Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 276-281

SOMATOSTATIN PRETREATMENT FACILITATES GRF-INDUCED GH RELEASE AND INCREASE IN FREE CALCIUM IN PITUITARY CELLS

Hideaki Soya*, Mitsuo Suzuki† and Masakatsu Kato

Department of Physiology, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

Received September 7, 1990

Summary: Somatostatin pretreatment sensitizes rat anterior pituitary to hGRF stimulation in vitro. The pretreatment (1 nM for 10 min) facilitated GH release response of dispersed rat anterior pituitary cells to hGRF (1 nM for 3 min) 2.04-fold in a perifusion system. The effect lasted even 20 min after the pretreatment. SRIF pretreatment decreased cAMP content in the cells after hGRF stimulation to 61% of the control value. When hGRF was replaced by 1 mM DBcAMP and 15 mM KCl, the pretreatment increased GH secretion 1.69- and 1.67-fold respectively. SRIF pretreatment (1 nM for 10 min) caused a larger increase in $(Ca^{2+})i$ by hGRF than that of control. The effect of SRIF pretreatment facilitates GRF-induced increase in GH secretion probably through the stimulation of increase in $(Ca^{2+})i$.

The inhibitory action of SRIF on hormone release (1-3) is exerted by multiple mechanisms in which adenylate cyclase activity (4-6) and cAMP-independent processes (7-9) are involved. The latter processes result in the membrane hyperpolarization and the decrease in intracellular concentration of free calcium [$(Ca^{2+})i$](6, 10), due to the activation of potassium channels via Ni protein (11). In contrast, an acute withdrawal of SRIF induces a rebound increase in GH release by secretagogues (12-13). In addition, SRIF pretreatment prevents or rapidly restores the responsiveness of rat pituitary desensitized by GRF continuously (15) or repetitively (16) applied. These findings indicate a dualistic action of SRIF, which consists of a well-known direct inhibitory action, and a facilitatory permissive effect due to pretreatment. In the present paper, to characterize the facilitatory effect of SRIF on GH secretion in response to hGRF, we employed dispersed rat anterior pituitary cells subjected to a perifusion system.

* Present address: Department of Physical and Health Education, Faculty of Education, Mie University, Tsu 514, Japan.
† To whom correspondence should be addressed.

Abbreviations: GH, growth hormone; SRIF, somatostatin; hGRF, human growth hormone-releasing factor; DBcAMP, dibutyryl-cAMP; $(Ca^{2+})i$, intracellular free calcium concentration.

0006-291X/90 $1.50

Materials and Methods

Perifusion system: Anesthetized male Wistar rats (pentobarbital sodium 50 mg/kg body weight) were transcardially perfused with phosphate-buffered saline (PBS) and 15 ml of 0.5% trypsin (Sigma type III). The pituitaries quickly removed, were further trypsinized in the 0.5% solution and the dispersed cells were prepared as described previously (17, 18). The cells were resuspended in medium A (Eagle's MEM containing 2 mM L-glutamine and 0.25% BSA (fraction V, Boehringer Mannheim). The population of the somatotrophs (GH cells) of the dispersed cells were more than 50% (17, 19). The cells were mixed with Bio-Gel P2 (200-400 mesh, Bio-Rad Lab.) and placed in a 2.5 ml disposable syringe. Each column contained 2.5-3.5 x 10^6 cells with a bed volume of 0.5 ml and a head volume of 100 µl. The column were continuously perifused with medium A (0.3 min/min) for the first 2 hr and with medium B (mM: 137.5 NaCl, 5 KCl, 25 $CaCl_2$, 0.8 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4) for the next 1 hr at a flow rate of 1.0 ml/min.

