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Somatostatin inhibition of Ca^{2+}-induced insulin secretion in permeabilized HIT-T15 cells

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Somatostatin inhibited Ca^{2+}-induced insulin secretion in permeabilized HIT-T15 cells, albeit with decreased sensitivity relative to intact cells. The inhibitory action required the presence of GTP, whereas GDP could not substitute for GTP. Pertussis-toxin treatment before cell permeabilization abolished the inhibition of secretion. Thus somatostatin, by activating a G-protein, interferes with exocytosis distal to the generation of soluble intracellular messengers.

INTRODUCTION

Somatostatin is a negative modulator of secretion from a variety of tissues, such as the pituitary, the endocrine and exocrine pancreas, the gastrointestinal tract and the central nervous system [1,2]. After binding to its receptor, somatostatin exerts various effects which have been implicated in the inhibition of secretion from the pituitary and the endocrine pancreas. On the one hand, the hormone inhibits adenylate cyclase and lowers cyclic AMP levels [3,4]. On the other hand, interference with plasma-membrane ion channels has also been described. Thus somatostatin hyperpolarizes pituitary cells [5] and causes transient inhibition of glucose-induced electrical activity in pancreatic β-cells [6]. The latter is probably due to the opening of ATP-sensitive K^+ channels, recently reported for the insulin-secreting cell line R1Nm5F [7]. The effect on membrane potential could explain the decrease in cytosolic Ca^{2+} observed in both pituitary cells [8] and insulin-secreting cells [9,10] after somatostatin exposure. In addition, direct inhibition of Ca^{2+} currents was found in a cell line of pituitary origin [11]. The different actions of somatostatin are mediated via one or several pertussis-toxin-sensitive G-proteins [3,7–9,11]. The inhibition of insulin secretion by somatostatin cannot be explained entirely by decreasing either cellular cyclic AMP or cytosolic Ca^{2+}. Thus the addition of cyclic AMP or Ca^{2+} ionophore, which by-pass adenylate cyclase and membrane ion channels respectively, still allowed somatostatin inhibition of insulin secretion [12,13]. Another finding arguing against changes in cytosolic Ca^{2+} as the main determinant of somatostatin action is the transient nature of the decrease in cytosolic Ca^{2+}, contrasting markedly with the prolonged inhibition of insulin secretion [9]. It therefore appears that somatostatin inhibits secretion primarily at a point distal to the generation of the cyclic AMP and Ca^{2+} signals.

In the present study, the early signal-transduction mechanisms were by-passed by using electrically permeabilized cells. This preparation permits the examination of stimulatory and inhibitory factors regulating the late secretory events leading to the exocytotic release of insulin. We show here that somatostatin is capable of inhibiting Ca^{2+}-induced insulin secretion in a newly established preparation of permeabilized cells of the hamster HIT-T15 cell line.

EXPERIMENTAL

Methods

HIT-T15 cells (passages 69–78) were cultured in RPMI-1640 medium containing 10% (v/v) fetal-calf serum, 11.1 mM-glucose, 0.1 μM-selenious acid, 10 μg of GSH/ml, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. After detachment of the cells from the culture flasks with 0.025% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, containing 137 mM-NaCl, 2.7 mM-KCl, 8 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4 and 0.25 mM-EDTA, pH 7.6, the cells were either seeded into 24-well culture plates (approx. 0.5×10^6 cells/0.5 ml) for the incubation of intact cells, or kept 3 h in spinner culture before permeabilization.

Insulin secretion from intact cells was measured during 30 min at 37 °C after a 30 min preincubation in glucose-free Krebs–Ringer bicarbonate–Hepes buffer, containing 25 mM-Hepes, 1 mM-$CaCl_2$, 126 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$ and 0.07% bovine serum albumin, pH 7.4.

