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Studies on Action of Somatostatin on Growth Hormone Release in Relation to Calcium and cAMP (40543)

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Somatostatin (SRIF) has been shown to inhibit the release of not only growth hormone (GH) (1) but also several other peptide hormones (2–6). Since SRIF inhibits the release of hormones from various secretory tissues, presence of a common step in the release process is suggested. However, there are discrepancies among the previous findings on the mechanism of action of SRIF. Some studies showed that SRIF decreases the concentration of cyclic 3′,5′-AMP (cAMP) through inhibition of adenylate cyclase in the pituitary gland (7, 8), whereas other studies suggested that it acts beyond the formation of cAMP in inhibiting the release of GH or insulin (4, 9). On the other hand, it was suggested that the inhibition by SRIF of the release of GH or insulin results from a decrease in calcium (Ca^{2+}) influx (5, 10–15). However, in other reports SRIF was shown to inhibit the release of these hormones without any detectable effect on Ca^{2+} efflux (16–18) or influx (18).

In the present work, we examined the effect of SRIF on GH release in relation to Ca^{2+} and cAMP metabolism. We used two types of secretagogue which are known to stimulate GH release *in vitro* (19). The first type includes agents which are known to increase intracellular cAMP levels, such as dibutyryl cAMP (DBcAMP) (a derivative of cAMP) and prostaglandin E_1 (PGE_1) (a stimulator of adenylate cyclase), and the second type includes agents which increase Ca^{2+} influx, such as high K^+ (a membrane depolarizer) and calcium ionophore A23187.

Materials and methods. Chemicals were obtained from the following sources: synthetic SRIF (20), a generous gift from Dr. S. Sawano, Toranomon Hospital; A23187, kindly donated by Dr. R. R. Hamill of Eli Lilly; DBcAMP, Boehringer-Mannheim; PGE_1, kindly supplied by Ono Pharmaceutical Company, Japan; Dulbecco's modified Eagle's Medium (DMEM), viokase and fetal calf serum, Grand Island Biological Company; collagenase, Sigma Chemical Company; horse serum, The Research Foundation for Microbial Diseases of Osaka University.

Primary cultures of anterior pituitary cells from adult male Wistar rats were performed according to the method of Vale *et al.* (21). The dispersed cells ($0.3–0.7 \times 10^6$ per hemipituitary) obtained by treatment with collagenase and viokase were distributed to 35 × 10-mm Falcon petri dishes containing 2.0 ml of the growth medium, DMEM (the concentration of $NaHCO_3$ reduced to 2.2 g/liter) supplemented with 10% horse serum, 2.5% fetal calf serum, 1% GIBCO-nonessential amino acid, 100 U/ml penicillin, and 100 μg/ml streptomycin. Then cells were incubated at 37° under water-saturated atmosphere of 5% CO_2 and 95% air. Culture medium was changed 3 days after plating. For studies of GH release, cells on day 4 were washed three times with DMEM without sera and preincubated for 1 hr at 37° in the same medium. Then the medium was replaced with the fresh serum-free medium containing substances to be tested and the cells were further incubated for 3 hr. Medium was removed, diluted with an equal volume of 1% BSA–phosphate-buffered saline, and stored at −20° until assayed. GH content in the medium was measured by double-antibody radioimmunoassay (22). Results were expressed as microgram equivalents of the rat GH reference standard (NIAMD rat GH-RP-1).

When the concentration of K^+ in the medium was raised, the concentration of Na^+ was decreased by equivalent amount so that isotonicity was maintained. A23187 and PGE_1 were dissolved in dimethyl sulfoxide (DMSO) to be used. DMSO was added for final concentrations which never exceed 0.1% to all incubation media. DMSO (0.1%) alone had no effect on the hormone release. A23187 was dissolved in the medium immediately

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prior to the incubation. To examine the effect of the concentration of extracellular Ca^{2+} (Fig. 1), the media containing different amounts of Ca^{2+} were made by adding appropriate amounts of $CaCl_2$ to Ca^{2+}-depleted DMEM which was prepared by omission of $CaCl_2$ and by replacement of Ca pantothenate with Na pantothenate. When Ca^{2+} concentration was indicated at 0 m*M*, 0.2 m*M* ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was further added to Ca^{2+}-depleted DMEM. Under this condition, pituitary cells remained as monolayer and were not detached from the dish although some cells became round.

