Somatostatin inhibits vasopressin-stimulated phosphoinositide hydrolysis and influx of extracellular calcium in clonal hamster β (HIT) cells

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Vasopressin (VP) stimulates insulin secretion and inositol phosphate (InsP) production in clonal hamster β cells (HIT) via a cyclic AMP-independent V1-receptor-mediated signal-transduction pathway. Somatostatin (SRIF) inhibited VP-stimulated insulin secretion, and the effects of SRIF were abolished by pretreatment with pertussis toxin. The Ca^{2+} -channel blockers verapamil and nifedipine also inhibited VP-stimulated insulin secretion during 20 min incubations, but verapamil was ineffective at 2 min, and the effects of SRIF and nifedipine together were not additive. SRIF failed to inhibit further the attenuated insulin response to VP in Ca^{2+} -free medium. VP-stimulated InsP production was also inhibited by SRIF in a pertussis-toxin-sensitive manner. Whereas VP-stimulated insulin secretion was almost completely inhibited by SRIF at an equimolar concentration, VP-stimulated InsP production was much less sensitive to inhibition by SRIF, even at a 100-fold excess concentration. VP increased cytosolic Ca^{2+} in HIT cells loaded with fura 2, the fluorescent Ca^{2+} indicator. The increase was biphasic, with an initial rapid spike increase followed by a prolonged second phase. Both SRIF, at a concentration which inhibited VP-stimulated insulin secretion but not InsP production, and verapamil failed to inhibit the rapid spike increase in intracellular Ca^{2+} , but did inhibit the second phase. We conclude that VP induces biphasic changes in cytosolic Ca^{2+} , secondary to mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+} . SRIF inhibits insulin secretion by interrupting influx of extracellular Ca^{2+} , likely by inhibiting G_i -subunit activity. Inhibition of VP-stimulated phosphoinositide hydrolysis, which is also pertussis-toxin-sensitive, may represent an additional mechanism of action of SRIF.

INTRODUCTION

SRIF, initially isolated from the hypothalamus on the basis of its ability to inhibit growth-hormone secretion, has a spectrum of inhibitory effects on the secretion of numerous peptide hormones, including insulin [1]. Receptor activation is linked to the inhibitory G-protein subunit G_i [2–4]. SRIF both decreases intracellular cyclic AMP levels and inhibits the influx of extracellular Ca^{2+} , by interactions with voltage-dependent Ca^{2+} channels or K^+ channels [5–9].

We studied the mechanisms of action of SRIF in clonal SV40-transformed hamster β cells (HIT cells) [10], which, like mouse islets, respond to VP, over a concentration range of 0.1–10 nM, with increased insulin secretion and PI hydrolysis and no changes in cellular cyclic AMP levels [11,12]. Stimulation of insulin secretion by VP in HIT cells, mouse islets and perfused rat pancreas [13] is mediated by V1 receptors, but the β -cell VP receptor has not yet been fully characterized. The stimulatory effects of VP on insulin secretion in HIT cells are poorly inhibited by V1a (pressor) antagonists, indicating that these receptors do not belong to the V1a class, found in liver and vascular tissue [14–16].

EXPERIMENTAL

HIT cells were grown as previously described in 16 mm polystyrene multiwell containers as monolayers in RPMI 1640, supplemented with 10% fetal-calf serum, penicillin (10^5 units/l), streptomycin (100 µg/l), amphotericin B (250 µg/l) and Hepes

(10 mM), in a humidified atmosphere of air/ CO_2 (19:1) at 37 °C [12].

Insulin secretion

On reaching near-confluence, cells were washed with 2×1 ml of incubation medium [Hanks' balanced salt solution (HBSS) containing 1 g of glucose/l, 60 mg of bacitracin/l and 20 mM-Hepes] and incubated in 1 ml of this medium for 20 min at 37 °C in the absence or presence of test substances. The media were centrifuged at 1000 g for 5 min (Sorvall GLC) and aspirated for immediate insulin radioimmunoassay [12]. Six wells were studied for each variable, and samples were assayed in triplicate for insulin content. In some experiments, cells were incubated for 24 h with pertussis toxin (100 ng/ml) before experimental incubation.

Radioimmunoassay data were processed with a Beckman DP5500 reduction unit. Assay sensitivity was 0.4 µ-units/tube with pig insulin as the standard; intra- and inter-assay variations were 4 and 9% respectively.

