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Transcriptional Regulation of Secretogranin II and Chromogranin B by Cyclic AMP in a Rat Pheochromocytoma Cell Line

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SUMMARY

When PC-12 cells were treated with 10 μM forskolin, the expression of two members of the granin family, secretogranin II (SgII) and chromogranin B (CgB), were differentially regulated. SgII mRNA levels declined progressively after forskolin treatment to reach a level of 22 ± 1% of control after 48 hr, whereas CgB mRNA levels increased more rapidly, reaching a maximum of 3-fold above control after 24 hr. The dependence of these changes on an increase in cellular cAMP levels, activation of cAMP-dependent protein kinase (PKA), protein synthesis, and changes in the rate of transcription was investigated. The effects of forskolin on SgII and CgB mRNAs were reproduced by 1 mM 8-bromo-cAMP but not by 10 μM 1,9-dideoxyforskolin, an inactive analog of forskolin. The actions of forskolin on SgII and CgB mRNAs were blocked by treatment with 60 μM H-89, a selective PKA inhibitor, and were blunted in PKA-deficient PC-12 cell clones. To examine whether forskolin action was dependent on ongoing protein synthesis, PC-12 cells were treated with 1 μg/ml cycloheximide before the addition of forskolin. The reduction in SgII mRNA levels by forskolin was not evident in PC-12 cells treated with cycloheximide. Rather, in the presence of cycloheximide, forskolin stimulated SgII mRNA levels 3.6 ± 0.7-fold above control. The induction of CgB mRNA by forskolin was not affected by cycloheximide treatment. The superinduction of SgII mRNA by cycloheximide and forskolin was related to the extent of protein synthesis inhibition, was observed in cells treated with forskolin and other protein synthesis inhibitors, and was blunted in PKA-deficient PC-12 cells, suggesting that this effect was dependent on inhibition of protein synthesis and activation of PKA. To determine whether changes in SgII and CgB mRNA levels resulted from changes in the rate of transcription, nuclear run-on assays were performed in nuclei isolated from PC-12 cells that had been treated for 2 hr with cycloheximide, forskolin, or the two combined. Transcription of the SgII gene was not significantly affected by treatment with either forskolin or cycloheximide alone but was increased 12.9 ± 1.0-fold above control in nuclei from cells treated with cycloheximide and forskolin together. Forskolin caused a 3.8 ± 0.8-fold induction of CgB transcription. The half-lives of SgII and CgB mRNAs are long (33 ± 8 and 19 ± 6 hr, respectively), suggesting that the stimulatory effects of forskolin on CgB mRNA levels and on SgII mRNA levels in the absence of protein synthesis are mediated predominantly by an increase in the transcription of these genes. These results also indicate that enhanced transcription of the SgII gene is repressed, such that a stimulatory effect of cAMP is not revealed unless protein synthesis is inhibited.

The granins are a family of immunologically distinct, secretory proteins. The three major components of this family are CgA, CgB, and SgII (1). The granin family may also include several other proteins, termed secretogranins III, IV, and V, which share many of the properties of the granins (1). Despite extensive investigation of the tissue distribution and physicochemical properties of the granins (2, 3), our understanding of the function and regulated synthesis of these proteins is incomplete. Functional analysis of the granins has focused on both extracellular and intracellular roles for these proteins. For example, the granins may serve as precursors of biologically active peptides such as pancreastatin and chromostatin, which have autocrine inhibitory activity on hormone secretion (4, 5). On the other hand, the propensity of the granins to aggregate in a high-calcium, low-pH environment has suggested a potential role in the concentration of granule contents and formation of dense core granules (6, 7).

The selective expression of the granins in endocrine and neuronal cells has led to the appreciation that these proteins can act as markers for neuroendocrine differentiation (2, 3). The molecular basis for tissue-specific expression of the granins

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ABBREVIATIONS: CgA, chromogranin A; CgB, chromogranin B; SgII, secretogranin II; PMA, phorbol-12-myristate-13-acetate; DMEM, Dulbecco's modified Eagle's medium; TSH, thyrotropin; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKA, cAMP-dependent protein kinase.

in neuroendocrine cells has not been investigated. In fact, the factors that normally regulate the granins are only beginning to be understood. These studies have been aided by the availability of cDNA probes for each of the major members of the granin family from several species, including rat (8–10). Furthermore, regulatory elements within the promoter regions of the mouse CgA (11), CgB (12), and SgII (13) genes have been tentatively identified and have provided keys to potential regulation of their expression. It is now appreciated that, in spite of the grouping of the granins as a family based on similar physicochemical characteristics and some structural homology, the granins may be differentially regulated. For example, the level of SgII mRNA is increased by histamine, phorbol esters, and elevations of cellular cAMP in bovine chromaffin cells, whereas the level of CgA mRNA is unaffected by these treatments (14, 15). On the other hand, glucocorticoids increase CgA but not SgII synthesis in the rat pituitary gland (16).

Our studies have focused on the rat PC-12 pheochromocytoma cell line, in which we also observed a differential regulation of granin expression. We found that forskolin, an activator of adenylate cyclase, increased the level of CgB mRNA but decreased the level of SgII mRNA (17). We have now furthered these findings by investigating 1) whether the different actions of forskolin on SgII and CgB mRNA levels are both mediated by an increase in cellular cAMP and subsequent activation of PKA, 2) whether the effects of forskolin on SgII and CgB mRNA levels are dependent on ongoing protein synthesis, and 3) whether changes in the rate of transcription of the SgII and CgB genes contribute to the changes in the steady state levels of their mRNAs. The results of these studies have revealed a complex regulation of SgII mRNA by cAMP in PC-12 cells.

Experimental Procedures

Materials. Culture medium was obtained from GIBCO/Bethesda Research Laboratories (Grand Island, NY). Horse serum and fetal calf serum were purchased from Central Biomedia (Irwin, MO) and Hyclone Laboratories (Logan, UT), respectively. Forskolin, 8-bromo-cAMP, antibiotics, cycloheximide, emetine, puromycin, PMA, and reagents for RNA isolation and hybridization were obtained from Sigma Chemical Co. (St. Louis, MO). Pactamycin was a gift from the Upjohn Company (Kalamazoo, MI). H-89 was purchased from Seikagaku Corporation (Rockville, MD). cDNAs for rat CgB (9), SgII (10), and cyclophilin (18) were prepared as hybridization probes by random priming as described previously (17).

Cell cultures. Wild-type PC-12 cells and the PKA-deficient PC-12 cell variant A126–1B2 cells (19) were maintained in culture in DMEM supplemented with 12.5% horse serum, 2.5% fetal calf serum, 50 units/ml penicillin G, and 0.05 mg/ml streptomycin, at 37° in a humidified atmosphere of 5% CO_2/95% air. PKA-deficient PC-12 cell variants AB.11 and 123.7 (20) were grown in the aforementioned medium with the addition of 100 μg/ml G-418 (GIBCO). All cells were subcultured into 100-mm dishes at a density of 500,000 cells/dish in the aforementioned medium and were grown for 2–3 days before treatment. The protein kinase activity of the PC-12 cell clones was measured in the presence of a maximally stimulating concentration of cAMP (10 μM) by a modification of the method of Roskoski (21), using 0.14 mM Kemptide (Pensinsula Laboratories, Belmont, CA) as the substrate, as described previously (22).

