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# SOMATOSTATIN PRETREATMENT DESENSITIZES SOMATOSTATIN RECEPTORS LINKED TO ADENYLATE CYCLASE AND FACILITATES THE STIMULATION OF CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE ACCUMULATION IN ANTERIOR PITUITARY TUMOR CELLS[1]

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## Abstract

Somatostatin-14 (SRIF) inhibits both hormone- and forskolin-stimulated cyclic adenosine 3′:5′-monophosphate (cyclic AMP) formation in tumor cells of the mouse anterior pituitary (AtT-20/D16-16). However, long-term pretreatment of cells with SRIF modifies the responsiveness of this system in two ways: The response of adenylate cyclase to stimulatory agents is enhanced, whereas the ability of SRIF to inhibit stimulated cyclic AMP formation is reduced. The supersensitive adenylate cyclase response and the SRIF desensitization were dependent on the concentration and duration of SRIF pretreatment. Enhancement of forskolin-stimulated cyclic AMP formation occurred within 4 hr, whereas that of corticotropin-releasing-factor-,(−)-isoproterenol-, and vasoactive intestinal peptide-induced cyclic AMP accumulation required 16 hr of pretreatment. The elevated responses to each of these stimulants were due to increases in their maximal ability to stimulate cyclic AMP formation. Cycloheximide treatment blocked the enhanced cyclic AMP response induced by SRIF pretreatment, suggesting a requirement for protein synthesis. In membrane preparations, SRIF pretreatment facilitated activation of adenylate cyclase by forskolin, sodium fluoride, and guanosine 5′-($\beta,\gamma$-imido)-triphosphate without affecting basal activity. These results suggest that desensitization of an inhibitory input to adenylate cyclase is accompanied by a supersensitivity of adenylate cyclase to stimulatory agents through a process requiring protein synthesis.

Somatostatin (SRIF) is a 14 amino acid-containing peptide found in the adenohypophysis where it is known to inhibit the release of several hormones including growth hormone (Brazeau et al., 1974), prolactin, and thyrotropin (Vale et al., 1974). In tumor cells of the mouse anterior pituitary (AtT-20/D16-16), which secrete adrenocorticotropin hormone (ACTH), SRIF blocks ACTH release induced by corticotropin-releasing factor (CRF), (−)-isoproterenol, and vasoactive intestinal peptide (Heisler et al., 1982). Furthermore, stimulation of ACTH secretion by forskolin, an agent proposed (Seamon and Daly, 1981) to activate directly the catalytic subunit of adenylate cyclase, was also antagonized by SRIF (Heisler et al., 1982). Because SRIF inhibits hormone- and forskolin-stimulated cyclic AMP accumulation in these cells (Heisler et al., 1982), it was proposed that SRIF receptors are negatively coupled to adenylate cyclase and that an inhibition of this enzyme's activity may be the principle mode of action of SRIF in modulating ACTH secretion.

Recent studies have shown that pituitary cells become refractory to SRIF following prior exposure to the peptide. Smith and Vale (1980) observed that long-term treatment of rat anterior pituitary cells with SRIF reduced the ability of this peptide to inhibit growth hormone and thyroid-stimulating hormone release. In the present study we report that long-term SRIF pretreatment induces SRIF desensitization, and in addition increases the response of adenylate cyclase to stimulatory agents. This latter effect is dependent on protein synthesis and may involve the generation of new adenylate cyclase molecules or proteins necessary for adenylate cyclase activation.

## Materials and Methods

Mouse AtT-20/D16-16 tumor cells were grown and subcultured in Dulbecco's modified Eagle's medium

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(DMEM) with 10% fetal calf serum as previously described (Hook et al., 1982). For measurement of intracellular cyclic AMP and cyclic GMP levels, cells were plated in 35-mm diameter culture cluster dishes (Costar) at an initial density of $1.5 \times 10^5$ cells/well and were used 5 days after subculturing (60 to 80% confluency). For adenylate cyclase and phosphodiesterase studies, cells were plated in 25-$cm^2$ culture flasks (Falcon) at an initial density of $1 \times 10^6$ cells/flask and were used 4 to 5 days after subculturing (60 to 80% confluency).

Somatostatin-14 and D-Trp$^8$-somatostatin (Peninsula) were prepared as stock solutions ($10^{-4}$ M) in acidified PBS (pH 1.0) and BSA (1%). For the pretreatment studies these peptides were added to DMEM containing 10% fetal calf serum and bacitracin (3 μg/ml). This medium was applied to cells for varying periods of time and incubated at 37°C in an atmosphere of 5% $CO_2$. For the measurement of intracellular cyclic AMP, the incubation medium was removed, and the cells were washed twice with fresh DMEM containing HEPES (25 mM), 2% fetal calf serum, bacitracin with or without 0.5 mM 3-isobutyl-methylxanthine (IBMX). Cells were equilibrated with this medium for 15 min. Then 1 ml of fresh medium with or without test agents was incubated with the cells for 30 min. The reaction was terminated by removing the medium and adding 1 ml of 0.5 N HCl to the cells. The cells were then sonicated, and the samples were frozen for later cyclic AMP analysis. Intracellular cyclic AMP was determined using the commercially available radioimmunoassay kit from New England Nuclear. Results are expressed as picomoles of cyclic AMP formed per well. Protein levels were not significantly different between wells nor changed by the SRIF pretreatment. Protein content was determined by a modification of the method of Lowry et al. (1951).