Inositol phosphate (InsP) production

We have previously reported an increase in InsP_3 levels in response to VP at 30 and 60 s, after which time, InsP_3 levels begin to increase in a time-dependent manner [12]. Since InsP is derived from the phospholipase C-induced breakdown of PtdInsP_2 to $\text{Ins}(1,4,5)\text{P}_3$, we measured the production of InsPs in the presence of 10 mM-LiCl to prevent the metabolism of InsP to inositol at 20 min, by which time there is marked accumulation of InsP_1 in the presence of VP.

Abbreviations used: VP, [arginine]vasopressin; SRIF, somatostatin (somatotropin-release-inhibiting factor); PI, phosphoinositide; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks' balanced salt solution.

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HIT cell monolayers in 22 mm polystyrene containers were prelabelled for 48 h with 20 μ Ci of [3 H]inositol (15 Ci/mmol)/well and InsP production was determined as previously reported [12], by the methodology described by Berridge *et al.* [17]. Before experimental incubation cells were washed twice, and 20 min incubations were performed. The reaction was halted by the removal of media and addition of 0.5 ml of methanol. To the methanol cell extracts, 2 ml of chloroform and 0.4 ml of water were added and InsPs were measured in the aqueous fractions by anion-exchange chromatography on Dowex AG1 X8 (200–400 mesh) [12,17]. Radiation was measured by liquid-scintillation spectroscopy. Variables were studied in triplicate.

Data are presented as means $\pm$ S.E.M. and statistical analysis used Student's *t* test or ANOVA when appropriate (N.S., not significant).

Ca²⁺ measurements

The method described by Rajan *et al.* [18] in HIT cells was followed, with minor modifications. Cells were detached from 75 cm² culture flasks by treatment with 0.05% trypsin. After detachment, 10 ml of RPMI 1640 culture medium (as above) was added, and cells were recovered by centrifugation (Sorvall GLC, 400 g for min), re-washed in 20 ml of HBSS incubation medium, and then incubated in 1 ml of HBSS containing the fura 2 precursor fura 2AM, at a final concentration of 1 μ M for 1 h at 37 °C. Cells were then washed in 3 $\times$ 10 ml of HBSS and resuspended in HBSS at a concentration of 1 $\times$ 10⁶ cells/ml. Cell viability was assessed by Trypan Blue staining and exceeded 90%.

The cell suspension was placed in a 3 ml quartz cuvette. Emission was measured at 505 nm in a Perkins Elmer LS 5 spectrofluorimeter equipped with a 3600 LS data station. The excitation wavelength was alternated between 340 and 380 nm, by using a fura 2 basic computer program (Perkins–Elmer) at 8 s intervals. Measurements were made at 22 °C, at which temperature dye leakage was negligible, in three studies. Representative results are shown. Test substances, added as concentrated solutions, did not affect fluorescence in the absence of cells. Maximum and minimum fluorescence were measured in the presence of brominated A23187 (10 μ M) and 10 mM MnCl₂ respectively.

Materials

HIT cells were kindly provided by Dr. A. E. Boyd III. Media, sera and antibiotics were purchased from GIBCO, Grand Island, NY, U.S.A. Peptides were bought from Peninsula Laboratories, Belmont, CA, U.S.A. and [125 I] and [3 H]inositol were supplied by New England Nuclear, Boston, MA, U.S.A. Fura 2AM and brominated A23187 were bought from Molecular Probes, Junction City, OR, U.S.A. Other reagents were purchased from Sigma, St. Louis, MO, U.S.A., except for the anti-insulin antibody and guinea-pig antiserum, obtained from Arnel Laboratories, Brooklyn, NY, U.S.A.

RESULTS

Insulin secretion

As shown in Table 1, SRIF inhibited both basal and VP-stimulated insulin secretion. In the presence of 10 nM-VP and SRIF at an equimolar concentration, insulin levels were only slightly above baseline unstimulated values.

The Ca²⁺-channel blockers verapamil and nifedipine inhibited VP-stimulated insulin secretion during 20 min incubations. However, verapamil did not significantly inhibit VP-stimulated insulin secretion during a 2 min incubation (Table 2).

In the presence of both 1 μ M-SRIF (maximal inhibitory concentration) and 100 μ M-nifedipine, no further inhibition was observed [control 44 $\pm$ 5, 10 nM-VP 152 $\pm$ 13, VP + SRIF 68 $\pm$ 3, VP + nifedipine 88 $\pm$ 14 μ -units/ml (*P* < 0.0005 and < 0.005 versus VP alone) and VP + SRIF + nifedipine 76 $\pm$ 8 μ -units/ml (N.S. versus VP plus SRIF or nifedipine alone)].

