Selective Regulation of $G_i\alpha_1$ Expression and Function in PC12 Cells by cAMP

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Hormone and neurotransmitter receptor systems regulate both the activity and expression of GTP-binding proteins (G-proteins). However, relatively little is known about the mechanism by which this regulation occurs. One G-protein subtype, $G_i\alpha_1$, is expressed primarily in neuronal cells. Here, we demonstrate the selective regulation of $G_i\alpha_1$ mRNA and protein levels by cAMP. Treatment of PC12 cells with forskolin increases $G_i\alpha$ protein levels. Similarly, incubation of PC12 cells with agents that increase intracellular levels of cAMP, including forskolin, dibutyryl-cAMP, and 8-bromo-cAMP, induce a two- to fourfold increase in $G_i\alpha_1$ mRNA levels. Furthermore, the effect of increased intracellular cAMP is specific for $G_i\alpha_1$ mRNA expression; the levels of mRNA encoding other G-protein subtypes remain unaltered. cAMP-stimulated $G_i\alpha_1$ expression occurs within hours of treatment and is sustained for days. Increasing intracellular cAMP by activation of cell surface adenosine receptors also increases $G_i\alpha_1$ mRNA levels. Treatment of PC12 cells with phorbol esters, NGF, or depolarizing concentrations of KCl did not increase $G_i\alpha_1$ mRNA expression, demonstrating that $G_i\alpha_1$ expression is specifically regulated by cAMP. Guanine nucleotide-mediated inhibition of adenylate cyclase activity was measured in order to determine if the change in $G_i\alpha$ protein expression was accompanied by a change in G-protein function. Adenylate cyclase activity in PC12 cells treated with an adenosine analog and therefore expressing higher levels of $G_i\alpha$ protein is more sensitive to inhibition by guanine nucleotides than in nontreated PC12 cells. *De novo* mRNA synthesis, but not protein synthesis, is required for the increase in $G_i\alpha_1$ mRNA expression. The rates of mRNA degradation of $G_i\alpha_1$ mRNA in control and cAMP-treated cultures are indistinguishable, suggesting that the increase in $G_i\alpha_1$ mRNA is due to an increased rate of synthesis, rather than a decreased rate of degradation. This may represent a neuronal-specific regulatory mechanism to increase selectively the expression and function of $G_i\alpha_1$ by neurotransmitters and synaptic activity.

G-proteins are a large family of closely related polypeptide complexes responsible for signal-effector coupling. G-protein complexes are composed of α -, β -, and γ -subunits. Each distinct G-protein is defined by its α -subunit, one of several polypeptides of 39–52 kDa. The α -subunit contains the site for ADP-ribosylation by bacterial toxins (cholera toxin and/or pertussis toxin) as well as the GTPase activity required for the deactivation of a G-protein following hormone binding to its receptor and consequent activation of the G-protein by GTP. Each α -subunit is coupled to a $\beta\gamma$ -complex composed of either a 35 or 36 kDa β -subunit and a γ -subunit of M_r 8000 (for review, see Spiegel, 1987; Casey and Gilman, 1988).

G-proteins couple membrane receptors to many effector systems involved in physiological signaling, such as the regulation of the activity of phospholipases (Cockcroft, 1987; Jelsema and Axelrod, 1987), ion channels (Pfaffinger et al., 1985; Yatani et al., 1987), retinal phosphodiesterase (Stryer, 1986), and adenylate cyclase (Gilman, 1987). Adenylate cyclase activity is directly modulated by stimulatory (G_s) and inhibitory (G_i) G-proteins that act as signal transducers between hormone receptors and adenylate cyclase (Gilman, 1987). Three distinct $G_i\alpha$ subunits have been described, $G_i\alpha_1$, $G_i\alpha_2$, and $G_i\alpha_3$. Of the three, $G_i\alpha_1$ is more selectively neuronal in its expression than either of the other two (Jones and Reed, 1987).

Although it is well established that G-proteins function as couplers in these effector systems to regulate signal transduction, the regulation of G-protein expression is less well understood. The expression of G-proteins is regulated through development, by hormones, and by transmitters. The amounts and types of G-protein subunits found in heart are differentially regulated through development (Luetje et al., 1987). Reithmann et al. (1990) have shown that stimulation of β -adrenergic receptors in rat heart results in upregulation of $G_i\alpha_2$ and $G_i\alpha_3$ proteins and a concomitant decrease in adenylate cyclase activity. Stimulation of adenylate cyclase activity of mouse S49 lymphoma cell induces an increase in $G_i\alpha_2$ mRNA and a decrease in the amount of $G_i\alpha$ (Haddock et al., 1990). In rat adipocytes A1-adenosine receptor agonists regulate $G_i\alpha_2$ and $G_i\alpha_3$ expression (Green, 1987; Longabaugh et al., 1989) and glucocorticoids regulate G_β mRNA levels (Ros et al., 1989), while in smooth muscle cells A1-adenosine agonists regulate both $G_i\alpha_2$ and β -adrenergic receptor expression (Haddock et al., 1991).

Regulation of neuronal G-protein expression has been studied in neuroblastoma $\times$ glioma (NG108-15) cells. When exposed to cAMP analogs, these cells “differentiate” by extending neurite-like processes (Mullaney et al., 1988). Upon differentiation, levels of $G_i\alpha$ proteins decrease and levels of $G_o\alpha$ protein increase (Mullaney et al., 1988; Mullaney and Mulligan, 1989). Asano et al. (1989) also reported an increase in levels of $G_o\alpha$ protein,

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but relatively small changes in G_{α_2} protein levels, when NG108-15 or PC12 cells were exposed to either NGF or forskolin. Cell density has been reported to influence the effect of NGF on G_{α} mRNA levels in PC12 cells (Tjaden et al., 1990). What remains unclear, however, is the molecular mechanism responsible for the regulated expression of G-protein subunits.

Rat pheochromocytoma (PC12) cells serve as a useful model system in which to study regulation of neuronal G-protein expression. PC12 cells were isolated from an adrenal chromaffin tumor (Greene and Tischler, 1976). When exposed to NGF or cAMP, PC12 cells differentiate into cells with a neuronal phenotype, sending out neurites (Greene and Tischler, 1976) and increasing the expression of a set of overlapping (but not identical) neuronal genes (Leonard et al., 1987; Kalman et al., 1990). As described below, cAMP also selectively regulates the function and expression of the neural-specific G-protein G_{α_1} .

