCCATG and 5′-GCG(A/G)GC(A/G)GT(A/G)GATTCCACCTG (8). To amplify AC5 and AC6 cDNA fragments from PC-12 cells, 1 μg of poly(A)$^+$ was used to prepare cDNA using Moloney murine leukemia virus reverse transcriptase [Riboclone oligo(dT) cDNA Syn-system; Promega, Madison, WI]. One twentieth of the cDNA product was then used as template in each 50-μl PCR, as described above.

Adenylyl cyclase assay. Adenylyl cyclase activity was assayed as described previously (4). In brief, cells were washed three times to remove agonist and were resuspended in lysis buffer (10 mM EDTA, 20 mM Tris·HCl, 250 mM sucrose, 1 mM 1,4-dithiothreitol, 0.1 mM PMSF, 40 μM leupeptin, pH 7.4). We then sonicated the cells using a W-380 sonicator (Ultrasonics), at a setting of 20% output power, for a total of 45 sec. The homogenate was centrifuged at 40,000 × *g* for 30 min to collect the membrane fraction. In those experiments with elevated calcium concentrations, membrane preparations were washed two more times with ice-cold HDLP buffer (20 mM HEPES, 0.5 mM 1,4-dithiothreitol, 0.1 mM leupeptin, 40 mM PMSF, pH 7) containing 1 mM EGTA, to remove the endogenous calcium and calmodulin (12). The adenylyl cyclase activity assay was performed at 37° for 10 min, in a 400-μl reaction mixture containing 1 mM ATP, 100 mM NaCl, 0.4 unit of adenosine deaminase, 50 mM HEPES, 6 mM $MgCl_2$, 1 μM GTP, and 20–50 μg of membrane protein. In some experiments, as indicated, 500 μM GDPβS and 10 mM $MnCl_2$ were used to substitute for GTP and $MgCl_2$, respectively, to minimize the influence of $G_{s\alpha}$ protein in forskolin-evoked adenylyl cyclase activity. Reactions were stopped with 0.6 ml of 10% trichloroacetic acid. The cAMP formed was isolated by Dowex chromatography (Sigma) and assayed by radioimmunoassay using a ^{125}I-cAMP assay system (Amersham International). No significant difference was found with addition of 20 mM creatine phosphate (Sigma), 100 units/ml creatine phosphokinase (Sigma), or 0.5 mM IBMX to the cyclase reaction, in the presence or absence of elevated calcium. IBMX was omitted from the cyclase assay unless indicated otherwise, because this phosphodiesterase inhibitor has additional modes of action, including inhibition of adenosine receptors (13) and interference with the binding of cAMP to protein kinase A (14). Also, addition of calcium to the cyclase assay did not evoke any detectable phosphodiesterase activity in control or desensitized membranes. Inclusion of an adenosine antagonist, 8-PT (10 μM), had no detectable effect on the basal or forskolin-evoked adenylyl cyclase activities. The enzyme activity was linear up to 20 min with membrane protein amounts up to 100 μg. All samples were assayed in triplicate.

Phosphodiesterase assay. PC-12 cells were washed twice with ice-cold phosphate-buffered saline, resuspended in lysis buffer (20 μM leupeptin, 1 mM PMSF, 10 mM NaF, 50 mM benzamidine, 50 mM Tris·HCl, pH 8), and then sonicated using a W-380 sonicator (Ultrasonics), at a setting of 20% output power, for a total of 45 sec. The homogenate was centrifuged at 800 × *g* for 10 min to remove insoluble materials. The supernatants were collected for the phosphodiesterase assay as described previously (4). In brief, the phosphodiesterase assay was carried out in phosphodiesterase buffer containing 45 mM Tris·HCl, pH 7.7, 0.1 mM $MgSO_4$, 1 μM [2,8-^{3}H]cAMP (31.4 Ci/mmol; NEN-DuPont), and 50 μg of protein, in a total volume of 200 μl. The reaction mixture was incubated for 5 min at 30°, frozen in a dry ice/ethanol bath to terminate the reaction, and boiled for 1 min. Snake (*Crotalus atrox*) venom (20 μg) was added to each tube, and the tubes were incubated at 30° for 30 min. [^{3}H]Adenosine was isolated using acidic alumina. The enzyme activity was linear up to 10 min with 50 μg of protein. All samples were assayed in triplicate.

Transfection. The rat AC6 cDNA was a generous gift from Dr. R. Iyengar (Mount Sinai School of Medicine, City University of New York) (15). The bovine PP2A cDNA was isolated from a bovine adrenal chromaffin cDNA library (16). The cDNAs for AC6 and PP2A were subcloned separately into the *Eco*RI site of pMT_2, a eukaryotic expression vector. Transient expression of cDNAs was performed using a DEAE-dextran protocol modified by 0.1 mM chloroquine treatment (17). Three days after transfection, the adenylyl cyclase activities in the transfected cells were assayed as described above.