For measurement of adenylate cyclase activity, preincubation medium (DMEM, 10% fetal calf serum with or without somatostatin) was removed, and the cells were rapidly washed three times with ice-cold phosphate-buffered saline (pH 7.4). The cells were detached from the flask, homogenized in 5 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol) with a Teflon-glass homogenizer and centrifuged at 100,000 × $g$ for 30 min at 4°C. The pellet was resuspended in buffer, and tissue (approximately 10 μg of protein in 10 μl) was added to tubes that contained buffer (40 mM Tris-HCl, 1 mM IBMX, 0.5 mM EGTA, 5 mM $MgCl_2$ at pH 8.0) and test agents at 4°C. The final volume was 50 μl. The reaction was started by the addition of $10^{-3}$ M ATP, and the mixture was incubated at 30°C for 5 min. The reaction was terminated by the addition of 250 μl of 0.05 M sodium acetate buffer (pH 6.2) and boiling for 3 min. The cyclic AMP in these samples was analyzed using the commercially available kit from Becton-Dickinson. Under the conditions used, enzyme activity was linear with time and protein concentration. The values are expressed as picomoles of cyclic AMP per milligram of protein formed in 5 min.

Phosphodiesterase activity was assayed using the two-step procedure of Thompson et al. (1979). The final reaction mixture contained 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 μM cyclic AMP, 0.5 μCi of [2,8-$^3$H] adenosine 3′,5′-cyclic phosphate, ammonium salt (New England Nuclear), and drug as indicated. Reactions were initiated by addition of enzyme, incubated at 30°C for 10 min, and stopped by boiling for 60 sec. Snake venom nucleotidase (100 μl of 1 mg/ml, *Ophiophagus hannah*) was added, and tubes were incubated at 30°C for 10 min. Methanol (1 ml) was added; samples were applied to AGl-X8 (200 to 400 mesh, 0.7 × 1.5 cm) columns and eluted with 1 ml of methanol. Radioactivity from the eluant was quantified by liquid scintillation counting (Beckman, LS-230). Conversion of 5′-AMP to adenosine by nucleotidase and recovery from the column were greater than 95%.

For the measurement of cyclic GMP levels, samples were prepared as described for the intracellular cyclic AMP studies. Cyclic GMP levels were determined using the commercially available kit from New England Nuclear.

## Results

Both CRF and forskolin stimulate cyclic AMP accumulation in AtT-20 cells. SRIF inhibits the effects of these two stimulants in a dose-dependent manner (Fig. 1). Two hours of SRIF pretreatment did not appear to alter either the ability of CRF or forskolin to stimulate

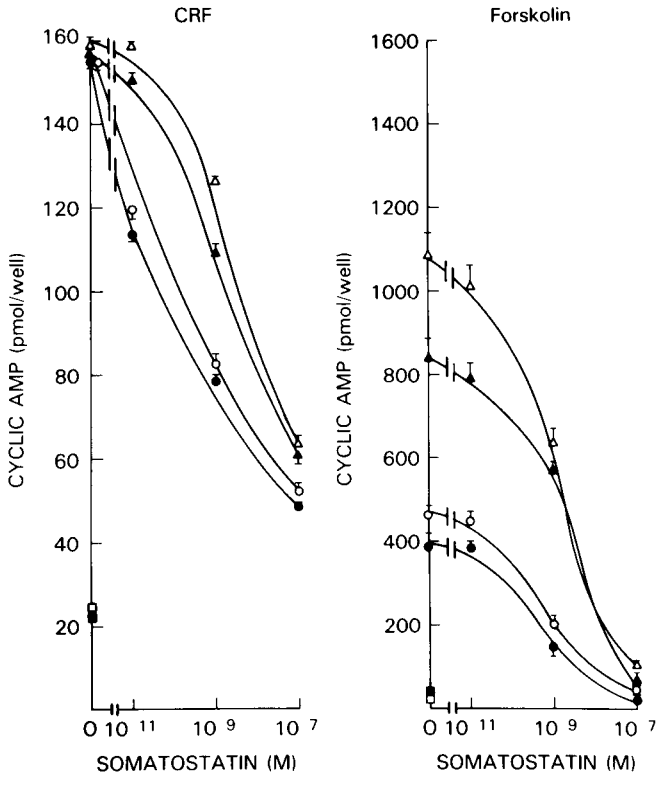


*Figure 1.* Effect of SRIF pretreatment on SRIF's inhibition of CRF- and forskolin-stimulated cyclic AMP accumulation. AtT-20 cells were pretreated for 8 (△), 4 (▲), 2 (○), and 0 (●) hr with SRIF ($10^{-7}$ M). At the end of this time the cells were washed twice and re-incubated with CRF ($10^{-7}$ M) (*left panel*) or forskolin ($10^{-5}$ M) (*right panel*) in the presence of different concentrations of SRIF. Cyclic AMP accumulation studies were performed as described under "Materials and Methods." Control (■) and treated (8 hr (□) basal cyclic AMP levels were not different. Each point represents the mean ± SEM of triplicate experiments repeated twice.

