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Multiple Mechanisms for Desensitization of A2a Adenosine Receptor-Mediated cAMP Elevation in Rat Pheochromocytoma PC12 Cells

YIJUANG CHERN, HSING-LIN LAI, JIM C. FONG, and YU LIANG

Institute of Biomedical Sciences (Y.C., H.-L.L., Y.L.), Academia Sinica, Taipei, Taiwan, Republic of China, and Institute of Biochemistry (J.C.F.), National Yang-Ming Medical College, Taipei, Taiwan, Republic of China

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SUMMARY

To understand the regulation of A2a adenosine receptor (A2a-R) response, we examined the molecular mechanisms underlying the desensitization of A2a response in rat pheochromocytoma PC12 cells, which possess an A2a-R identical with the A2a receptor we recently cloned from rat brain. Prolonged exposure of PC12 cells to adenosine agonists significantly inhibited the response of the cells to subsequent stimulation with an A2a-selective adenosine agonist (CGS21680). No significant change in the number of binding sites and affinity for CGS21680 was observed in desensitized cells, nor did we find any significant change in the transcript level of A2a-R in cells pretreated with adenosine agonists. However, the basal adenylyl cyclase activity and the cyclase activities stimulated by adenosine agonists, by GTPγS, and by forskolin were reduced in desensitized cells. Prolonged exposure of PC12 cells to dibutyryl-cAMP did not significantly change either the basal or the adenosine agonist-evoked adenylyl cyclase activity. Therefore, elevation of cellular cAMP content is by itself not sufficient to produce the observed reductions of adenylyl cyclase activity with A2a desensitization. Inhibition of adenylyl cyclase activity in desensitized cells occurred after short-term (30 min) incubation with CGS21680 and could be blocked by the adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine. Gsα protein levels did not significantly change after a 30-min exposure to CGS21680. In contrast, long-term exposure (12–20 hr) of PC12 cells to adenosine agonists resulted in a slight further reduction of adenylyl cyclase activity and a consistent decline in the Gsα protein level. In addition, long-term incubation with adenosine agonists or with forskolin-enhanced phosphodiesterase (PDE) activity in the cytosolic and membrane fractions by 57 ± 9% and 53 ± 18%, respectively. Hydrolysis of cAMP was significantly faster in agonist-desensitized cells than in control cells. PDE might therefore play an important role in desensitization of the A2a response in PC12 cells. Polymerase chain reaction-based analysis of the mRNA for A2a-R and A2b-R indicated that both A2a-R and A2b-R were present in PC12 cells; the A2b response was also diminished in A2a-desensitized cells. Our data suggest that inhibition of adenylyl cyclase after short-term agonist treatment, down-regulation of Gsα protein level after long-term agonist treatment, and activation of PDE after long-term agonist treatment account for desensitization of the A2a-mediated response in PC12 cells.

Adenosine has been reported to modulate a wide variety of physiological responses through specific receptors (1). Molecular cloning of at least four distinct adenosine receptor subtypes has recently been reported (2–5). These adenosine receptors associate with adenylyl cyclase to regulate intracellular cAMP content in response to extracellular adenosine. Receptors that mediate inhibition of adenylyl cyclase have been designated A1, whereas receptors that mediate stimulation of the cyclase have been designated A2. A1 and A2 adenosine receptors can also be distinguished by the relative agonist potencies of certain adenosine analogues that bind to them (1). Furthermore, there are two different A2 adenosine receptors—A2a and A2b. Both A2 adenosine receptors stimulate adenylyl cyclase, but they have very different affinities for adenosine agonists (4). We recently cloned the rat A2a-R, which contains seven transmembrane domains and belongs to the G protein-coupled receptor family (2). The recently cloned rat A3 adenosine receptor (5) inhibits adenylyl cyclase but differs from both A1 and A2 receptors in its relative agonist potency. However, the action of adenosine is not always cAMP dependent (6).

Reduction of cellular response to adenosine after prolonged stimulation with adenosine agonists has been described in several different cell lines (7–9). Uncoupling, down-regulation,

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ABBREVIATIONS: A2a-R, A2a adenosine receptor; A2b-R, A2b adenosine receptor; PDE, phosphodiesterase; PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphate; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; SSC, standard saline citrate; EDTA, ethylenediaminetetraacetic acid; ADA, adenosine deaminase; DTT, 1,4-dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BCIP, bromochloroindolyl phosphate; NBT, nitro blue tetrazolium; NECA, *N*-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

and phosphorylation of the A1 adenosine receptor have been proposed to mediate the A1 desensitization process. In contrast, no significant change in receptor number and affinity have been found during A2 desensitization (8). The molecular mechanisms underlying A2 desensitization therefore remain largely unknown.

In the rat adrenal tumor cell line PC12, adenosine enhances catecholamine secretion and causes concomitant increases in cellular cAMP (10). In addition, adenosine has been demonstrated to inhibit cell division and to promote neurite extension in PC12 cells (11). Therefore, the PC12 cell line serves as a very good model for studying the physiological role of adenosine in modulating neurotransmitter release and neuronal differentiation. In the present study, we selected these cells for studying A2a desensitization because we found that PC12 cells possess an A2a-R identical with that from rat brain. Our data suggest that inhibition of adenylyl cyclase, down-regulation of Gsα protein level, and activation of PDE might contribute to desensitization of A2a-R-mediated cAMP elevation in PC12 cells.

Experimental Procedures

Materials. Adenosine agonists and antagonists were obtained from Research Biochemical Inc. (Natick, MA). Milrinone, cAMP, and cGMP were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. PC12 cells were originally obtained from ATCC (CRL1721) and maintained in Dulbecco's minimal essential 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°.

Polymerase chain reaction. 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 of each dNTP, DNA template, and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) per 50 μl of reaction solution. The reaction proceeded for 40 PCR cycles (94°, 1 min; 55° for A2a-R and 72° for A2b-R, both 1 min; 72°, 4 min). To amplify adenosine receptor cDNA fragments from PC12 cells, 1 μg of polyA was used to prepare cDNA using MoMuLV reverse transcriptase (Riboclone oligo-dT-cDNA Synsystem, Promega, Madison, WI). One-twentieth of the cDNA product was then used as template in each 50-μl PCR reaction as described above.