Materials and Methods

Materials. Forskolin was purchased from CalBiochem. 8-Bromo-cAMP, dibutyryl-cAMP, cycloheximide, actinomycin D, and $(-)^N$ -(2-phenylisopropyl)adenosine (PIA), and sodium citrate were obtained from Sigma Chemicals. 35 S-UTP and 3 H-UTP were from New England Nuclear–Du Pont; α - 32 P-ATP and 3 H-cAMP were from ICN. SP6 and T7 polymerase were purchased from Promega. RPMI 1640 medium, fetal bovine serum, and horse serum were obtained from GIBCO/Bethesda Research Labs or Hazeltine. 2.5S NGF, purified by the method of Longo et al. (1980), and AS7 antiserum were the generous gifts of M. Bothwell (University of Washington) and A. Spiegel (National Institutes of Health), respectively.

Cell culture. Rat pheochromocytoma (PC12) cells were obtained from the laboratory of Dr. M. Bothwell (University of Washington) and were cultured according to Greene and Tischler (1976). Briefly, cells were cultured in 85% RPMI, 10% horse serum, 5% fetal bovine serum, and penicillin–streptomycin (100 U/ml and 100 μ g/ml final concentrations, respectively) at 5% CO_2 . Cells were split 1:4 every 5–7 d. For all experiments, cells were plated on poly-L-lysine (50 μ g/ml)-coated tissue culture plates, cultured overnight, and treated with agents for the indicated amount of time. With extended time in culture (20–25 passages), PC12 cells became less responsive to forskolin stimulation and demonstrated more morphological variants than did cells from younger cultures (Greene and Tischler, 1976). Early passages resulted in a three- to fourfold stimulation of G_{α_1} mRNA, whereas later passages showed only a twofold stimulation. Therefore, after 3–4 months in culture, new cells were routinely thawed. The level of G_{α_1} mRNA varied significantly for different batches of thawed cells but remained quite consistent for parallel cultures and for cells cultured from the same batch of thawed cells.

Immunoblot analysis. Immunoblot analysis of G_{α} was performed according to Luetje et al. (1987). Membranes were prepared from treated and control cell and subjected to SDS-PAGE, and immunoblot analysis was performed using the antiserum AS7 (obtained from Dr. Allen Spiegel, National Institutes of Health). This antiserum, raised against a decapeptide corresponding to the C-terminus of transducin, reacts strongly with G_{α_1} and G_{α_2} , but only weakly with G_{α_3} .

Northern blot analysis of total cellular RNA. Total cellular RNA was isolated by the method of Poppel and Baglioni (1990) or by homogenization in 0.01 *N* sodium acetate, pH 4.5, plus 0.5% SDS. Homogenates were extracted twice with phenol at 50°C for 5 min prior to isopropanol precipitation. RNA was size fractionated under denaturing conditions on agarose-formaldehyde gels and hybridized to cDNA probes for G-protein α - and β -subunits as described by Uhler et al. (1986) and Luetje et al. (1988).

The G-protein cDNA probes were obtained as follows: full-length cDNA clone for rat G_{α_2} and a near full-length clone for G_{α_1} were obtained from Drs. H. Itoh and Y. Kaziro (University of Tokyo; Itoh et al., 1986); a 0.6 kilobase XbaI–EcoRI fragment was isolated from a full-length clone for rat G_{α_1} , and a 0.4 kb BamHI fragment was isolated from a full-length clone for rat G_{α_3} [full-length G_{α_1} and G_{α_3} clones were obtained from Dr. R. Reed (Jones and Reed, 1987)]; a full-length cDNA clone for bovine $G\beta$ -1 was obtained from Dr. J. Hurley (University of Washington; Fong et al., 1986). All DNA fragments were subcloned into pGEM-3Z.

After denaturing in buffer containing 2.2 M formaldehyde and 50% formamide, the samples were subjected to electrophoresis on a 1% agarose gel, transferred to nitrocellulose, baked, and hybridized under conditions of moderately high stringency to 32 P-labeled, nick-translated probes (10⁷ cpm/ml). [The hybridization conditions were 50% formamide, 6 $\times$ saline–sodium citrate (SSC; 1X SSC: 0.15 M NaCl, 0.015 M), 50 mM NaHPO₄, pH 7.4, 5.0 mM EDTA, 0.1% SDS, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 100 μ g/ml salmon sperm DNA, at 37°C. The filters were washed in 2X SSC, 0.5% SDS, 0.1% sodium pyrophosphate at room temperature followed by washing at 42°C.]

Solution hybridization analysis of total cellular RNA. To obtain quantitative information on changes in mRNA levels, solution hybridization analysis was used. RNA was isolated from treated or control cultures and incubated overnight with 35 S-labeled antisense riboprobes made by standard procedures (Melton et al., 1984). Briefly, cDNA fragments were subcloned into pGEM-3Z plasmids that contain both T7 and SP6 promoter sequences. Single-stranded, 35 S-labeled RNA probes were generated by transcription of linearized plasmids using either SP6 or T7 polymerase and 35 S-UTP. Radiolabeled probes (2500–4500 cpm) were then incubated with cellular RNA (7.0 μ g) under stringent hybridization conditions according to McKnight et al. (1988). Nonhybridized single-stranded RNA was then digested with RNases A and T1; samples were trichloroacetic acid (TCA) precipitated, filtered over glass–fiber filters (Whatman GF/C), and counted in a scintillation counter. Standard curves were generated by hybridization with 3 H-labeled sense riboprobes. Background was determined by incubating probe without additional RNA and RNase and by including 7.0 μ g of yeast RNA as an additional control. The standard curve was linear in the range of 6×10^5 to 3×10^8 molecules of G_{α_1} mRNA per assay. Sample signals were typically 2–10 times higher than background, and increased linearly as cellular RNA was varied from 2–10 μ g per assay.