cyclic AMP synthesis or the inhibitory effect of SRIF. However, after 4 hr of SRIF exposure, the potency of SRIF to inhibit the cyclic AMP responses of CRF or forskolin was reduced ($IC_{50}$ value for SRIF inhibition of CRF-stimulated cyclic AMP accumulation: control, $1.4 \pm 0.3 \times 10^{-10}$ M; 2-hr treated, $5.7 \pm 1.4 \times 10^{-10}$ M; 4-hr treated, $1.2 \pm 1.8 \times 10^{-9}$ M; 8-hr treated, $5.4 \pm 0.6 \times 10^{-9}$ M; SRIF inhibition of forskolin-stimulated cyclic AMP accumulation: control, $3.7 \pm 1.1 \times 10^{-10}$ M; 2-hr treated, $6.2 \pm 1.4 \times 10^{-10}$ M; 4-hr treated, $1.2 \pm 0.8 \times 10^{-9}$ M; 8-hr treated, $3.4 \pm 1.7 \times 10^{-9}$ M). This is illustrated as a rightward shift in the dose response curve for SRIF (Fig. 1). CRF-stimulated cyclic AMP formation was elevated after 16 hr of SRIF pretreatment (Table I). In contrast, the cyclic AMP response to forskolin was increased after only 4 hr of SRIF treatment (Table I). The ability of (−)-isoproterenol and vasoactive intestinal peptide (VIP) to stimulate cyclic AMP accumulation was also enhanced following 16 hr of pretreatment but not after shorter periods of exposure. The ability of SRIF to inhibit VIP- and (−)-isoproterenol- as well as CRF- and forskolin-stimulated cyclic AMP accumulation was significantly reduced after 8 hr of SRIF pretreatment, at which time only forskolin exhibited a greater level of stimulation of cyclic AMP formation (Fig. 2). The enhanced response of VIP and (−)-isoproterenol (Fig. 3 and Table I) as well as that of CRF and forskolin (Fig. 4 and Table I) was due to a greater maximal ability of each agent to stimulate cyclic AMP formation with no apparent change in their respective receptor affinities. The density of β-adrenergic receptors on AtT-20 cells as assessed by [$^3$H] dihydroalprenolol binding to intact cells was not altered after 16 hr of SRIF pretreatment (control $B_{max}$ = 106.5 fmol/mg of protein, $K_d$ = 5.8 nM; treated $B_{max}$ = 98.7 fmol/mg of protein, $K_d$ = 6.7 nM). Basal cyclic AMP and cyclic GMP levels (Table II) and phosphodiesterase activity (Table III) were not affected by acute SRIF exposure nor SRIF pretreatment.

The increase in CRF- and forskolin-stimulated cyclic AMP synthesis was dependent on the concentration of SRIF to which the cells were exposed (Fig. 4). Little change in CRF- or forskolin-stimulated cyclic AMP accumulation was induced by 16 hr of treatment with $10^{-11}$ M SRIF, whereas higher concentrations of this peptide did cause a significant increase. D-Trp$^8$-somatostatin, a more potent analogue of SRIF in inhibiting growth hormone release (Vale et al., 1976), is about 10-fold more effective than SRIF in increasing forskolin's stimulation of cyclic AMP accumulation (Fig. 5).

Forskolin stimulates cyclic AMP accumulation in AtT-20 cells either in the presence or absence of phosphodiesterase inhibitors, although the magnitude of stimulation is much greater with IBMX (Fig. 6). In either condition, SRIF pretreatment still increases forskolin's ability to stimulate cyclic AMP production. Furthermore, exposure of AtT-20 cells to SRIF for 16 hr increased forskolin's ability to activate adenylate cyclase in membrane preparations (Fig. 7). Sodium fluoride-(Fig. 7) as well as guanosine 5′-(β,γ-imido)-triphosphate (Gpp(NH)p)-stimulated adenylate cyclase activity was also increased following such pretreatment (Fig. 8). Basal adenylate cyclase (Fig. 7) activity and protein content were not affected by SRIF pretreatment or by SRIF itself.

Somatostatin-induced increase of responsiveness of adenylate cyclase to forskolin was reversible (Table IV). Pretreatment for 20 hr produced a 3-fold increase in forskolin's effect. Complete return to control level stimulation occurred 6 hr after removal of the peptide. Four hours of pretreatment with SRIF caused a 2-fold greater stimulation by forskolin and required over 3 hr of SRIF withdrawal for recovery to control responsiveness. Control forskolin-stimulated and basal cyclic AMP formation were not altered during the time course of recovery (Table IV).

The long delay observed in the sensitization of ade-

TABLE I

*Time course for the increase in stimulation of cyclic AMP accumulation following SRIF pretreatment*

The cyclic AMP values represent the mean ± SEM (picomoles of cyclic AMP per well) of three different experiments. Protein content (micrograms) is the average amount of protein in the wells for each time point. No difference in protein content between the wells was observed during the stimulation period (30 min) as compared to basal conditions. In the experimental design, on the fifth day after subculture, the cells had their medium changed. For some, medium with $10^{-7}$ M SRIF was added for 24 hr. For others, control medium was added for 23, 22, 20, 16, or 8 hr, and then medium with SRIF was added for 1, 2, 4, 8, and 16 hr, respectively. In control cells, SRIF-free medium was added for 24 hr. At the end of this time, all of the cells were washed twice with fresh medium and stimulated with CRF ($10^{-7}$ M), forskolin ($10^{-5}$ M), isoproterenol ($10^{-6}$ M), or VIP ($10^{-7}$ M). This protocol allowed for all the cells to live for the same amount of time. Experiments with forskolin and CRF were done on different subcultures than isoproterenol or VIP, which may explain the different protein levels per well and basal cyclic AMP levels. The procedure for stimulating cyclic AMP formation and its measurement is described under "Materials and Methods."

| Treatment | Time of SRIF Pretreatment (hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 | 16 | 24 |
| Basal | 25.4 ± 3 | 22.6 ± 3 | 28.1 ± 3 | 27.3 ± 3 | 28.2 ± 4 | 29.9 ± 2 | 32.4 ± 3 |
| CRF | 155 ± 22 | 160 ± 25 | 163 ± 27 | 162 ± 27 | 169 ± 22 | 228 ± 31 | 265 ± 38 |
| Forskolin | 391 ± 35 | 405 ± 38 | 460 ± 31 | 844 ± 57 | 1092 ± 91 | 1483 ± 122 | 1450 ± 133 |
| Protein content/well | 171 ± 10 | | | 185 ± 10 | 174 ± 22 | 169 ± 10 | 178 ± 10 |
| Basal | 6.8 ± 1 | | | 7.4 ± 1 | 7.0 ± 1 | 7.9 ± 2 | 7.2 ± 1 |
| Isoproterenol | 71.6 ± 9 | | | 73.4 ± 7 | 79.8 ± 8 | 116.6 ± 9 | 128.2 ± 10 |
| VIP | 20.4 ± 4 | | | 22.4 ± 3 | 25.3 ± 4 | 40.8 ± 5 | 47.2 ± 5 |
| Protein content/well | 55 ± 4 | | | 62 ± 6 | 53 ± 3 | 59 ± 4 | 65 ± 6 |