Preparation of total cellular RNA and Northern blot analysis. Cytoplasmic RNA was prepared using the acid guanidinium thiocyanate-phenol-chloroform extraction method (12). Of total RNA, 50 μg were fractionated using 1.2% agarose containing 5.5% formaldehyde and transferred to GeneScreen Plus (DuPont, MA). The filter was hybridized with a ^{32}P-labeled, 1.8-kb A2a-R cDNA fragment (2) and a "housekeeping" gene, GAPDH (13). The radiolabeled probes were generated by a multiprime DNA labeling system (Amersham, England). Hybridization was done at 42° in 1% SDS, 10% dextran sulfate, and 50% formamide. Filters were washed in 2× SSC twice for 5 min at room temperature, 2× SSC plus 1% SDS twice for 30 min at 60°, and then in 0.1 × SSC twice for 30 min at room temperature.

Radioligand binding assay. Membrane fractions were prepared by disrupting cells using a Polytron homogenizer for 30 sec. The homogenate was then centrifuged at 500 × g for 20 min at 4°. The supernatant was collected and centrifuged again at 45,000 × g for 15 min at 4°. The pellet was washed twice with ice-cold buffer A (50 mM Tris, 150 mM NaCl, 0.1 mM EGTA, 5 mM EDTA, pH 7.5), resuspended in the same buffer, and stored at −80°. Assays for binding of adenosine agonists were performed in triplicate at room temperature in the presence of ADA (0.2 units/ml; Sigma) for 1 hr. The binding assays were terminated by vacuum filtration on Whatman FC filters. Three milliliters of ice-cold washing buffer (20 mM Tris-HCl, pH 7.5, and 2.5% bovine serum albumin) were added to the sample tube, and the mixture was poured onto the filter. The filters then were washed with 3 ml of ice-cold washing buffer five times, and the radioactivity on the filters was measured. Nonspecific binding was obtained in the presence of a 100-fold excess of unlabeled agonist.

Measurement of cAMP content and decay. Intracellular cAMP content of PC12 cells was determined as described by Brooker *et al.* (14) with slight modification. In brief, PC12 cells were washed twice using Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 10 mM glucose, 5 mM HEPES buffer adjusted to pH 7.4), and resuspended in the same solution at 1×10^6 cells per 0.9 ml in each 1.5-ml tube. To assess the cAMP accumulation on stimulation, 0.1 ml of 10-fold concentrated test reagent was added to each tube and gently mixed at room temperature for 10 min. Cells were then washed twice rapidly in ice-cold Locke's solution. We extracted cellular cAMP by adding 0.3 ml of 0.1 N HCl to each tube. For cAMP decay experiments, cells were treated with adenosine of the indicated concentration for 10 min at room temperature as described above. ADA (2 units) was then immediately added to the medium in the presence of the indicated reagent. After various time intervals, the cAMP decay was terminated by vacuum filtration on Whatman FC filters. Five milliliter of ice-cold Locke's buffer were poured on the filter to remove any residual extracellular adenosine and ADA. The filters were then soaked in 1 ml of 0.1 N HCl to extract the cellular cAMP. The cAMP was assayed using the cAMP [^{125}I] assay system (Amersham, England).

Adenylyl cyclase assay. Adenylyl cyclase activity was assayed as described previously (15) with slight modification. In brief, cells were washed three times to remove agonist and resuspended in the lysis buffer (10 mM EDTA, 20 mM Tris, 250 mM sucrose, 1 mM DTT, 0.1 mM PMSF, 40 μM leupeptin, pH 7.4). We then sonicated the cells using a W-380 sonicator (Ultrasonics, Inc.) 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 and cytosolic fractions. 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 units of ADA, 50 mM HEPES, 6 mM $MgCl_2$, 1 μM GTP, and 20–50 μg of membrane protein. Reactions were stopped by the addition of 0.6 ml of 10% TCA. The cAMP formed was isolated by Dowex chromatography (Sigma) and assayed by radioimmunoassay as described above. No significant difference was found by adding 20 mM creatine phosphate (Sigma), 100 units/ml of creatine phosphokinase (Sigma), or 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) to the cyclase reaction. The enzyme activity was linear for as long as 20 min with membrane protein up to 100 μg. All samples were assayed in triplicate.

PDE assay. The membrane and cytosolic fractions from PC12 cells were prepared as for adenylyl cyclase assay. The PDE assay was carried out in PDE buffer containing 48 mM Tris-HCl (pH 7.7), 0.1 mM $MgSO_4$, 1 μM [2,8-^{3}H]cAMP (31.4 Ci/mmol; Dupont-New England Nuclear, Boston, MA) 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. We then added 20 μg of snake venom (from *Crotalus atrox*; Sigma) to each tube and incubated the tubes at 30° for 30 min. [^{3}H]Adenosine was isolated using acidic alumina as described elsewhere (16). The enzyme activity was linear for as long as 10 min with 50 μg of protein. All samples were assayed in triplicate.

SDS-PAGE and Western blotting. We determined protein concentration by a simple colorimetric assay based on the Bradford dye binding procedure (17) using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard. For SDS-PAGE, membrane fractions were combined with two times the sample buffer containing 125 mM Tris-HCl (pH 6.8), 20% glycerol, 1% SDS, 15% 2-mercaptoethanol, 200 mM DTT, and 0.01% bromphenol blue; boiled for 5 min; centrifuged to remove the insoluble material; and separated on 10% separating gels according to the method of Laemmli (18). After electrophoresis, the gel was transferred to a PVDF membrane, blocked with 5% skim milk in PBS, and then incubated with antiserum against Gsα (DuPont New England

Nuclear) at 4° overnight. Typically, we used a 1:1000 dilution for antiserum against Gsα unless stated otherwise. After three 5-min washes in PBS, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) at a 1:2000 dilution for 1 hr at room temperature. The membranes were washed (3 times with PBS and stained with BCIP/NBT) in a reaction buffer containing 100 mM Tris base, 100 mM NaCl, 5 mM $MgCl_2$, 0.4 mM NBT, and 0.45 mM BCIP.

Results

To study the desensitization of A2a-R-mediated cAMP elevation, PC12 cells at 70% confluency were treated with the nonselective adenosine agonist NECA (100 nM) or the A2a-selective agonist CGS21680 (1 μM) for the indicated period of time (Fig. 1). After a 4-hr incubation with either adenosine agonist, approximately 90% of the cAMP accumulation in response to activation of A2a-R was inhibited. No significant changes in the morphology and the proliferation rate of the desensitized cells as determined by [^{3}H]thymidine uptake were observed (data not shown).

To compare the effects of different adenosine agonists (NECA, CGS21680, and N^6-cyclohexyladenosine) on desensitization of the response to activation of A2a-R in PC12 cells, we tested the desensitizing abilities of these three adenosine agonists. As demonstrated in Fig. 2, a good correlation between the affinities of the three adenosine agonists for A2a-R and their ability to desensitize the A2a response in PC12 cells was observed. The relative potency of these agonists to desensitize the A2a response was as follows: NECA = CGS21680 ≫ N^6-cyclohexyladenosine, which is the same order as their potencies for activating A2a-R.