Northern blot analysis. Total RNA was isolated from wild-type and PKA-deficient PC-12 cells by the method of Chirgwin *et al.* (23). RNA was size-fractionated on 1.4% agarose/2.2 M formaldehyde gels, transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) by capillary blotting in 10× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), and fixed by baking of the Nytran at 80° for 2 hr. For slot blots, RNA was isolated by the method of Gough (24), serially diluted (highest concentration, 6 μg), blotted onto Nytran membranes, and hybridized as described previously (17). Bands were visualized by autoradiography. Hybridizing band intensities of the slot blots were determined by scanning densitometric analysis, using an LKB Ultroscan XL laser densitometer and Gel Scan XL (version 1.2) software. Intensities of granin mRNAs were standardized to the level of cyclophilin mRNA, a ubiquitously expressed mRNA that codes for a cyclosporin-binding protein (25). None of the treatments significantly altered the steady state levels of cyclophilin mRNA.

Nuclear run-on transcription assays. The nuclear run-on assays were performed by the method of McKnight and Palmiter (26). Certain modifications were necessary to optimize our reaction conditions, as described below. Nuclei were isolated from PC-12 cells by lysis of cells with Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.4). The nuclei were washed once in Nonidet P-40 lysis buffer and resuspended at 1.5–2.5 × 10^7 nuclei/205 μl in a solution of 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 50 mM Tris·HCl, pH 8.3. Each reaction consisted of 205 μl of nuclei in a 300-μl reaction volume containing 0.25 mM concentrations each of ATP, GTP, and CTP (Pharmacia LKB Biotechnology, Piscataway, NJ), 300 μCi of [^{32}P]UTP (3000 Ci/mmol; New England Nuclear, Boston, MA), 1.5 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.15 M KCl, 200 units of RNasin (Promega Corporation, Madison, WI), and 5 mM Tris·HCl, pH 8.0. The reaction mixture was incubated for 45 min at 30°. Twenty units of RQ1 DNase (Promega) were added and the reaction was incubated for an additional 10 min at 30°. The mixture was adjusted to 1× SET (1% SDS, 5 mM EDTA, 10 mM Tris·HCl, pH 7.4) by the addition of 5× SET buffer, and proteinase K (GIBCO-BRL, Gaithersburg, MD) was added to a final concentration of 250 μg/ml. After incubation at 50° for 30 min, the RNA was extracted by addition of an equal volume of phenol/chloroform/isoamyl alcohol (50:49:1), followed by precipitation with an equal volume of isopropanol in the presence of 2.3 M ammonium acetate and 50 μg of tRNA (Sigma) at −70° for 20 min. The RNA was centrifuged at 12,000 × *g* for 20 min at 4°. The pellets were dried and then resuspended in 100 μl of TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.4), followed by centrifugation through Quick Spin G-50 Sephadex columns (Boehringer Mannheim, Indianapolis, IN). The eluate was adjusted to 0.2 N NaOH, and HEPES and sodium acetate were added to reach final concentrations of 0.24 M and 0.3 M, respectively. Fifty micrograms of tRNA were added, and the RNA was precipitated with 3 volumes of ethanol at −20° overnight. After centrifugation at 12,000 × *g* for 20 min at 4°, the RNA pellets were dried and resuspended in hydridization solution containing 50% formamide, 5× SSC, 0.1% Ficoll, 0.01% polyvinylpyrrolidine, 1% SDS, 100 μg/ml salmon sperm DNA (Pharmacia LKB Biotechnology), 1 μg/ml poly(A)$^+$ (United States Biochemical Corporation, Cleveland, OH), 1.5 mM dithiothreitol, 400 units of RNasin, and 50 mM sodium phosphate, pH 7.4, as described by Brown *et al.* (27). Before hybridization, the RNA was heated at 65° for 15 min.

Preparation of filters. Nytran membranes containing 4 μg of CgB, SgII, and cyclophilin cDNAs, pGEM4Z plasmid (Promega), and a 5.8-kilobase *Hind*III fragment of the rat 18 S ribosomal gene were prepared as suggested by Schleicher and Schuell. Four micrograms of DNA in 200 μl of TE buffer were made 0.3 N in NaOH, incubated at 65° for 30 min, and cooled to 22°, and 1 volume of 2 M ammonium acetate was added. The DNA was slot blotted using a GIBCO-BRL Hybri-Slot manifold, and the membranes were baked at 80° for 1 hr. The filters were prehybridized in hybridization solution, but without dithiothreitol and RNasin, for a minimum of 4 hr at 42°.

Hybridization conditions. The filters were hybridized to the run-on products (2–9 × 10^6 cpm/ml) in 1.5 ml of hybridization solution for 36 hr at 42°. Standardization was achieved by adding the same quantity of radioactivity to all hybridizations in a single experiment. After hybridization, the filters were washed in 0.1% SDS/2× SSC at 22°, in the same buffer at 42°, and finally in 0.1% SDS/0.1× SSC at 42°. After two incubations (for 30 min each) with 50 μg/ml RNase A (5 Prime →

3 Prime, Boulder, CO) in 1× SSC/TE buffer at 37°, the filters were washed again as described above. The filters were then exposed to Kodak XAR film, in cassettes with intensifying screens, for 3 days at −80°. Autoradiographs for 18 S rRNA were obtained from films exposed for 1–2 hr. Data were quantitated by scanning densitometric analysis as described above. The hybridization intensities of CgB, SgII, and cyclophilin RNAs were normalized to the intensity of 18 S rRNA.

mRNA decay. Pulse labeling of PC-12 cells was performed as described by Perrone-Bizzozero *et al.* (28). Briefly, the cells were pulsed with 10 μCi/ml [^{32}P]orthophosphoric acid (carrier-free; New England Nuclear) for 16 hr in phosphate-free DMEM (GIBCO) containing 12% dialyzed horse serum, 2.5% dialyzed fetal calf serum, and antibiotics. After the pulse, the cells were washed twice in DMEM with 0.01 M sodium phosphate, pH 7.7, and were incubated in standard culture medium containing an additional 0.01 M sodium phosphate, pH 7.7, for a chase period of 0, 3, 6, or 24 hr. The cells were then harvested and total cytoplasmic RNA was isolated by the method of Gough (24), with the following modifications. After extraction with chloroform/phenol/isoamyl alcohol, total cytoplasmic RNA was precipitated on dry ice for 20 min with an equal volume of isopropranol, in the presence of 50 μg of tRNA. After centrifugation at 11,400 × *g* at 4° for 20 min, the pellet was dried and resuspended in TE buffer, pH 7.4. The RNA was separated from unincorporated nucleotides by centrifugation through Quick Spin RNA columns, and NaOH was added to 0.2 N. HEPES was added to 0.24 M and the RNA was then precipitated and resuspended in hybridization solution without dithiothreitol and RNasin, as described above.

