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Somatostatin inhibits multireceptor stimulation of cyclic AMP formation and corticotropin secretion in mouse pituitary tumor cells

(corticotropin-releasing factor/vasoactive intestinal peptide/β_2-adrenergic agonists)

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ABSTRACT **The AtT-20/D16-16 mouse pituitary tumor cell secretes corticotropin (ACTH) in response to corticotropin-releasing factor (CRF), (−)-isoproterenol, and vasoactive intestinal peptide (VIP). These responses are associated with a rapid increase in cyclic AMP formation. Somatostatin (SRIF) markedly decreases the stimulatory effect of CRF, (−)-isoproterenol, and VIP on both cyclic AMP formation and immunoreactive ACTH secretion. Forskolin and cholera toxin, adenylate cyclase activators, also stimulate cyclic AMP formation and ACTH secretion in AtT-20 cells and these responses are all inhibited by SRIF. The ACTH secretory responses to melittin and to the calcium ionophore A23187, neither of which increases cyclic AMP in AtT-20 cells, were not inhibited by SRIF. SRIF did not affect the binding of a tritiated β-adrenergic receptor antagonist to AtT-20 membranes nor did it decrease basal cyclic AMP formation even in the presence of excess phosphodiesterase inhibitor, indicating that the reduction of cyclic AMP levels by SRIF did not involve either an interference with β-adrenergic agonist binding to receptors or stimulation of cyclic AMP degradation. These results indicate that the inhibition of CRF-, (−)-isoproterenol-, and VIP-stimulated ACTH secretion by SRIF may be regulated by its inhibitory action on adenylate cyclase.**

The AtT-20 mouse pituitary cell line has been extensively used to investigate the regulation of synthesis, storage, and secretion of the corticotropin (ACTH)/β-endorphin family of peptides (1–8). Recent studies in our laboratory have demonstrated the responsiveness of this clonal cell line to synthetic corticotropin-releasing factor (CRF) (9), to β_2-adrenergic agonists, and to vasoactive intestinal peptide (VIP) (unpublished data). All three substances increase cyclic AMP formation in the tumor cells and the combination of any two of the agents on cyclic AMP synthesis is additive (unpublished data).

The tetradecapeptide somatostatin (SRIF) is known to inhibit ACTH secretion in AtT-20 tumor cells stimulated by high concentrations of potassium or by hypothalamic extracts (10). SRIF has a marked inhibitory effect on the elevated ACTH levels found in patients with Nelson syndrome (11, 12), with Addison disease (13), or after bilateral adrenalectomy (12). In nonpathologic states, basal ACTH appears to be unaffected by SRIF (14). SRIF receptors have been identified in both anterior pituitary membranes (15) and in pituitary tumor cells (14, 16). We report that SRIF inhibits ACTH secretion stimulated by CRF, (−)-isoproterenol, and VIP from mouse pituitary tumor cells, and we present evidence suggesting that this effect is caused by SRIF-induced inhibition of cyclic AMP synthesis.

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MATERIALS AND METHODS

Materials. A23187 and forskolin were obtained from Calbiochem–Behring. Synthetic CRF, SRIF, and VIP (porcine) were purchased from Peninsula Laboratories (San Carlos, CA). Bacitracin, barbital, cholera toxin, human serum albumin, (−)-isoproterenol (+)-bitartrate, 3-isobutyl-1-methylxanthine (IBMX), and melittin were from Sigma. [^{3}H]Dihydroalprenalol ([^{3}H]DHA; 43 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and cyclic AMP radioimmunoassay kit were from New England Nuclear. Dulbecco's modified Eagle's medium (DME medium) was obtained from GIBCO and trypsin was from Worthington. Fetal calf serum was purchased from North American Biologicals (Miami, FL). ACTH standard (human) and antiserum were generous gifts from the National Pituitary Agency (Baltimore, MD). ^{125}I-labeled ACTH was obtained from Immunonuclear (Stillwater, MN). Goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). All other chemicals were of reagent grade.

Cell Culture Methods. AtT-20/D16-16 cells were grown in DME medium containing 10% fetal calf serum at 37°C in a humidified atmosphere of 10% CO_2/90% air as described (9).