Assays for rat GH, cAMP and intracellular free calcium: Rat GH in the effluents was assayed by a double antibody method with materials supplied by NIDDK. rGH RP-2 was used as the reference standard. The minimal detectable value was 7.8 ng/ml. The intra- and interassay coefficients of variation were 4% and 9%, respectively. For the determination of cAMP, the perifusion columns were removed 2 min before or 3.5 min after the introduction of hGRF and added with perchloric acid (1.5 N, 0.25 ml). The extracts were neutralized with KOH (1.5 N) and the concentration of cAMP in supernatants was determined with a Yamasa cAMP assay kit (17, 18). The dispersed cells were incubated in medium A at 37°C for 2 hr and spun down, resuspended in medium B containing 0.1% BSA and loaded with 1 µM fura 2/AM (Dojindo Laboratories) for 15 min at 37°C. The loaded cells were washed and resuspended in medium B (2 x 10^6/1.5 ml) with or without SRIF (1 nM or 10 nM). After 10 min incubation, the cells were centrifuged at 400 x g for 5 min and resuspended in medium B and transferred into a 1 cm quartz cuvette. The fluorescence was monitored at 37°C under agitation with a Hitachi fluorescence spectrophotometer 650-60 (9).

Results

When the cells were preperifused with SRIF (1 nM for 10 min), GH secretion in response to hGRF (1 nM for 3 min) was markedly facilitated (Fig. 1, upper panel), whereas a small rebound increase in GH secretion after SRIF withdrawal lasted for 13 min (Fig. 1, lower panel) as reported previously (20). To examine the dependency of the facilitatory effect on the concentration of SRIF and the duration of its application, the area under the curve was calculated. Among those examined the most effective dose of SRIF in facilitating hGRF-induced GH secretion was 1 nM, which augmented GH secretion 2.04-fold to the control response. A comparable and smaller facilitation was also observed at 0.1 nM and 10 nM of SRIF (Fig. 2, A). We therefore adopted this concentration for pretreatment in the subsequent experiments. The concentrations of SRIF and hGRF used in the present experiments were comparable to those determined in hypophyseal portal blood of male rats (20). The degree of facilitation also depended on the duration of SRIF pretreatment (Fig. 2, B). SRIF pretreatment for 10 min was the most effective, when compared with those for 3 and 20 min (Fig. 2, B). The facilitation was observed 2 min after with-

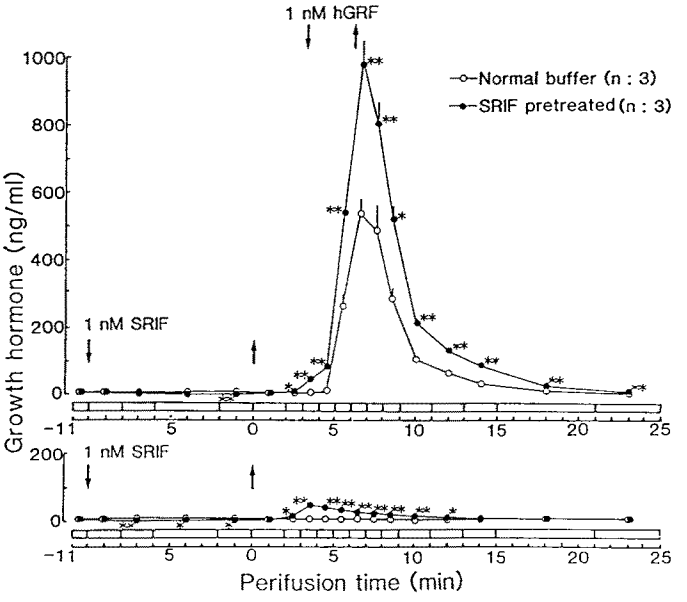

Fig. 1. Effect of SRIF pretreatment on hGRF-induced GH secretion. Upper panel, facilitation of hGRF-induced GH secretion by the preperifusion with SRIF. SRIF (1 nM) was introduced at -10 min (↓) and withdrawn at 0 time (↑). hGRF (1 nM) was introduced at 3 min and withdrawn at 6 min (↑). Each point represents the mean ± S.E. of triplicate determination as the same as following perifusion experiments. Each perifusion column contained 3 x 10^6 dispersed pituitary cells. Lower panel, a rebound increase in GH secretion caused by SRIF withdrawal. SRIF (1 nM) applied in each column was identical to that used in the upper panel. * P<0.05 and ** P<0.01 vs. the control values respectively (Student's t test).