Due to the quantity of cDNA required for these experiments, linearized plasmids containing each cDNA were used. Each cDNA was ligated into the plasmid pGEM4Z, which was also used without insert to detect nonspecific hybridization to the plasmid. The CgB, SgII, and 18 S cDNA-containing plasmids were linearized with *Sac*I, *Acc*I, and *Sac*I, respectively. pTHg6.3 in pGEM4Z, a recombinant plasmid containing approximately 6300 base pairs of the rat tyrosine hydroxylase gene (29), was linearized with *Kpn*I. pGEM4Z was linearized with *Bam*HI. Fifteen micrograms of linearized plasmid DNA in 200 μl of TE buffer, pH 7.4, were applied to Nytran membranes as described above. The immobilized plasmids were incubated in prehybridization buffer for 4 hr and hybridized to the RNA (2–4 × 10^6 cpm/ml) as described above. The same quantity of radioactivity was used for all hybridizations carried out in a single experiment.

Statistical analysis. Statistical analysis was by analysis of variance, followed by *post hoc* analysis with the Tukey-Kramer multiple-comparison test, using GB-STAT software (Dynamic Microsystems, Silver Spring, MD). When multiple drug treatments were compared only with control, Dunnett's multiple-comparison test was used. Relationships between variables were examined by Pearson correlation and regression analyses. Unless indicated otherwise, each experiment was performed three times on separate days with different batches of cells. These data are presented as a percentage of control, which was set at 100% for each experiment.

Results

Mediation by cAMP of the effect of forskolin on SgII mRNA levels. The administration of forskolin to PC-12 cells differentially regulated the mRNA levels of two members of the granin family in the cells. The steady state level of CgB mRNA increased relatively rapidly in PC-12 cells treated with forskolin, reaching a maximum of 3-fold above control levels after 24 hr (Fig. 1). On the other hand, forskolin treatment of PC-12 cells caused a progressive decrease in SgII mRNA levels, which had a longer time course than that observed for CgB mRNA and which reached a level of 22 ± 1% of control after 48 hr (Fig. 1). The response of SgII mRNA to forskolin in PC-12 cells was reproducibly seen in three separate experiments.

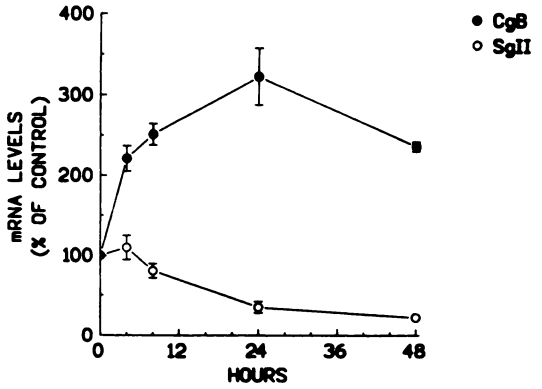

Fig. 1. Time course of forskolin-induced changes in the steady state levels of CgB and SgII mRNA in PC-12 cells. PC-12 cells were treated with 10 μM forskolin for 4–48 hr, after which the cells were collected for determination of CgB, SgII, and cyclophilin mRNA levels by slot blot analysis. Each *point* represents the mean ± standard error of three separate determinations.

The changes in CgB and SgII mRNAs in response to forskolin were reflected in changes in the cellular contents of CgB and SgII proteins. When we examined protein levels in control and forskolin-treated PC-12 cells by Western blot analysis using antibodies to rat CgB and SgII (17, 30), we found that treatment with forskolin for 24 hr increased CgB (apparent molecular mass, 110 kDa) to 307 ± 18% of control (three experiments) and decreased SgII (apparent molecular mass, 86 kDa) to 43 ± 12% of control (three experiments).

A number of cAMP-independent actions of forskolin have been reported, including effects in PC-12 cells (31). To investigate whether the action of forskolin on SgII mRNA in PC-12 cells was mediated by cAMP, we examined the effects of the active cAMP analog 8-bromo-cAMP and an analog of forskolin, 1,9-dideoxyforskolin, which is unable to activate adenylate cyclase (32), on granin mRNA levels. We found that the steady state levels of SgII mRNA were significantly reduced (to 64 ± 1% of control) by the administration of 1 mM 8-bromo-cAMP to PC-12 cells for 24 hr (Fig. 2A). In the same experiment, SgII mRNA levels were reduced to 33 ± 4% of control by 10 μM forskolin but were not significantly affected by 10 μM 1,9-dideoxyforskolin (Fig. 2A). These data suggest that the down-regulation of SgII mRNA levels by forskolin is mediated by an increase in cellular cAMP levels. CgB mRNA levels were increased by both 8-bromo-cAMP and forskolin, whereas 1,9-dideoxyforskolin had no effect on CgB mRNA levels (Fig. 2B), consistent with our previous findings that the action of forskolin on CgB mRNA is mediated by activation of adenylate cyclase (17).

Mediation by PKA activation of the effect of forskolin on SgII mRNA levels. We determined by two methods whether the effects of forskolin on SgII and CgB mRNA levels were mediated by activation of PKA. First, we examined the effects of forskolin in PC-12 cells that had been pretreated with H-89, a selective inhibitor of PKA (33). H-89 has a K_i for PKA that is 10-fold lower than that for cGMP-dependent protein kinase and is >500 times less potent in inhibiting other kinases (33). Whereas treatment of PC-12 cells with H-89 alone did not significantly affect the steady state levels of SgII and CgB mRNA, H-89 blocked both the forskolin-induced decrease in SgII mRNA and the increase in CgB mRNA (Table 1). Second, the effect of forskolin was evaluated in PKA-deficient PC-12