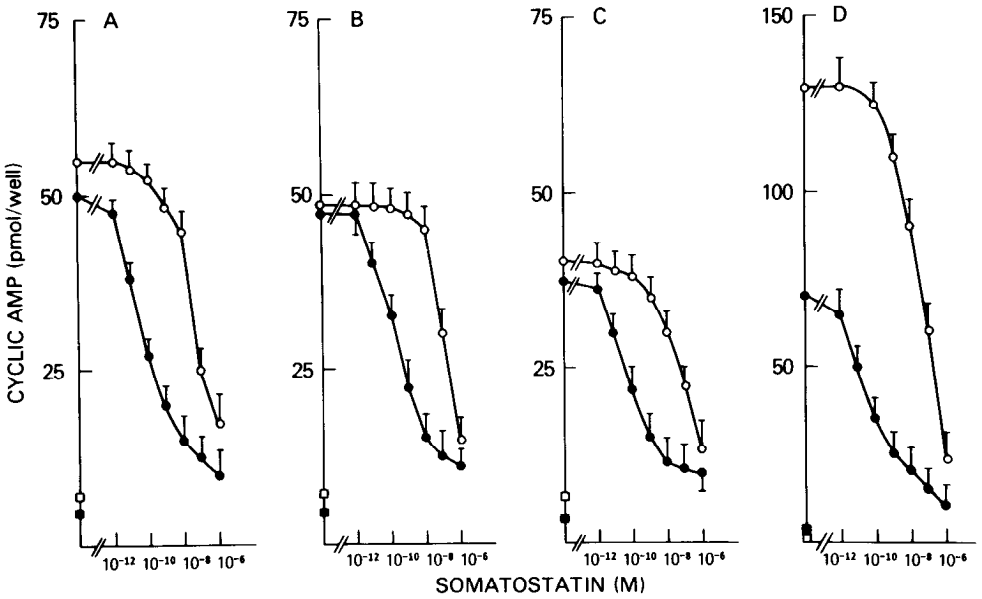


*Figure 2.* SRIF pretreatment desensitizes SRIF receptors negatively linked to the multihormonal stimulation of cyclic AMP accumulation. Cells were pretreated with (○) or without (●) SRIF ($10^{-7}$ M) for 16 hr. At the end of this time the cells were washed, and either CRF ($10^{-7}$ M) (*A*), (−)-isoproterenol ($10^{-6}$ M) (prepared in 100 μM ascorbic acid) (*B*), VIP ($10^{-7}$ M) (Peninsula, prepared as a 100 μM stock solution in acidified PBS (pH 10) and BSA (1%)) (*C*), or forskolin ($10^{-5}$ M) (prepared as a 10 mM solution in ethanol) (*D*) was applied with different concentrations of SRIF. Cyclic AMP accumulation was determined as described under "Materials and Methods." Basal cyclic AMP levels in control (■) and treated (□) cells were not different. The $IC_{50}$ values for SRIF inhibition were: CRF—control, $1.8 \pm 2.1 \times 10^{-10}$ M; treated, $1.2 \pm 0.9 \times 10^{-8}$ M; isoproterenol—control, $4.5 \pm 1.8 \times 10^{-10}$ M, treated, $9.5 \pm 2.1 \times 10^{-9}$ M; VIP—control, $1.1 \pm 1.2 \times 10^{-10}$ M, treated, $2.2 \pm 1.3 \times 10^{-8}$ M; forskolin—control, $1.4 \pm 0.8 \times 10^{-10}$ M; treated, $2.9 \pm 1.4 \times 10^{-8}$ M. Results are the means of three separate experiments.

TABLE II

*Effect of somatostatin pretreatment on cyclic GMP levels*

Cells were pretreated with or without SRIF ($10^{-7}$ M) for 16 hr. At the end of this time the cells were washed and either exposed or not exposed to SRIF ($10^{-7}$ M). The cyclic GMP studies were performed as described under "Material and Methods." Results are the mean ± SEM of three experiments. None of the values are significantly different from each other using a Student's *t* test.

| | Control | Pretreated |
|---|---|---|
| | *pmol/well* | |
| Basal | 3.91 ± 0.1 | 3.9 ± 0.2 |
| Somatostatin ($10^{-7}$ M) | 3.98 ± 0.5 | 4.06 ± 0.3 |

TABLE III

*Lack of effect of SRIF pretreatment on phosphodiesterase activity*

Cells were treated for 20, 4, or 0 hr with SRIF ($10^{-7}$ M). The cells were then processed for use in the phosphodiesterase assay as described under "Materials and Methods." In control tissue SRIF was also applied to test its direct effect on phosphodiesterase activity. Values represent the mean ± SEM of three experiments.

| Treatment | Phosphodiesterase Activity (nmol cyclic AMP/min/mg protein) |
|---|---|
| Control | |
| Basal | 4.86 ± 0.47 |
| SRIF ($10^{-7}$ M) | 4.88 ± 0.08 |
| 4-hr treatment | |
| Basal | 4.42 ± 0.23 |
| 20-hr treatment | |
| Basal | 4.46 ± 0.15 |

nylate cyclase to forskolin by SRIF pretreatment, as well as the slow recovery following this treatment, suggested a requirement for protein synthesis. To test this hypothesis, cycloheximide was co-incubated with SRIF for 4 hr (Table V). At a concentration of 10 μg/ml, cycloheximide inhibits 95% of the incorporation of [$^3$H]leucine into trichloroacetic acid-precipitable protein in corticotrophs (Vale and Rivier, 1977; S. H. Heisler, unpublished results). This agent totally blocked the sensitization of adenylate cyclase to forskolin activation but did not alter basal or control forskolin-stimulated cyclic AMP production after 4 hr of treatment (Table V). A lower concentration of cycloheximide produced less blockade of this effect (Table V). Longer periods (6 to 16 hr) of incubation with cycloheximide also blocked the somatostatin-induced sensitization of adenylate cyclase; however, basal and control forskolin-stimulated cyclic AMP levels were also reduced (Table VI). No visible cellular damage was observed after long-term (6 to 16 hr) cycloheximide treatment (such as marked morphological changes or detachment of the cells from the culture plates); however, the occurrence of cell toxicity or changes in the cell cycle induced by long-term cycloheximide treatment cannot be excluded.