Results

To study the desensitization of A2a adenosine receptor-mediated cAMP elevation, PC-12 cells were treated with an A2a-selective agonist (CGS21680, 2.5 μM) for 30 min (Fig. 1). As we reported earlier (4), the adenylyl cyclase activity evoked by forskolin was significantly inhibited in the CGS21680-desensitized cells (Fig. 1). To ensure that the changes in cAMP levels reflected changes in adenylyl cyclase activity and not changes in phosphodiesterase or adenosine

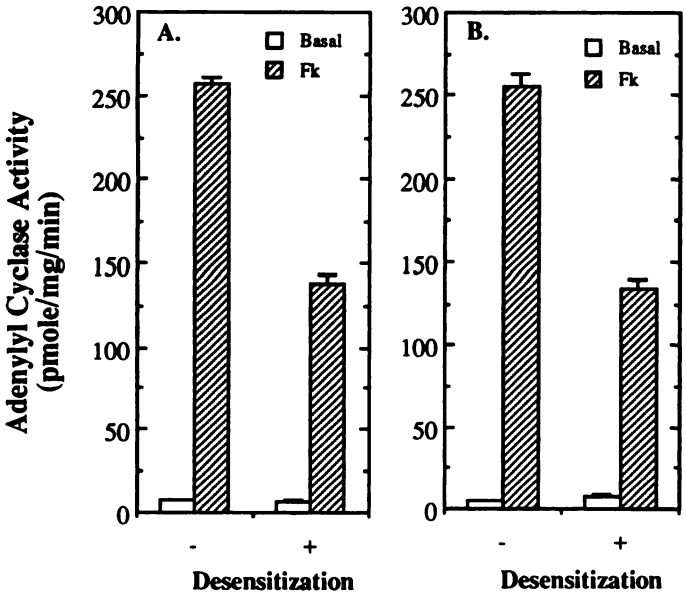

Fig. 1. Effects of a phosphodiesterase inhibitor (IBMX) and an adenosine antagonist (8-PT) on inhibition of adenylyl cyclase activity during A2a desensitization. Membrane fractions were prepared from PC-12 cells that had been pretreated with or without CGS21680 (2.5 μM) for 30 min to trigger A2a desensitization, as indicated. Basal and forskolin (*Fk*) (5 μM)-evoked adenylyl cyclase activities were measured in the absence (A) or presence (B) of IBMX (0.5 mM) and 8-PT (10 μM). The results are from one representative experiment of three independent experiments performed.

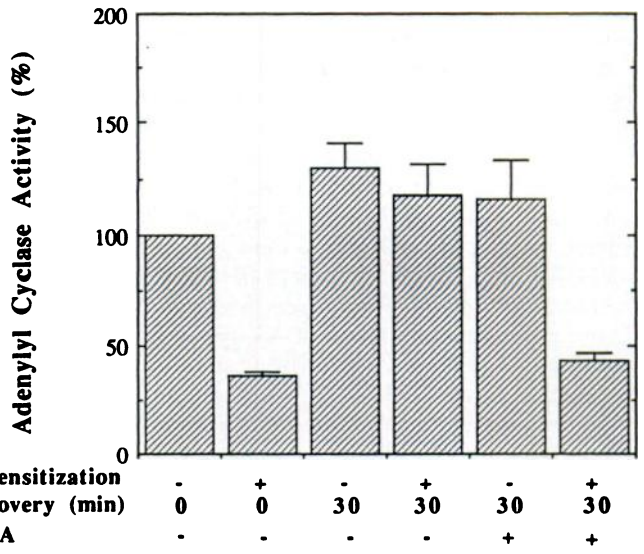

Fig. 2. Reversibility of the inhibition of adenylyl cyclase activity during A2a desensitization. Membrane fractions were prepared from PC-12 cells that had been pretreated with or without CGS21680 (2.5 μM) for 30 min, as indicated. To allow for recovery of adenylyl cyclase activity after A2a desensitization, the membrane fractions were resuspended in assay buffer and allowed to remain on ice for the indicated periods of time to recover in the absence of agonist. Where indicated, OKA (10 nM) was added to the assay buffer to prevent dephosphorylation. Adenylyl cyclase activities in response to forskolin (5 μM) were then assayed as described in Experimental Procedures. Values represent the mean ± standard error of at least nine determinations (three determinations in three independent experiments) and are expressed as percentages of the adenylyl cyclase activity (217 ± 18 pmol/mg/min) in control non-desensitized membranes without the recovery incubation.

receptor-mediated adenylyl cyclase activity, we examined the effects of a phosphodiesterase inhibitor (IBMX) and an adenosine antagonist (8-PT) in our adenylyl cyclase assays. As shown in Fig. 1, addition of 8-PT and IBMX in our adenylyl cyclase assays had no detectable effect on the basal or forskolin-stimulated adenylyl cyclase activities. Most importantly, these reagents also had no effect on the inhibition of adenylyl cyclase activity during A2a desensitization.

To explore the molecular mechanism underlying the inhibition of adenylyl cyclase activity during A2a desensitization, PC-12 cells at 70% confluency were treated with an A2a-selective agonist (CGS21680, 2.5 μM) for 30 min to trigger desensitization. A typical 64 ± 1% inhibition of the forskolin-evoked adenylyl cyclase activity was observed in desensitized membranes (Fig. 2). This inhibition of adenylyl cyclase activity appeared to be reversible, because incubation of the desensitized membranes in the absence of agonist for 30 min restored the adenylyl cyclase activity to a level comparable to that of the control membranes. Interestingly, addition of a potent and relatively selective PP2A inhibitor (OKA, 10 nM) completely blocked the recovery of adenylyl cyclase activity from A2a desensitization. These data imply that protein phosphorylation may be involved in the regulation of adenylyl cyclase activity during A2a desensitization.