Results. None of four secretagogues tested stimulated GH release under Ca^{2+}-free condition, indicating that they require Ca^{2+} in the media for stimulation of GH release (Fig. 1). The basal GH release increased with the concentration of Ca^{2+} up to 1.8 m*M* and leveled off above this concentration. Maximum release of GH in the presence of DBcAMP or PGE_1 was also obtained at 1.8 m*M* Ca^{2+}. Further increase of GH release was not observed by increasing the concentration of Ca^{2+} up to 7.2 m*M*. On the other hand, GH release caused by high K^+ continued to increase with the concentration of Ca^{2+} up to 10.8 m*M*. GH release by A23187 was maximally stimulated at 3.6 m*M* or higher concentration of Ca^{2+}. Maximal stimulatory effect of A23187 was obtained at 5 to 10 *μM*.

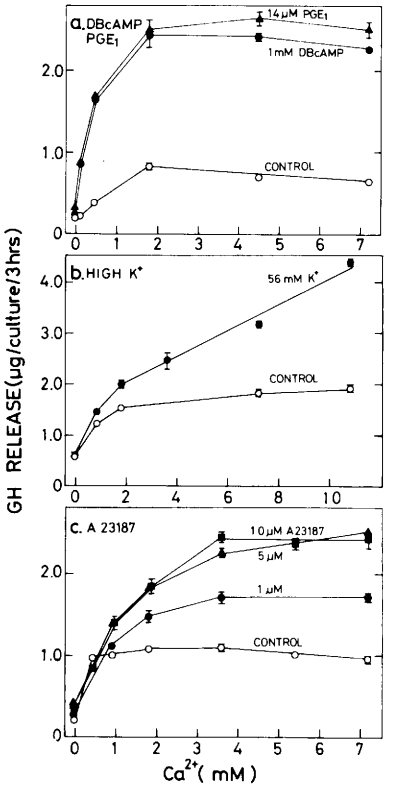

FIG. 1. Effect of extracellular calcium on GH release stimulated by (a) DBcAMP (1 m*M*), PGE_1 (14 *μM*), (b) high K^+ (56 m*M*), or (c) A23187 (1, 5, 10 *μM*). Incubations and assays were performed as described under Materials and Methods. Control represents the value in the absence of stimulant. Each point represents the mean ± SEM of three cultures.

Dose–response curves for the effect of SRIF on the basal and stimulated GH release, which were determined by 3-hr incubation, are shown in Fig. 2. Both the basal GH release and the DBcAMP- or PGE_1-induced GH release were almost completely inhibited by the simultaneous addition of 0.1 *μM* SRIF. On the other hand, the increment of GH release induced by high K^+ or A23187 was not prevented by high dose (0.1 to 1 *μM*) of SRIF both at normal and high concentrations of Ca^{2+}, although total GH release was decreased by SRIF. At 1.8 m*M*, not at 5.4 m*M*, the increment of GH release induced by A23187 was somewhat inhibited by high dose of SRIF. That is, the increment of GH release induced by A23187 in the presence of high dose of SRIF was 60–70% of that without SRIF.