To understand the molecular mechanism involved in A2a desensitization, we measured the numbers of A2a-R in control

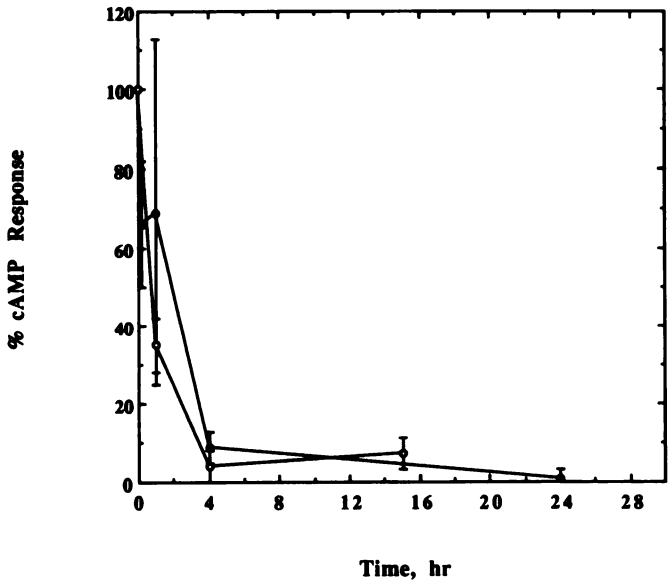

Fig. 1. Time course of A2a desensitization in PC12 cells. PC12 cells were pretreated with 100 nM NECA (○) or 1 μM CGS21680 (●) for the indicated period of time, washed twice to remove the agonist, and stimulated with 1 μM CGS21680 for 10 min at room temperature. The accumulation of cAMP was measured as detailed in Materials and Methods. Values represent the mean ± standard error of the mean of at least 12 determinations (four determinations in three independent experiments) and are expressed as percentages of the cAMP accumulation (560 ± 112 pmol/million cells) in control cells.

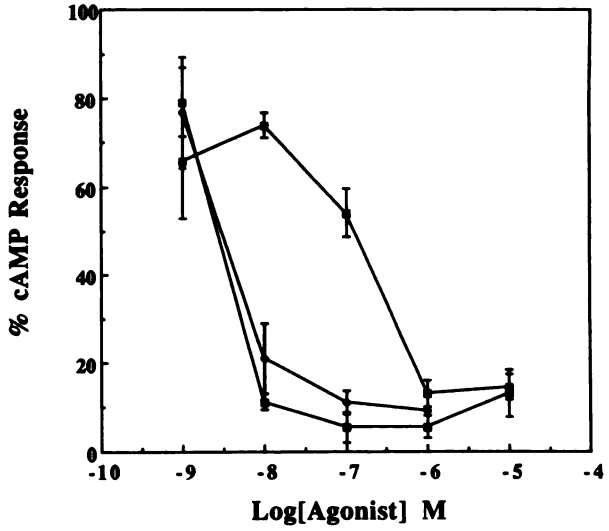

Fig. 2. The affinities of the three adenosine agonists correlate well with their ability to desensitize the CGS21680-evoked cAMP accumulation in PC12 cells. PC12 cells of 70% confluency were treated with CGS21680 (□), NECA (●), and N^6-cyclohexyladenosine (■) at the concentration indicated for 14 hr. Cells were then washed twice and stimulated with 1 μM CGS21680 for 10 min at room temperature. Data are mean ± standard error of the mean and expressed as percentages of the cAMP accumulation (419 ± 138 pmol/million cells) in control cells (0 hr). The results are from one representative experiment of three experiments performed.

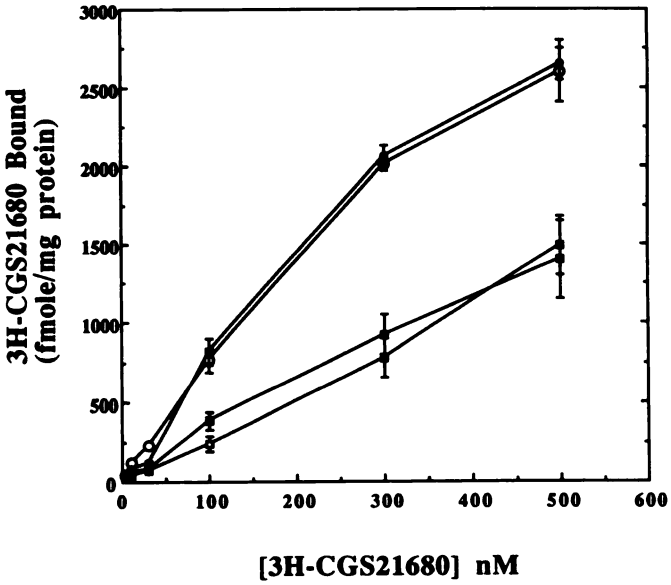

Fig. 3. No significant change in the number of binding sites and affinity for CGS21680 was observed in desensitized cells. PC12 cells were incubated with 100 nM NECA for 14 hr (closed symbols) or left untreated (control cells; open symbols). Saturation curves for the A2a-selective agonist [^{3}H]CGS21680 were obtained using binding analysis as detailed in Materials and Methods. ○ and ●, total binding, □ and ■, nonspecific binding. The results are from one representative experiment of three experiments performed.

and in desensitized cells. Plasma membranes from PC12 cells treated with or without NECA (100 nM) for 14 hr were harvested, and the receptor binding assay was performed as described in Materials and Methods. Binding of the A2a-selective adenosine agonist [^{3}H]CGS21680 was saturable (Fig. 3). The maximum numbers of binding sites for CGS21680 in control

and desensitized PC12 cells were 1380 ± 132 and 1452 ± 215 fmol/mg of protein, respectively. No significant change in the equilibrium dissociation constants (K_d; 115 ± 25 nM for control cells and 140 ± 20 nM for desensitized cells) was observed. The values for K_d and the maximum number of binding sites were calculated from Scatchard analyses of binding data from three independent experiments. Under the same conditions, 90% of the A2a response as determined by cAMP accumulation was desensitized (Fig. 2). We also examined the gene expression of A2a-R during this desensitization process (Fig. 4, A and B). Of total RNA collected from PC12 cells treated with or without 1 μM CGS21680 for the indicated period of time, 50 μg were loaded in each lane. The Northern blot was then hybridized with ^{32}P-labeled A2a-R cDNA and a 1.3-kb fragment of GAPDH to demonstrate the equal loading of RNA in each lane. In agreement with the insignificant change in numbers of A2a-R in desensitized cells, we found no significant change in the transcript levels of A2a-R. Therefore, down-regulation of A2a-R expression is unlikely to account for the desensitization of A2a-R-mediated cAMP elevation.