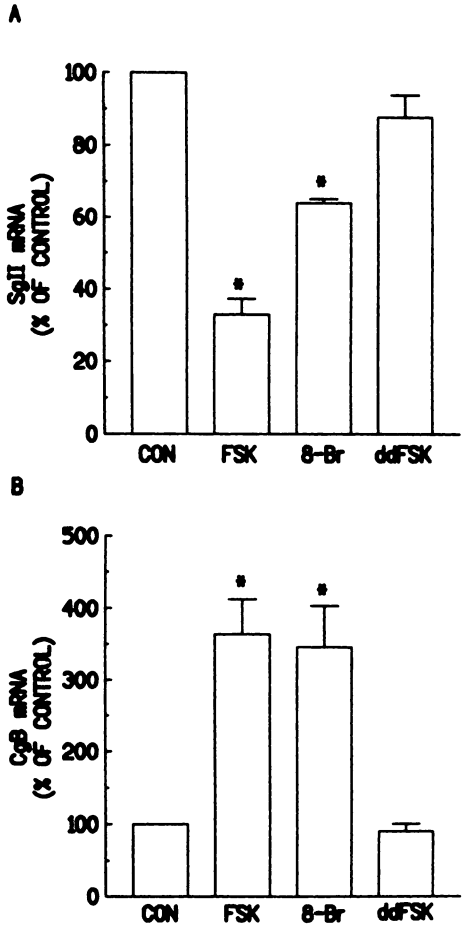

Fig. 2. Effect of 8-bromo-cAMP and 1,9-dideoxyforskolin on the steady state levels of SgII (A) and CgB (B) mRNAs. PC-12 cells were treated with 10 μM forskolin (*FSK*), 1 mM 8-bromo-cAMP (*8-Br*), or 10 μM 1,9-dideoxyforskolin (*ddFSK*) for 24 hr, after which the cells were collected for determination of SgII, CgB, and cyclophilin mRNA levels by slot blot analysis. Each *point* represents the mean ± standard error of three separate determinations. *, $p < 0.01$, different from control (*CON*).

TABLE 1

Effect of pretreatment with H-89 on the changes in SgII and CgB mRNA induced by forskolin in PC-12 cells

PC-12 cells were treated with 60 μM H-89 for 45 min before the addition of 10 μM forskolin to one half of the dishes. After further incubation for 40 hr, the cells were collected for determination of SgII, CgB, and cyclophilin mRNA levels by slot blot analysis. Each value represents the mean ± standard error of three separate determinations.

	Control	Forskolin	H-89	H-89 + Forskolin
			% of control	
SgII mRNA	100	41 ± 5[a]	115 ± 20	89 ± 15
CgB mRNA	100	349 ± 27[b]	69 ± 7	80 ± 10

[a] $p < 0.05$, different from control, H-89-treated, and H-89- plus forskolin-treated cells.
[b] $p < 0.001$, different from control, H-89-treated, and H-89- plus forskolin-treated cells.

cells. A126–1B2 cells were isolated after nitrosoguanidine mutagenesis and are deficient in PKA-II (19), whereas 123.7 and AB.11 clones were established by transfection of cells with vectors encoding mutant regulatory subunits of PKA-I and have reduced PKA-I and PKA-II activities (20). The total PKA activities of 123.7, A126–1B2, and AB.11 cells were 25%, 24%, and 27% of wild-type PC-12 cell activity, respectively. We found that the effects of 10 μM forskolin on SgII and CgB mRNA levels were significantly blunted in the PKA-deficient cell clones (Table 2). The SgII and CgB mRNAs, represented in each of the PKA-deficient cell lines by single mRNA species of approximately 2.4 kilobases, were as abundant as in the wild-type PC-12 cells (Fig. 3). This suggests that the diminished response of the PKA-deficient mutants to forskolin does not result from expression of other forms of SgII or CgB mRNAs that might be regulated differently or from under- or over-expression of these mRNAs such that their response to external factors may be masked. Rather, these results suggest that the effects of forskolin to decrease SgII mRNA and increase CgB mRNA levels are mediated by activation of PKA.

Forskolin action in the presence of cycloheximide. Because there was a delay in the response of PC-12 cells to forskolin, especially in the case of SgII mRNA, for which

TABLE 2

Effect of forskolin on the steady state levels of SgII and CgB mRNA in PKA-deficient PC-12 cells

Wild-type PC-12 cells and PKA-deficient PC-12 cell lines 123.7, A126-1B2, and AB.11 were treated with 10 μM forskolin for 24 hr before collection for determination of SgII, CgB, and cyclophilin mRNA levels by slot blot analysis. The data are presented as a percentage of control, which was set at 100% for each cell strain. Each value represents the mean ± standard error of three separate determinations.

Cells	mRNA levels	
	SgII	CgB
	% of control	
PC-12	29 ± 1[a]	253 ± 13[b]
123.7	97 ± 3	147 ± 10
A126-1B2	101 ± 9	131 ± 6
AB.11	74 ± 20	161 ± 17[c]

[a] $p < 0.01$, different from untreated wild-type cells and different from forskolin-treated 123.7 and A126-1B2 cells.
[b] $p < 0.01$, different from untreated wild-type cells and different from forskolin-treated 123.7, A126-1B2, and AB.11 cells.
[c] $p < 0.05$, different from untreated AB.11 cells.

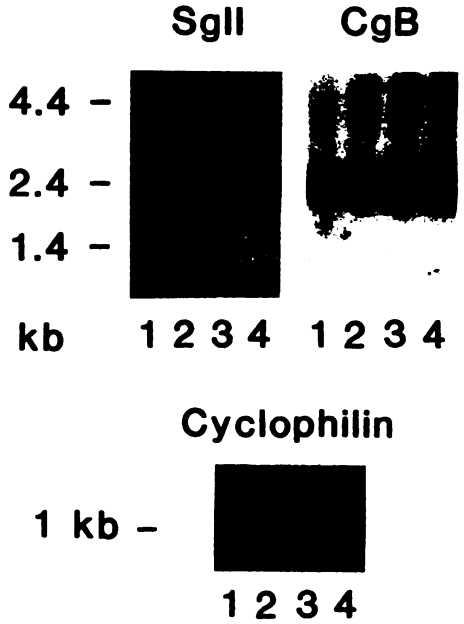

Fig. 3. Northern blot analysis of SgII and CgB mRNA in wild-type and PKA-deficient PC-12 cells. Total RNA was isolated from wild-type PC-12 (*lanes 1*), 123.7 (*lanes 2*), A126–1B2 (*lanes 3*), or AB.11 (*lanes 4*) cells, hybridized (10 μg/lane) with randomly primed SgII, CgB, or cyclophilin cDNAs, and autoradiographed.

maximal effects were not observed until at least 48 hr after forskolin administration (Fig. 1), we investigated whether the effects of forskolin were dependent on protein synthesis. PC-12 cells were pretreated for 2 hr with 1 μg/ml cycloheximide, which inhibited the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable counts by >90%, after which the effect of forskolin on SgII and CgB mRNAs was determined. Because of the difference in the time course of the response of SgII mRNA and CgB mRNA to forskolin, PC-12 cells were treated with forskolin for 12 hr for determination of SgII mRNA or for 4 hr for determination of CgB mRNA. Cycloheximide was present throughout the course of the experiments. Whereas treatment with cycloheximide alone had no effect on SgII mRNA levels in PC-12 cells, it completely eliminated the decrease in SgII mRNA observed with forskolin treatment alone (Fig. 4A). In fact, in the presence of cycloheximide forskolin increased the level of SgII mRNA, reaching a level of 361 ± 69% above control, compared with a level of 66 ± 7% of control in cells treated with forskolin alone (Fig. 4A). The superinduction of SgII mRNA was also observed in cells treated with cycloheximide and 1 mM 8-bromo-cAMP but not in cells treated with cycloheximide and 1,9-dideoxyforskolin (data not shown), suggesting that this effect of forskolin was indeed mediated by an increase in cellular cAMP levels. In contrast, the effect of forskolin on CgB mRNA levels was not affected by treatment with cycloheximide (Fig. 4B).