Time courses of GH release by secretagogues in the absence and presence of SRIF are shown in Fig. 3. GH release stimulated by DBcAMP or PGE_1 increased linearly with incubation time in the absence of SRIF. This release was completely inhibited by 0.1 *μM* SRIF, although the levels of GH in the media rose slightly after 3 hr of incubation. On the other hand, high K^+ induced a largest rate of GH release during the first 30 min. Then the rate of release decreased to a basal release rate at 3 hr after the start of incubation. During the initial rapid release SRIF decreased the increment of GH accumulation induced by high K^+ by about 60% at both 1.8 m*M* and high Ca^{2+}. At 3 hr SRIF showed little inhibitory effect on the increment of GH release induced by high K^+ in the presence of both concentrations of Ca^{2+}, similar results being shown in Fig. 2. GH release stimulated

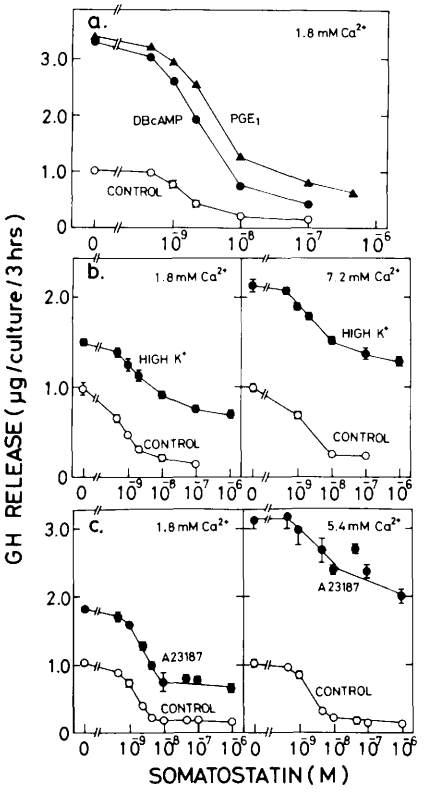

FIG. 2. Effect of SRIF on GH release stimulated by (a) DBcAMP (1 m*M*), PGE_1 (14 μM), (b) high K^+ (56 m*M*), or (c) A23187 (10 μM). Incubations and assays were performed as described under Materials and Methods. Each point represents the mean ± SEM of three cultures.

by A23187 increased with incubation time till about 3 hr at both 1.8 m*M* and high Ca^{2+}. The increment of GH accumulation induced by A23187 in the presence of 1.8 m*M* Ca^{2+} was inhibited by SRIF by about 60 and 40% during the incubation of 30 min and 3 hr, respectively. At 5.4 m*M* Ca^{2+}, however, SRIF affected GH release induced by A23187 in a similar fashion to that observed in the presence of high K^+.

High K^+ or A23187 together with DBcAMP or PGE_1 stimulated GH release more than that obtained with individual stimulus alone, although the increment caused by the combination of A23187 and PGE_1 was only slight. GH releases in the presence of SRIF were always higher when two agents, each of cAMP-related group and Ca^{2+} influx group, were combined than when single agent was used. This suggests Ca^{2+} influx group tended to counteract the action of SRIF to cAMP-related group. Especially in the case of high K^+, the inhibitory effect of SRIF on both DBcAMP and PGE_1 was completely abolished (Fig. 4). Similar results were obtained when these agents were added to the cultures which were preincubated with SRIF for 3 hr (Fig. 5). The amount of released GH during the 30 min from pituitary-cultured cells preincubated with SRIF was higher than that from cells preincubated without it. This fact indicates the rebound release occurred in the cultured cells after removal of SRIF, as in the case of the perfused anterior pituitary (23). Under these conditions, the inhibitory action of SRIF on PGE_1-induced GH release