To assess the role of protein phosphorylation in regulating the activity of adenylyl cyclase during A2a desensitization, PC-12 cells were exposed to various concentrations of OKA for 30 min to block the endogenous protein phosphatase activity. We then measured the adenylyl cyclase activity of the cells. As shown in Fig. 3, OKA inhibited the adenylyl cyclase activity in a dose-dependent manner. Maximal inhibition (83 ± 1% inhibition) occurred at 0.3 nM OKA. No

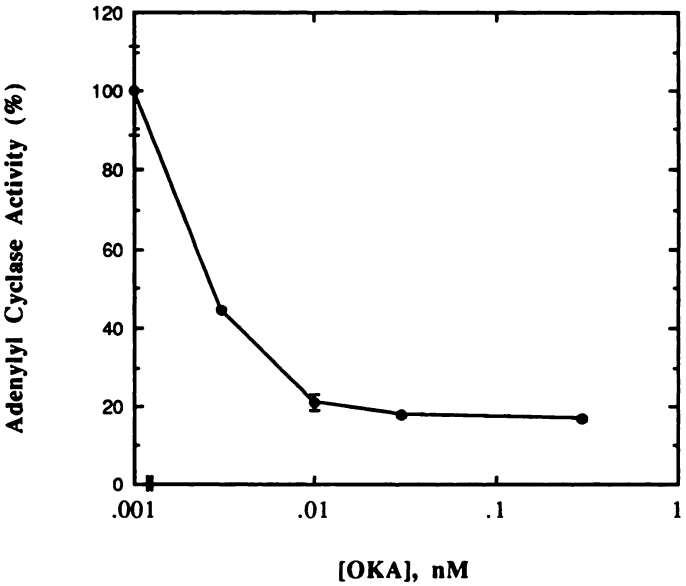

Fig. 3. Dose-response curve for OKA inhibition of adenylyl cyclase activity in PC-12 cells. Cells were treated with OKA at the indicated concentration for 30 min. Membrane fractions were collected. Adenylyl cyclase activities in response to forskolin (5 μM) were then assayed. Values represent the mean ± standard error of three determinations and are expressed as percentages of the adenylyl cyclase activity (233 ± 7 pmol/mg/min) in control non-OKA-treated membranes. The results are from one representative experiment of three independent experiments performed.

significant effect of the carrier (0.01% ethanol) on the cyclase activity was observed.

Given that inhibition of adenylyl cyclase activity occurred both in A2a-desensitized cells and in OKA-treated cells, it is reasonable to hypothesize that protein phosphorylation may mediate the suppression of adenylyl cyclase activity that occurs during A2a desensitization. To investigate that possibility, cells were treated with or without OKA for 30 min and then exposed to CGS21680 to induce desensitization. At 0.3 nM, OKA alone caused a typical 70 ± 2% inhibition of forskolin-stimulated adenylyl cyclase activity. Most interestingly, no further inhibition of adenylyl cyclase activity was observed in OKA-treated cells after a subsequent 30-min incubation with CGS21680, whereas the same treatment resulted in a 68 ± 9% inhibition in non-OKA-treated cells (Fig. 4). Because the inhibition of adenylyl cyclase activity by OKA treatment and by prolonged exposure of PC-12 cells to CGS21680 appeared to give similar results, these two treatments may regulate adenylyl cyclase activity through the same mechanism, which results in enhancement of the level of phosphorylation of adenylyl cyclase.

To further investigate the effect of phosphorylation on adenylyl cyclase activity, we identified the adenylyl cyclase profile in PC-12 cells. The effect of Ca^{2+}, which activates type I, III, and VIII adenylyl cyclases and inhibits AC6, on the adenylyl cyclase activity in PC-12 cells was examined. As shown in Fig. 5A, the cyclase activities stimulated by CGS21680 and by forskolin were both significantly reduced in the presence of calcium. The CGS21680-induced adenylyl cyclase activity was reduced essentially to basal levels, whereas only 76 ± 2% of the forskolin-induced adenylyl cyclase activity was inhibited. These data demonstrated the existence of AC6 in PC-12 cells. In addition, AC6 is likely to be the predominant adenylyl cyclase associated with the A2a adenosine receptor in PC-12 cells. To verify the existence of AC6 in PC-12 cells, we used PCR to detect the transcripts of both AC5 and AC6 (Fig. 5B). A 1095-bp DNA fragment was amplified from PC-12 mRNA by the reverse transcription-PCR technique, using a set of primers that can amplify a 1095-bp DNA fragment from either AC5 or AC6. This 1095-bp DNA fragment amplified from AC6 cDNA can be digested into 300-bp and 795-bp fragments by *Xho*I, whereas that amplified from AC5 cDNA can be digested into 220-bp and 875-bp bands by *Sac*I (15). Our data suggest that AC6 but not AC5 exists in PC-12 cells. Because 24 ± 2% of the forskolin-evoked adenylyl cyclase activity was insensitive to calcium (Fig. 5A), other adenylyl cyclase subtypes, in addition to AC6, might exist in PC-12 cells.