drawal, peaked at 3 min and then gradually decreased, but it was still evident 20 min after withdrawal (Fig. 2, C), when the rebound increase completely disappeared (Fig. 1, lower panel). These results indicate that the facilitation by SRIF pretreatment rapidly takes place after a short term application and lasts for a considerable duration, 20 min, after the withdrawal.

To investigate the mechanism underlying in the facilitation caused by the SRIF pretreatment, we examined the effect of SRIF pretreatment on the hGRF-induced accumulation of cellular cAMP, since cAMP is thought to mediate GRF action in the somatotrophs. Although there was no apparent effect of preperifusion with 1 nM SRIF on the basal cAMP level 2 min before hGRF application, the pretreatment with SRIF decreased the hGRF-induced accumulation of cAMP to 61% of control level 3.5 min after the introduction of 1 nM hGRF. Thus it is likely that the pretreatment with SRIF augments the sensitivity of somatotrophs to cAMP in the process of GH secretion. This possibility was also confirmed, when DBcAMP was substituted for hGRF. DBcAMP (1 mM)-induced GH secretion was increased 1.69-fold by the SRIF pretreatment (Table 1). A number of findings support the view that voltage-sensitive Ca^{2+} channels are involved in the hGRF-induced GH secretion (9, 17, 21). In addition, these

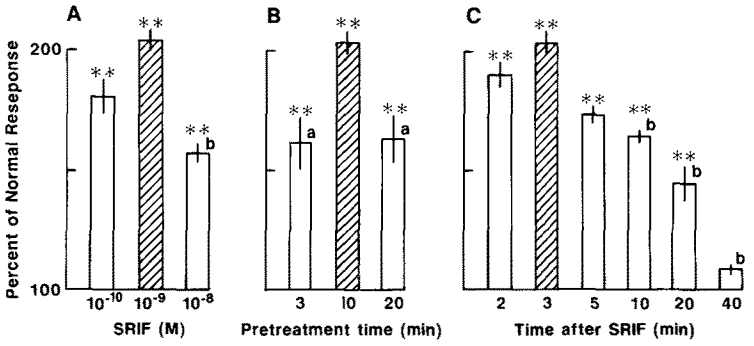

Fig. 2. Characterization of the facilitation of hGRF-induced GH release by the pretreatment with SRIF. The amount of GH released by hGRF was integrated as the area under the curve above the basal level of GH secretion at 1 min before hGRF application. A, Effect of varying concentrations of SRIF during pretreatment for 10 min on hGRF-induced GH release from dispersed pituitary cells. Procedures for SRIF application were the same as shown in Fig. 1, except the concentrations of SRIF. B, Effect of duration of SRIF pretreatment on hGRF-induced GH release. The application procedures were the same as shown in Fig. 1, except the duration of pretreatment. C, Effect of elapsed time after withdrawal of SRIF pretreatment. But elapsed time between SRIF (1 nM, 10 min) withdrawal and hGRF (1 nM, 3 min) application varied. In the figure, in panels A, B and C, the hatched column shows the maximal facilitation by SRIF (204% of the control) in the procedures shown in Fig. 1. Significant differences from the control values observed without hGRF stimulation were at the levels of *, $P<0.05$ and **, $P<0.01$ respectively. Significantly different from maximum facilitation (hatched column): at the levels of a, $P<0.05$ and b, $P<0.01$, respectively.

recent reports suggest that via cAMP hGRF activates less selective Na^+ channels which depolarize the somatotrophs and activate voltage-sensitive Ca^{2+} channels. If this is the case, the level of depolarization of the somatotrophs should be closely related to the magnitude of hGRF-induced GH