In the absence of extracellular Ca²⁺ in the incubation media, 10 nM-VP induced an attenuated insulin response (96 $\pm$ 8 versus 152 $\pm$ 14 μ -units/ml with Ca²⁺; *P* < 0.002), and under these conditions neither SRIF (1 μ M) nor nifedipine (100 μ M) significantly inhibited insulin secretion [control 28 $\pm$ 2, VP 96 $\pm$ 8, VP plus SRIF 78 $\pm$ 10 (N.S.), VP plus nifedipine 116 $\pm$ 10 μ -units/ml (N.S.)].

Overnight pretreatment with pertussis toxin (100 ng/ml) had no effect on 10 nM-VP-stimulated insulin secretion, but the inhibitory effects of 10 nM-SRIF on VP-stimulated insulin secretion were abolished by pertussis-toxin pretreatment [VP 370 $\pm$ 21 and VP + SRIF 236 $\pm$ 14 (*P* < 0.005), compared with VP 351 $\pm$ 13 and VP + SRIF 346 $\pm$ 10 μ -units/ml after pertussis toxin (N.S.)].

Table 1. Effects of SRIF on basal and VP-stimulated insulin secretion

SRIF caused dose-dependent inhibition of basal insulin secretion (*P* < 0.001). Insulin secretion, stimulated by 10 nM-VP (*P* < 0.0001), was slightly inhibited by 1 nM-SRIF (*P* < 0.025) and almost completely by SRIF at concentration of 10–1000 nM (all *P* < 0.005 versus VP alone).

Basal secretion: SRIF (nM)	Insulin (μ -units/ml)
0	53 $\pm$ 3
1	37 $\pm$ 4
10	33 $\pm$ 3
100	33 $\pm$ 4
1000	25 $\pm$ 2
SRIF (nM) + VP (10 nM)	
0	143 $\pm$ 7
1	119 $\pm$ 6
10	66 $\pm$ 5
100	60 $\pm$ 3
1000	55 $\pm$ 3

Table 2. Effects of Ca²⁺-channel blockers on VP-stimulated insulin secretion

Verapamil and nifedipine inhibited VP-stimulated insulin secretion (both *P* < 0.0005) during 20 min incubations. In the presence of VP, verapamil failed to lower insulin levels significantly during a 2 min incubation (N.S.).

	Insulin (μ -units/ml)
20 min	
Control	26 $\pm$ 8
VP (10 nM)	152 $\pm$ 14
VP + verapamil (50 μ M)	62 $\pm$ 2
VP + nifedipine (10 μ M)	52 $\pm$ 4
2 min	
Control	9 $\pm$ 2
VP	51 $\pm$ 5
VP + verapamil	42 $\pm$ 3

Table 3. Effects of SRIF on basal and VP-stimulated InsP production

SRIF caused significant inhibition of basal InsP production only at a concentration of 1000 nM ($P < 0.01$). In a separate experiment, 10 nM-VP stimulated InsP production ($P < 0.001$). SRIF significantly inhibited stimulated InsP production at concentrations of 100 nM ($P < 0.05$) and 1000 nM ($P < 0.01$).

Basal production:		InsP (c.p.m.)
SRIF (nM)		
0		638 ± 42
0.1		580 ± 83
10		451 ± 96
1000		457 ± 21

SRIF (nM)	VP	InsP (c.p.m.)
0	—	360 ± 42
0	+	3260 ± 369
1	+	3598 ± 178
10	+	3100 ± 181
100	+	2143 ± 265
1000	+	2117 ± 187

Table 4. Effects of pertussis toxin on InsP production

SRIF (1 μ M) significantly inhibited the increment in InsP₁ ($P < 0.01$), InsP₂ ($P < 0.05$) and InsP₃ ($P < 0.05$) production induced by VP (10 nM). After 24 h of exposure to pertussis toxin (PT), SRIF failed to inhibit VP-stimulated production of InsP, InsP₂ or InsP₃.

	InsP production (c.p.m.)		
	InsP ₁	InsP ₂	InsP ₃
Control (PT)	388 ± 25	43 ± 5	17 ± 1
Control	380 ± 28	59 ± 3	22 ± 3
VP (PT)	2056 ± 87	77 ± 8	35 ± 8
VP	2410 ± 207	80 ± 12	45 ± 5
VP + SRIF (PT)	2091 ± 136	71 ± 12	34 ± 3
VP + SRIF	1500 ± 127	50 ± 5	31 ± 4

Table 5. Interactions of SRIF and PMA on InsP production

VP stimulated InsP production ($P < 0.0005$) and this increase was significantly inhibited by 100 nM-PMA or 1000 nM-SRIF (both $P < 0.0005$). Coincubation of cells with PMA and SRIF caused further inhibition of InsP production ($P < 0.005$ versus VP + PMA).