To further investigate the mechanism underlying the loss of A2a responsiveness in PC12 cells, we measured their adenylyl cyclase activity. Interestingly, the basal cyclase activity and the activities evoked by adenosine agonists, by GTPγS, or by forskolin were all inhibited to a very similar extent (60–70% inhibition) in the adenosine agonist-desensitized cells (Table 1). When cells were pretreated with the cAMP analogue dibutyryl-cAMP, which permeates membranes, no inhibition of the basal or adenosine agonist-stimulated adenylyl cyclase activity was observed. Pretreating PC12 cells with forskolin also failed to down-regulate the adenosine agonist-stimulated adenylyl cyclase activity (data not shown). Therefore, the elevation in cellular cAMP content in response to an A2a agonist was apparently not sufficient to induce reduction of adenylyl cyclase activity during desensitization of A2a response. The time course of this down-regulation of forskolin-evoked adenylyl cyclase activity during desensitization of the A2a response is shown in Fig. 5. After the first 30-min incubation with CGS21680, 52 ± 6% of the forskolin-induced adenylyl cyclase activity was inhibited in PC12 cells. Longer incubation (17 hr) with the adenosine agonist resulted in only slight further inhibition of the adenylyl cyclase activity.

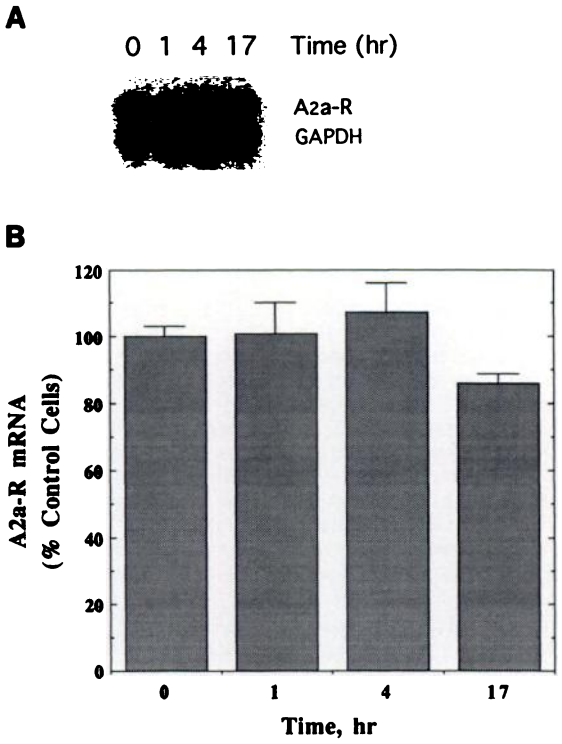

Fig. 4. No significant change occurred in the transcript level of A2a-R in desensitized cells. A, PC12 cells were treated with or without CGS (1 μM) for the periods of time indicated. Cytoplasmic RNA was prepared from these cells using the acid guanidiniumthiocyanate-phenol-chloroform extraction method as described in Materials and Methods. Fifty micrograms of total RNA were fractionated electrophoretically on 1.2% agarose containing 5.5% formaldehyde, transferred to GeneScreen Plus, and hybridized with ^{32}P-labeled cDNA fragments of A2a-R and GAPDH gene. The results are from one representative experiment of four independent experiments that gave similar results. B, Values for mRNA content are expressed as percentages of mRNA in control cells: (integrated absorbance units of A2a-R signal + GAPDH signal at the indicated time)/(A2a-R + GAPDH at 0 hr) × 100. The data were generated by quantitative computing densitometry of autoradiograms from at least three independent Northern blots using the image analysis software package ImageQuant v. 3.15 (Molecular Dynamics).

Because the amount of Gsα protein might significantly affect adenylyl cyclase activity, we measured Gsα protein levels during desensitization of the A2a response by immunoblot analysis (Fig. 6). Although a 30-min incubation with CGS21680 resulted in a 52 ± 6% inhibition of the forskolin-induced adenylyl cyclase activity (Fig. 5), no significant change in the Gsα protein level was found. However, Gsα protein level was considerably reduced when cells were treated with CGS21680 for 16 hr. This down-regulation of Gsα protein levels after long-term (16 hr) exposure to CGS21680 might be in part responsible for down-regulation of the adenylyl cyclase activity.

To determine whether this down-regulation of adenylyl cyclase activity was mediated through adenosine receptors, the adenosine antagonist DPCPX was used to block the CGS21680-evoked down-regulation of adenylyl cyclase activity (Table 2). We observed a typical 49 ± 5% inhibition of the forskolin-induced adenylyl cyclase activity in CGS21680-pretreated cells. In the presence of 100 μM DPCPX, which blocked the CGS21680-induced cAMP elevation by 97 ± 0.3% (data not shown), treatment with CGS21680 did not significantly inhibit the forskolin-induced adenylyl cyclase activity. The antagonist itself also slightly inhibited the adenylyl cyclase activity.

When we compared the extent of A2a desensitization as determined by cAMP accumulation (91 ± 4% inhibition, Fig.

TABLE 1

Inhibition of adenylyl cyclase activity by prolonged exposure of PC12 cells to adenosine agonists

Cells were treated with the indicated reagents for 15 hr. Membrane fractions were prepared from these cells as described in Materials and Methods. Adenylyl cyclase activity at basal level (no addition) and in response to NECA (100 μM), CGS21680 (100 μM), GTPγS (50 μM), or forskolin (10 μM) was assayed. Each assay was carried out in triplicate. The values are mean ± standard error from one representative experiment of four experiments performed with similar results.