Adenylate cyclase activity assays. Inhibition of adenylate cyclase activity by guanyl-5'-yl imidodiphosphate (GppNHp) was measured as previously described (Halvorsen and Nathanson, 1984). Briefly, PC12 cells were cultured overnight on poly-L-lysine-coated plates, 10 μ M PIA was added to half of the cultures, and cells were harvested 48 hr later. Cells were homogenized by 20 passes through a glass–glass tissue grinder in 1.0 mM dithiothreitol (DTT), 200 mM sucrose, and 10 mM HEPES (pH 7.4). Membranes were pelleted, washed twice, and resuspended in the same buffer. The adenylate cyclase assay buffer contained 0.25 mM α - 32 P-ATP (14–22 Ci/mmol), 10 mM creatine phosphate, 50 U/ml creatine phosphokinase, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM theophylline, 0.1% BSA, 5 mM MgCl₂, 0.05 mM sodium ascorbate, 75 mM NaCl, 80 mM sucrose, and 0.4 mM DTT in 10 mM HEPES (pH 7.4). A 2 mM concentration of 3 H-cAMP (to monitor recovery) and GppNHp (10^{-9} – 10^{-5} M) were added to a final assay volume of 0.25 ml.

Adenylate cyclase activity was initiated by the addition of 50 μ l membranes (~2 mg/ml) to assay tubes pre-equilibrated to 30°C. Activity was assayed in triplicate for 5 min at 30°C and stopped by the addition of 0.75 ml 6.7% TCA followed by heating to 100°C for 2 min. cAMP was isolated by the method of Salomon et al. (1974), as modified by Halvorsen and Nathanson (1984).

Measurement of inhibition of protein synthesis. To determine whether protein synthesis was required for the increase in G_{α_1} mRNA expression, cycloheximide (12 μ g/ml) and forskolin (10 μ M) were added simultaneously to PC12 cell cultures. After 8 hr, cells were harvested, total RNA isolated, and solution hybridization analysis performed as described above. To measure the inhibition of protein synthesis by cycloheximide, 3 H-leucine was included for the duration of the 8 hr incubation. Cells were harvested, washed, TCA precipitated, and filtered (Whatman GF/C). 3 H-leucine incorporated was determined by liquid scintillation counting and compared with forskolin-treated PC12 cell cultures that were incubated in the absence of cycloheximide.

Results

cAMP has previously been shown to alter the expression of G_{α} proteins in a number of cell systems (e.g., Green, 1982; Longabaugh et al., 1989; Hadcock et al., 1990). The diterpene forskolin directly activates adenylate cyclase and is an efficient means by which to increase intracellular levels of cAMP maximally. In order to determine the effect of cAMP on G_{α} -protein expression in PC12 cells, immunoblot analysis was performed using a polyclonal antiserum specific for G_{α} . Incubation of

Figure 1. Regulation of G_{α} protein expression by forskolin. PC12 cells were incubated in the presence (+) or absence (-) of $10 \mu\text{M}$ forskolin. After 18 hr, cells were harvested. Total cellular protein (25 mg) was subjected to immunoblot analysis as described in Materials and Methods. Data are representative of at least four separate experiments. Standards (Bio-Rad) are shown on the left as 110 kDa, 47 kDa, 33 kDa, 24 kDa, and 16 kDa.

PC12 cells with forskolin ($10 \mu\text{M}$) increases the level of G_{α} protein (Fig. 1). This increase occurs within the first 1–2 d of exposure to forskolin.

A common mechanism by which cAMP increases protein expression is through regulation of gene transcription. Northern blot analysis of total RNA isolated from control or forskolin treated cells was performed to determine if forskolin treatment also caused an increase in the levels of G_{α} mRNA. Figure 2 demonstrates that cAMP selectively increases G_{α_1} mRNA expression; the levels of the other G_{α} -protein subtypes remain unaltered, as do levels of G_{α_2} and G_{β} mRNA. To quantitate the changes in mRNA expression, solution hybridization analysis was performed (Table 1). Forskolin treatment of PC12 cells increases G_{α_1} mRNA to a far greater extent (three- to four-

Figure 2. Specificity of increase of G_{α_1} mRNA expression as determined by Northern blot analysis. PC12 cells were incubated in the presence (+) or absence (-) of $10 \mu\text{M}$ forskolin for 12 hr. Total RNA was isolated and Northern blot analysis performed with ^{32}P -labeled nick-translated probes specific for each G-protein subtype. Data are representative of at least three separate experiments. Exposure times are for 24 hr.

fold) than that G_{α_2} , G_{α_3} , G_{α_4} , or G_{β} mRNA, thereby confirming the data obtained by Northern analysis (Fig. 2) and demonstrating that increased intracellular cAMP selectively regulates G_{α_1} mRNA expression.

Treatment of PC12 cells with NGF also induces neuronal differentiation and alters gene expression (Leonard et al., 1987). Therefore, it was important to determine whether NGF affected G-protein expression in PC12 cells. NGF (50 ng/ml) was added to the culture medium and G_{α_1} mRNA levels were measured by solution hybridization. In contrast to forskolin-treated cells, NGF-treated PC12 cells do not exhibit an increase in G_{α_1} mRNA levels (Table 1), even after 72 hr of exposure (data not shown).

Table 1. Selectivity of increase of G_{α_1} mRNA expression as measured by solution hybridization analysis

Treatment	G_{α_1}	G_{α_2}	G_{α_3}	G_{α_4}	β_1
$10 \mu\text{M}$ forskolin	384%	106%	123%	117%	118%
50 ng/ml NGF	99%	84%	76%	84%	94%

PC12 cells were cultured in the presence of forskolin (for 8 hr) or NGF (for 48 hr) at the indicated concentrations. Total RNA was isolated and solution hybridization analysis performed with ^{32}S -labeled RNA probes specific for each G-protein subtype. Data are the average of three experiments each performed in duplicate or triplicate. Data are presented as the percentage of control (PC12 cells cultured without additions). SD was always less than 12% of the mean, with the exception of G_{α_3} (123 ± 28).