Incubation Procedure. Cells were plated in 35-mm diameter culture dishes (Costar) at an initial density of $1.5–2 \times 10^5$ cells per well and were used 5–7 days after subculturing (60–80% confluency). Before use, cells were equilibrated for 60 min at 37°C in 1 ml of DME medium/25 mM Hepes, pH 7.4, containing 2% fetal calf serum and bacitracin at 3 μg/ml. For ACTH secretion studies, the medium was aspirated and replaced with 1 ml of the same medium containing added test agent(s), and the cells were incubated for an additional 60 min. An aliquot of the medium was removed and centrifuged to remove detached cells and debris. The supernatant was frozen at −20°C until used for ACTH measurement. For cyclic AMP studies, cells were preincubated for 30 min with 0.5 mM IBMX in fresh medium. This medium then was aspirated and replaced with 1 ml of fresh culture medium containing 0.5 mM IBMX and added test agent(s) and the cells were incubated for 15 min. The medium was aspirated and 1 ml of 10 mM acetic acid containing 0.5 mM IBMX was added to the cells. The cells were detached with a rubber policeman and the suspension was sonicated. A 0.1–0.2 ml aliquot was dried by vacuum centrifugation and the pellets were resuspended in 0.1 ml of 50 mM sodium acetate buffer (pH 6.2) and used directly for cyclic AMP measurement. In experiments in which (−)-isoproterenol was used, 10 μM ascorbic acid was added to the medium. Forskolin was

Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; DHA, dihydroalprenolol; DME medium, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine; SRIF, somatostatin; VIP, vasoactive intestinal peptide.

dissolved in ethanol and diluted in phosphate-buffered saline (pH 7.4). The highest concentration of ethanol to which cells were exposed did not alter unstimulated cyclic AMP synthesis or ACTH secretion (data not shown). All other test agents were added from 100-fold concentrated stock solutions.

[^{3}H]DHA Binding Assay. AtT-20 tumor cell membranes were prepared as follows. Plated cells were washed with phosphate-buffered saline, homogenized (Brinkmann Polytron, setting 3 for 5 sec), centrifuged at 100,000 × *g*, and the supernatant then was decanted. The pellet was resuspended in 50 mM Tris·HCl buffer (pH 7.8), homogenized, and recentrifuged. After resuspension of the pellet in Tris·HCl buffer, aliquots (≈50 μg of protein) of the tissue homogenate were incubated in polystyrene tubes for 30 min at 25°C with [^{3}H]DHA (8 nM) and either varying concentrations of SRIF or 10 μM *d,l*-propranolol, which was used to define specific tissue binding. The reaction was terminated by vacuum filtration over GF/C (Whatman) glass fiber filters. The dried filters were placed in Hydrofluor (New England Nuclear) and radioactivity was measured by liquid scintillation spectrometry.

ACTH and Cyclic AMP Assays. ACTH immunoreactivities were assayed in secreted medium as described (9) by using antiserum specific for the 14–24 region of ACTH. Cyclic AMP levels were measured by using a commercially available assay kit from New England Nuclear.

RESULTS

The effect of SRIF on basal and (−)-isoproterenol-induced release of immunoreactive ACTH is illustrated in Fig. 1. SRIF alone had little effect on basal secretion of ACTH in concentrations between 10^{-11} and 10^{-8} M but decreased basal secretion at 10^{-7} M by about 50%. (−)-Isoproterenol increased ACTH secretion about 2.5-fold. SRIF had a marked inhibitory effect on (−)-isoproterenol-induced ACTH secretion. At the highest concentration of SRIF tested, (−)-isoproterenol-stimulated secretion was decreased to control levels. (−)-Isoproterenol caused a 9-fold increase in cyclic AMP levels. SRIF markedly decreased (−)-isoproterenol-stimulated cyclic AMP formation within the same concentration range that effectively decreased the ACTH secretory response to (−)-isoproterenol (Fig. 1). At all concentrations utilized, SRIF alone had no effect on cyclic AMP synthesis in the absence of (−)-isoproterenol.

The two highest effective concentrations of SRIF from the above study were tested on CRF- and VIP-stimulated increases in cyclic AMP formation and ACTH secretion. CRF and VIP alone increased ACTH secretion 3- and 2.5-fold, respectively (Fig. 2). These effects were decreased by SRIF. CRF and VIP increased cyclic AMP levels about 9- and 3.5-fold, respectively, and these effects were also blocked by SRIF.