Permeabilization by high-voltage discharge (30 exposures to an electrical field of 3 kV/cm each of 30 μs duration) was carried out in mannitol buffer (pH 7.0), consisting of 270 mM-mannitol, 10 mM-potassium glutamate, 0.4 mM-EGTA and 20 mM-Hepes. This treatment results in a cell population of which 79 ± 1% ($n = 5$ independent preparations) of the cells do not exclude Trypan Blue after staining, indicating cell permeabilization. The procedures of incubation and determination of immunoreactive insulin released into the supernatant (measured at the end of the incubation) are identical with those described previously for electrically permeabilized RINm5F cells [14,15]. Briefly, permeabilized cells [$0.77(\pm 0.06) \times 10^6$ cells/0.1 ml; $n = 10$ independent experiments] were incubated in potassium glutamate buffer (pH 7.0), containing 140 mM-potassium glutamate, 5 mM-NaCl, 7 mM-$MgSO_4$, 5 mM-Na_2ATP, 10.2 mM-EGTA and Ca^{2+} and other test substances at appropriate concentrations for 15 min at 4 °C and subsequently for 5 min at 37 °C. This protocol was chosen to ascertain the uptake of extracellularly added solutes. When present, the ATP-regenerating system consisted of 21 units of creatine kinase/ml and 2 mM-phosphocreatine.

Materials

GTP, guanosine 5′-[γ-thio]triphosphate (GTP[S]) and GDP were purchased from Sigma (St. Louis, MO, U.S.A.). Na_2ATP was from either Sigma or Boehringer Mannheim (Mannheim, Germany). Pertussis toxin was from List Biochemical Labs (Campbell, CA, U.S.A.). All experiments were performed with somatostatin 14 (S-14), except for the studies in intact cells where somatostatin 28 (S-28) and Sandostatin [SMS, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr] were also used. All somatostatin analogues were generously given by Dr. C. Bruns, Sandoz A.G. (Basel, Switzerland).

Abbreviation used: GTP[S], guanosine 5′-[γ-thio]triphosphate.

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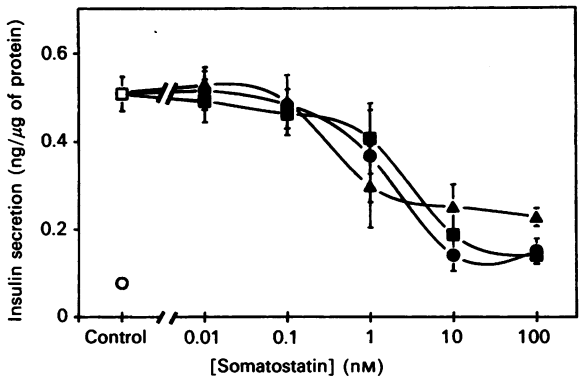

Fig. 1. Somatostatin inhibition of nutrient-induced insulin secretion in intact HIT cells

Cells were cultured and incubated as described in the Experimental section. Insulin secretion was stimulated during 30 min by the combination of glucose (10 mM), glutamine (5 mM) and leucine (5 mM). Somatostatin analogues (●, somatostatin 14; ■, somatostatin 28; ▲, Sandostatin) were tested as indicated. Results are means ± S.E.M. of three observations from three independent experiments.

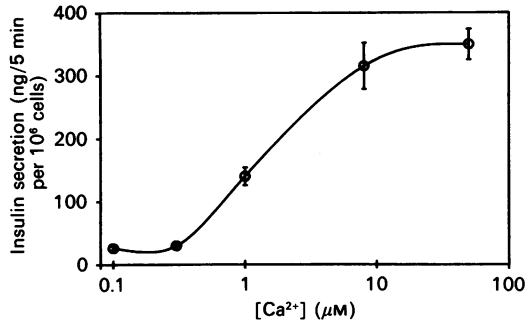

Fig. 2. Ca^{2+}-induced insulin secretion from electrically permeabilized HIT cells

Cells were prepared as described in the Experimental section, and insulin release into the supernatant was measured after 5 min incubation at 37 °C. The Ca^{2+} concentrations, measured with a Ca^{2+}-selective micro-electrode [33], were as indicated. GTP (100 μM) and an ATP-regenerating system were present throughout. Results are means ± S.E.M. of five observations from one experiment.

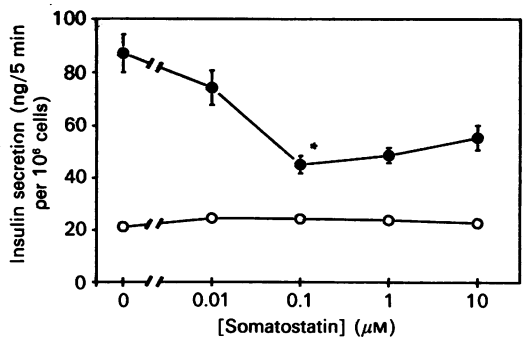

Fig. 3. Concentration-dependency of somatostatin-induced inhibition of insulin secretion in permeabilized HIT cells

Cells were incubated at 0.1 μM-Ca^{2+} (○) and 8 μM-Ca^{2+} (●) and increasing amounts of somatostatin as indicated; 100 μM-GTP and an ATP-regenerating system were present throughout. For details see the legend to Fig. 2. Results are the mean ± S.E.M. of ten observations from two independent experiments. Statistical analysis was by Student's t test for unpaired data; * $P < 0.001$. Where not shown, the S.E.M. is masked by the size of the symbols.