We further evaluated the superinduction of SgII mRNA in PC-12 cells by forskolin in the presence of cycloheximide by answering four questions. 1) What duration of cycloheximide treatment is necessary for superinduction with forskolin to be observed? 2) What is the relationship between the extent of superinduction of SgII and the extent of protein synthesis inhibition by cycloheximide? 3) Is superinduction of SgII mRNA also observed when PC-12 cells are pretreated with protein synthesis inhibitors with mechanisms of action different from that of cycloheximide? 4) Is the superinduction of SgII mRNA by forskolin in the presence of cycloheximide mediated by activation of PKA? We incubated PC-12 cells with cycloheximide for various lengths of time to determine the time necessary to observe the superinduction of SgII mRNA with forskolin. In all cases cells were treated with forskolin for 4 hr, during which time the decrease in SgII mRNA with forskolin is not observed (Fig. 1). The maximal induction of SgII mRNA was achieved when cells were treated with cycloheximide for 2 hr before the addition of forskolin (Fig. 5). When cells were treated with cycloheximide for shorter periods, the extent of induction of SgII mRNA was progressively less.

Some protein synthesis inhibitors, including cycloheximide, can influence steady state mRNA levels by altering the rate of transcription of some genes (34). Because this effect occurs at concentrations of cycloheximide that do not inhibit protein synthesis, it was important to compare the extent of superinduction of SgII mRNA by forskolin and different concentrations of cycloheximide with the extent of protein synthesis inhibition. PC-12 cells were treated with 0.01–1 μg/ml cycloheximide, which inhibited protein synthesis to increasing extents (Fig. 6). We found that there was a significant relationship between the extent of superinduction of SgII mRNA by forskolin in the presence of different concentrations of cycloheximide and the extent to which protein synthesis was inhibited ($r = 0.85$, $p < 0.01$) (Fig. 6). Maximum induction of SgII mRNA was not achieved until protein synthesis was inhibited by >90%, suggesting that the action of cycloheximide here is dependent on its ability to arrest translation.

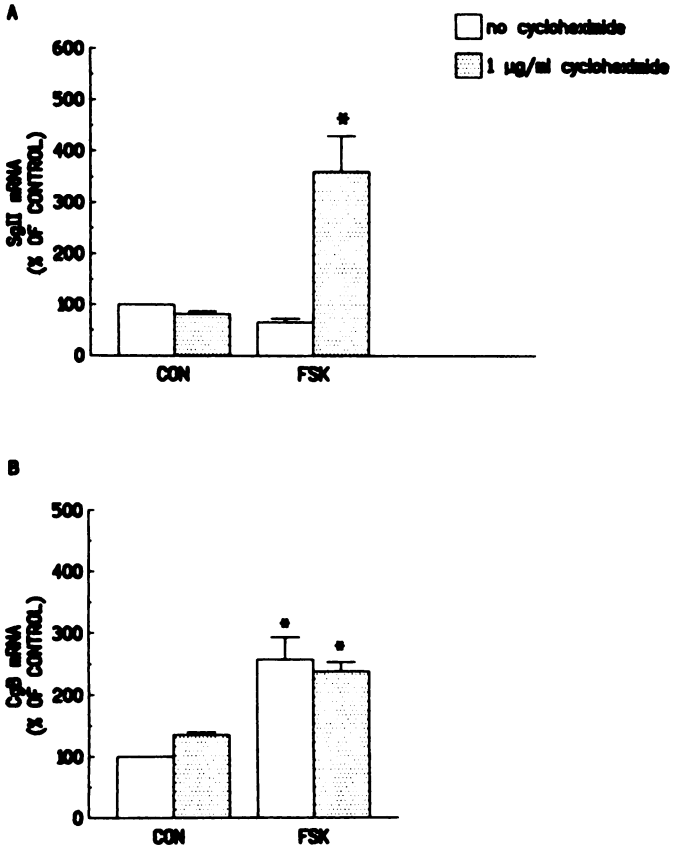

Fig. 4. Effect of forskolin in the presence of cycloheximide on the steady state levels of SgII (A) and CgB (B) mRNAs. PC-12 cells were incubated with or without 1 μg/ml cycloheximide for 2 hr, after which one half of each group also received 10 μM forskolin (*FSK*). The cells were further incubated for either 12 or 4 hr, after which they were collected for determination of SgII or CgB mRNA levels, respectively, by slot blot analysis. Each *point* represents the mean ± standard error of three separate experiments, standardized to the level of cyclophilin mRNA. *, $p < 0.01$, different from control (*CON*).

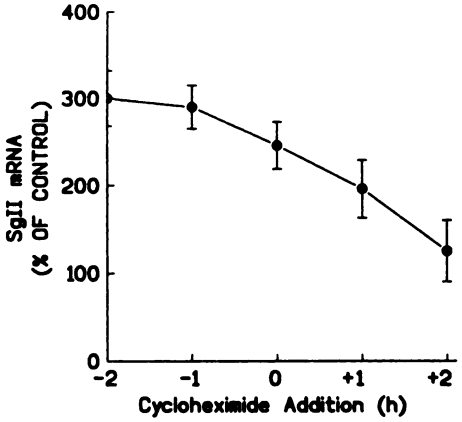

Fig. 5. Time frame for cycloheximide superinduction of SgII mRNA in PC-12 cells. All cells were treated for 4 hr with 10 μM forskolin. Cycloheximide (1 μg/ml) was added either 2 hr or 1 hr before (−2, −1), at the same time as (*0*), or 1 hr or 2 hr after (+1, +2) the addition of forskolin. The cells were then collected for the determination of SgII and cyclophilin mRNA levels by slot blot analysis. Each *point* represents the mean ± standard error of three separate determinations.

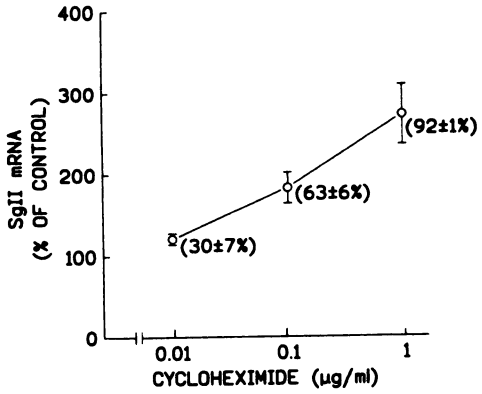

Fig. 6. Effect of different concentrations of cycloheximide on the induction of SgII mRNA by forskolin in PC-12 cells. PC-12 cells were treated with cycloheximide (0.01–1 μg/ml) for 2 hr before the addition of 10 μM forskolin. After 4 hr the cells were collected for determination of SgII and cyclophilin mRNA levels by slot blot analysis. In each of the experiments the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable counts was determined. *Values in parentheses*, extent of protein synthesis inhibition at each concentration of cycloheximide. Each *point* represents the mean ± standard error of three separate determinations.