The interaction of SRIF with forskolin and cholera toxin, compounds which activate adenylate cyclase and bypass cell surface receptors (17–22), was investigated. Both forskolin and cholera toxin strongly stimulated cyclic AMP formation and ACTH release from mouse pituitary tumor cells (Fig. 3). Forskolin increased cyclic AMP levels 25-fold and caused a 5-fold increase in ACTH release. These effects were almost completely blocked by 10^{-7} M SRIF. Cholera toxin elevated cyclic AMP levels 35-fold and ACTH release 7.5-fold. SRIF decreased both responses by 50–60%.

Melittin, a phospholipase A_2 activator (23, 24), was also found to evoke ACTH release from mouse pituitary tumor cells (Fig. 4). The calcium ionophore, A23187, which elicits secretion from numerous endocrine and exocrine cell systems, also stimulates ACTH secretion from the tumor cells (Fig. 4). Unlike (−)-isoproterenol, CRF, or VIP, melittin did not increase cyclic AMP levels in the mouse pituitary tumor cells (Fig. 4). The calcium ionophore had a marginal effect on cyclic nucleotide levels. These observations suggest that melittin and A23187 stimulate ACTH secretion by a cyclic AMP-independent pathway. SRIF did not antagonize the ACTH secretory response to either melittin or A23187 (Fig. 4).

To test whether SRIF decreased cyclic AMP formation in the

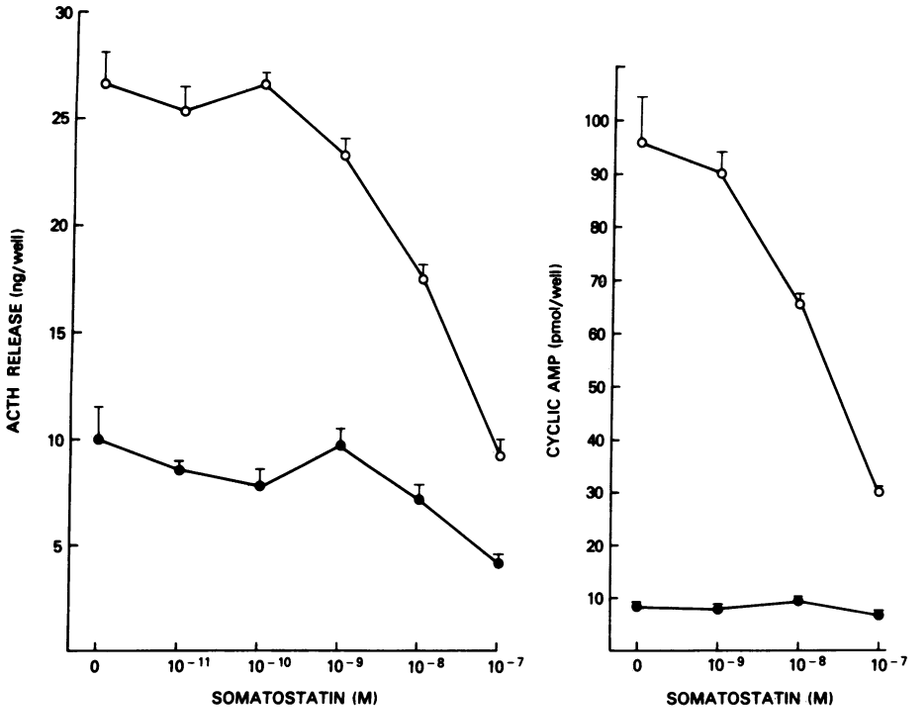

FIG. 1. Somatostatin-induced inhibition of (−)-isoproterenol-stimulated ACTH secretion and cyclic AMP formation. Immunoreactive ACTH secretion was measured after a 60-min incubation with or without test agents; cyclic AMP was measured after a 15-min incubation. ●, Somatostatin; ○, 10^{-6} M (−)-isoproterenol and somatostatin. Values are means ± SEM.