RESULTS

HIT cells secrete insulin in response to glucose and other nutrient secretagogues [16,17]. The combination of 10 mM-glucose, 5 mM-leucine and 5 mM-glutamine caused a 7-fold stimulation of insulin secretion from intact cells (Fig. 1), corresponding to approx. 14% of cellular hormone content. Stimulated secretion was inhibited by all three somatostatin analogues in a dose-dependent manner. With somatostatin 14 and somatostatin 28 the maximal inhibition was about 85% of the stimulated release seen at hormone concentrations of 10 and 100 nM. Sandostatin inhibited secretion by 65%. Half-maximal effects were reached at concentrations of the inhibitor of 1–5 nM (Fig. 1).

An increase in the concentration of cytosolic Ca^{2+} is thought to be the main mediator of nutrient-stimulated insulin secretion, and such an increase has also been observed in glucose-stimulated HIT cells [10,18]. In permeabilized cells, Ca^{2+} directly stimulates exocytosis of insulin. When electrically permeabilized HIT cells were incubated in solutions containing Ca^{2+} in increasing amounts from 0.1 to 50 μM, release of insulin was stimulated up to 11-fold (Fig. 2). Secretion was augmented half-maximally by 1.6 μM-Ca^{2+} and maximally by about 20 μM-Ca^{2+} (Fig. 2), representing fractional release of up to 40% of cellular hormone content. Ca^{2+}-induced insulin secretion is temperature-dependent, since it was inhibited by over 90% at 4 °C (results not shown). To test the dependency on ATP, three separate experiments were performed in which the Ca^{2+} concentration was raised from 0.1 to 50 μM in the presence or absence of ATP. The incremental insulin secretion was 41.6 ± 2.2 and 99.7 ± 13.3 ng of insulin/5 min per 10^6 cells in the absence and presence of 5 mM-ATP respectively. Under standard conditions (with ATP) the stimulation of secretion was rapid and reached maximal levels at 2 min. Ca^{2+}-induced release was 31.2 ± 1.4, 36.5 ± 1.9, 46.7 ± 3.5, 42.6 ± 2.1 and 39.1 ± 1.8% of cellular insulin content at 0.5, 1, 2, 5 and 10 min respectively ($n = 10$, from two separate experiments). The contribution to the secretory response from cells remaining intact after the permeabilization procedure is negligible. In two experiments where intact and permeabilized cells were incubated in parallel, secretion rose by 63 ± 15% and 1160 ± 97% ($n = 10$) over basal, respectively, when the Ca^{2+} concentration was increased from 0.1 to 10 μM.

The potency of the different somatostatin analogues to inhibit secretion in intact HIT cells was similar (Fig. 1); therefore in all subsequent experiments in permeabilized cells only somatostatin 14 was tested. In the presence of GTP (100 μM) and a nucleotide-regenerating system, somatostatin inhibited Ca^{2+}-induced secretion in a dose-dependent manner (Fig. 3). Concentrations lower than 10 nM did not affect secretion significantly (results not shown), whereas the hormone inhibited secretion by 24, 68, 62 and 50% at 0.01, 0.1, 1 and 10 μM respectively. The E_{50} was estimated as approx. 50 nM.

Several experimental approaches were used to investigate the involvement of a G-protein in somatostatin action. First, the dependency on exogenous GTP was examined. In the absence of GTP, somatostatin (0.1 μM) failed to inhibit Ca^{2+}-induced insulin secretion (12% attenuation; Fig. 4). The hormone caused inhibitions of 22, 40, 40 and 39% at GTP concentrations of 10, 100, 500 and 1000 μM respectively (Fig. 4). Second, the somatostatin effect on Ca^{2+}-induced secretion was completely overcome when cells were treated with pertussis toxin (100 ng/ml) during the 3 h spinner culture before permeabilization (Table 1, A). The negative value represents a slight