Table 2. Regulation of G_{α_1} expression by increased intracellular cAMP

Treatment	G_{α_1} expression (% control)
1.0 mM dibutyryl-cAMP	$325 (\pm 62)$
1.0 mM 8-bromo-cAMP	$148 (\pm 19)$
$10 \mu\text{M}$ forskolin	$384 (\pm 59)$
$10 \mu\text{M}$ PIA	$358 (\pm 35)$
50 ng/ml NGF	$99 (\pm 0.5)$
$0.1 \mu\text{M}$ PMA	$90 (\pm 6.4)$

PC12 cells were cultured with the indicated concentrations of additions for 8 hr. Total RNA was isolated and solution hybridization analysis performed with ^{32}S -labeled RNA probes specific for G_{α_1} . Data are the average of two or three experiments, each performed in triplicate. Data are presented as the percentage of control G_{α_1} mRNA expression (PC12 cells without additions; $\pm$ SD). The level of G_{α_1} mRNA expression in control cells was $2.75 (\pm 0.20) \times 10^3$ molecules G_{α_1} mRNA/ μg total RNA.

Figure 3. Regulation of GppNHP-mediated inhibition of adenylate cyclase activity by increased G_{α_i} mRNA expression. PC12 cells were cultured in the absence (solid symbols) or in the presence (open symbols) of $10 \mu\text{M}$ PIA [(-)- N^6 (2-phenylisopropyl)adenosine] for 48 hr. Cells were harvested, membranes prepared, and adenylate cyclase activity measured as described in Materials and Methods. Data are presented as the percentage of control (no GppNHP) adenylate cyclase activity and are the average of four separate experiments ($\pm$ SEM), each performed in triplicate. In a typical experiment, the basal adenylate cyclase activity was $46.4 (\pm 2.7)$ and $48.1 (\pm 4.0)$ pmol cAMP/mg protein/5 min ($\pm$ SD), for PIA-treated and control cultures, respectively.

Indeed, there was comparatively little effect on overall G-protein expression of any of the other G-protein mRNAs examined in PC12 cells cultured in the presence of NGF (Table 1).

To determine if the effects of forskolin on G_{α_i} mRNA levels were directly related to increases in cAMP, other methods of increasing intracellular cAMP levels were used. Addition of 1.0 mM dibutyryl-cAMP or 1.0 mM 8-bromo-cAMP to cultured PC12 cells also increases G_{α_i} mRNA levels (Table 2), although 8-bromo-cAMP is less effective.

In addition to forskolin and cAMP analogs, hormone receptor-mediated increases in cAMP levels also increase G_{α_i} mRNA expression. The addition of the adenosine analog PIA [(-)- N^6 (2-phenylisopropyl)adenosine] to PC12 cells has been shown pre-

viously to stimulate the adenosine receptor to rapidly increase cAMP levels (Gatti et al., 1988). Activation of adenylate cyclase by this mechanism is as effective as forskolin at increasing levels of G_{α_i} mRNA (Table 2).

In order to determine if the increase in G_{α_i} resulted in an alteration of G-protein function in PC12 cells, adenylate cyclase activity was measured. Seamon and Daly (1982) demonstrated that at relatively low concentrations and over short incubation periods the nonhydrolyzable GTP analog GppNHP will activate G_i to cause inhibition of adenylate cyclase activity in a receptor-independent fashion. Higher concentrations of GppNHP cause an increase in adenylate cyclase activity by activating G_s (Seamon and Daly, 1982; Subers and Nathanson, 1989). As shown in Figure 3, PC12 cells cultured in the presence of PIA (and therefore expressing higher levels of G_{α_i}) are more sensitive to inhibition of adenylate cyclase by GppNHP. Concentration-response curves for GppNHP demonstrate that the maximal inhibition of adenylate cyclase activity is slightly greater for PIA-treated cells as compared to control cells ($18\% \pm 6$ and $6\% \pm 3$, respectively; percent control inhibition $\pm$ SEM). PIA-treated PC12 cells expressing higher levels of inhibitory G_{α_i} are also less sensitive to the stimulation of adenylate cyclase activity that occurs at high concentrations of GppNHP. The maximal stimulation of adenylate cyclase activity by high concentrations of GppNHP is significantly less for PIA-treated cultures as compared to control ($110\% \pm 8$ and $181\% \pm 8$, respectively; percent control stimulation $\pm$ SEM; Fig. 3).

Solution hybridization analysis was performed to quantitate changes in mRNA levels of G_{α_i} over time. cAMP levels in PC12 cells were stimulated by treatment with $10 \mu\text{M}$ forskolin, and levels of G_{α_i} mRNA measured and compared to control (nontreated) cells. As demonstrated in Figure 4, G_{α_i} mRNA levels in cAMP-induced PC12 cells rapidly increase three- to fourfold as compared with control cells; new steady state levels of G_{α_i} mRNA are reached within 6–8 hr of treatment (Fig. 4). Higher concentrations of forskolin do not increase the magnitude of induction or alter the kinetics of induction; concentrations of forskolin ranging from $1.0 \mu\text{M}$ to $100 \mu\text{M}$ all increase G_{α_i} mRNA expression to the same extent (data not shown). The increased expression of G_{α_i} mRNA persists for at least 72 hr (data not shown).

Figure 4. Time course of regulation of G_{α_i} mRNA expression by forskolin or by stimulation of the adenosine receptor. PC12 cells were incubated in the presence of $10 \mu\text{M}$ PIA [(-)- N^6 (2-phenylisopropyl)adenosine] (solid squares), $10 \mu\text{M}$ forskolin (open squares), or $0.1 \mu\text{M}$ PMA (solid diamonds) for the indicated times. Total mRNA was isolated and solution hybridization analysis was performed with ^{35}S -labeled RNA probes specific for G_{α_i} . Data are the average of three experiments ($\pm$ SD), each performed in triplicate. Data are presented as the percentage of control (PC12 cells cultured without additions). The level of G_{α_i} mRNA expression in control cells was $2.75 (\pm 0.20) \times 10^5$ molecules G_{α_i} mRNA/ μg total RNA.

Figure 5. Effects of depolarization on G_{α_i} expression in PC12 cells. PC12 cells were incubated in the presence of 44 mM KCl $\pm$ 10 μ M forskolin (F) for 8 hr. Total RNA was isolated and solution hybridization analysis was performed with 35 S-labeled RNA probes specific for G_{α_i} . Data are the average of two experiments, each performed in triplicate. Data are presented as the percentage of control (PC12 cells without additions; $\pm$ SD). The level of G_{α_i} mRNA expression in control cells was $3.43 (\pm 0.27) \times 10^5$ molecules G_{α_i} mRNA/ μ g total RNA.