Pretreatment	Adenylyl cyclase activity				
	Basal	NECA	CGS	GTPγS	Forskolin
	pmol/min/mg of protein				
None	30 ± 15	97 ± 5	78 ± 9	154 ± 4	676 ± 39
CGS21680 (100 nM)	11 ± 1	33 ± 5	37 ± 6	64 ± 4	273 ± 35
NECA (10 μM)	13 ± 9	31 ± 9	26 ± 9	33 ± 1	175 ± 34
Dibutyryl-cAMP (1 mM)	37 ± 1	110 ± 6	70 ± 5	298 ± 19	1330 ± 326

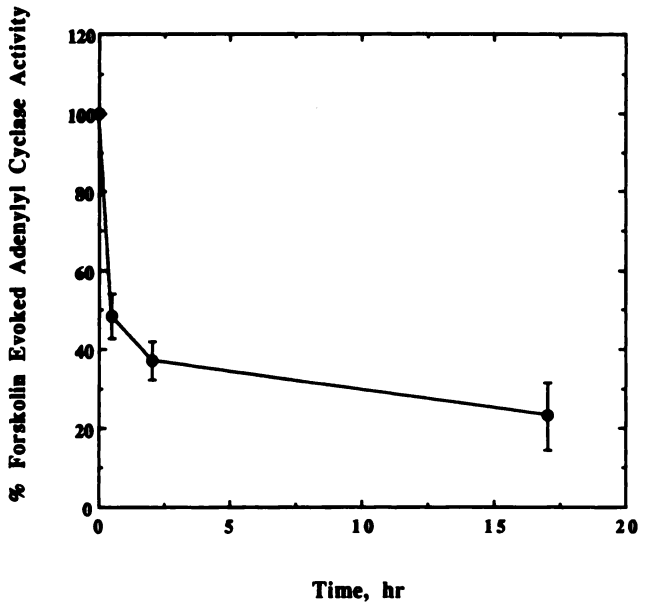

Fig. 5. Time course of the inhibition of forskolin-evoked adenylyl cyclase activity during A2a desensitization in PC12 cells. Membrane fractions were prepared from these cells as described in Materials and Methods. Adenylyl cyclase activities in response to forskolin (5 μM) were assayed. Each assay was carried out in triplicate. Values represent the mean ± standard error of the mean of at least 12 determinations (triplicate determinations in four or five independent experiments) and are expressed as percentages of the adenylyl cyclase activity in untreated cells.

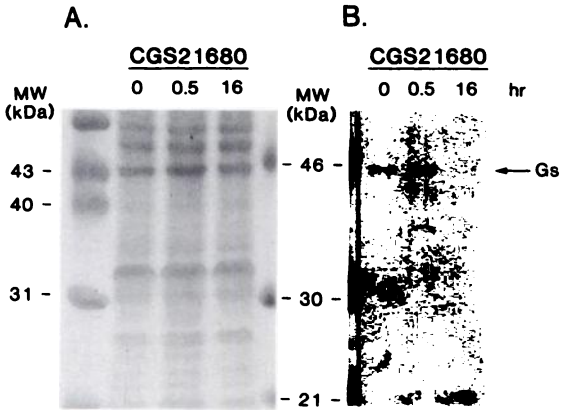

Fig. 6. Effect of CGS21680 pretreatment on Gsα protein levels. Membrane fractions were collected from PC12 cells pretreated with CGS21680 (1 μM) for the indicated periods of time. A, SDS-PAGE of membrane fractions demonstrate the equal loading of membrane proteins in each *lane*. Proteins (100 μg/*lane*) were visualized by Coomassie staining. B, Immunoblot analysis of membrane fractions (100 μg/*lane*) using a 1:1000 dilution of the anti-Gsα antiserum. The results are from one representative experiment of three independent experiments that gave similar results.

1) or adenylyl cyclase assay (75 ± 5% inhibition, Fig. 6), the cAMP accumulation appeared to be inhibited more than the cyclase activity. This indicated that multiple mechanisms may be involved in A2a adenosine desensitization. We therefore assessed the role of PDE in A2a desensitization. When PC12 cells were treated with CGS21680 for 16 hr, PDE activity was increased by 43 ± 22% (seven experiments; $p < 0.005$, two-tailed Student's *t* test) and 53 ± 18% (seven experiments; $p < 0.001$), respectively, in the cytosolic and membrane fractions. Because an increase in the PDE activity in cells pretreated

TABLE 2

Effect of an adenosine antagonist, DPCPX, on the inhibition of forskolin-evoked adenylyl cyclase activity in desensitized cells

Cells were treated with or without an adenosine antagonist, DPCPX (100 nM), for 10 min, and then with the A2a-selective agonist (CGS21680, 100 nM) for an additional 30 min. Membrane fractions were prepared from these cells as described in Materials and Methods. Adenylyl cyclase activities evoked by forskolin (5 μM) were assayed. Each assay was carried out in triplicate. The values are mean ± standard error from one representative experiment of three experiments performed with similar results.

Pretreatment	Forskolin-evoked adenylyl cyclase activity
	pmol/min/mg protein
None	264 ± 9
CGS21680	134 ± 14
DPCPX	200 ± 7
DPCPX/CGS21680	198 ± 18

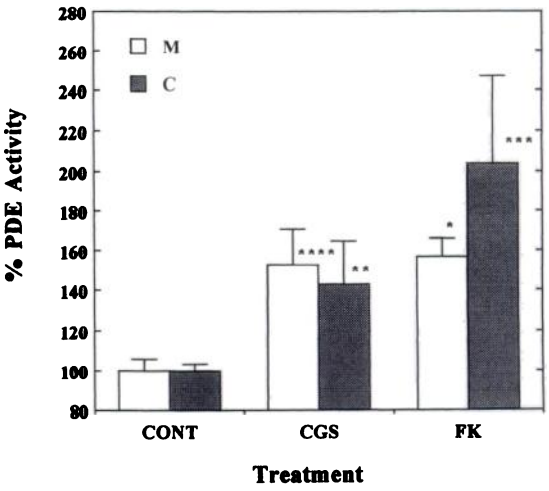

Fig. 7. Activation of PDE activity in PC12 cells treated with CGS21680 (1 μM) and forskolin (1 μM) for 16 hr. PDE activity was determined in both cytosolic (*C*, shaded bars) and membrane (*M*, open bars) fractions of cells treated with the indicated reagent for 14 hr as detailed in Materials and Methods. Values represent the mean ± standard error of the mean of at least 12 determinations (triplicate determinations in four to seven independent experiments) and are expressed as percentages of the PDE activity in untreated cells (*CONT*). The PDE activities in membrane and cytosolic fractions of untreated cells were 16 ± 2.2 and 32 ± 2.8 pmol/mg/min, respectively. Statistical significance: *, **, ***, and **** indicate differences between untreated cells and treated cells ($p < 0.05$, 0.005, 0.0025, 0.001, respectively; two-tailed Student's *t* test).

with forskolin for 16 hr was also observed (Fig. 7), we conclude that PDE is involved in the desensitization of the A2a response, probably in a cAMP-dependent manner. Short-term (30-min) exposure to adenosine agonists or forskolin did not result in a significant increase in PDE activity (data not shown). To characterize the PDE subtype involved in desensitization of the A2a response, we examined the effect of milrinone (a PDE III inhibitor) and cGMP on the increase in PDE activity in desensitized cells. Although milrinone inhibited cAMP hydrolysis by 54 ± 4% (three experiments, $p < 0.005$) in control PC12 cells, it did not block the PDE activity activated by NECA or forskolin treatment (Fig. 8A). In addition, cGMP inhibited cAMP hydrolysis by 28 ± 4% (three experiments, $p < 0.005$) in control PC12 cells. However, cGMP did not affect the increase in PDE activity in desensitized cells (Fig. 8B).