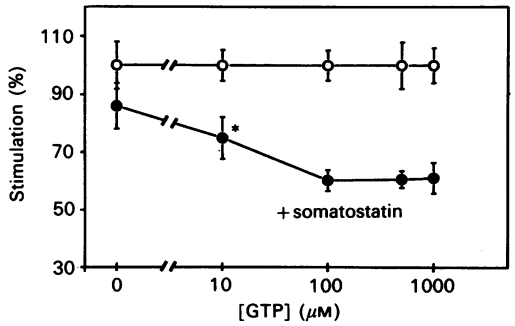

Fig. 4. Effect of GTP on somatostatin-induced inhibition of insulin secretion in electrically permeabilized HIT cells

Cells were incubated as described in the Experimental section. Somatostatin was tested at 0.1 μM, GTP was added in increasing amounts as indicated and an ATP-regenerating system was present throughout. Secretion owing to the increase of Ca^{2+} from 0.1 to 10 μM in the presence of the respective GTP concentration was set at 100%. None of the tested GTP concentrations significantly altered Ca^{2+}-induced secretion (results not shown). Results are means ± S.E.M. of 15 observations from three independent experiments. Statistical analysis was by Student's *t* test for unpaired data: $*P < 0.02$.

Table 1. Effects of guanine nucleotides and pertussis-toxin pretreatment on somatostatin-induced inhibition of Ca^{2+}-induced insulin secretion in permeabilized HIT cells

HIT cells were permeabilized and incubated as described in the Experimental section. Results are expressed as percentage inhibition of insulin secretion owing to the increase in Ca^{2+} from 0.1 to 10 μM in the presence of the respective guanine nucleotide. In Experiment A half of the cells were pretreated with pertussis toxin (100 ng/ml during the 3 h spinner culture) before permeabilization. GTP (100 μM) and the ATP-regenerating system were present during incubation. Somatostatin was tested at 0.1 μM throughout. When GTP[S] or GDP was added (B), the regenerating system was omitted. The guanine nucleotides did not affect Ca^{2+}-stimulated secretion significantly. Data are means ± S.E.M. for the numbers of observations (shown in parentheses) from one (A) or two (B and C) experiments.

	Condition	Somatostatin effect (% inhibition of Ca^{2+}-induced release)
A	Control cells; 100 μM-GTP	57.9 ± 5.9 (5)
	Pertussis-toxin-treated cells; 100 μM-GTP	−15.0 ± 10.6 (5)
B	100 μM-GTP	28.4 ± 4.6 (10)
	10 μM-GTP[S]	24.6 ± 4.4 (10)
	100 μM-GDP	−9.1 ± 6.8 (10)
C	200 μM-GTP	39.7 ± 6.3 (9)
	200 μM-GTP + 100 μM-GDP	17.3 ± 4.8 (10)

augmentation of Ca^{2+}-induced secretion by somatostatin in pertussis-toxin-treated cells. Third, if activation of a G-protein mediates the effect of somatostatin, maintenance of the G-protein in its GDP-liganded form should interfere with the action of the hormone. Indeed, when GTP was replaced by GDP (100 μM), somatostatin no longer inhibited insulin secretion (Table 1, B). Moreover, a competition between GTP and GDP is suggested from the results depicted in Table 1(C), where GDP attenuated the effect seen in the presence of GTP. Finally, G-proteins can also be activated by the stable GTP analogue GTP[S]. In the presence of 10 μM-GTP[S], inhibition was not significantly different from that obtained in the presence of 100 μM-GTP (Table 1, B). In all experiments illustrated in Fig. 4 and Table 1, GTP, GDP and GTP[S] did not significantly alter basal and Ca^{2+}-stimulated insulin secretion (results not shown).

DISCUSSION

We demonstrate here that somatostatin, an important physiological inhibitory hormone in a variety of systems, not only abolishes stimulated insulin secretion in intact HIT cells (Fig. 1, and refs. [16,19]), but also Ca^{2+}-induced hormone release in electrically permeabilized cells (Fig. 3). Therefore, somatostatin action on exocytosis occurs distal to the generation of small soluble second messengers such as cyclic AMP, which leaks out of the permeabilized cells [14], and Ca^{2+}, whose concentration is buffered under the present experimental conditions. Previous indirect studies in islet cells with the ionophore A23187 or $^{45}Ca^{2+}$-flux measurements, as well as addition of exogenous cyclic AMP, had already pointed to a distal site of action of somatostatin [12,13,20]. The preserved efficacy of somatostatin in permeabilized cells, although not denying a possible modulation of ion channels in intact cells, makes it unlikely that ion channels are the main effector system of the hormone.