The time course of G_{α_i} mRNA expression stimulated by activation of the adenosine receptor with PIA was also determined. As demonstrated in Figure 4, the kinetics of induction of G_{α_i} mRNA levels by PIA are indistinguishable from those induced by forskolin. The level of G_{α_i} mRNA begins to increase within 1 hr, becomes maximal by 6 hr, and persists for days.

In addition to regulation of gene expression by cAMP, presumably through the cAMP-dependent protein kinase pathway, at least two other protein kinase cascades are involved in the regulation of gene expression, protein kinase C and calcium/calmodulin kinase. To measure the effect of the stimulation of protein kinase C on G_{α_i} mRNA expression, the phorbol ester phorbol 12-myristate 13-acetate (PMA) was added to cultures of PC12 cells and solution hybridization analysis was performed. Even at high concentrations of PMA (0.1 μ M), there was no effect on G_{α_i} mRNA expression (Fig. 4, Table 2). Similarly, depolarization of cells by KCl to open voltage-gated calcium channels had no effect (Fig. 5), demonstrating that increasing intracellular calcium levels was ineffective at increasing G_{α_i} mRNA levels in PC12 cells. Membrane depolarization has been shown previously to act synergistically with forskolin to increase expression of the *c-fos* gene in PC12 cells due to increased levels of intracellular calcium (Sheng et al., 1990). Even when depolarizing concentrations of KCl and forskolin are added simultaneously to PC12 cultures, there is little additional effect of membrane depolarization of G_{α_i} mRNA expression (Fig. 5). Therefore, G_{α_i} appears to be induced specifically by cAMP.

Models of the regulation of gene expression due to activation of protein kinases suggest a mechanism that relies on phosphorylation of previously synthesized nuclear proteins. These models predict that *de novo* protein synthesis is not required for the increase in G_{α_i} mRNA. In support of this prediction, concentrations of cycloheximide that inhibit 97% of total protein synthesis in PC12 cells do not inhibit the forskolin-stimulated increase in G_{α_i} mRNA (Fig. 6).

Increases in G_{α_i} mRNA levels can be the result of either increased mRNA synthesis or decreased mRNA degradation. Because cAMP-induced changes in mRNA levels are most often

Figure 6. Effects of cycloheximide on forskolin-stimulated G_{α_i} mRNA expression in PC12 cells. PC12 cells were incubated in the presence of cycloheximide (10 mg/ml) $\pm$ forskolin (10 μ M) for 8 hr. Total RNA was isolated and solution hybridization analysis performed with 35 S-labeled RNA probes specific for G_{α_i} . Data are the average of two experiments, each performed in triplicate. Data are presented as the percentage of control (PC12 cells incubated without additions; $\pm$ SD). Ninety-seven percent of total PC12 cell protein synthesis was inhibited by 10 mg/ml cycloheximide. The level of G_{α_i} mRNA expression in control cells was $3.43 (\pm 0.27) \times 10^5$ molecules G_{α_i} mRNA/ μ g total RNA.

achieved through increased synthesis and G_{α_i} mRNA levels rapidly reach new steady state levels (Fig. 4), one would predict that increased synthesis is the mechanism by which cAMP alters G_{α_i} expression. As shown in Figure 7, mRNA synthesis is required for the cAMP-induced G_{α_i} mRNA levels measured. When the potent inhibitor of mRNA synthesis actinomycin D (16 μ M) is added simultaneously with forskolin to PC12 cell cultures, the increased levels of G_{α_i} mRNA are abolished (Fig. 7).

To determine if the G_{α_i} mRNA in forskolin-treated cells has an altered half-life, rates of G_{α_i} mRNA degradation were measured and compared between forskolin-treated and control cul-

Figure 7. Inhibition of forskolin-induced stimulation of G_{α_i} mRNA expression by actinomycin D. PC12 cells were cultured in the presence of 16 μ M actinomycin D (A) $\pm$ 10 μ M forskolin (Forsk) for 8 hr. This concentration of actinomycin D has been shown previously to inhibit RNA synthesis in PC12 cells (Edgar and Thoenen, 1978). After 8 hr, cells were harvested and total RNA was isolated. Solution by hybridization analysis was performed with 35 S-labeled RNA probes specific for G_{α_i} . Data are the average of three experiments, each performed in triplicate. Data are presented as the percentage of control (PC12 cells without additions; $\pm$ SD). The level of G_{α_i} mRNA expression in control cells was $5.84 (\pm 0.59) \times 10^6$ molecules G_{α_i} mRNA/ μ g total RNA.

Figure 8. Degradation rates of G_{α_1} mRNA. PC12 cells were cultured in the presence (solid symbols) or absence (open symbols) of forskolin ($10 \mu\text{M}$) for 12 hr. Actinomycin D ($16 \mu\text{M}$) was then added to treated and control cultures for the indicated amount of time. Cells were subsequently harvested and total RNA isolated. Solution hybridization analysis was performed with ^{35}S -labeled RNA probes specific for G_{α_1} . Each time point is the average of three to five experiments, each performed in triplicate. Data are presented as the percentage of control (PC12 cells without actinomycin D; $\pm$ SD). The level of G_{α_1} mRNA expression was $1.66 (\pm 0.08) \times 10^7$ and $6.75 (\pm 0.53) \times 10^6$ molecules G_{α_1} mRNA/ μg total RNA in forskolin treated and control cultures, respectively.

tures. After 12 hr of forskolin treatment to permit maximal induction of G_{α_1} mRNA, actinomycin D was added to treated and control cultures of PC12 cells. Cells were harvested, total RNA isolated, and samples analyzed by solution hybridization at the indicated times. As demonstrated in Figure 8, the rates of degradation of G_{α_1} mRNA in forskolin treated and control cultures are indistinguishable. Because of the toxic effects of actinomycin D treatment that appear after 8 hr of drug treatment, the relatively long apparent half-life of approximately 40–45 hr may not be an accurate measure of the true half-life of the G_{α_1} mRNA. These data do, however, suggest that increased intracellular cAMP raises levels of G_{α_1} mRNA by increasing the rate of gene transcription.