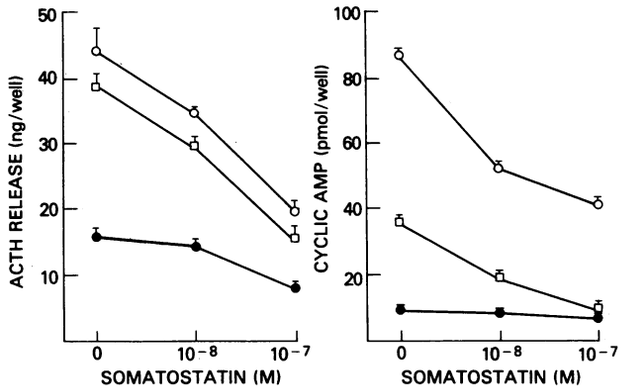

FIG. 2. Somatostatin-induced inhibition of CRF- and VIP-stimulated ACTH secretion and cyclic AMP formation. Immunoreactive ACTH secretion was measured after 60 min of somatostatin incubation with 10^{-7} M CRF (○) or 10^{-7} M VIP (□) or somatostatin without either CRF or VIP (●). Values are means ± SEM.

cells by interfering with agonist binding to receptors, the effect of SRIF on binding of a β-adrenergic antagonist, [^{3}H]DHA, was measured. Specific [^{3}H]DHA binding to AtT-20 pituitary tumor cell membranes was completely inhibited by 10 μM *d,l*-propranolol and accounted for 70% of total tissue binding. In a concentration range of 10^{-10} to 10^{-6} M, SRIF did not alter [^{3}H]DHA binding to AtT-20 cell membranes nor did it affect nonspecific, nontissue binding (data not shown).

SRIF had no effect on basal cyclic AMP synthesis in the presence or absence of IBMX, indicating that SRIF inhibited only agonist-stimulated cyclic AMP synthesis.

DISCUSSION

ACTH secretion in mouse AtT-20 pituitary cells is under multireceptor control, as illustrated in Fig. 5. Synthetic CRF, (−)-isoproterenol, or VIP stimulates an increase in cyclic AMP formation that is accompanied by an increase in ACTH secretion. CRF was also reported to elevate cyclic AMP formation and increase ACTH secretion in primary cultures of dispersed rat anterior pituitary cells (26, 27). Our observations that ACTH secretion is stimulated by forskolin and cholera toxin, compounds that stimulate adenylate cyclase (17–22), and by 8-bromo-cyclic AMP (28) indicate that intracellular cyclic AMP mediates, in part, the ACTH secretory response to hormones. In a separate study (29) we found that CRF and (−)-isoproterenol share a convergent secretory pathway, distal to the production of cyclic AMP, whereas VIP and CRF or VIP and (−)-isoproterenol stimulate ACTH secretion in an additive manner. Calcium also is essential for CRF, VIP, or (−)-isoproterenol-induced secretion (7, 29).

In the present study, SRIF decreased CRF-, (−)-isoproterenol-, and VIP-elevated intracellular cyclic AMP levels and ACTH secretion. That SRIF probably exerts its effects via a specific receptor interaction is supported by the observation that SRIF receptors are present on AtT-20 cells (10, 16).

The site or mechanism of action by which SRIF inhibits cyclic AMP formation is unknown. We have observed that SRIF does not decrease basal (unstimulated) cyclic AMP levels in the cells, suggesting that the peptide exerts a regulatory influence on cyclic AMP formation only in the presence of extracellular stimulants. It is unlikely that SRIF acts to decrease cyclic AMP formation by interfering with agonist binding to receptors because it did not block the binding of the β-antagonist dihydroalprenolol to AtT-20 cells. It is improbable that SRIF lowers agonist-stimulated cyclic AMP synthesis by increasing the activity of phosphodiesterase because IBMX, a potent phosphodiesterase inhibitor, was present in great excess in incubation media during cyclic AMP studies. The lack of a SRIF effect on basal cyclic AMP synthesis, together with the above observations, suggests that SRIF acts by inhibiting adenylate cyclase.