To further establish that the superinduction of SgII mRNA by cycloheximide was due to inhibition of protein synthesis, we examined the effect of forskolin in the presence of other protein synthesis inhibitors, which act by different mechanisms (35). Emetine and cycloheximide cause polysome stabilization, whereas puromycin and pactamycin cause disaggregation of polysomes. We found that superinduction of SgII mRNA in PC-12 cells was observed after treatment with forskolin in the presence of all of the protein synthesis inhibitors examined (Fig. 7A). The incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable counts was inhibited by at least 92% by each of the agents at the concentrations used. The induction of CgB mRNA by forskolin was not affected by preincubation with any of the protein synthesis inhibitors (Fig. 7B).

We also determined whether the superinduction of SgII mRNA by forskolin and cycloheximide was dependent on activation of PKA. Wild-type PC-12 cells and the 123.7 and A126–1B2 PKA-deficient PC-12 cell mutants were incubated with 1 μg/ml cycloheximide for 2 hr before the addition of 10 μM forskolin, and the level of induction of SgII mRNA over untreated controls was evaluated after 4 hr. In this experiment, SgII mRNA levels in wild-type PC-12 cells were elevated to 209 ± 19% of control ($p < 0.001$ versus control cells) after cycloheximide and forskolin treatment. The extent of induction of SgII mRNA with cycloheximide and forskolin was significantly lower in 123.7 and A126–1B2 cell mutants, reaching only 121 ± 9% and 111 ± 7% of their respective controls ($p < 0.01$ versus cycloheximide- and forskolin-treated wild-type cells). These results suggest that the induction of SgII mRNA by cycloheximide and forskolin in PC-12 cells is mediated by activation of PKA. We also found that the induction of SgII mRNA by cycloheximide and forskolin in wild-type PC-12 cells was blocked by 60 μM H-89 (data not shown), further supporting this notion.

The results of these studies suggest that under normal conditions a protein factor represses the stimulated expression of SgII mRNA in PC-12 cells, such that induction of SgII mRNA, for example by elevated cellular cAMP levels, is revealed only after protein synthesis is inhibited. We therefore investigated

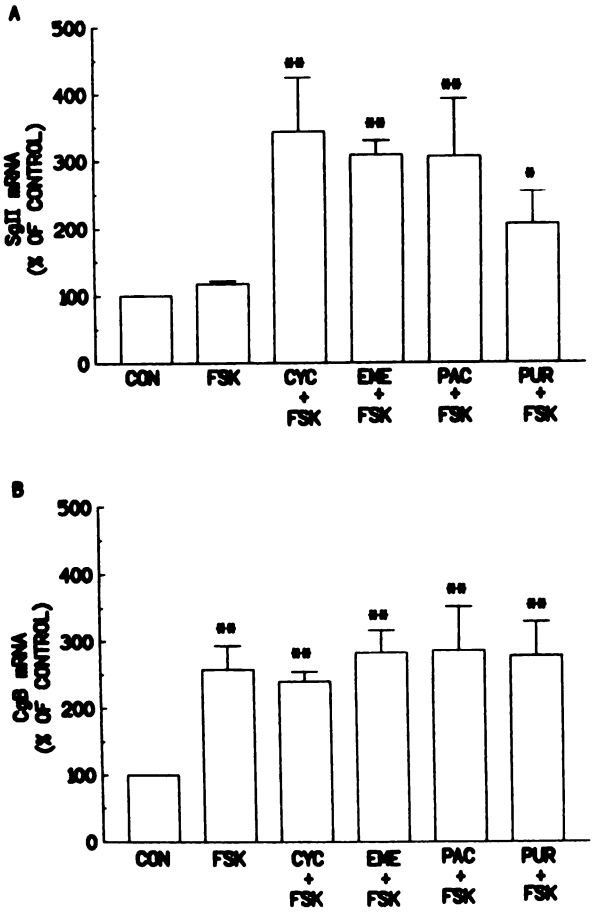

Fig. 7. Effect of forskolin in the presence of different protein synthesis inhibitors on the steady state levels of SgII (A) and CgB (B) mRNAs. PC-12 cells were treated for 2 hr with either 1 μg/ml cycloheximide (*CYC*), 1 μg/ml emetine (*EME*), 0.6 μg/ml pactamycin (*PAC*), or 100 μg/ml puromycin (*PUR*), after which the cells were treated with 10 μM forskolin (*FSK*). After 4 hr the cells were collected for determination of SgII, CgB, and cyclophilin mRNA levels by slot blot analysis. Each *point* represents the mean ± standard error of three separate determinations. **, $p < 0.01$, different from control (*CON*); *, $p < 0.05$, different from control.

the effects of stimulatory factors, which have been shown to affect SgII levels in bovine chromaffin cells (15), on SgII mRNA levels in control PC-12 cells and in cells in which protein synthesis was inhibited. PC-12 cells were pretreated with 1 μg/ml cycloheximide for 2 hr, after which they were treated for 4 hr with either 100 nM PMA to activate protein kinase C, 50 mM KCl to depolarize the cells, or 10 μM forskolin. Neither PMA nor KCl treatment had a significant effect on SgII mRNA levels in either control PC-12 cells (97 ± 4% and 101 ± 6% of control, respectively) or PC-12 cells that were also treated with cycloheximide (116 ± 8% and 98 ± 10% of control, respectively). No effect was observed even when PMA concentrations as high as 1 μM were used or when the time course for PMA and KCl effects was extended to 24 hr. In the same experiments, SgII mRNA levels were significantly increased by treatment for 4 hr with cycloheximide and forskolin together (235 ± 13% of control). Thus, even in the presence of protein synthesis inhibition we did not detect an effect of activation of protein kinase C or depolarization on SgII mRNA levels.