To determine whether AC6 activity is reduced during A2a desensitization, we examined the effect of calcium on adenylyl cyclase activity in control cells and in desensitized cells. Again, a typical 70 ± 10% inhibition of adenylyl cyclase activity was observed during A2a desensitization (Fig. 6). In control membranes, calcium suppressed 62 ± 13% of the forskolin-evoked cyclase activity. When calcium was added to the desensitized membranes, only a very slight decrease in the total cyclase activity was found, compared with the de-

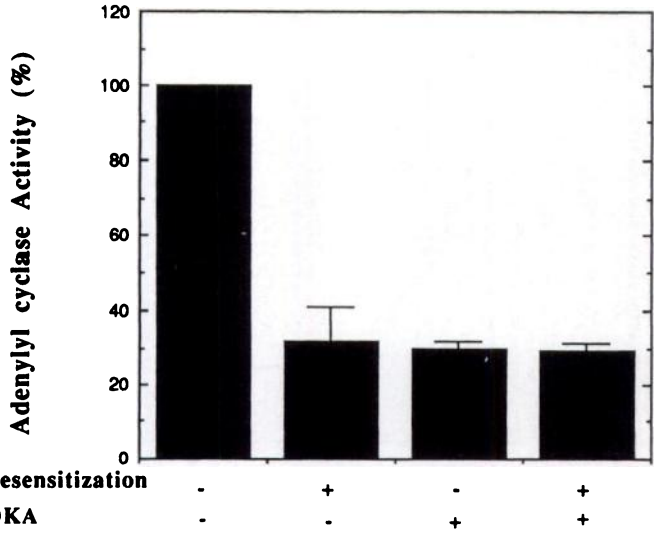

Fig. 4. Effect of OKA on A2a desensitization. Cells were treated with or without OKA for 30 min, as indicated. The A2a-selective adenosine agonist CGS21680 was then added to the medium for another 30 min to induce desensitization. Adenylyl cyclase activity was then measured in membrane fractions prepared from these cells. Values represent the mean ± standard error of at least nine determinations (three determinations in three independent experiments) and are expressed as percentages of the adenylyl cyclase activity (184 ± 8 pmol/mg/min) in control nontreated membranes.

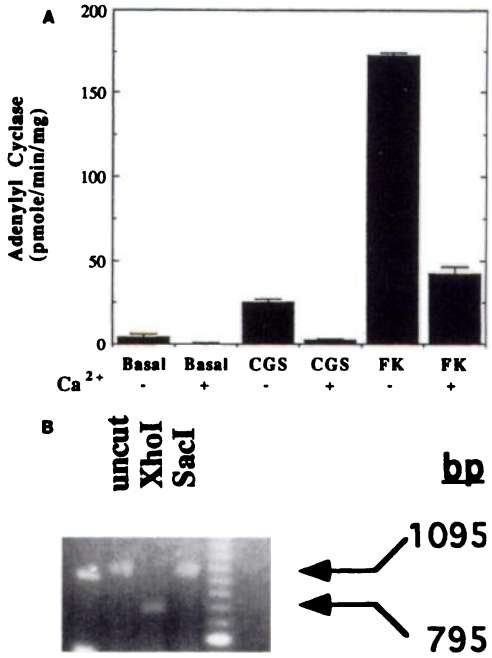

Fig. 5. Existence of AC6 activity in PC-12 cells. A, Membrane fractions were collected from PC-12 cells, washed twice in an EGTA-containing buffer to remove endogenous calcium, and resuspended in an EGTA/calcium-containing buffer to control the free calcium concentration at 0 or 30 μM for adenylyl cyclase assays. Values represent the mean ± standard error of three determinations. The results are from one representative experiment of three independent experiments performed. *CGS*, CGS21680; *FK*, forskolin. B, PCR-based detection of the mRNA for AC5 and AC6 is shown. Two oligonucleotides were used as primers to specifically amplify DNA fragments from both AC5 and AC6, as described in Experimental Procedures. The predicted size of the amplified DNA fragments from both AC5 and AC6 is 1095 bp. The AC6 1095-bp band can be digested into 300-bp and 795-bp fragments by *Xho*I, whereas the AC5 1095-bp band can be digested into 220-bp and 875-bp bands by *Sac*I. One third of the PCR product was used in the digestion reactions with the indicated restriction enzyme and was examined by ethidium bromide-agarose (1%) electrophoresis. The leftmost and rightmost lanes are size markers. These data suggest the existence of AC6 but not AC5 in PC-12 cells.

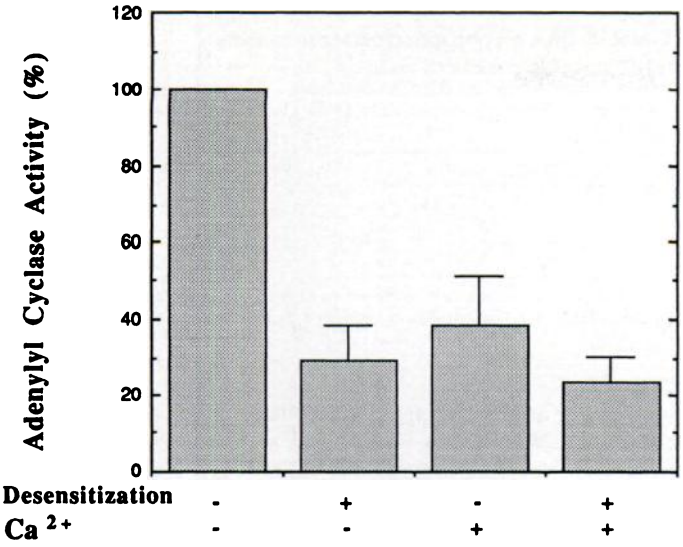

Fig. 6. Inhibition of AC6 activity during A2a desensitization. Membrane fractions were collected from control or agonist-desensitized (2.5 μM CGS21680 for 30 min) cells. Adenylyl cyclase activity was assayed in the presence or absence of Ca^{2+}, as described for Fig. 5A. Values represent the mean ± standard error of at least 15 determinations (three determinations in five independent experiments) and are expressed as percentages of the adenylyl cyclase activity (191.5 ± 39 pmol/mg/min) in control membranes.

crease in control membranes in the presence of calcium. These data suggest that the activity of AC6 is markedly inhibited during A2a desensitization.