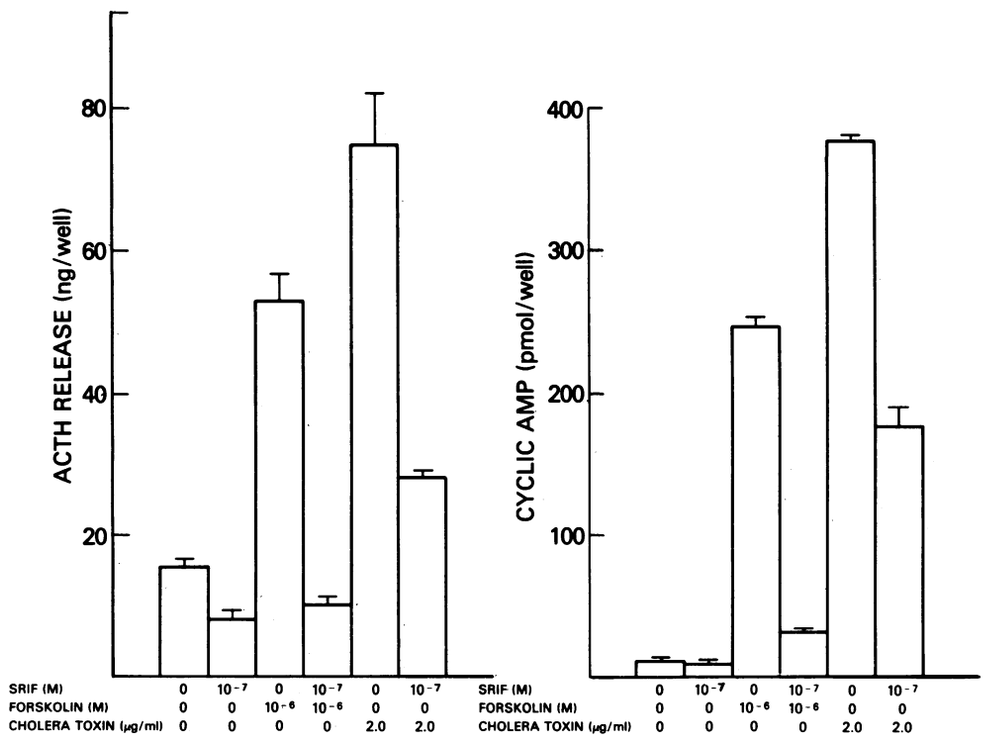

FIG. 3. Somatostatin-induced inhibition of forskolin- and cholera toxin-stimulated ACTH secretion and cyclic AMP formation. Immunoreactive ACTH secretion was measured after a 60-min incubation with or without test agents; cyclic AMP was measured after a 15-min incubation. Values are means ± SEM.

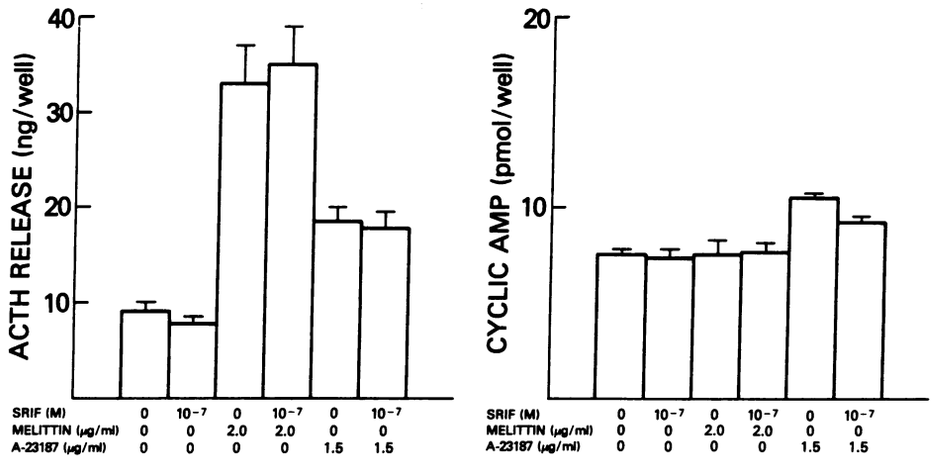

FIG. 4. Melittin- and A23187-stimulated ACTH secretion: lack of a somatostatin inhibitory action. Immunoreactive ACTH secretion was measured after a 60-min incubation with or without added test agents; cyclic AMP was measured after a 15-min incubation. Values are means ± SEM.