It should be noted that the sensitivity of secretion to somatostatin is lowered (about 10-fold) after permeabilization. This could be due to both the loss of functional receptors and depletion from the permeabilized cells of essential factors involved in the regulation of secretion. Accordingly, the variation in stimulated secretion and degree of inhibition by somatostatin between preparations could be an indication of such depletion. One factor identified as essential to support inhibition of secretion is GTP, known to be required for the activation of G-proteins. Consequently, inhibition of Ca^{2+}-induced secretion by somatostatin depends on the presence of exogenously added GTP and, as expected, GDP does not substitute for GTP. The G-proteins involved remain to be identified, but they belong to the pertussis-toxin-sensitive group. Although binding of the hormone in permeabilized cells was not assessed in the present work, a study in a pituitary cell line demonstrated that pertussis toxin decreased somatostatin binding only to isolated membranes, not to intact cells [21].

The sensitivity to pertussis toxin of somatostatin effects is shared by adrenaline acting via α_2-adrenoceptors [3,9,14] and by the neuropeptide galanin [9,15,22], two other potent inhibitors of insulin secretion. Like somatostatin, these hormones not only inhibit insulin secretion but also lower cyclic AMP levels [3,22]. They also transiently lower cytosolic Ca^{2+} and membrane depolarization in nutrient-stimulated cells [9]. In addition, noradrenaline and adrenaline have been shown to inhibit Ca^{2+}-stimulated insulin secretion from permeabilized islets [23,24], and we have previously demonstrated such effects of adrenaline [14] and galanin [15] in permeabilized RINm5F cells. Our studies revealed that, although adrenaline and somatostatin effects depend on the presence of GTP, such was not the case for galanin inhibition of secretion. This difference cannot be explained by the two cell systems used (RINm5F and HIT), but rather suggests intrinsic differences in the transduction of the receptor activation. Recently, somatostatin was shown to inhibit cyclic AMP- and Ca^{2+}-ionophore-induced corticotropin release from intact AtT-20 cells, a pituitary cell line. The report also demonstrates a small decrease in Ca^{2+}-stimulated hormone release from digitonin-permeabilized cells, sensitive to guanine nucleotides and pertussis toxin [25]. These findings make the concept of the distal action of the inhibitory hormones more general.

The attenuation by pertussis toxin of the actions of somato-

statin [3,7,9], of α_2-adrenergic agonists [3,9,14] and of galanin [9,15,22,26] on adenylate cyclase, on ion channels and on exocytosis suggests, but does not prove, that the dissociation of the pathways occurs distal to hormone–receptor binding and G-protein activation. It is of interest in this context that neither somatostatin inhibition of neurotensin secretion from enteric endocrine cells nor the inhibition of Na^+/H^+ exchange was affected by pertussis toxin [27]. Despite this, pertussis toxin blocked the somatostatin inhibition of adenylate cyclase in these cells [27], possibly suggesting the involvement of an additional G-protein or another mechanism regulating Na^+/H^+ exchange. Interference with Na^+/H^+ exchange cannot explain the inhibition of insulin secretion, since this process is no longer operative in permeabilized cells.

The effector systems involved in the modulation of exocytosis are still unknown. Apart from a possible modulation by G-proteins of the putative fusion pore [14,28], the influence could also be exerted via phosphorylation/dephosphorylation reactions. However, in permeabilized islets noradrenaline failed to change basal as well as Ca^{2+}- and cyclic AMP-dependent phosphorylation patterns analysed by one-dimensional gel electrophoresis [29]. More detailed examination of the phosphorylation reactions may be necessary, as suggested by a recent report, describing the effects of the inhibitory peptide Phe-Met-Arg-Phe (FMRF) in *Aplysia* neurons. The peptide prevented phosphorylation of a limited number of protein spots, as revealed by two-dimensional gel electrophoresis [30]. The inhibitory hormones may well act to influence protein kinases or phosphatases, in particular, since somatostatin has been shown to promote phosphotyrosine phosphatase activity in membranes of a pancreatic cancer-cell line [31,32]. However, in the latter report [32] the somatostatin analogue Sandostatin (SMS) was practically inefficient in stimulating phosphatase activity. This compound is fully active in inhibiting insulin secretion (Fig. 1), perhaps indicating the involvement of different receptors in the two processes. The further identification of subtypes of somatostatin receptors and the delineation of post-receptor events are thus required.

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