Table 1. Effect of SRIF pretreatment on dibutyryl cAMP (DBcAMP) and high KCl-induced GH secretion

Stimulation	ng GH release[a]	
	Control	SRIF
DBcAMP		
1 mM	160.6 ± 17.2[b]	270.4 ± 23.7*
KCl		
15 mM	69.6 ± 4.8[c]	116.3 ± 13.4*
20 mM	1610.7 ± 64.3	1650.6 ± 180.0
50 mM	2406.9 ± 382.8	2765.8 ± 313.1

SRIF (1 nM) was applied for 10 min and 3 min after the withdrawal of SRIF, DBcAMP or KCl was applied for 3 min.
a, Mean ± S.E. of the integrated amount of GH released (triplicate determinations) above the level at 4 min after SRIF withdrawal, DBcAMP for 11 min (b) and KCl for 3 min (C) respectively.
* Significantly different from the control values observed without pretreatment ($P<0.05$).

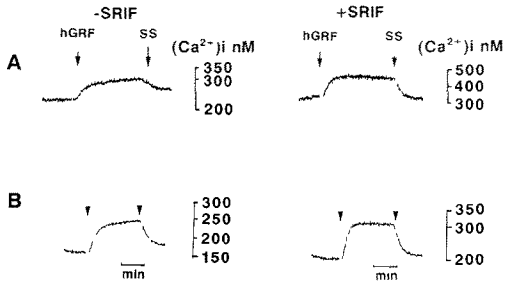

Fig. 3. Effect of SRIF pretreatment on the intracellular Ca^{2+} concentration in dispersed rat pituitary cells. A, representative recordings of $(Ca^{2+})i$ monitored with fura 2, when hGRF (1 nM) and SRIF (SS, 1 nM) were applied. Arrows and arrow heads indicate the time of application. B, The concentrations of SRIF and hGRF were both increased to 10 nM respectively.

secretion. To examine the effect of the perifusion with SRIF on depolarization-induced GH secretion, we used excess K^+ stimulation. GH secretion induced by 15 mM K^+ which causes a small depolarization was 1.67-fold facilitated by the pretreatment with 1 nM SRIF (Table 1), whereas 20 or 50 mM K^+-induced GH secretion was not significantly increased by the pretreatment. These relevant findings indicate a possible mechanism in the effect of SRIF pretreatment, and that this takes place at a step beyond cAMP generation, closely related to a process of GRF-induced Ca^{2+} influx or exocytosis of GH secretory granules.

Finally, we tried to observe the effect of SRIF pretreatment on the hGRF-induced increase in $(Ca^{2+})i$ in the somatotrophs with fura 2 (9, 22) in a static incubation system. The pretreatment with SRIF (1 nM) for 10 min clearly facilitated an increase in $(Ca^{2+})i$ and also a SRIF (1 nM)-induced decrease in $(Ca^{2+})i$ (Fig. 3, A). The percent increase over the prestimulatory basal level of SRIF-pretreated cells at the peak value, by 3 min after the addition of hGRF was significantly higher than that of control (119.0 ± 3.3 vs. 141.5 ± 5.0%; $P<0.01$, n=6), although there was no significant change in basal $(Ca^{2+})i$ by the pretreatment (-SRIF: 217.7 ± 11.3 vs. +SRIF: 263.3 ± 21.0 nM; $P>0.4$, n=6). A similar representative case was also observed, when the concentrations of SRIF and hGRF were increased to 10 nM respectively and hGRF stimulation was introduced shortly after resuspension (Fig. 3, B). The pretreatment clearly facilitated the velocity of the increase in $(Ca^{2+})i$ by hGRF. It was also clear that the pretreatment caused a rapid decrease in $(Ca^{2+})i$ following the addition of SRIF as well. The effect of SRIF pretreatment is in striking contrast to a well-known acute action of the peptide in inhibiting the increase in adenylate cyclase activity, the membrane depolarization, $(Ca^{2+})i$ and GH secretion in response to GRF stimulation (7, 8, 9, 10).