## Discussion

In anterior pituitary tumor cells the sensitivity of adenylate cyclase to different stimulatory agents is mod-

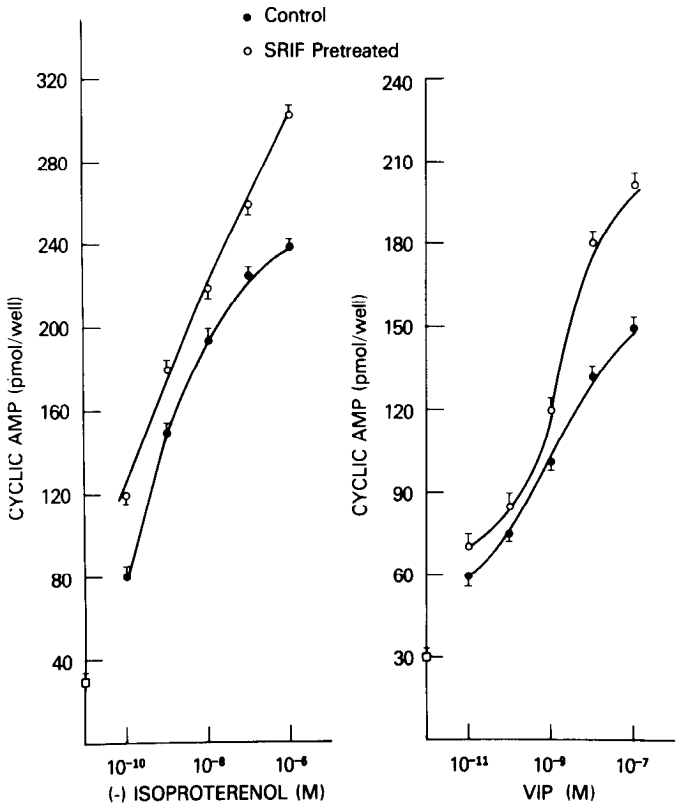


*Figure 3.* SRIF pretreatment increases the maximal ability of (−)-isoproterenol (*left*) or VIP (*right*) to stimulate cyclic AMP formation. Cells were pretreated with (○) or without (●) SRIF ($10^{-7}$ M) for 16 hr. After washing, the cells were incubated with different concentrations of (−)-isoproterenol (in $10^{-4}$ M ascorbic acid) or VIP. Basal cyclic AMP levels (□) were not affected by the pretreatment. The results are the mean ± SEM of three experiments.

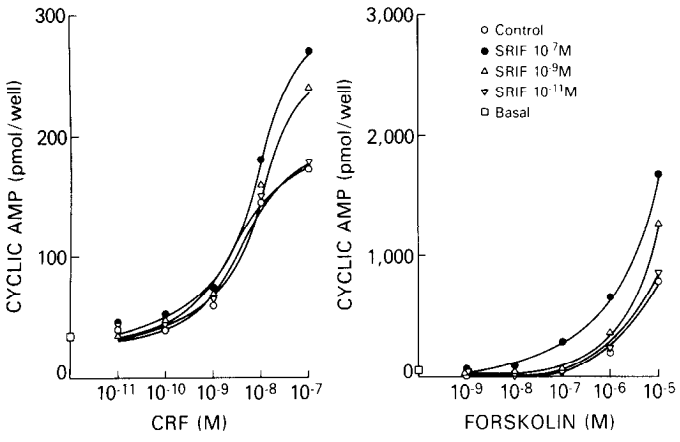


*Figure 4.* Dose-dependent increase in CRF- (*left*) or forskolin-stimulated (*right*) cyclic AMP formation. Cells were treated for 16 hr with 0.01 nM (▽), 1 nM (△), 100 nM (●), or 0 nM (○) SRIF. After washing, the cells were exposed to different concentrations of CRF or forskolin. Results are the means of three experiments. The SEM did not vary by more than 5% for each point. Basal cyclic AMP levels (□) were not affected by the pretreatment.