Adenylate cyclase is part of a complex regulatory system mediating the effect of numerous hormones and neurotransmitters. In general, the system (Fig. 5) consists of a membrane receptor (R), a catalytic unit (C), and a guanyl nucleotide regulatory (N) unit (see ref. 25). Two functional types of N units have been described, one mediating the stimulation (N_s) and the other mediating the inhibition (N_i) of adenylate cyclase by GTP. Both forskolin and cholera toxin activate adenylate cyclase through different mechanisms. Forskolin appears to activate the cyclase at the catalytic unit independently of cell surface receptors, guanyl nucleotide, or calcium/calmodulin (17–22). On the other hand, cholera toxin stimulates adenylate cyclase in a variety of cell membranes by covalently modifying the N_s unit and by allowing GTP to stimulate the cyclase (20–22). The ability of SRIF to block the effects of both forskolin and cholera toxin on cyclic AMP synthesis (and ACTH secretion) suggests that SRIF has a direct effect on the catalytic unit of the cyclase or, alternately, acts on an inhibitory N unit (N_i) coupled to the catalytic unit of the enzyme (Fig. 5).

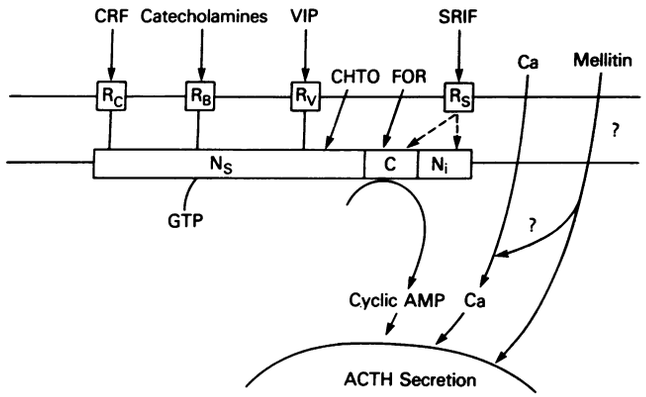

FIG. 5. Multireceptor regulation of ACTH secretion. Regulation of ACTH secretion may involve the following molecular and cellular events. CRF, catecholamines (β_2-agonists), and VIP bind to their respective receptors (R_C, R_B, and R_V), allowing the guanyl nucleotide unit (N_S) of the adenylate cyclase system, along with GTP, to stimulate the catalytic unit (C) (see ref. 25). This leads to the production of intracellular cyclic AMP and, in the presence of calcium, an enhanced release of ACTH. (For simplicity, a single N_S unit is depicted for all three agonists.) Cholera toxin (CHTO) and forskolin (FOR) bypass surface receptors and activate adenylate cyclase at the N_S and C units, respectively. SRIF binds to a specific receptor (R_S), possibly allowing a different guanyl nucleotide unit (N_i) to inhibit adenylate cyclase activity in the presence of agonist and GTP. Alternatively, SRIF may directly inhibit the C unit of adenylate cyclase. The mechanism of action of melittin is unknown but it may trigger ACTH secretion like the calcium ionophore A23187 by facilitating calcium entry or by activating phospholipase A_2, or both.

Both melittin, a phospholipase A_2 stimulator (23, 24) and a secretogogue in the exocrine pancreas (30), and the calcium ionophore A23187 cause release of granule-stored products from numerous cells. Both of these substances stimulated ACTH secretion from AtT-20 cells without an increase in the production of cyclic AMP. SRIF did not block either of these secretory responses, again suggesting that activation of adenylate cyclase is a specific prerequisite for the inhibitory effect of SRIF. Richardson and Schonbrunn (10) reported that the ability of SRIF to inhibit ACTH secretion in AtT-20 cells, stimulated either by high concentrations of potassium or by hypothalamic extracts, was reversed by increasing extracellular calcium. We have found that both cyclic AMP and calcium are required for ACTH secretion in AtT-20 cells (unpublished data). The inhibition of either cyclic AMP synthesis or calcium utilization therefore would be expected to result in the blockade of ACTH secretion. Our findings are consistent with those of Richardson and Schonbrunn (10) but show that the site of action of SRIF is on cyclic AMP synthesis.

Abnormalities of ACTH secretion are found in a wide array of pathologic conditions, including Cushing disease, Addison disease, and endogenous depression. In addition, plasma ACTH is often elevated during stress. Among the many varieties of stress are those caused by somatic pain or emotional distress. Our results suggest that hypersecretion of ACTH might be linked to the stimulatory influence of CRF, catecholamines, or VIP, acting alone or in combination depending upon the pathologic state or the type of stress, and that SRIF acts by modulating the exaggerated release of ACTH.

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