Discussion

Regulation of G-protein expression in neuronal systems has been investigated only recently (Mullaney et al., 1988; Asano et al., 1989; Mullaney and Mulligan, 1989; Tjaden et al., 1990). Here we demonstrate the selective upregulation of G_{α_1} gene expression by cAMP in PC12 cells. When these cells are cultured in the presence of agents that stimulate intracellular cAMP levels, G_{α_1} mRNA levels are increased three- to fourfold in a relatively short time period. This increase is selective for G_{α_1} and is induced by cAMP-dependent protein kinase activation, but not by activation of other protein kinase pathways. Direct activation of adenylate cyclase by forskolin, use of permeable cAMP analogs, or activation of cell surface receptors that stimulate cyclase activity through G_s are all effective in upregulating G_{α_1} mRNA expression.

Increased levels of intracellular cAMP stimulate G_{α_1} protein expression in PC12 cells. Immunoblot analysis using polyclonal antisera to G_{α_1} protein demonstrates a substantial increase in G_{α_1} protein (Fig. 1). Because G_{α_1} mRNA levels, but not the levels of other G-protein mRNAs (Fig. 2, Table 1), are increased by forskolin treatment, the increase in G_{α_1} protein expression is most likely a reflection of increased G_{α_1} protein levels relative

to G_{α_2} and G_{α_3} . This is consistent with the results of Asano et al. (1989), who found relatively little change in the levels of G_{α_2} protein when PC12 cells were exposed to forskolin.

The ability of guanine nucleotides to inhibit adenylate cyclase activity in membrane homogenates was measured to determine if the increase in G_{α_1} expression was accompanied by increased functional activity. It has been demonstrated that overnight treatment of cells in culture with forskolin can result in an increase in basal adenylate cyclase activity, which was suggested to be due to an inability to remove the lipophilic forskolin completely from membranes prior to assay (Haddock et al., 1990). We therefore used the adenosine analog PIA instead of forskolin to stimulate intracellular cAMP levels to increase expression of G_{α_1} . The basal activity of PIA-treated PC12 cells was not significantly different from nontreated, control cells ($101\% \pm 2.2$; percent control basal activity $\pm$ SEM, $n = 4$). However, the cAMP-induced increases in G_{α_1} protein levels in PC12 cells result in significant changes in the sensitivity of adenylate cyclase to G_i -mediated inhibition of enzyme activity (Fig. 3). Thus, the increase in G_{α_1} expression results in altered G-protein function.

NGF treatment of PC12 cells induces extensive neurite outgrowth (Greene and Tischler, 1976), indicative of neuronal differentiation. The mechanism by which NGF alters gene expression and neuronal differentiation remains unclear (for review, see Halegoua et al., 1991), as does the role of cAMP in NGF signaling. cAMP as a second messenger for NGF action has been proposed by many groups (e.g., Schubert and Whitlock, 1977; Schubert et al., 1978), although others have failed to detect rises in cAMP following NGF treatment (Frazier et al., 1973; Hatanaka et al., 1978; Lakshmanan et al., 1978; for review, see Halegoua et al., 1991). Recently, Kalman et al. (1990) demonstrated that both NGF and cAMP increase the expression of voltage-gated sodium channels in PC12 cells. In contrast, our data indicate that G_{α_1} mRNA expression is regulated by cAMP, but not by NGF (Table 1). Therefore, the increase in G_{α_1} mRNA expression does not appear to be a secondary effect of the morphological differentiation that occurs following cAMP treatment.

The results of the actinomycin D experiments suggest that cAMP increases G_{α_1} mRNA levels by increasing the rate of gene transcription. While transcriptional nuclear runoff assays are required to confirm this conclusion, these experiments are complicated by the very long half-life for G_{α_1} mRNA and the concomitant very low rate of RNA synthesis. The increase in G_{α_1} mRNA by cAMP suggests the involvement of a transcriptional element regulated by cAMP. Recently, a number of *cis*-acting elements have been described that are responsible for the regulation of gene expression by cAMP. The cAMP regulatory element (CRE) was first identified as a regulatory region of the rat PEPCK gene (phosphoenolpyruvate carboxykinase). Subsequently, this 8 base pair sequence [T(G/T)ACGTCA] was demonstrated to act as a functional CRE in a number of other genes (for review, see Roesler et al., 1988). The second cAMP-responsive element described was the activator protein 2 (AP-2) binding site (consensus sequence CCCCAGGC). Like CRE, this *cis*-acting element can act as a basal enhancer of gene transcription and as an inducer of gene transcription when stimulated by cAMP. In addition, AP-2 increases gene expression in the presence of phorbol esters (Imagawa et al., 1987). Furthermore, there are a number of additional elements that mediate transcriptional regulation by cAMP, including sites that bind the

factor PEA-2 and NF-1 related proteins (Albanese et al., 1991; Bowlus et al., 1991; Chu et al., 1991).

The involvement of potential cAMP-induced regulatory elements in G-protein expression has only recently been examined. Transcription of the $G_{i\alpha_2}$ gene has been shown to be regulated by cAMP in S49 mouse lymphoma cells (Hadcock et al., 1990), and the $G_{i\alpha_2}$ gene contains a potential AP-2 binding site (Weinstein et al., 1988, 1990), but not a CRE. Long-term exposure of PC12 cells to NGF has been shown to alter protein levels of G_{α} (but not affect amounts of $G_{i\alpha_2}$ protein; Asano et al., 1989); other G-protein subtypes and mRNA levels had not been studied previously. The isolation and characterization of the genomic sequence of rat $G_{i\alpha_1}$ have not been published, although both the bovine and human $G_{i\alpha_1}$ genes contain sequences similar (but not identical) to a CRE (Itoh et al., 1988). Because membrane depolarization of PC12 cells has been shown to mimic the effects of cAMP on gene induction by calmodulin kinase activation of CRE binding protein (CREB) and because PMA should cause activation of AP-2, the lack of effect of membrane depolarization (Fig. 5) or PMA treatment (Fig. 4, Table 2) on $G_{i\alpha_1}$ expression suggests that the effects of cAMP are not mediated by CREB at a CRE or by AP-2 at its response element.