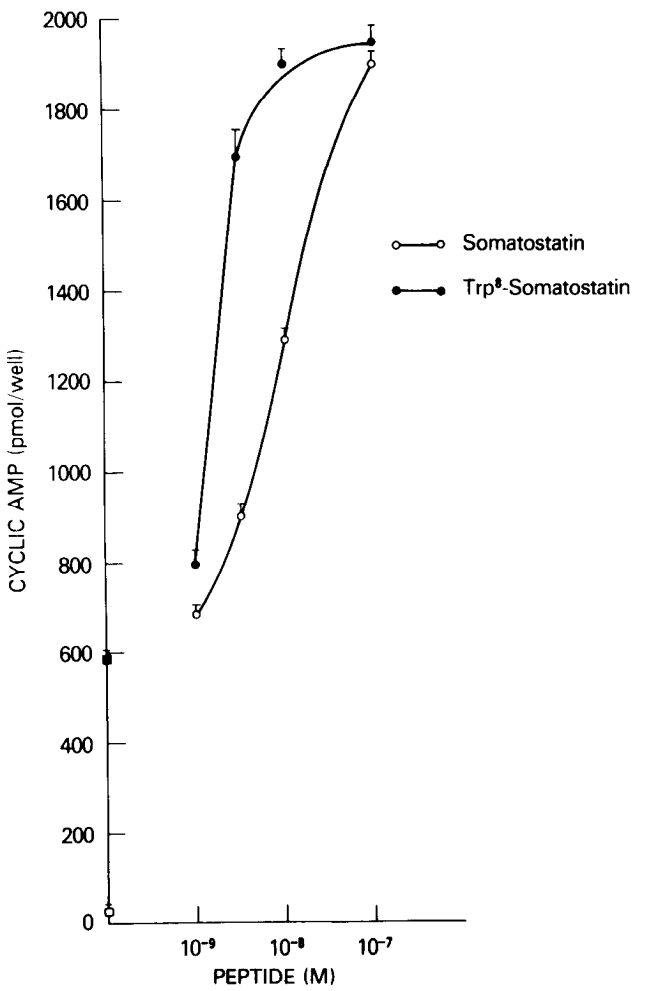


*Figure 5.* Trp$^8$-somatostatin increases forskolin-stimulated cyclic AMP synthesis. Cells were pretreated for 16 hr with different concentrations of SRIF (○) or Trp$^8$-somatostatin (●). At the end of this time the cells were washed and stimulated with forskolin ($10^{-5}$ M). Control forskolin-stimulated (■) and basal (□) cyclic AMP accumulations are also presented. The results are the mean ± SEM of three experiments.

ified by SRIF. The ability of several hormones to increase cyclic AMP accumulation is inhibited acutely by SRIF. Furthermore, SRIF blocks the effects of forskolin. This inhibitory effect requires simultaneous addition of SRIF with the stimulatory agents. In contrast, when AtT-20 cells are pre-exposed to SRIF, the ability of forskolin and hormones to increase intracellular cyclic AMP formation is enhanced. This enhancement occurs after only 4 hr of pretreatment for forskolin and is dependent on the concentration of SRIF to which the cells were exposed. In AtT-20 cell membranes, forskolin-, Gpp(NH)p-, and NaF-stimulated adenylate cyclase activities are increased by SRIF pretreatment; whereas basal adenylate cyclase activity was not altered. Thus, the supersensitive cyclic AMP response appears to result from changes in adenylate cyclase activity and not from a reduction of phosphodiesterase activity.

The cycloheximide blockade of the SRIF-induced increase in forskolin-stimulated cyclic AMP formation suggests that protein synthesis is required for the SRIF-

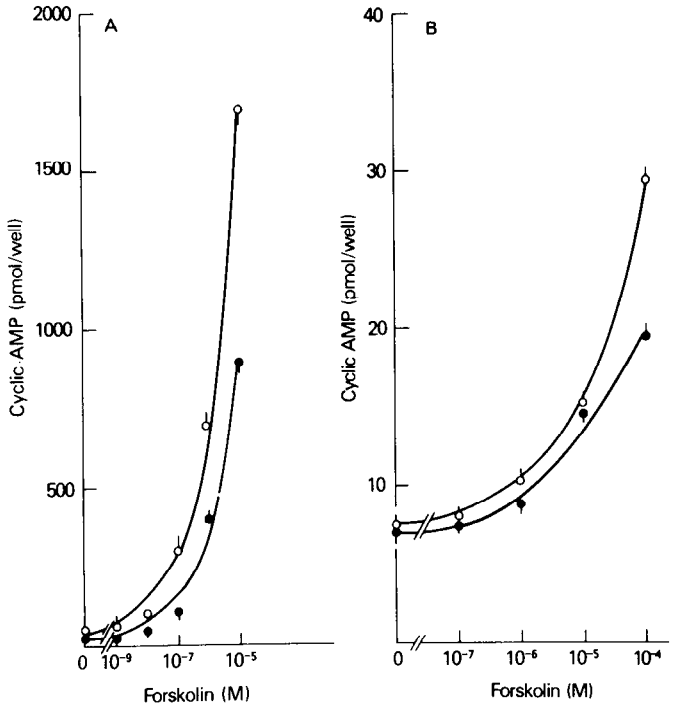


*Figure 6.* Increased forskolin response in the absence of phosphodiesterase inhibitors. Cells were pretreated for 8 hr with (○) or without (●) SRIF ($10^{-7}$ M). After washing, the cells were incubated with different concentrations of forskolin in the presence (*A*) or absence (*B*) of 0.5 mM IBMX. Cyclic AMP formation was then measured as described under "Materials and Methods." Results are the mean ± SEM of three experiments.

induced supersensitive response of adenylate cyclase. Treatment for more than 4 hr with cycloheximide reduced basal and forskolin-stimulated cyclic AMP production and blocked SRIF's enhancing effects on forskolin-stimulated cyclic AMP production. The reduced basal and forskolin-stimulated cyclic AMP responses following cycloheximide treatment longer than 4 hr may reflect the cytotoxic actions of this drug. However, Barovsky et al. (1983) found that treatment of C6 glioma cells with cycloheximide for 24 hr reduced, apparently in a specific manner, forskolin-stimulated cyclic AMP accumulation. These authors suggested from this observation as well as other data that a protein(s) is necessary for forskolin's activation of adenylate cyclase. It is possible that SRIF pretreatment alters the turnover of such a protein or alternatively, SRIF could induce the synthesis of components of the adenylate cyclase holoenzyme.

Barovsky et al. (1983) also observed that cycloheximide treatment did not reduce prostaglandin-stimulated cyclic AMP accumulation and only slightly decreased (−)-isoproterenol's effects on adenylate cyclase. This suggests that forskolin activates adenylate cyclase through mechanisms different from those associated with hormones. Similarly, changes induced by SRIF pretreatment on forskolin- and hormone-stimulated cyclic AMP production were not the same. Much longer pretreatment with SRIF was necessary to increase CRF's, VIP's, and isoproterenol's cyclic AMP response than forskolin's. Furthermore, the magnitude of the increase was greater for forskolin. Thus, SRIF may differentially regulate the mechanisms involved in hormone versus forskolin's activation of adenylate cyclase.