Transcriptional regulation of SgII and CgB genes. A change in the steady state level of SgII and CgB mRNAs in

PC-12 cells may result from a change in the rate of transcription of the SgII gene and/or from a change in the stability of the mRNAs. We therefore evaluated whether changes in the level of transcription of either the SgII or CgB gene contributed to the observed changes in the steady state levels of mRNA. Nuclear run-on assays were performed in nuclei isolated from PC-12 cells that had been treated for 2 hr with forskolin in the presence or absence of cycloheximide. Representative autoradiograms of the hybridization signals for SgII and CgB from one of the nuclear run-on assays are shown in Figs. 8 and 9, respectively. Quantitation of the signals from three independent experiments is shown in Fig. 10. The ratios of the densitometric intensities of the SgII and CgB signals to that of the 18 S rRNA signal were set at 100%. Neither forskolin treatment nor cycloheximide treatment consistently affected the level of transcription of the SgII gene (to 126 ± 31% and 267 ± 36% of control, respectively) (Figs. 8 and 10A). On the other hand, treatment of PC-12 cells with cycloheximide and forskolin together increased the level of transcription of the SgII gene to 1290 ± 96% of control. A different pattern of regulation by cAMP was observed for CgB. After 2 hr of forskolin treatment, the level of transcription of CgB increased to 601 ± 87% of control (Figs. 9 and 10B). No change in the transcription of the cyclophilin gene was observed after any of the treatments (Figs. 8 and 9). The stimulation of CgB transcription by forskolin was not affected by coadministration of cycloheximide (data not shown).

The stimulatory effects of cAMP on the steady state levels of SgII and CgB mRNAs observed after 4 hr of treatment result predominantly from changes in the rate of transcription, because we found that these mRNAs are extremely stable. The half-lives of SgII and CgB mRNA were determined after labeling of PC-12 cells with [^{32}P]orthophosphoric acid. The decrease in the amount of radiolabeled SgII, CgB, tyrosine hydroxylase, and 18 S RNAs over time followed a simple exponential function (Fig. 11). The half-lives of these RNAs were calculated using the equation $t_{1/2} = 0.693/\lambda$, where λ is the decay constant derived from the slope of the line of the natural logarithm of the proportion of RNA remaining at a given time. We found the half-lives of the SgII and CgB mRNAs to be long (Fig. 11, *inset*). The half-lives calculated for tyrosine hydroxylase mRNA and 18 S RNA were consistent with the results of previous studies showing that the tyrosine hydroxylase mRNA is relatively short-lived (36), whereas the 18 S RNA is extremely stable (37). Thus, even complete stabilization of the SgII and CgB mRNAs during the time course of the experiments would increase the steady state levels of these mRNAs by only 10%.

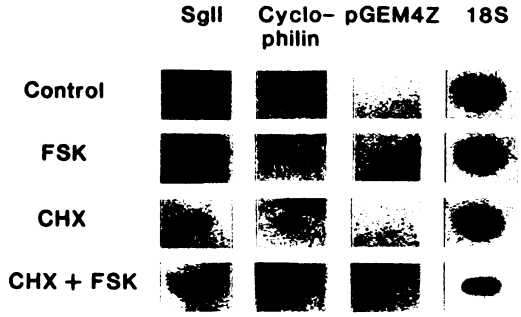

Fig. 8. Representative autoradiogram of SgII, cyclophilin, and 18 S transcripts from nuclear run-on assays in PC-12 cell nuclei. PC-12 cells were treated with 10 μM forskolin (*FSK*), 1 μg/ml cycloheximide (*CHX*), or 10 μM forskolin and 1 μg/ml cycloheximide together for 2 hr, after which the nuclei were isolated and nuclear run-on assays were performed as described in Experimental Procedures. pGEM4Z was used to determine the level of nonspecific hybridization.

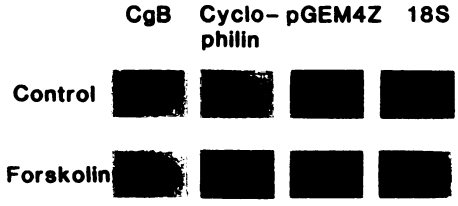

Fig. 9. Representative autoradiogram of CgB, cyclophilin, and 18 S transcripts from nuclear run-on assays in PC-12 cell nuclei. PC-12 cells were treated with 10 μM forskolin for 2 hr, after which the nuclei were isolated and nuclear run-on assays were performed as described in Experimental Procedures. pGEM4Z was used to determine the level of nonspecific hybridization.

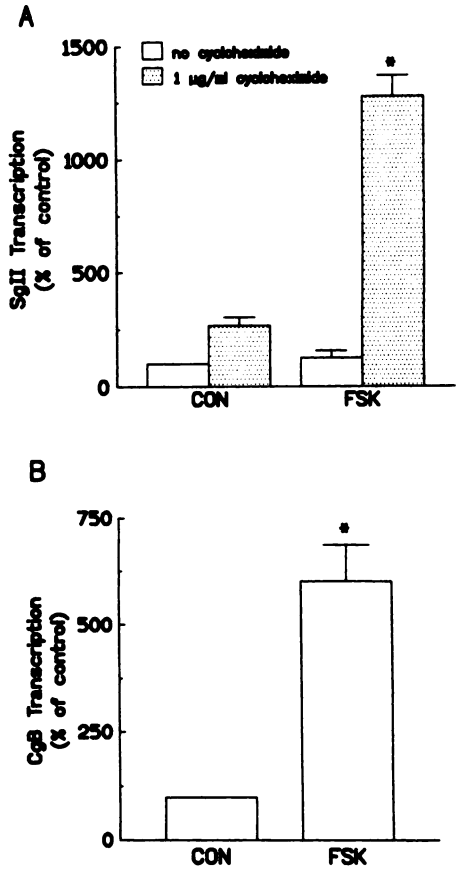

Fig. 10. Effects of forskolin (*FSK*), cycloheximide, or the two combined on SgII gene transcription (A) and the effect of forskolin on CgB gene transcription (B) in PC-12 cells. Cells were treated as described in the legends to Figs. 8 and 9, respectively; the nuclei were isolated and the nuclear run-on assays were performed as described in Experimental Procedures. The hybridization intensities of SgII and CgB mRNAs were normalized to the intensity of 18 S RNA. Each *point* represents the mean ± standard error of three separate determinations. *, $p < 0.001$, different from control (*CON*).

Discussion

We have shown that the actions of forskolin on the steady state levels of SgII and CgB mRNAs in PC-12 cells are mediated by an increase in the cellular level of cAMP and subsequent activation of PKA. These conclusions are based on the following evidence: 1) the cAMP analog 8-bromo-cAMP had actions

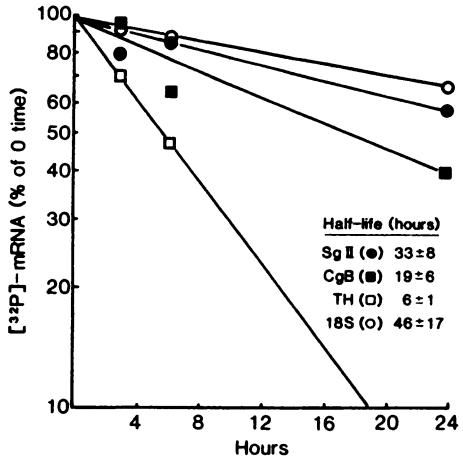

Fig. 11. Half-lives of SgII, CgB, and tyrosine hydroxylase (*TH*) mRNAs and 18 S RNA. PC-12 cells were labeled for 16 hr with [^{32}P]orthophosphoric acid, washed, and incubated for a chase period of up to 24 hr. The cells were collected and the amounts of radiolabeled granin and tyrosine hydroxylase mRNAs and 18 S RNA were quantitated. Each *point* represents the mean of three separate determinations. Standard errors did not exceed 15% of any mean. *Inset*, calculated half-lives of the RNAs.