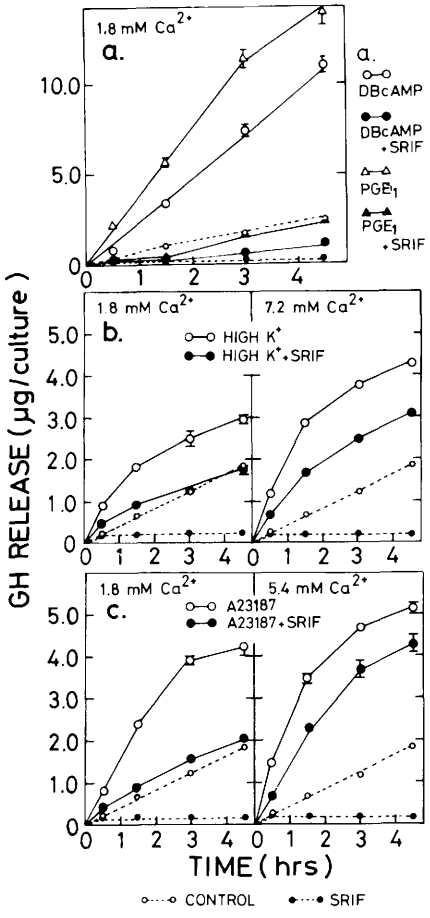

FIG. 3. Time course of the effect of (a) DBcAMP (1 m*M*), PGE_1 (14 μM), (b) high K^+ (56 m*M*), or (c) A23187 (10 μM) on GH release in the absence or presence of 0.1 μM SRIF. The basal GH release in the absence or presence of 0.1 μM SRIF was shown by the dotted line.

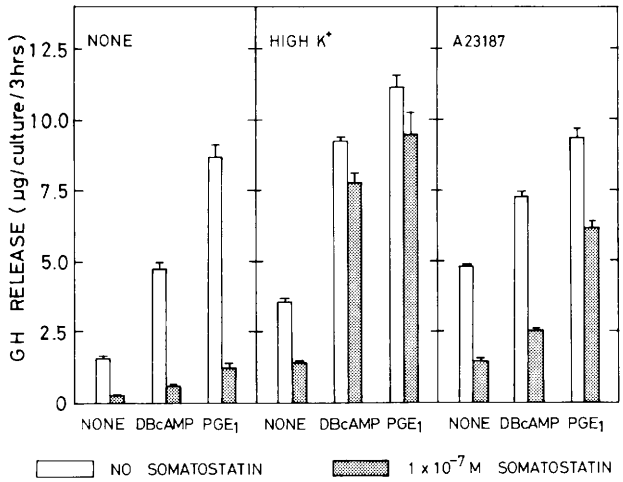

FIG. 4. Effect of SRIF (0.1 μM) on GH release induced by the combination of DBcAMP (1 mM) or PGE_1 (14 μM) with high K^+ (56 mM) or A23187 (10 μM) at 1.8 mM Ca^{2+}. Incubations and assays were performed as described under Materials and Methods. Each point represents the mean ± SEM of four cultures.

was abolished completely by simultaneous addition of high K^+ and partially by A23187 during the incubation of both the first 30 min and next 1 hr. Similar results were obtained when DBcAMP was used instead of PGE_1 (data not shown).

Discussion. Although GH releases stimulated by two types of secretagogues absolutely required extracellular Ca^{2+} as reported previously (19, 24), those from cultured cells showed a difference in calcium dependency (Fig. 1). Both the basal GH release and the DBcAMP- or PGE_1-induced GH release were maximal at 1.8 mM Ca^{2+} and were unaffected by further increase of Ca^{2+} concentration. In contrast, GH release stimulated by high K^+ or A23187 was progressively enhanced by the increase of the Ca^{2+} concentration above physiological level. These observations indicate that cells, under the condition stimulated by cAMP as well as at rest, are insensitive to supraphysiological concentration of external Ca^{2+}. On the other hand, in the presence of high K^+ or A23187, pituitary cells seem to become sensitive to high Ca^{2+}. This difference in Ca^{2+} sensitivity may be due to that of Ca^{2+} influx, because it has been shown that DBcAMP increased GH release without affecting both the amount of ^{45}Ca incorporation and intracellular calcium content in a pituitary organ (25, 26). On the other hand, high K^+ which is known to have no effect on pituitary cAMP level (27) has been shown to increase both the amount of ^{45}Ca incorporation and calcium content in the pituitary gland (25, 26). A23187 has been also shown to facilitate the transport of divalent cations across plasma membrane (28). However, recent studies (19) have shown that an increase of intracellular level of cAMP enhances cytoplasmic Ca^{2+} concentration by mobilizing calcium from intracellular reservoirs. Therefore, although these two types of secretagogues seem to affect calcium metabolism differently, either type of them increases cytoplasmic Ca^{2+} concentration, resulting in increased release of hormone as is thought to occur in chromaffin cells (19, 29).