	InsP (c.p.m.)
Control	1155 ± 91
VP (10 nM)	3725 ± 172
VP + SRIF	2822 ± 31
VP + PMA	1549 ± 74
VP + SRIF + PMA	1171 ± 43

Effects of SRIF on PI hydrolysis

SRIF caused dose-dependent inhibition of the increment in InsP stimulated by VP (Table 3). Whereas SRIF almost completely inhibited VP-stimulated insulin secretion when present with VP at an equimolar concentration, SRIF was less effective in inhibiting VP-stimulated PI hydrolysis.

Pretreatment of cells overnight with 100 ng of pertussis toxin/

Fig. 1. Effects of VP on intracellular Ca²⁺

VP (10 nM) caused a biphasic increase in intracellular Ca²⁺, as indicated by the 340 nm/380 nm signal intensity, in HIT cells loaded with fura 2. The initial rapid spike was followed by a prolonged second Ca²⁺ response.

Fig. 2. Effects of SRIF on intracellular Ca²⁺

Pretreatment with 10 nM-SRIF failed to inhibit the first-phase increase in intracellular Ca²⁺, but attenuated the second-phase response to VP. Ca²⁺ influx secondary to 56 mM-KCl-induced membrane depolarization was not inhibited by SRIF.

ml abolished the inhibitory effects of SRIF on VP-stimulated production of InsP₁, InsP₂ and InsP₃, but did not interfere with the stimulatory effects of VP (Table 4).

The phorbol ester phorbol 12-myristate 13-acetate (PMA) also inhibited VP-stimulated InsP production (control 356 ± 14, 10 nM-VP 1051 ± 96, VP + 10 nM-PMA 515 ± 36, VP + 100 nM-PMA 212 ± 9, VP + 1000 nM-PMA 285 ± 24 c.p.m. respectively). The inhibitory effects of 1000 nM-SRIF and 100 nM-PMA (maximal concentration) were additive (Table 5), suggesting that these two inhibitors of PI hydrolysis act via different mechanisms.

Effects of SRIF on intracellular Ca²⁺

VP (10 nM) induced a rapid spike increase in intracellular Ca²⁺, which was followed by a prolonged second phase (Fig. 1). SRIF at a concentration of 10 nM, which almost completely abolishes the insulin response to 10 nM-VP but has minimal effects on VP-stimulated InsP production, failed to inhibit the rapid Ca²⁺ spike induced by 10 nM-VP, but attenuated the second-phase Ca²⁺ response (Fig. 2). In the presence of 50 μ M-verapamil, a similar rapid increase in cytosolic Ca²⁺ was observed in response

Fig. 3. Effects of verapamil on intracellular Ca^{2+}

VP (0.1 nM) caused a sustained rise in intracellular Ca^{2+} , and subsequent exposure to 10 nM-VP caused a biphasic Ca^{2+} response. In the lower panel, 50 μM -verapamil, added before VP, did not inhibit the first-phase Ca^{2+} response to 0.1 nM- or 10 nM-VP or to 100 nM-gastrin-releasing peptide (GRP), but did inhibit the rapid Ca^{2+} spike induced by membrane depolarization in response to 56 mM-KCl.

to 0.1 nM or 10 nM-VP, but again the second-phase Ca^{2+} response was inhibited (Fig. 3). Verapamil, but not SRIF, inhibited the rapid increase in intracellular Ca^{2+} secondary to Ca^{2+} influx, induced by membrane depolarization in response to 56 mM-KCl (Figs. 2 and 3), indicating different specificities of SRIF and verapamil for membrane Ca^{2+} channels.

DISCUSSION

The activation of V1 receptors is believed to stimulate phospholipase C via a mechanism dependent on a stimulatory G-protein subunit [14,19]. Hydrolysis of PtdInsP_2 leads to the formation of two second messengers, $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol, which mobilize intracellular Ca^{2+} and activate protein kinase C respectively. SRIF inhibited VP-stimulated insulin secretion and InsP production, and both actions were abolished by pretreatment with pertussis toxin, suggesting mediation by a G_i -subunit membrane protein, previously shown to be related to the mechanism of action of SRIF [2–4].

No added inhibitory effects of SRIF on VP-stimulated insulin secretion were apparent in the presence of the Ca^{2+} -channel blocker nifedipine, likely indicating a shared mechanism of action, i.e. inhibition of VP-induced influx of extracellular Ca^{2+} . This concept is supported by the observation that SRIF had no further inhibitory effect on the attenuated insulin response to VP when extracellular Ca^{2+} was omitted from the incubation media. Since VP has no effect on cAMP levels in HIT cells [13], these findings corroborate many previous observations that SRIF

inhibits peptide-hormone secretion by limiting Ca^{2+} influx via voltage-dependent Ca^{2+} channels or by opening K^+ channels and causing membrane hyperpolarization [6–9].