To evaluate the contribution of the increase in PDE activity to desensitization of the A2a response, we compared the cAMP decay in control cells with that in cells pretreated with

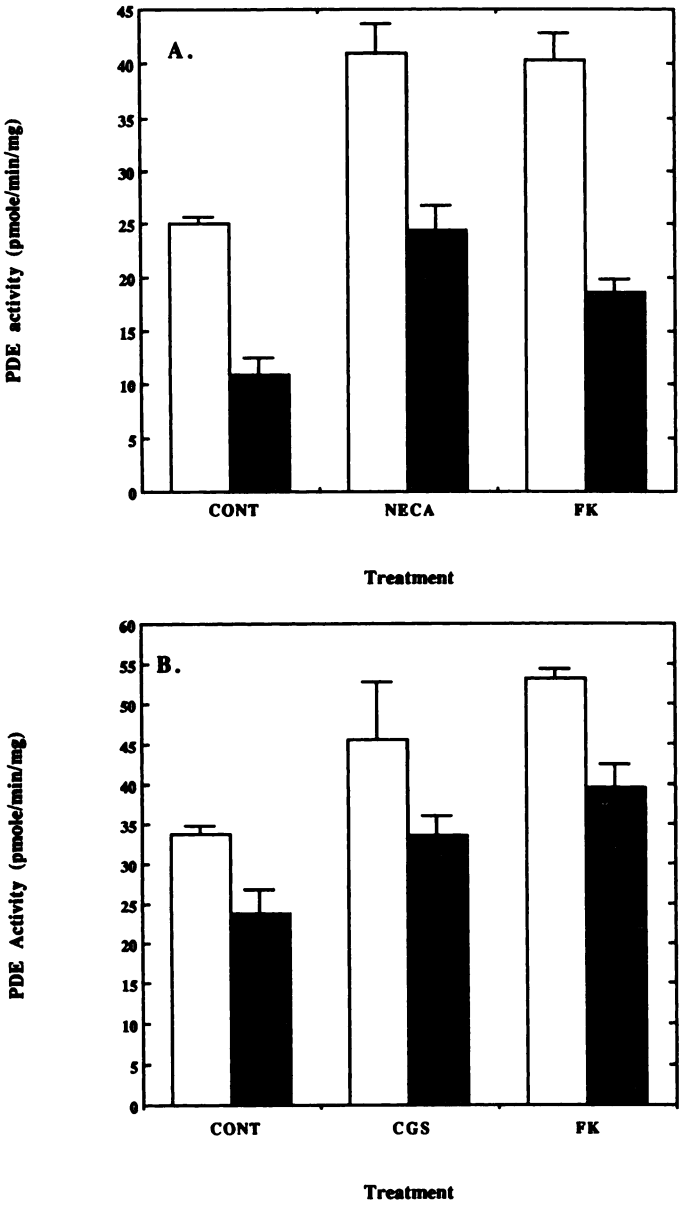

Fig. 8. Effect of milrinone and cGMP on the PDE activities in control cells and desensitized cells. A, Effect of milrinone, a type III PDE inhibitor. PC12 cells were pretreated with NECA (1 μM) or forskolin (1 μM) as indicated for 12 hr. PDE activities in the cytosolic fractions were determined as detailed in Materials and Methods in the absence (open bars) or presence of milrinone (10 μM, shaded bars). Each assay was carried out in triplicate. The values are mean ± standard error from one representative experiment of four experiments performed with similar results. B, Effect of cGMP. PC12 cells were pretreated with CGS21680 (1 μM) or forskolin (1 μM) as indicated for 12 hr. PDE activities in the cytosolic fractions were determined as detailed in Materials and Methods in the absence (open bars) or presence of cGMP (1 μM, shaded bars). Each assay was carried out in triplicate. The values are mean ± standard error from one representative experiment of four experiments performed with similar results.

CGS21680 for 14 hr. To increase the cellular cAMP content, cells were stimulated with 5 or 500 μM adenosine as indicated for 10 min at room temperature. In control cells, 5 μM adenosine increased the cellular cAMP content to a level very similar to that accomplished by 500 μM adenosine in CGS21680-treated cells. cAMP accumulation was terminated by adding ADA, and the cAMP decay following the addition of ADA was determined as described in Materials and Methods. As shown in Fig. 9, 35 ± 6% (500 μM adenosine stimulation) and 13 ± 8% (5 μM adenosine stimulation) of the cAMP content were hydrolyzed within 5 sec after treatment with ADA in control cells, whereas 85 ± 9% of the cAMP content was degraded in desensitized cells. These findings suggest that the increase in PDE activity in cells exposed to long-term CGS21680 treatment results in much more rapid cAMP degradation.

Hide *et al.* (19) recently showed that PC12 cells contain both A2a-R and A2b-R by comparing the activations of adenylyl cyclase using a nonselective adenosine agonist (NECA) and an A2a-selective agonist (CGS21680). To confirm their finding, we first amplified the DNA fragments of A2a-R and A2b-R from the first-strand cDNA of PC12 cells using the PCR technique (Fig. 10). We then subcloned and sequenced these two PCR fragments (data not shown) to show that there were indeed two A2 adenosine receptors in PC12 cells that are identical with those recently cloned (2, 4). Although binding of both A2 adenosine receptors leads to the activation of adenylyl cyclase, it takes a 100-fold higher concentration of NECA to stimulate the A2b-R than to stimulate the A2a-R. We therefore examined whether the A2b response was also inhibited during A2a desensitization. To do this, PC12 cells were pretreated with or without CGS21680 (2.5 μM) for 14 hr. Dose-response curves for the effects of NECA and CGS21680 on cAMP accumulation in control and desensitized cells are shown in Fig. 11. In control cells, NECA induced a higher maximum cAMP response than CGS21680, because both A2a-R and A2b-R were activated by NECA, whereas CGS21680 activated only