In the case of insulin or glucagon release (10–12), high Ca^{2+} has been shown to enhance both the basal and the stimulus-induced hormone release and to reverse the inhibitory effect of SRIF on the release of these hormones. However, in this work, high Ca^{2+} did not reverse the action of SRIF on the basal GH release. This difference may be due to that of permeability of Ca^{2+} in plasma membrane of nonstimulated cells.

Belanger *et al.* (30) reported that SRIF is unable to inhibit GH release induced by high K^+ during 90-min incubation. On the other hand, Schofield *et al.* (16, 17) reported that SRIF inhibits GH release induced by A23187 and partly inhibits that by high K^+ during 20- to 30-min incubation. To clarify this discrepancy, we have studied the time course of

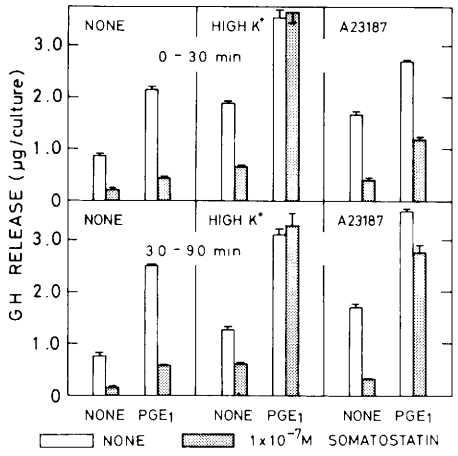

FIG. 5. Effect of high K^+, A23187, and/or PGE_1 on SRIF inhibition of GH release at 1.8 m*M* Ca^{2+}. After preincubation with 0.1 μM SRIF for 3 hr, the cells were incubated for 30 min in fresh DMEM containing 56 m*M* K^+, 10 μM A23187, and/or 14 μM PGE_1 in the absence or presence of 0.1 μM SRIF and the media were removed for assay of GH content (0–30 min, upper). Then the cells were further incubated for 1 hr in the fresh media containing the same agents (30–90 min, lower). The basal amount of GH which was released during the first 30-min incubation from cells preincubated without SRIF was 0.440 ± 0.038 μg/culture. When SRIF was removed from the cultures, cells were washed three times with DMEM. Each value represents the mean ± SEM of four cultures.

effect of SRIF on the stimulated GH release by high K^+ or A23187. SRIF showed a partial inhibitory effect on the increment of GH release during the first 30 min. However, SRIF was shown to have little effect on the increment of GH release induced by these agents during 3-hr incubation, although the total amount of GH release was decreased (Figs. 2 and 3). Therefore, the previous discrepancy concerning the effect of SRIF on GH release induced by high K^+ may be due to difference in an incubation period. In this respect, a similar result was obtained by Wollheim *et al.* (31) that insulin release stimulated by high concentration of A23187 was not inhibited by SRIF over 60 min but inhibited by it during 10-min incubation. They suggested that the inability of inhibitory effect of SRIF under a long exposure of A23187 may be due to damage to SRIF receptors. However, this possibility is excluded by our 3-hr-incubation experiments where SRIF partly inhibited GH release induced by high K^+ or A23187 in such a dose-dependent manner as it inhibited basal GH release (Fig. 2). The reason why there is a difference between short and prolonged incubation experiments in sensitivity to SRIF of GH release induced by high K^+ or A23187 remains to be elucidated.