Furthermore, down-regulation of AC6 activity took place in the same time frame as did the down-regulation of forskolin-evoked total adenylyl cyclase activity during A2a desensitization (Fig. 7). After the first 30 min of incubation with CGS21680, 86 ± 4% of AC6 activity and 80 ± 2% of the total adenylyl cyclase activity were inhibited in PC-12 cells. Longer incubation (17 hr) with CGS21680 resulted in only a slight additional inhibition of both AC6 activity and total adenylyl cyclase activity. These data strengthened our hypothesis that AC6 is the major adenylyl cyclase that is suppressed during A2a desensitization.

To investigate whether the inhibition of AC6 activity in desensitized PC-12 cells is mediated through protein phosphorylation, we examined the effect of OKA on AC6 activity. The calcium-inhibitable AC6 activity represented 70 ± 9% of total adenylyl cyclase activity in control membranes. In addition, OKA inhibited 74 ± 8% of the total adenylyl cyclase activity. Importantly, only a very minor portion of the adenylyl cyclase was further inhibited by calcium in the OKA-treated membranes. Thus, AC6 appears to be the major target for protein phosphorylation, because inhibition of adenylyl cyclase activity in PC-12 cells by OKA almost completely diminished the Ca^{2+}-inhibitable adenylyl cyclase activity (Fig. 8).

To reduce the possible influence of $G_{s\alpha}$ on adenylyl cyclase activity (18), we substituted GDPβS and $MnCl_2$ for GTP and $MgCl_2$, respectively, in our adenylyl cyclase assay (Fig. 9). Although GDPβS/$MnCl_2$ markedly reduced the Ca^{2+}-inhibitable, forskolin-stimulated adenylyl cyclase (AC6) activity, suppression of AC6 activity during A2a desensitization (Fig. 9A) and during OKA treatment (Fig. 9B) remained largely the same. These results suggest that a change in $G_{s\alpha}$ activity is not necessary, under these conditions, to inhibit AC6 activity. We have also measured the effect of OKA on phosphodiesterase, which is responsible for the hydrolysis of cAMP. As shown in Table 1, only a very slight (~10%) inhibition by OKA (10 nM) was consistently observed.

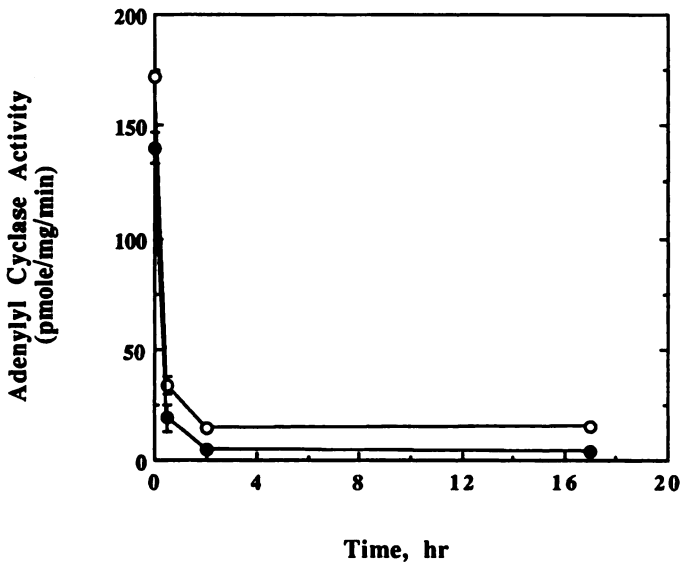

Fig. 7. Time course of inhibition of AC6 activity during A2a desensitization. Membrane fractions were prepared from these cells as described in Experimental Procedures. Adenylyl cyclase activities in response to forskolin (5 μM) were assayed. The AC6 activity in these membranes represents the difference between the forskolin (5 μM)-evoked adenylyl cyclase activities assayed in the absence and in the presence of 30 μM free calcium, as described for Fig. 4. Each assay was carried out in triplicate. The results are from one representative experiment of at least three independent experiments performed. ●, AC6 activity; ○, forskolin-stimulated total adenylyl cyclase activity.

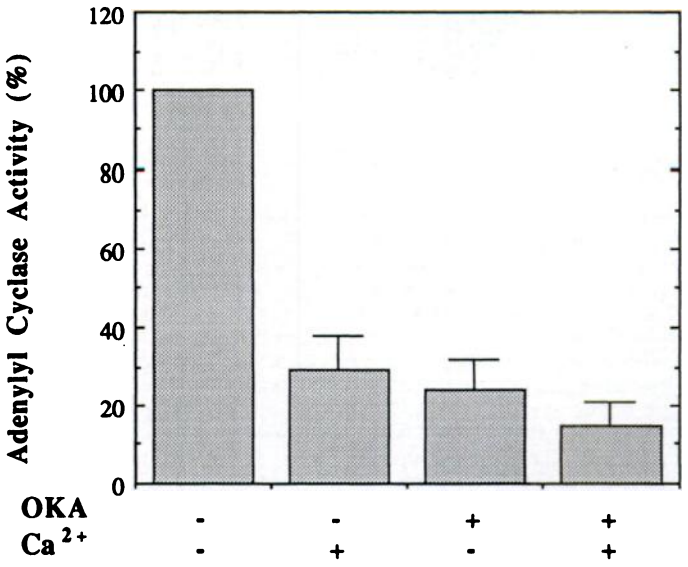

Fig. 8. OKA inhibition of the AC6 activity in PC-12 cells. Membrane fractions were collected from control or OKA-treated (0.3 nM OKA for 30 min) cells. Adenylyl cyclase activity was assayed in the presence or absence of calcium as indicated. Values represent the mean ± standard error of at least 15 determinations (three determinations in five independent experiments) and are expressed as percentages of the adenylyl cyclase activity (197 ± 22 pmol/mg/min) in control membranes.