SRIF was capable of abolishing the insulin response induced by VP, whereas the InsP response was only partially inhibited by SRIF. At concentrations of SRIF 10- or 100-fold greater than those of VP, InsP levels did not fall to unstimulated values. These findings indicate that the major inhibitory actions of SRIF on VP-stimulated insulin secretion are not secondary to inhibition of PI hydrolysis and are mediated by effects on the influx of extracellular Ca^{2+} . However, it is possible that inhibition of PI hydrolysis by SRIF may be, at least in part, responsible for some of its inhibitory effects on insulin secretion, since $\text{Ins}(1,3,4,5)\text{P}_4$, which is derived from $\text{Ins}(1,4,5)\text{P}_3$, has been reported to increase the influx of extracellular Ca^{2+} [20,21]. Using Dowex chromatography, we have been unable to demonstrate that VP stimulates InsP_4 production in HIT cells and believe that a more sensitive technique, such as reverse-phase h.p.l.c., will be required to examine this possibility. This technique was employed by Li *et al.* [22], who recently reported that VP increased production of InsP_4 isomers, including $\text{Ins}(1,3,4,5)\text{P}_4$, in RINm5F cells.

VP had biphasic effects on cytosolic Ca^{2+} . A rapid and transient rise in intracellular Ca^{2+} occurred in tandem with the rapid transient rise in InsP_3 levels, which we have previously reported in HIT cells [12]. Pre-exposure of cells to SRIF at an equimolar concentration (10 nM) failed to inhibit the rapid spike in intracellular Ca^{2+} but did attenuate the prolonged second-phase calcium response. Under these conditions, SRIF causes only a minor inhibition of stimulated InsP production and would not be expected to interfere with the mobilization of intracellular Ca^{2+} in response to VP. However, SRIF almost completely abolishes VP-stimulated insulin secretion under these conditions, presumably by inhibiting the influx of extracellular Ca^{2+} . Likewise, verapamil did not inhibit the Ca^{2+} spike induced by VP, but did inhibit the second-phase Ca^{2+} response. These observations thus suggest that the rapid spike increase in cytosolic Ca^{2+} induced by VP represents mobilization of intracellular Ca^{2+} . This step in the signal-transduction sequence, mediated by phospholipase C, typifies V1-receptor-mediated pathways in vascular smooth muscle or liver [14–16]. Verapamil also failed to inhibit significantly insulin secretion at 2 min, although its effects were significant at 20 min, providing further evidence for biphasic effects of VP to mobilize intracellular Ca^{2+} and increase Ca^{2+} influx.

Verapamil, but not SRIF, inhibited the rapid rise in intracellular Ca^{2+} in response to KCl-induced membrane depolarization. These results are similar to those of Hsu *et al.* [9], who reported that addition of SRIF before exposure to 15 mM-KCl did not attenuate the rise in intracellular Ca^{2+} induced by KCl in HIT cells, whereas the addition of SRIF after KCl inhibited Ca^{2+} influx via voltage-dependent Ca^{2+} channels [9].

PMA also inhibited VP-stimulated PI hydrolysis, and when cells were exposed to both PMA (at a maximal effective concentration) and SRIF, an additive response was observed, indicating that SRIF and PMA inhibit InsP production via different mechanisms. This is not unexpected, since it is well recognized that phorbol esters, such as PMA, activate protein kinase C [23,24]. Ryn *et al.* [25] have reported that protein kinase C activation causes feedback inhibition of phospholipase C. SRIF does not interfere with the binding of [^3H]VP to HIT cells (S. B. Richardson & T. Laya, unpublished work). Taken together, our findings provide further evidence that SRIF inhibits PI hydrolysis by inhibiting G-protein subunit activity, rather than by interfering with earlier or later events in the signal-transduction cascade.

SRIF is present in the delta cells of pancreatic islets [1,26] and immunoreactive VP has been detected in mammalian pancreas

[27]. We have found that acid/acetone pancreatic extracts displace [³H]VP in a radioreceptor assay in HIT cells, suggesting, although not proving, that pancreatic VP-like material is bioactive (S. B. Richardson & T. Laya, unpublished work). Both SRIF and VP might be capable of influencing physiological insulin secretion. At sites of paracrine interaction in the islets (β - δ), where high concentrations of SRIF would be expected, inhibition of PI hydrolysis may represent an additional mechanism of action of SRIF.

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