Previous studies have shown that SRIF decreases the accumulation of cAMP by inhibiting its production (7, 8) and also that SRIF depresses the release of hormones induced by agents which increase intracellular cAMP levels without affecting cAMP production, and thus SRIF is thought to act upon or distal to the cAMP-dependent process(es) (4, 12, 15, 32). In this study, although GH release induced by PGE_1 or DBcAMP was suppressed by the presence of SRIF, its inhibitory effect was almost completely cancelled by the further addition of high K^+ (Fig. 4). The complete antagonism by the simultaneous addition of PGE_1 and high K^+ against the action of SRIF was still shown when 3-hr preincubation with SRIF was made to exert its inhibitory effect fully (Fig. 5). These observations suggest that SRIF is likely to exert its inhibitory effect beyond the cAMP-dependent process(es) rather than on this process(es) directly. Moreover, PGE_1 can potentiate high K^+-induced GH release during a short-term incubation (Fig. 5), suggesting that the effect of cAMP is rapid, and thus that GH synthesis may not be involved in the stimulation of GH release induced by cAMP. This suggestion is supported by the observation obtained by Stachura (33) that DBcAMP stimulates the release of stored GH during an initial burst for about 30 min.

Similar tendency was observed in the study using A23187. The inhibitory effect of SRIF on the increment of GH release caused by cAMP-related compounds was partially abolished by A23187. The effect of A23187 on SRIF was less potent than that of high K^+. This may be due to the difference of Ca^{2+} influx mechanism of these two agents. However, it would be probable that at higher concentration of Ca^{2+} the similar result will be obtained by A23187 as that by high K^+, because SRIF did not counteract the increase of GH release caused by A23187 at 5.4 m*M* Ca^{2+} as shown in the dose–response and time course studies (Figs. 2 and 3).

Although the present study could not fully

show the action mechanism of SRIF on GH release, one of possible mechanisms is its hyperpolarizing effect on the plasma membrane. Recently, Pace *et al.* (34) reported that the action of SRIF was to hyperpolarize the transmembrane potential presumably by increasing the K^+ conductance of the plasma membrane in rat pancreatic islet cells. This hyperpolarization would reduce the voltage-dependent permeability of the plasma membrane to Ca^{2+} which results in decrease in Ca^{2+} influx, and thus suppresses insulin release. In our results, the effect of SRIF was completely abolished by high K^+ treatment together with addition of PGE_1. This fact suggests that the inhibitory effect of SRIF on GH release may partly be explained by hyperpolarizing the transmembrane potential which is overcome by high K^+ in the anterior pituitary cells, as suggested in the pancreatic islet cells. In case of A23187, it may partly compensate the inhibition of Ca^{2+} entry caused by hyperpolarization.

Summary. The effect of SRIF on GH release was studied in monolayer-cultured cells of rat anterior pituitary with respect to Ca^{2+} and cAMP metabolism. Both the basal GH release and the DBcAMP- or PGE_1-stimulated GH release showed the requirement for Ca^{2+} in the medium but were not affected by increasing Ca^{2+} over 1.8 m*M*. On the other hand, GH release induced by high K^+ or A23187 also required the presence of Ca^{2+} but showed a different dependency on Ca^{2+} concentration, being increased by high Ca^{2+}. SRIF inhibited almost completely both the basal and DBcAMP- or PGE_1-stimulated GH release. Although SRIF showed a partial inhibitory effect on the increment of GH release induced by high K^+ during a short-term incubation at both 1.8 m*M* and high Ca^{2+}, little inhibitory effect of SRIF on that was observed in 3-hr-incubation experiments. Similar results were obtained when A23187 was used in the presence of 5.4 m*M* Ca^{2+}. In the presence of 1.8 m*M* Ca^{2+}, however, the increment of GH release induced by A23187 was inhibited partly during the incubation of both 30 min and 3 hr. When high K^+ or A23187 was added together with DBcAMP or PGE_1, an additional increase in GH release occurred. Inhibition by SRIF of GH release enhanced by DBcAMP or PGE_1 was almost completely reversed by the simultaneous presence of high K^+. Similar tendency, but less potently, was observed when A23187 was used instead of high K^+. In regard to dependency on extracellular Ca^{2+} concentration and sensitivity to SRIF, high K^+ and A23187 appear to enhance GH release by a mechanism different from that of DBcAMP and PGE_1. Moreover, our results confirm the previous findings that SRIF seemed to inhibit the hormone release by acting distal to the cAMP-dependent process(es) of the releasing pathway.

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