similar to those of forskolin on SgII and CgB mRNA levels; 2) 1,9-dideoxyforskolin, an analog of forskolin that does not activate adenylate cyclase, had no effect on SgII and CgB mRNA levels; 3) the effects of forskolin were blocked by pretreatment with H-89, a selective inhibitor of PKA; and 4) the effects of forskolin were significantly blunted in PKA-deficient PC-12 cell strains. However, the responses of these two granins to cAMP otherwise differ in PC-12 cells. First, forskolin down-regulated the level of SgII mRNA but up-regulated the level of CgB mRNA. Second, the maximum effect of forskolin on SgII mRNA levels was not reached for at least 48 hr, whereas the maximum increase in CgB mRNA levels was reached within 24 hr of forskolin administration. Third, whereas the protein synthesis inhibitor cycloheximide had no effect on the induction of CgB mRNA by forskolin, we found that cycloheximide masked the response of SgII mRNA to forskolin. In fact, instead of the forskolin-mediated decrease in SgII mRNA observed under control conditions, we found that forskolin in the presence of cycloheximide induced a marked increase in SgII mRNA levels. Because administration of cycloheximide alone did not affect the steady state levels of SgII mRNA in PC-12 cells, the combined effect of cycloheximide and forskolin on SgII mRNA levels may be considered superinduction.

Regulation of specific mRNAs in the manner shown here for SgII has been reported only rarely in the endocrinological literature. For example, the levels of fibronectin mRNA in bovine BGC-1 granulosa cells (38) and of TSH receptor mRNA in rat FRTL-5 thyroid cells (39) are decreased by cAMP analogs and forskolin. These effects are reversed to stimulatory effects when protein synthesis is inhibited by cycloheximide. Those investigators showed that both the down-regulation of these mRNAs by cAMP and their up-regulation by cAMP in the presence of cycloheximide are mediated at least in part by a change in transcription of the respective genes (38, 39). We found that the induction of SgII mRNA by cAMP in the presence of cycloheximide in PC-12 cells is also mediated by an increase in the transcription of the SgII gene. We do not yet know the mechanism by which increased cellular cAMP levels decreased the level of SgII mRNA in protein synthesis-replete PC-12 cells. We did not observe a change in the level of transcription of the SgII gene by nuclear run-on analysis, suggesting that a decrease in *de novo* synthesis of the SgII mRNA may not mediate the effect of cAMP. However, considering the low level of transcription of the SgII gene in PC-12 cells, compared with, for example, that of the CgB gene, subtle changes in the level of SgII gene transcription might not have been detected. It is also possible that cAMP increases the post-transcriptional processing of SgII pre-mRNA and/or destabilizes cytoplasmic SgII mRNA.

The mechanism by which pretreatment with cycloheximide uncovers a stimulatory effect of cAMP on the transcription of the SgII gene is unknown. The effect of cycloheximide is most likely mediated by translational arrest, because it was closely associated with the extent of protein synthesis inhibition and was reproduced by several protein synthesis inhibitors. Thus, the action of cycloheximide in PC-12 cells may be to inhibit the synthesis of labile proteins that control the level or duration of cAMP-stimulated transcription of the SgII gene. Treatment with cycloheximide alone did not affect SgII mRNA levels, suggesting that the basal expression of SgII mRNA in PC-12 cells is not under negative control by labile factors. However, the labile repressor hypothesis is controversial (34), and information on specific repressor proteins is limited. Nevertheless, the results of these studies suggest that the expression of the SgII gene is repressed, so that the stimulatory effect of cAMP is not revealed unless protein synthesis is inhibited. Like the effects of forskolin in the absence of cycloheximide (Tables 1 and 2), the stimulatory effect of cAMP on SgII mRNA accumulation is likely mediated by activation of PKA, because the induction was significantly blunted in PKA-deficient PC-12 cell mutants. However, treatment with either phorbol ester or KCl did not affect SgII mRNA levels in either control PC-12 cells or cells in which protein synthesis was inhibited. These results suggest that the inability to observe induction of SgII mRNA with these agents, which increase SgII mRNA levels in bovine chromaffin cells (15), is not due to an overriding repression of SgII mRNA expression in PC-12 cells. The reasons for the different effects of phorbol ester and KCl on SgII mRNA levels in bovine chromaffin cells and PC-12 cells are unknown.

PC-12 cells and bovine adrenal chromaffin cells also differ in their response to cAMP. Only a positive regulation of SgII mRNA by cAMP, which occurred in the presence of ongoing protein synthesis, was observed in bovine chromaffin cells (15). The reason for the different effects of cAMP on PC-12 cells versus primary cultures of adrenal cells depending on the level of protein synthesis is not known. It may simply result from species differences in the regulation of SgII expression. Alternatively, it may reflect cellular changes that accompany adaptation to long term culture conditions such that PC-12 cells express factors important for negative regulation, whereas primary cultures of adrenal cells do not. Similar adaptive changes have been postulated to occur in thyroid cells in long term culture. Only positive regulation of TSH receptor mRNA was observed by cAMP in primary cultures of human thyroid cells (40), whereas both positive and negative regulation of TSH receptor mRNA occurs in the FRTL-5 thyroid cell line (39). It was argued that regulation of the TSH receptor in the FRTL-5 cell line actually reflects normal physiology and that factors important for negative regulation of the TSH receptor may be

lost in thyroid cells in the course of initial culture with fetal calf serum (41). The physiological significance of our findings on SgII regulation in PC-12 cells remains to be determined and must await a better understanding of the function of SgII. However, the finding that SgII mRNA levels are subject to both negative and positive regulation suggests that the expression of SgII, like other proteins whose expression is similarly regulated in cultured cells, is tightly controlled.

The regulation of CgB mRNA by cAMP in PC-12 cells appears to follow the well defined pathway of transcriptional regulation by cAMP. It is independent of ongoing protein synthesis and therefore falls into the class 1 category of cAMP-regulated genes, as defined by Roesler *et al.* (42). According to this model, cAMP binds to the regulatory subunits of PKA, resulting in dissociation of the holoenzyme and activation of the catalytic subunits. The catalytic subunits enter the nucleus and phosphorylate the cAMP-responsive element-binding protein [reviewed recently by Spaulding (43)], resulting in activation of the CgB gene and increased transcription. These results are consistent with the presence of a consensus cAMP-responsive element in the 5′ flanking region of the CgB gene (12).

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