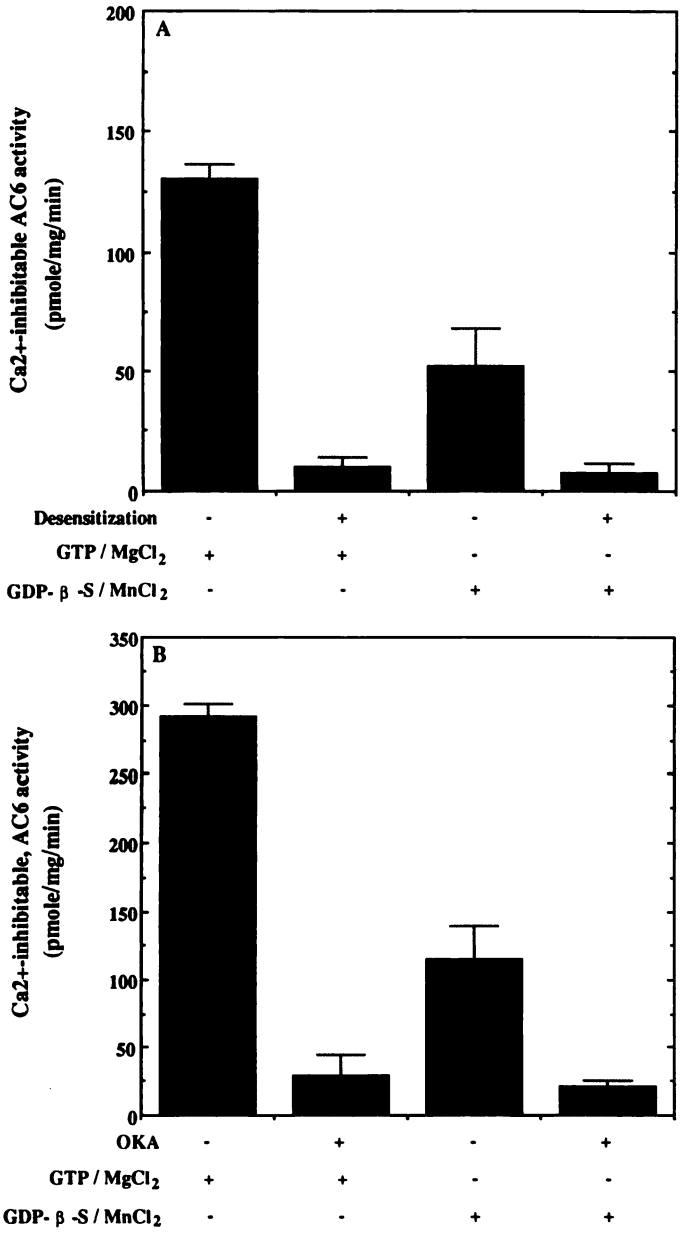

Fig. 9. Evidence that suppression of forskolin-evoked AC6 activity was largely independent of a change in $G_{s\alpha}$ activity. A, PC-12 cells were treated with or without CGS21680 (2.5 μM) for 30 min to evoke A2a desensitization. Adenylyl cyclase activity was then measured in the presence of GTP (1 μM)/$MgCl_2$ (6 mM) or GDPβS (500 μM)/$MnCl_2$ (10 mM) as indicated. AC6 activity in these membranes represents the difference between the forskolin (5 μM)-evoked adenylyl cyclase activities assayed in the absence and in the presence of Ca^{2+}. The results are from one representative experiment of three independent experiments performed. B, PC-12 cells were treated with or without OKA (10 nM) for 60 min. Adenylyl cyclase activity was then measured in the presence of GTP (1 μM)/$MgCl_2$ (6 mM) or GDPβS (500 μM)/$MnCl_2$ (10 mM) as indicated. AC6 activity in these membranes represents the difference between the forskolin (5 μM)-evoked adenylyl cyclase activities assayed in the absence and in the presence of Ca^{2+}. The results are from one representative experiment of four independent experiments performed.

To further examine the effect of protein phosphorylation on AC6, we incubated purified human PP2A with the membrane fraction of PC-12 cells and then measured the adenylyl cyclase activity in the presence of GTP/$MgCl_2$ or GDPβS/$MnCl_2$. Under either set of conditions, PP2A dramatically increased the AC6 activity (Table 2). In addition, we co-transfected COS-1 cells with AC6 and PP2A. Both AC6 and PP2A were subcloned separately into the pMT_2 expression vector, which contains a simian virus 40 origin of replication and therefore allows high-level expression of the desired protein in COS-1 cells. These cells were chosen because they are relatively easily transfected. In COS-1 cells transfected with PP2A, the cellular phosphatase activity was increased to twice the level in untransfected cells, using *p*-nitrophenyl phosphate as the substrate (data not shown). As shown in Table 3, AC6 was also expressed in COS-1 cells. Expression of PP2A alone in COS-1 cells enhanced the endogenous AC6 activity (Table 3). Overexpression of the rat AC6 cDNA in COS-1 cells increased the total AC6 activity to almost twice that in untransfected cells. More importantly, coexpression of AC6 with PP2A significantly enhanced the transfected

TABLE 1

Effect of OKA on phosphodiesterase activity

PC-12 cells were treated with or without OKA (10 nM) for 30 min. The phosphodiesterase activity was assayed as described in Experimental Procedures. The results are from one representative experiment of three independent experiments performed.