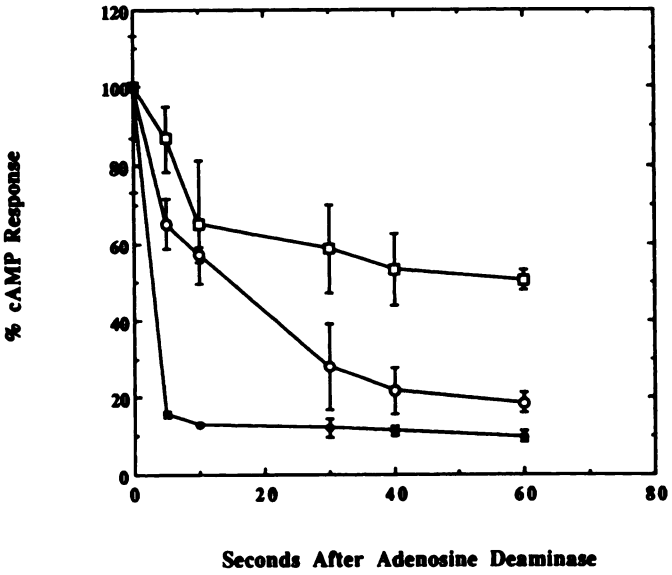

Fig. 9. cAMP decay was much faster in desensitized cells than in control cells. PC12 cells were pretreated with (●) or without (○, □) CGS21680 (2.5 μM) for 14 hr, washed twice to remove the agonist, and then stimulated with 500 μM (circles) or 5 μM (squares) adenosine for 10 min at room temperature. cAMP accumulation was terminated by adding ADA (2 units). At the indicated time point, cAMP decay was terminated by vacuum filtration on Whatman FC filters as described. Cellular cAMP content is plotted as the percentage of the cAMP response at the time of ADA addition. The cAMP accumulations in response to 500 μM adenosine in control cells, 5 μM adenosine in control cells, and 500 μM adenosine in desensitized cells are 581 ± 77, 212 ± 33 and 226 ± 1 pmol/10^6 cells, respectively. The values are mean ± standard error from one representative experiment of three experiments performed with similar results.

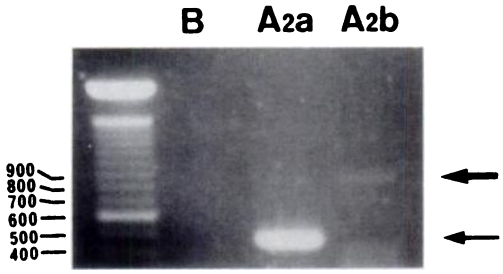

Fig. 10. Two A2 adenosine receptors (A2a and A2b) are present in PC12 cells. Two pairs of oligonucleotides were used as primers to specifically amplify DNA fragments of the A2a-R and A2b-R as described in Materials and Methods. These primers were as follows: for A2a, 5′-GCCAACGGAAGTGCCA-3′ and 5′-GCTTTTGGGACGCTTCTGGA; and for A2b, 5′-GCCTCGAGTGCTTTACAGACCCCC-3′ and 5′-GAAAGTTGACTGTCCCCCGGCCTG-3′. The predicted sizes of the amplified DNA fragments for A2a-R and A2b-R are 466 bp (thin arrow) and 886 bp (thick arrow), respectively. One-tenth of the PCR product was examined by ethidium bromide/agarose (1%) electrophoresis. B, Negative control.

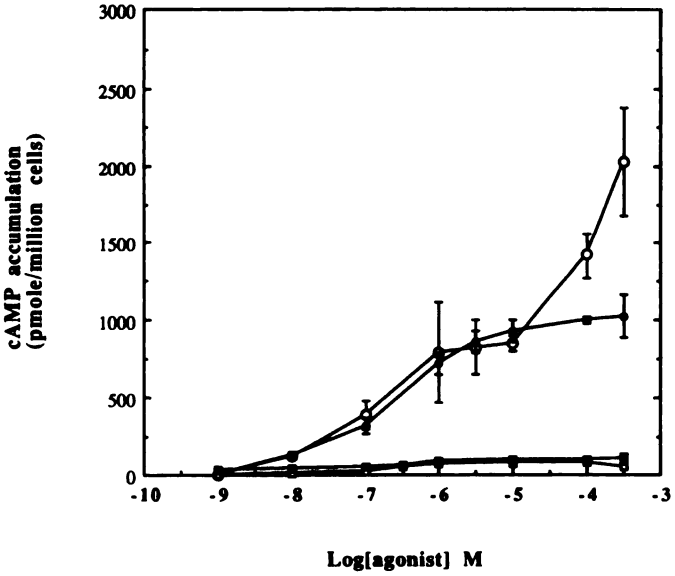

Fig. 11. The A2b adenosine response was also diminished in PC12 cells desensitized with the A2a-specific agonist. Cells were treated with 2.5 μM CGS21680 for 14 hr (squares) or left untreated (circles), washed twice, and stimulated with CGS21680 (closed symbols) or NECA (open symbols) at the concentrations indicated for 10 min at room temperature. The accumulation of cAMP was then determined. Data are mean ± standard error. The results are from one representative experiment of three experiments performed.

the former. Most interestingly, the difference between the abilities of NECA and CGS21680 to increase the cellular cAMP level disappeared in desensitized cells, suggesting that the A2b adenosine response was also diminished in A2a-desensitized cells.

Discussion

Prolonged exposure of PC12 cells to adenosine agonists significantly inhibited their response to subsequent stimulation with an A2a-selective adenosine agonist. Our data suggest that at least three different mechanisms may contribute to the above phenomenon. Short-term (30-min) exposure to adenosine agonists inhibited adenylyl cyclase activity, which led to a marked decrease in the response of the cells to activation of A2a-R. Further reduction of the A2a response could be achieved by long-term (12–20-hr) exposure to adenosine agonists and was accompanied by a slight decrease in the Gsα protein level and the activation of PDE.

Our proposed model for A2a desensitization differs from the mechanism established using the β_2-adrenergic receptor and rhodopsin systems, in which agonist-induced reduction in the number of cell surface receptors as well as receptor phosphorylation have been suggested to be the major mechanisms involved (20, 21). In contrast, we found no significant decrease in the expression of A2a-R. Importantly, the relative potency of these adenosine agonists in desensitizing the A2a response was in the same order as their potencies for activating A2a-R (Fig. 2). In addition, we have demonstrated that an adenosine antagonist (DPCPX) was able to block the desensitization of adenylyl cyclase activity in CGS21680-treated cells (Table 2). Therefore, it appears that desensitization of the A2a response is mediated through prolonged activation of the A2a receptor. Interestingly, the adenylyl cyclase activities evoked by adenosine agonists, by GTPγS, or by forskolin were down-regulated to a very similar extent (60–70% inhibition) in the adenosine agonist-desensitized cells, indicating that the coupling between A2a-R and its effectors was probably not affected. Moreover, there is no potential phosphorylation site for the cAMP-dependent kinase in the deduced amino acid sequence of A2a-R (2). Therefore, the cAMP-dependent kinase is unlikely to directly modulate A2a-R during A2a desensitization. Both elevation of cellular calcium concentration by ionomycin and activation of protein kinase C by a phorbol ester failed to induce the desensitization of A2a response in PC12 cells (data not shown). Nevertheless, we cannot exclude entirely the possibility that receptor phosphorylation may play a role in A2a desensitization.