Regulation of a neural-specific G-protein, $G_{i\alpha_1}$, presumably by protein kinase A, but not by Ca^{2+} or protein kinase C, presents an intriguing model for the molecular mechanism of regulation of G-protein expression by neurotransmitters and hormones. The specific upregulation of $G_{i\alpha_1}$ by increased mRNA expression will occur when surface receptors that couple to G_i are stimulated, but not by other receptor or ion channel subtypes.

Furthermore, this mechanism represents an increasingly complex theme in signal transduction pathways—the cross-regulation between related families of proteins. Recently, Morris et al. (1991) demonstrated the upregulation of α_1 -adrenergic receptors by activation of β_2 -adrenergic receptors. Cross-regulation of G-proteins has also been reported. Hadcock et al. (1990) reported the increased expression of $G_{i\alpha_2}$ by stimulation of the $G_{s\alpha}$ pathway in S49 mouse lymphoma cells. The regulation of $G_{i\alpha_2}$ by cAMP may be cell specific, since we are unable to detect changes in $G_{i\alpha_2}$ mRNA levels in PC12 cells treated with forskolin or cAMP analogs (Fig. 2, Table 1). The regulation of the expression and function of $G_{i\alpha_1}$ by cAMP may represent a selective mechanism for the regulation of a neural-specific G-protein in response to neurotransmitters and synaptic activity.

References

- Albanese C, Kay TWH, Troccoli NM, Jameson JL (1991) Novel cyclic adenosine 3',5'-monophosphate response element in the human chorionic gonadotropin β -subunit gene. *Mol Endocrinol* 5:693–702.
- Asano T, Morishita R, Sano M, Kato K (1989) The GTP-binding proteins G_o and G_{i2} of neural cloned cells and their changes during differentiation. *J Neurochem* 53:1195–1198.
- Bowlus CL, McQuillan JJ, Dean DC (1991) Characterization of three different elements in the 5'-flanking region of the fibronectin gene which may mediate a transcriptional response to cAMP. *J Biol Chem* 266:1122–1127.
- Casey PJ, Gilman AG (1988) G-Protein involvement in receptor-effector coupling. *J Biol Chem* 263:2577–2580.
- Chu H-M, Fischer WH, Osborne TF, Comb MJ (1991) NF-1 proteins from brain interact with the proenkephalin cAMP inducible enhancer. *Nucleic Acids Res* 19:2721–2728.
- Cockcroft S (1987) Polyphosphoinositide phosphodiesterase regulation by a novel guanine-nucleotide binding protein-Gp. *Trends Biochem Sci* 12:75–78.
- Edgar DH, Thoenen H (1978) Selective enzyme induction in a nerve growth factor-responsive pheochromocytoma cell line (PC12). *Brain Res* 154:186–190.
- Fong HKW, Hurley JB, Hopkins RS, Miake-Lye R, Johnson MS, Doolittle RF, Simon MI (1986) Repetitive segmental structure of the transducin β -subunit: homology with the CDC4 gene and identification of related mRNAs. *Proc Natl Acad Sci USA* 83:2162–2166.
- Frazier WA, Olendorf CE, Boyd LF, Aloe L, Johnson EM, Ferrendelli JA, Bradshaw RA (1973) Mechanism of action of NGF and cAMP on neurite outgrowth in embryonic chick sensory ganglia: demonstration of independent pathways of stimulation. *Proc Natl Acad Sci USA* 70:2448–2452.
- Gatti G, Madaddu L, Pandiella A, Pozzan T, Meldolesi J (1988) Second messenger generation in PC12 cells: interactions between cAMP and Ca^{2+} signals. *Biochem J* 255:753–760.
- Gilman AG (1987) G-proteins: transducers of receptor-generated systems. *Annu Rev Biochem* 56:615–649.
- Green A (1987) Adenosine receptor down regulation and insulin resistance following prolonged incubation of adipocytes with an A1 adenosine receptor agonist. *J Biol Chem* 262:577–579.
- Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which responds to nerve growth factor. *Proc Natl Acad Sci USA* 73:2424–2428.
- Hadcock JR, Ros M, Watkins DC, Malbon CC (1990) Cross-regulation between G-protein mediated pathways. Stimulation of adenylyl cyclase increases expression of the inhibitory G-protein $G_i\alpha_2$. *J Biol Chem* 265:14784–14790.
- Hadcock JR, Port JD, Malbon CC (1991) Cross-regulation between G-protein-mediated pathways. Activation of the inhibitory pathway of adenylyl cyclase increases the expression of beta 2-adrenergic receptors. *J Biol Chem* 266:11915–11922.
- Halegoua S, Armstrong RC, Kremer NE (1991) Dissecting the mode of action of a neuronal growth factor. In: *Current topics in microbiology and immunology*, Vol. 165, pp 119–170. New York: Springer.
- Halvorsen SW, Nathanson NM (1984) Ontogenesis of physiological responsiveness and guanine nucleotide sensitivity of cardiac muscarinic receptors during chick embryonic development. *Biochemistry* 23:5813–5821.
- Hatanaka H, Otten U, Thoenen H (1978) NGF-mediated selective induction of ornithine decarboxylase in rat pheochromocytoma: a cAMP-independent mechanism. *FEBS Lett* 92:313–316.
- Imagawa M, Chiu R, Karin M (1987) Transcriptional protein AP-2 mediates induction by 2 different signal transduction pathways—PKC and cAMP. *Cell* 51:251–260.
- Itoh H, Kozasa T, Nagata S, Nakamura S, Katada T, Ui M, Iwai S, Ohtsuka E, Kawasaki H, Suzuki K, Kaziro Y (1986) Molecular cloning and sequence determination of cDNAs for alpha subunits of the guanine nucleotide binding proteins-Gs, proteins-Gi, and proteins Go from rat brain. *Proc Natl Acad Sci USA* 83:3776–3780.
- Itoh H, Toyama R, Kozasa T, Tsukamoto T, Matsuoka M, Kaziro Y (1988) Presence of three distinct molecular species of Gi protein α subunits. *J Biol Chem* 263:6656–6664.
- Jelsema CL, Axelrod J (1987) Stimulation of phospholipase-A2 activity in rod outer segments of bovine retina and its modulation by GTP-binding proteins. *Proc Natl Acad Sci USA* 84:3623–3627.
- Jones DT, Reed RR (1987) Molecular cloning of 5 GTP-binding protein cDNA species from rat olfactory epithelium. *J Biol Chem* 262:14241–14249.
- Kalman D, Wong B, Horvai AE, Cline MJ, O'Laugh PH (1990) Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. *Neuron* 4:355–366.
- Kislauskis E, Dobner PR (1990) Mutually dependent response elements in the *cis*-regulatory region of the neurotensin/neuromedin N gene integrate environmental stimuli in PC12 cells. *Neuron* 4:783–795.
- Lakshmanan J (1978) Is there a second messenger for NGF-induced phosphatidylinositol turnover in rat superior cervical ganglia? *Brain Res* 157:173–177.
- Leonard DGB, Ziff EB, Greene LA (1987) Identification and characterization of mRNAs regulated by NGF in PC12 cells. *Mol Cell Biol* 7:3156–3167.
- Longabaugh JP, Didsbury J, Spiegel AM, Stiles GL (1989) Modification of the rat adipocyte A1 adenosine receptor adenylate cyclase system during chronic exposure to an A1 adenosine receptor agonist—