Treatment	Phosphodiesterase activity
	pmol/mg/min
None	25.6 ± 0.9
OKA	22.3 ± 0.8[a]

[a] Value that is significantly different ($p \leq 0.05$) between untreated cells and treated cells, based on two-tailed Student's *t* test.

TABLE 2

Increase of AC6 activity by PP2A in PC-12 cells

Membrane fractions (120 μg/reaction) were collected from PC-12 cells and incubated with or without PP2A (0.1 unit) on ice for 30 min. AC6 activity in these membranes represents the difference between the forskolin (5 μM)-evoked adenylyl cyclase activities assayed in the absence and in the presence of Ca^{2+}. To reduce the possible influence of $G_{s\alpha}$ protein in the forskolin-evoked adenylyl cyclase activity, GDPβS (500 μM) and $MnCl_2$ (10 mM) were substituted for GTP (1 μM) and $MgCl_2$ (6 mM), respectively, in the cyclase assay reactions, as indicated. The results are from one representative experiment of at least four independent experiments performed. One unit of PP2A releases 1 nM phosphate/min from 15 mM phosphorylase at 30°.

Exogenous PP2A	GTP/$MgCl_2$	GDPβS/$MnCl_2$	Ca^{2+}-inhibitable AC6 activity
			pmol/mg/min
0	+	–	232 ± 16
0.1 unit	+	–	411 ± 11
0	–	+	99 ± 10
0.1 unit	–	+	166 ± 27

TABLE 3

Enhancement of the AC6 activity by coexpression of PP2A and AC6 in COS-1 cells

COS-1 cells were transfected with pMT_2-based construct(s) containing the indicated cDNA(s). After 72 hr, membrane fractions were prepared from these cells. The AC6 activity in these membranes represents the difference between the forskolin (5 μM)-evoked adenylyl cyclase activities assayed in the absence and in the presence of Ca^{2+}. The results are from one representative experiment of six independent transfection experiments performed.

DNA transfected	Ca^{2+}-inhibitable AC6 activity
	pmol/mg/min
Vector	31 ± 1
AC6	59 ± 5
PP2A	55 ± 7
AC6 + PP2A	115 ± 4

AC6 activity. These results are in good agreement with our hypothesis that protein phosphorylation regulates AC6 activity. Again, $G_{s\alpha}$ appeared to play no significant role in the enhancement of AC6 activity by PP2A.

Discussion

Prolonged exposure of PC-12 cells to adenosine agonists significantly inhibits the adenylyl cyclase activity, which contributes to a lower response to subsequent stimulation with adenosine agonists (A2a desensitization). In this report, our data suggest that protein phosphorylation may mediate the suppression of adenylyl cyclase activity during A2a desensitization. In addition, AC6 appears to be the major adenylyl cyclase involved in A2a desensitization in PC-12 cells. Furthermore, PP2A plays a very important role in the regulation of AC6 activity during A2a desensitization.

Regulation of the eight different adenylyl cyclase activities has lately attracted much attention in the field of signal transduction. Although these adenylyl cyclases all exhibit the same enzyme activity and have very similar topologies (6), each adenylyl cyclase may undergo distinct regulation and may thus serve as a specialized coincidence detector (19). In PC-12 cells, we have demonstrated the existence of a Ca^{2+}-inhibitable adenylyl cyclase, AC6 (Fig. 5). Furthermore, AC6 appears to be the dominant adenylyl cyclase associated with the A2a adenosine receptor-mediated signal transduction pathway in PC-12 cells, because calcium almost completely abolished CGS21680-stimulated adenylyl cyclase activity (Fig. 5A). In addition, the time course of AC6 inhibition during A2a desensitization correlated very well with that of forskolin-evoked total adenylyl cyclase activity (Fig. 7). When the cytosolic Ca^{2+} concentration was raised in intact PC-12 cells by ionomycin in the presence of extracellular calcium, the CGS21680-induced increase in cellular cAMP content was also inhibited, to almost basal levels (data not shown). The existence of AC6 in PC-12 cells provides an important pathway to integrate calcium levels and adenosine-induced cAMP signaling in PC-12 cells. Furthermore, regulation of AC6 activity may result in modification of the adenosine response in these cells. Using the same PCR technique as described for Fig. 5B with two other sets of primers, for AC2 and AC4, we have also identified the presence of AC4, but not AC2, in PC-12 cells (data not shown), which is consistent with our observation of "Ca^{2+}-insensitive" adenylyl cyclase activity in PC-12 cells (Fig. 5A).

PKA-mediated protein phosphorylation has recently been suggested to regulate the lowered responsiveness of adenylyl cyclase to glucagon stimulation during heterologous desensitization in chick hepatocytes (8). There is at least one predicted PKA phosphorylation site in all adenylyl cyclases, except for AC4. In addition, protein kinase C has been reported to markedly enhance AC2 activity (9). Because activation of the A2a adenosine receptor results in an increase in the intracellular cAMP content, PKA must be activated during A2a desensitization. However, treatment with dibutyryl-cAMP or forskolin did not reproduce the inhibition of adenylyl cyclase activity during A2a desensitization in PC-12 cells (4). Thus, it is unlikely that PKA plays a role in the inhibition of AC6 during A2a desensitization. Regulation of the phosphorylation state of AC6 may result from activation of an unknown kinase or inactivation of PP2A during A2a desensitization. Inactivation of PP2A by phosphorylation in response to insulin has recently been reported (20). To demonstrate directly that the phosphorylation level of AC6 is elevated during A2a desensitization, we are in the process of preparing a polyclonal antibody against AC6. Further characterization of AC6 phosphorylation is essential for studying the regulation of AC6 during A2a desensitization.