Desensitization of the adenosine response in several different cell lines has been reported since 1981 (7–9). Recently, Ramkumar *et al.* (8) demonstrated that phosphorylation of the A1 receptor is involved in a slow desensitization process of the A1 adenosine receptor in DDT1 MG-2 smooth muscle cells. In contrast, homologous desensitization of the A2 receptor occurs much faster and without down-regulating the number of cell surface receptors (8). However, it is not clear which A2 adenosine receptors are present in those cell lines or which molecular mechanism(s) underlie the desensitization of the A2 adenosine response. In NG108 cells, desensitization of A2 adenosine receptors has been reported to be fully dependent (7) or only partially dependent (9) on the cAMP-mediated pathway. Those observations agree well with our finding that elevation of cellular cAMP content resulted in activation of PDE activity in PC12 cells (Fig. 7), which in turn should lead to lowered cAMP response. The desensitization of A2b response in cells desensitized to an A2a agonist may be due, at least in part, to a generalized depression of the cAMP system in the A2a desensitized cells, including increased PDE activity and down-regulation of Gsα protein level. In addition, A2a-R and A2b-R may be coupled to the same adenylyl cyclase subtype that is down-regulated in desensitized cells. It would be very interesting to examine whether cAMP elevation elicited by agonists other than adenosine is also reduced in PC12 cells pretreated with the A2a adenosine agonist. Unfortunately, we were unable to find an agonist other than adenosine to increase the cellular cAMP content in our PC12 cells; we stimulated PC12 cells with

prostaglandin E_1 and isoproterenol but found no increase in the cellular cAMP content.

Genes of at least six distinct mammalian adenylyl cyclases have been isolated (22). These enzymes can be activated by the α-subunit of Gs proteins. In addition, some adenylyl cyclases (types I, III, and V) can be modulated by Ca^{2+}/calmodulin in a cooperative feedback manner (23, 24). Overexpression of $pp60^{c\text{-}src}$ has also been found to have a negative effect on adenylyl cyclase in fibroblasts (25). Recently, several lines of evidence have suggested that the $\beta\gamma$-subunits of GTP-binding proteins can inhibit type I and stimulate type II adenylyl cyclases (23). Such interactions provide an additional pathway for cross-talk between various G protein-coupled receptors. In chick hepatocytes, phosphorylation of adenylyl cyclase by PKA has been proposed to mediate glucagon-induced desensitization (26). In PC12 cells, our data show that decreases occur in basal adenylyl cyclase activity and in cyclase activity stimulated by adenosine agonists, by GTPγS, or by forskolin during A2a desensitization. The molecular basis underlying the reduction of adenylyl cyclase activity is under investigation. A decrease in the Gsα protein level accounts for the inhibition of adenylyl cyclase activity after long-term but not short-term exposure of cells to adenosine agonists (Fig. 6). Interestingly, Kenimer and Nirenberg (7) also reported gradual losses of basal and prostaglandin E_1-, 2-chloroadenosine-, and NaF-stimulated adenylyl cyclase activities after prolonged activation of adenylyl cyclase by prostaglandin E_1 in NG108-15 cells, whereas prolonged activation by 2-chloroadenosine resulted in only slight decreases in basal and prostaglandin E_1-stimulated enzyme activities. Because dibutyryl-cAMP failed to reduce agonist-stimulated adenylyl cyclase activity, whereas adenosine agonists did reduce it (Table 1), elevation of cAMP in itself appears to be insufficient to mediate the down-regulation of adenylyl cyclase activity in PC12 cells. Interestingly, we have consistently found that the adenylyl cyclase activity stimulated by GTPγS and by forskolin, but not by adenosine agonists, was significantly enhanced in cells pretreated with dibutyryl-cAMP (Table 1) or forskolin (data not shown). Several different subtypes of adenylyl cyclase probably exist in PC12 cells, and each subtype may undergo a distinct mode of regulation on prolonged activation of the adenylyl cyclase. For example, there is at least one predicted cAMP-dependent kinase (PKA) phosphorylation site in all adenylyl cyclases except type 4 (26). Phosphorylation of each individual adenylyl cyclase by PKA may distinctly affect their activity. Long-term activation of PKA by dibutyryl-cAMP may potentiate the activity of a certain adenylyl cyclase subtype that is not coupled to A2a-R in PC12 cells. Therefore, the adenylyl cyclase activities elicited by GTPγS and by forskolin, but not by adenosine agonists, were significantly enhanced in dibutyryl-cAMP-pretreated cells (Table 1).

Considerable evidence has demonstrated that hormonal modulation of PDEs can be mediated through G proteins, cAMP, and Ca^{2+}/calmodulin-dependent protein kinases (27). A total of five subclasses of PDE, each containing several isoforms, have been described (27). In rat fat cells, insulin and isoproterenol activate a cGMP-inhibited low-K_m cAMP PDE through phosphorylation by cAMP-dependent and insulin-dependent serine kinases (28). In addition, gene expression of a high-affinity cAMP PDE can be up-regulated more than 100-fold by follicle-stimulating hormone or by the cAMP analogue dibutyryl-cAMP (29). Selective inhibitors of some PDEs are also available. In PC12 cells, Whalin *et al.* (30) recently demonstrated the existence of the type II PDE in both cytosolic and particulate compartments. Our data indicate that activation of PDE, which may be mediated through a cAMP-dependent pathway, results in heterologous desensitization (Figs. 7 and 11). The increase in PDE activity in desensitized cells could not be blocked by a type III PDE inhibitor (milrinone) or by cGMP (Fig. 8, A and B). Thus, activation of PDE II or PDE III is unlikely to contribute to desensitization of the A2a response in PC12 cells. The PDE isozyme profile in PC12 cells is under investigation to identify which PDE subtype is involved in desensitization of the A2a response.

In summary, our data suggest that coordinated regulation of cAMP synthesis (by adenylyl cyclase) and hydrolysis (by PDE) leads to desensitization of the A2a response in PC12 cells. This desensitization process is very complex and can be explained only in part by an elevation of cellular cAMP content on stimulation by adenosine agonists.

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Send reprint requests to: Dr. Yijuang Chern, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.