- alterations in the quantity of Gs- α and Gi- α are not associated with changes in their mRNAs. *Mol Pharmacol* 36:681–688.
- Longo FM, Woo JE, Mobley WC (1980) Purification of nerve growth factor. In: *Nerve growth factors* (Rush RA, ed), pp 3–30. New York: Wiley.
- Luetje CW, Gierschik P, Milligan G, Unson C, Spiegel A, Nathanson NM (1987) Tissue specific regulation of GTP-binding proteins and muscarinic acetylcholine receptor levels during cardiac development. *Biochemistry* 26:4876–4884.
- Luetje CW, Tietje KM, Christian JL, Nathanson NM (1990) Differential tissue expression and developmental regulation of guanine nucleotide binding regulatory proteins and their messenger RNAs in rat heart. *J Biol Chem* 263:13357–13369.
- McKnight GS, Uhler MD, Clegg CH, Correll LA, Cadd GG (1988) Application of molecular genetics techniques to the cAMP-dependent protein kinase system. *Methods Enzymol* 159:299–311.
- Melton DA, Krieg PA, Rebagliatti MR, Zinn K, Green MR (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res* 12:7035–7056.
- Morris GM, Hadcock JR, Malbon CC (1991) Cross-regulation between G-protein coupled receptors. *J Biol Chem* 266:2233–2238.
- Mullaney I, Mulligan G (1989) Elevated levels of the guanine-nucleotide-binding protein, Go, are associated with the differentiation of neuroblastoma $\times$ glioma hybrid cells. *FEBS Lett* 244:113–118.
- Mullaney I, Magee AI, Unson CG, Mulligan G (1988) Differential regulation of the guanine-nucleotide-binding proteins Gi and Go in neuroblastoma $\times$ glioma hybrid cells in response to dibutyryl cyclic AMP. *Biochem J* 256:649–656.
- Peppel K, Baglioni C (1990) A simple and fast method to extract RNA from tissue culture cells. *Biotechniques* 9:711–713.
- Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B (1985) GTP-binding proteins coupled muscarinic receptor to a K channel. *Nature* 317:536–538.
- Reithmann C, Gierschik P, Muller U, Werdan K, Jakobs KH (1990) *Pseudomonas* endotoxin A prevents β -adrenoceptor-induced up-regulation of Gi protein α subunits and adenylyl cyclase desensitization in rat heart muscle cells. *Mol Pharmacol* 37:631–638.
- Roesler WJ, Vandenbark GR, Hanson RW (1988) cAMP and the induction of eukaryotic gene transcription. *J Biol Chem* 263:9063–9066.
- Ros M, Watkins DC, Rapiejko PJ, Malbon CC (1989) Glucocorticoids modulate messenger-RNA levels for G-protein β -subunits. *Biochem J* 260:271–275.
- Salomon Y, Londos C, Dodkell M (1974) A highly sensitive adenylyl cyclase assay. *Anal Biochem* 58:541–548.
- Seamon KB, Daly JW (1982) Guanosine 5'-(β , γ -imido)triphosphate inhibition of forskolin-activated adenylyl cyclase is mediated by the putative guanine nucleotide regulatory protein. *J Biol Chem* 257:11591–11596.
- Sheng M, McFadden G, Greenberg ME (1990) Membrane depolarization and calcium induce *c-fos* transcription via phosphorylation of transcription factor CREB. *Cell* 4:571–582.
- Spiegel AM (1987) Signal transduction by guanine-nucleotide binding proteins. *Mol Cell Endocrinol* 49:1–16.
- Stryer L (1986) Cyclic-GMP cascade of vision. *Annu Rev Neurosci* 9:87–119.
- Subers EM, Nathanson MM (1989) Regulation by high density lipoproteins of muscarinic acetylcholine receptor function in chick heart cells cultured in defined medium. *J Biol Chem* 264:19685–19693.
- Tjaden G, Aguanno A, Kumar R, Benincasa D, Gubits RM, Yu H, Dolan KP (1990) Density-dependent NGF regulation of Gs α in PC12 cells. *Mol Cell Biol* 10:3277–3279.
- Uhler MD, Carmichael DF, Lee DC, Chivria JC, Krebs EG, McKnight GS (1986) Expression of cDNAs for 2 isoforms of the catalytic subunit of cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 83:1300–1304.
- Weinstein LS, Spiegel AM, Carter AD (1988) Cloning and characterization of the human gene for the α -subunit of Gi2, a GTP-binding signal transduction protein. *FEBS Lett* 232:333–340.
- Weinstein LS, Kats I, Spiegel AM, Carter AD (1990) Characterization of the promoter of the human Gi2 α -subunit gene. *Mol Endocrinol* 4:958–964.
- Yatani A, Codina J, Brown AM, Birnbaumer L (1987) Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein, Gk. *Science* 235:207–211.