Agonist-induced reduction of the number of cell surface receptors, as well as receptor phosphorylation, has been implicated in desensitization of many G protein-coupled receptor-mediated responses (21). In PC-12 cells, we found no significant decrease in the expression of endogenous A2a adenosine receptors during A2a desensitization. Instead, inhibition of adenylyl cyclase after short term agonist treatment, down-regulation of $G_{s\alpha}$ protein levels after long term agonist treatment, and activation of phosphodiesterase after long term agonist treatment resulted in A2a desensitization (4). Although agonist-stimulated *in vivo* phosphorylation of transfected canine A2a adenosine receptors in CHO cells was recently reported (22), the relevance of this process to functional A2a desensitization remains to be proven. Those authors suggested that phosphorylation of A2a adenosine receptors may selectively diminish productive interaction between the A2a adenosine receptor and $G_{s\alpha}$, although direct measurements of the A2a adenosine receptor-stimulated GTPase activity in control and desensitized cells were not available. Intriguingly, the adenylyl cyclase activity triggered by sodium fluoride or forskolin was not at all affected in desensitized CHO cells (22). Such discrepancy between the mechanisms underlying A2a desensitization characterized in PC-12 cells (with endogenous A2a adenosine receptors) and in CHO cells (with transfected canine A2a adenosine receptors) is not totally surprising and may result from differences in the cAMP signaling systems in these cells. For example, each cell line might contain different subtypes of adenylyl cyclase and thus undergo distinct regulation, as discussed above.

Although much less well known, protein phosphatases are as important as protein kinases in cellular regulation. At least four PP1s, two PP2As, three PP2Bs, and a phosphatase X exist in PC-12 cells (23). Each phosphatase might play a distinct role in the control of phosphoprotein functions. Until now, very little information has been available concerning the structural basis for the substrate specificity of the serine/threonine protein phosphatases. The physiological mechanisms that might activate these protein phosphatases are also largely unknown. Although OKA inhibits both PP2A and PP1, the affinity of OKA for PP2A is at least 100-fold higher than that for PP1 (11). Our use of a subnanomolar concentration of OKA to inhibit the AC6 activity suggests that PP2A, but not PP1 or other protein phosphatases, plays an important role in the regulation of adenylyl cyclase activity during A2a desensitization. This hypothesis is further strengthened by the observations that PP2A enhanced the AC6 activity in PC-12 cells (Table 2) and COS-1 cells (Table 3).

PP2A has been suggested to exist largely in the cytoplasm (24). Because the OKA-sensitive recovery of adenylyl cyclase activity after A2a desensitization can be observed in desensitized membrane fractions (Fig. 2), PP2A may also exist in the membrane fractions of PC-12 cells, to regulate the recovery of AC6 activity after the removal of adenosine agonists.

The existence of PP2A in the pellet after high-speed centrifugation has also been observed with COS-1 and Rat-1a cells (25). Although our data demonstrated that PP2A enhanced the AC6 activity both *in vitro* (Table 2) and *in vivo* (Table 3), we cannot completely exclude the possibility that PP2A caused dephosphorylation of another component in the cyclase signaling pathway and thereby led to the increase in AC6 activity. However, coexpression of PP2A with AC2 or AC4 in COS-1 cells did not cause an increase in the AC2 or AC4 activity (data not shown), indicating that this increase in AC6 activity by PP2A is relatively AC6 specific.

Although forskolin stimulates adenylyl cyclase directly, such stimulation has been shown to synergize with $G_{s\alpha}$ (26). Thus, modification of $G_{s\alpha}$ protein may also play an important role in the regulation of AC6 activity during A2a desensitization. Phosphorylation, by several different kinases, of $G_{s\alpha}$ proteins has been reported to change their efficiency of coupling to receptors or to adenylyl cyclase (27–29). Although we reported that no significant decrease in $G_{s\alpha}$ protein levels occurred during short term A2a desensitization (4), it was unclear whether the interaction between $G_{s\alpha}$ protein and adenylyl cyclase was affected. In the present study, we have substituted GDPβS and Mn^{2+} for GTP and Mg^{2+}, respectively, in some of our cyclase assays, to eliminate the potential influence of $G_{s\alpha}$ in forskolin-stimulated adenylyl cyclase activity. GDPβS is a GDP analog and is widely used to reduce G protein activity by occupying the guanine nucleotide binding site of G proteins. MnATP, instead of MgATP, can be used as a substrate by adenylyl cyclase in the absence of $G_{s\alpha}$. As shown in Fig. 9 and Table 2, a change in $G_{s\alpha}$ activity appeared to be unnecessary for the regulation of AC6 during A2a desensitization and after OKA treatment in PC-12 cells. Therefore, suppression of AC6 activity is likely to be mediated through protein phosphorylation of the cyclase itself and not through protein phosphorylation of $G_{s\alpha}$ protein.

In summary, our data suggest that protein phosphorylation mediates the inhibition of AC6 in PC-12 cells during A2a desensitization. In addition, PP2A might play a critical role in the regulation of AC6 activity in these cells.

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Send reprint requests to: Yijuang Chern, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.