Previous studies have also observed that chronic exposure of tissues to inhibitory hormones can enhance adenylate cyclase activity. Sharma et al. (1977) found that long-term (24 hr) treatment of neuroblastoma cells with morphine increased basal and prostaglandin-stimulated adenylate cyclase activity. Although this increase was partially blocked by cycloheximide, it was not possible to propose that morphine induced the synthesis of new adenylate cyclase molecules because neither NaF- nor Gpp(NH)p-stimulated adenylate cyclase activity was altered by the pretreatment. Likewise, prolonged treatment of neuroblastoma with noradrenaline increased basal and prostaglandin- but not NaF- or Gpp(NH)p-stimulated adenylate cyclase activity (Sabol and Nirenberg, 1979). Noradrenaline's increase of adenylate cyclase activity was not prevented by cycloheximide (Sabol and Nirenberg, 1979). As a result, it was suggested that long-term treatment of neuroblastoma with inhibitory

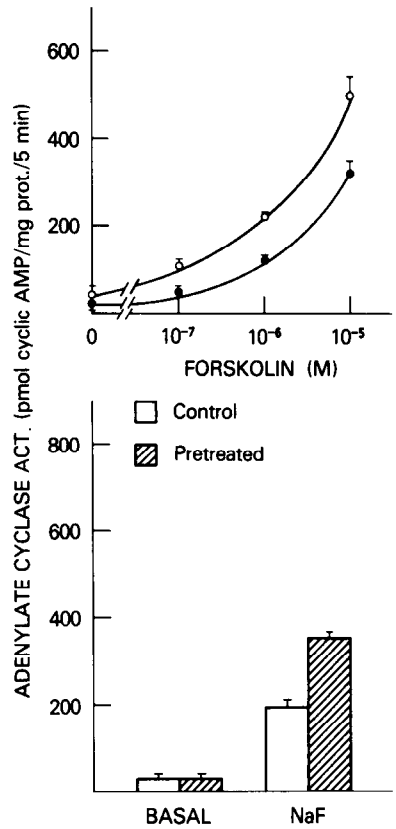


*Figure 7.* Effect of SRIF pretreatment on adenylate cyclase activity. Cells were pretreated with (○) or without (●) SRIF ($10^{-7}$ M) for 16 hr. Tissue was then prepared for adenylate cyclase studies as described under "Materials and Methods." *Top panel,* Membranes were exposed to different concentrations of forskolin for 5 min. Results are the mean ± SEM of six experiments each done in triplicate. *Bottom panel,* Membranes of control (□) and treated (▨) cells were exposed to $10^{-2}$ M sodium fluoride. Values are the mean ± SEM of six separate experiments done in triplicate. Protein content in control tissue was 18.2 ± 1.2 μg; in treated tissue it was 18.5 μg.

TABLE IV

*Recovery of foreskolin-stimulated cyclic AMP formation following SRIF pretreatment*

Values represent the mean ± SEM cyclic AMP levels (picomoles per well) of three different experiments. Some cells were pretreated for 20 hr with SRIF ($10^{-7}$ M). Another group of cells was exposed to control medium for 16 hr and then treated for 4 hr with SRIF-containing medium. Sham-treated cells had control medium for 20 hr. At the end of this pretreatment, cells were washed twice and incubated with control medium for different periods of time. Following each recovery period, cells were stimulated with forskolin ($10^{-5}$ M).

| Treatment | Recovery time (hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Control | | | | | | | |
| Basal | 21 ± 3 | 24 ± 3 | 28 ± 3 | 24 ± 3 | 24 ± 2 | 27 ± 2 | 24 ± 3 |
| Forskolin | 671 ± 50 | 690 ± 71 | 640 ± 56 | 650 ± 60 | 670 ± 69 | 660 ± 71 | 690 ± 53 |
| 4-hr SRIF | | | | | | | |
| Basal | 27 ± 3 | 21 ± 3 | 25 ± 3 | 28 ± 4 | | | |
| Forskolin | 1121 ± 90 | 1132 ± 98 | 1140 ± 81 | 670 ± 93 | | | |
| 20-hr SRIF | | | | | | | |
| Basal | 27 ± 3 | 28 ± 4 | 30 ± 3 | 31 ± 2 | 29 ± 4 | 24 ± 3 | 30 ± 4 |
| Forskolin | 1982 ± 200 | 1720 ± 180 | 1599 ± 190 | 1610 ± 210 | 1615 ± 180 | 1570 ± 140 | 710 ± 80 |

TABLE V

*Effect of cycloheximide on SRIF-induced increase of forskolin-stimulated cyclic AMP accumulation*

Cells were treated with either SRIF, cycloheximide (Cx), or their combination for 4 hr. The cells were washed twice with fresh medium and then stimulated with $10^{-5}$ M forskolin. The cycloheximide was prepared in PBS. The values represent the mean ± SEM cyclic AMP levels of three separate determinations. The values are expressed as the picomoles of cyclic AMP per well formed in 30 min.

| | Control | SRIF ($10^{-7}$ M) | Cx (10 μg/ml) | Cx (5 μg/ml) | Cx (10 μg/ml) + SRIF ($10^{-7}$ M) | Cx (5 μg/ml) + SRIF ($10^{-7}$ M) |
|---|---|---|---|---|---|---|
| Basal | 21 ± 1.5 | 23 ± 3.1 | 21 ± 2.7 | 22 ± 3.0 | 19 ± 2.1 | 21 ± 2.5 |
| Forskolin | 541 ± 41.0 | 886 ± 53.0[a] | 496 ± 59.0 | 507 ± 34.0 | 524 ± 34.0 | 771 ± 70.0[a] |

[a] Values significantly different from control forskolin-stimulated cyclic AMP formation at the level of $p \leq 0.05$ using the Student's $t$ test.