There are reports that SRIF pretreatment of GH_4C_1 cells for more than 10 hr (23), AtT-20 cells for 4 hr (24) and female rat pituitary cells for 2 hr (25) sensitize VIP, forskolin-stimulated cAMP accumulation and activin A-

induced FSH secretion respectively. In contrast to those found in long term incubations, the present findings were observed even 3 min after SRIF pretreatment for 3 min and 20 min after withdrawal of the pretreatment for 10 min, indicating a rapid and long-lasting event. The precise mechanism of the effect of pretreatment remains to be clarified.

Acknowledgments: We wish to thank the U.S. National Hormone and Pituitary Program and Dr. A.F. Parlow of the NIDDK for providing rat GH RIA kit. Thanks are also due to Dr. K. Wakabayashi for supplying goat antimonkey immunoglobulin G serum.

References

1. Krulich, L., Dhariwal, A.P.S. & McCann, S.M. (1968) Endocrinology 83, 783-790.
2. Brazeau, P.W., Vale, W., Brugus, R., Ling, N., Butcher, M., Rivier, J. & Guillemin, R. (1973) Science 179, 77-79.
3. Reichlin, S. (1983) New Engl. J. Med. 309, 1495-1501, 1556-1563.
4. Rouleau, D. & Barden, N. (1981) Can. J. Biochem. 59, 307-310.
5. Harwood, J.P., Grewe, C. & Aguilera, G. (1984) Mol. Cell Endocrinol. 37, 277-284.
6. Reisine, T.D. (1985) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 19, 169-177.
7. Koch, B.D., Blalock, J.B. & Schonbrunn, A. (1988) J. Biol. Chem. 263, 216-225.
8. Koch, B.D. & Schonbrunn, A. (1988) J. Biol. Chem. 263, 226-234.
9. Kato, M. and Suzuki, M. (1989) Brain Res. 476, 145-148.
10. Holl, R.W., Thorner, M.O. & Leong, D.F. (1988) Endocrinology 122, 2927-2929.
11. Koch, B.D., Dorfinger, K.J. & Schonbrunn, A. (1985) J. Biol. Chem. 260, 13138-13145.
12. Stachura, M.E. & Fitzer, R. (1977) Endocrinology 101, 1044-1053.
13. Kraicer, J., Cowan, J.S., Sheppard, M.S., Lussier, B. & Moor, B.C. (1986) Endocrinology 119, 2047-2051.
14. Weiss, J., Crodonin, M.J. & Thorner, M.O. (1989) Am. J. Physiol. 253, (Endocrinol. Metab. 16): E508-E514.
15. Clayton, R.N. & Bailey, L.C. (1987) J. Endocr. 112, 69-76.
16. Soya, H. & Suzuki, M. (1988) Endocrinology 122, 2492-2498.
17. Kato, M. & Suzuki, M. (1986) Japan. J. Physiol. 36, 1225-1239.
18. Kato, M., Hattori, M-A. & Suzuki, M. (1988) Am. J. Physiol. 254 (Endocrinol. Metab. 17), E476-E481.
19. Surks, M.I. & Defesi, C.R. (1977) Endocrinology 101, 946-958.
20. Plotzky, P.W. & Vale, W. (1985) Science 230, 461-463.
21. Cowan, J.S., Moor, B.C., Chow, A. & Kracier, J. (1983) Endocrinology 113, 1056-1061.
22. Grynniewicz, G., Poenie, M. & Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
23. Presky, D.H. & Schonbrunn, A. (1988) J. Biol. Chem. 263, 714-721.
24. Heisler, S. & Srikant, C.B. (1985) Endocrinology 117, 217-225.
25. Kitaoka, M., Takano, K., Kojima, I. & Ogata, E. (1989) Biochem. Biophys. Res. Commun. 162, 958-962.