TABLE VI

*Time course of cycloheximide's effect on the somatostatin-induced increase in forskolin-stimulated cyclic AMP accumulation*

Cells were treated with either SRIF, cycloheximide (10 μg/ml), or their combination for 4, 6, 8, or 16 hr. At the end of this time the cells were washed and stimulated with forskolin ($10^{-5}$ M). The values represent the mean ± SEM of three separate experiments and are expressed as picomoles of cyclic AMP per well formed in 30 min.

| Time | Control | | Somatostatin | | Somatostatin + Cycloheximide | | Cycloheximide | |
|---|---|---|---|---|---|---|---|---|
| | Basal | Forskolin | Basal | Forskolin | Basal | Forskolin | Basal | Forskolin |
| *hr* | | | | | | | | |
| 4 | 9.2 ± 0.2 | 300 ± 20.0 | 11.0 ± 1.7 | 537 ± 30[a] | 9.3 ± 1.1 | 333 ± 30 | 8.3 ± 0.9 | 295 ± 15 |
| 6 | 13.3 ± 1.3 | 319 ± 27 | 11.7 ± 1.1 | 602 ± 41[a] | 5.8 ± 0.7 | 304 ± 35 | 9.4 ± 1.0 | 255 ± 38 |
| 8 | 12.1 ± 2.0 | 370 ± 20 | 11.1 ± 1.0 | 704 ± 45[a] | 9.7 ± 0.2 | 344 ± 18 | 8.3 ± 2.0 | 170 ± 0.4[b] |
| 16 | 12.2 ± 2.1 | 261 ± 20 | 17.9 ± 1.6 | 874 ± 40[a] | 10.0 ± 1.0 | 461 ± 25[a] | 5.5 ± 0.5[c] | 139 ± 18[b] |

[a] Significantly greater ($p \leq 0.05$) than control forskolin values at correspondingly similar pretreatment times using the Student's $t$ test.

[b] Significantly less ($p \leq 0.05$) than control forskolin values at correspondingly similar pretreatment times using the Student's $t$ test.

[c] Significantly less ($p \leq 0.05$) than control basal values at the 16-hr time point using the Student's $t$ test.

hormones produces a prolonged activation of adenylate cyclase rather than changes in the synthesis rate of this enzyme (Sharma et al. 1977; Sabol and Nirenberg, 1979).

These previous findings in neuroblastoma cells clearly differ from the effects of SRIF pretreatment on AtT-20 cells. SRIF pretreatment increases forskolin-, NaF- and Gpp(NH)p-stimulated adenylate cyclase activity as well as forskolin- and hormone-stimulated cyclic AMP formation. Neither basal adenylate cyclase activity nor basal cyclic AMP accumulation was changed by SRIF treatment. The time course for the increased cyclic AMP response was more rapid than the morphine effect. Furthermore, SRIF pretreatment also desensitized SRIF receptors. Whether this desensitization is directly linked to the enhancement of adenylate cyclase activity or is unrelated is not clear, although the time courses for these processes are similar. These data indicate that the effects of SRIF pretreatment on the regulation of adenylate cyclase activity are not the same as those previously reported for other hormones (Sharma et al., 1977; Sabol and Nirenberg, 1979) and probably involve a unique alteration of the interplay between stimulatory and inhibitory influences on adenylate cyclase activity.

Both SRIF self-regulation and the accompanying rises in cyclic AMP synthesis are not a phenomenon restricted to tumor cells. Recently, Smith and Vale (1980) observed

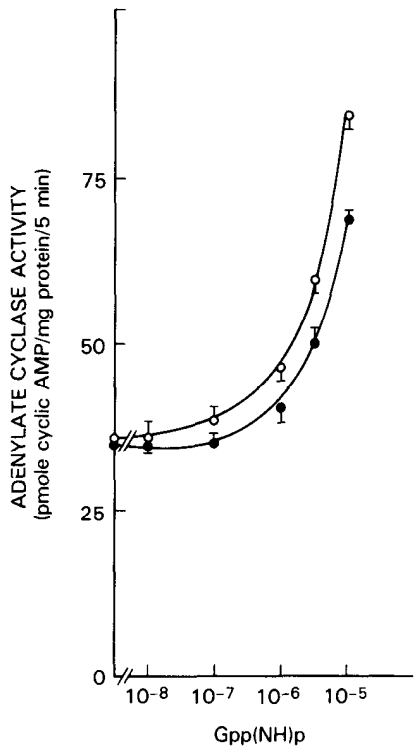


*Figure 8.* Effect of SRIF pretreatment on Gpp(NH)p-stimulated adenylate cyclase activity. Cells were pretreated with (○) or without (●) SRIF ($10^{-7}$ M) for 16 hr. Cells were then rinsed with PBS (pH 7.4) and processed for adenylate cyclase studies. Gpp(NH)p at different concentrations was added to the tissue under nonequilibrium conditions (30-min pre-incubation at 4°C). Results are the mean ± SEM of three experiments done in triplicate. Protein content was 13.2 ± 1.0 μg and 13.1 ± 1.1 μg in control and treated tissue, respectively.

that continuous but not pulsatile application of SRIF to rat anterior pituitary cells made those cells refractory to SRIF's inhibitory effects on growth hormone and thyroid-stimulating hormone release. Interestingly, cyclic AMP levels appeared higher in the SRIF-pretreated cells. These results, as well as those obtained with AtT-20 cells, suggest that the cellular mechanisms of adaptation to SRIF involve both SRIF desensitization which is due to either a downregulation of SRIF receptors or their uncoupling from adenylate cyclase and a compensatory enhancement of adenylate cyclase's sensitivity to stimulants. Such adaptive changes may, as previously proposed (Sharma et al. 1977), provide a mechanism for long-term cellular memory.

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