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A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary cell line

(cytosolic calcium/patch clamp/corticotropin/guanosine 5'-[γ-thio]triphosphate/AtT-20 cells)

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ABSTRACT **Somatostatin reduces voltage-dependent Ca^{2+} current (I_{Ca}) and intracellular free Ca^{2+} concentration in the AtT-20/D16-16 pituitary cell line. We tested whether guanine nucleotide-binding proteins (G or N proteins) are involved in the signal transduction mechanism between the somatostatin receptor and voltage-dependent Ca^{2+} channels. Treatment of the cells with pertussis toxin, which selectively ADP ribosylates the GTP binding proteins G_i and G_o and suppresses the ability of G_i to couple inhibitory receptors to adenylate cyclase, abolished the action of somatostatin on both I_{Ca} and intracellular free Ca^{2+}. Intracellular application of the nonhydrolyzable guanine nucleotide analog guanosine 5'-[γ-thio]triphosphate (GTP[γS]), which irreversibly activates G proteins, changed the somatostatin effect on I_{Ca} from a reversible to an irreversible inhibition. Intracellular GTP[γS] alone caused a very slowly developing inhibition of I_{Ca}. When I_{Ca} was inhibited by GTP[γS] (alone or with somatostatin), it failed to respond to subsequent applications of somatostatin. The effect of GTP[γS] on the inhibition of I_{Ca} by somatostatin was not altered by the intracellular application of cAMP and 3-isobutyl-1-methylxanthine. The results suggest that a GTP-binding protein is directly involved in the cAMP-independent receptor-mediated inhibition of voltage-dependent Ca^{2+} channels.**

Somatostatin is a peptide widely distributed in the body (1), which has inhibitory effects on the secretory response of a variety of cells in the gut, pancreas, and anterior pituitary (2, 3). In view of the importance of Ca^{2+} in the control of secretion, it has been suggested that somatostatin might act, in part, by regulating cytosolic Ca^{2+} in target organs (2, 4, 5). This hypothesis has received experimental support in some cell types. Somatostatin decreases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in two related clonal pituitary cell lines that secrete prolactin, GH_3 (6) and GH_4C_1 (7) cells. In another pituitary cell line, the corticotropin-secreting AtT-20 cells, somatostatin decreases $[Ca^{2+}]_i$ and inhibits the voltage-dependent Ca^{2+} current, I_{Ca} (8). In these cells, somatostatin also suppresses receptor-induced stimulation of adenylate cyclase by activating G_i, the GTP-dependent inhibitory subunit of the receptor–cyclase complex (9, 10). Recent evidence indicates that the effect of somatostatin on $[Ca^{2+}]_i$ and I_{Ca} is not mediated via inhibition of adenylate cyclase, since it is independent of cellular cAMP levels (8).

It has been shown recently that G proteins, originally described as regulatory components of adenylate cyclase, can also participate in the control of cellular events unrelated to cyclase activity (11–23). We have examined in AtT-20 cells the possibility that G proteins may represent the link between somatostatin receptors and voltage-dependent Ca^{2+} channels. We now report that the inhibition of the Ca^{2+} current by somatostatin is abolished by pertussis toxin, a protein isolated from *Bordetella pertussis*, which selectively ADP ribosylates the GTP-binding proteins G_i and G_o (24–27) and suppresses the ability of G_i to couple inhibitory receptors to adenylate cyclase (28). We also report that the intracellular application of the nonhydrolyzable GTP analog guanosine 5'-[γ-thio]triphosphate (GTP[γS]) renders irreversible the somatostatin-induced inhibition of I_{Ca}. This behavior would be expected of a process involving G_i or a similar molecule, since G_i activation is GTP dependent and is terminated by GTP hydrolysis (29, 30). These observations suggest the direct involvement of a G protein in the receptor-mediated regulation of voltage-dependent Ca^{2+} channels.

MATERIALS AND METHODS

The following substances were obtained from the companies indicated: synthetic somatostatin-14 and [D-Trp^8]somatostatin (Bachem Fine Chemicals, Torrance, CA); bovine serum albumin, cAMP, 3-isobutyl-1-methylxanthine (IBMX), tetrodotoxin, and 5'-guanylyl imidodiphosphate (p[NH]ppG) (Sigma); forskolin, quin-2, and quin-2 tetraacetoxymethylester (Calbiochem–Behring); Dulbecco's modified Eagle's medium (DMEM) (GIBCO); fetal calf serum (M. A. Bioproducts, Walkersville, MD, and KC Biological, Lenexa, KS); creatine phosphate, creatine kinase, and GTP[γS] (Boehringer Mannheim); pertussis toxin (List Biologicals, Campbell, CA).

Cell Culture Methods. AtT-20/D16-16 cells were subcultured and grown in DMEM with 10% fetal calf serum as described (31, 32). Cells were plated in 75-cm^2 tissue culture flasks at an initial density of 2×10^5 cells per cm^2 for quin-2 and in 35-mm diameter tissue culture dishes for patch-clamp experiments. Cells were used 5–7 days after plating (60–80% confluency).

Determination of Cytosolic Calcium. Cytosolic Ca^{2+} was measured by using the fluorescent Ca^{2+} probe quin-2 (33) as described (31). Briefly, the cells were detached from the growth flask, washed with DMEM containing 1 mg of bovine serum albumin per ml and incubated for 15 min at 37°C with 50 μM quin-2 acetoxymethylester. After washing with Hanks' medium with 0.2 mg of bovine serum albumin per ml, the cells were resuspended in Hanks' medium bovine serum albumin at 10^6 cells per ml and placed in a thermostated spectrophotometer cuvette. Cytosolic Ca^{2+} concentrations were calculated by the calibration procedure of Tsien *et al.*

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Abbreviations: G_i, the inhibitory guanyl nucleotide-binding regulatory protein subunit of adenylate cyclase (also referred to as N_i); G_o, a GTP-binding protein from bovine brain (also referred to as N_o); GTP[γS], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, 5'-guanylyl imidodiphosphate; IBMX, 3-isobutyl-1-methylxanthine.

(33). For pertussis toxin experiments, cells were incubated overnight with 50 or 100 ng of pertussis toxin per ml.

Patch-Clamp Methods. The patch-clamp method was used to record Ca^{2+} current in the whole-cell voltage-clamp mode (34) as described (8). Briefly, currents were recorded in an external solution containing 150 mM tetraethylammonium chloride, 0.8 mM $MgCl_2$, 5.4 mM KCl, 10 mM $CaCl_2$, 10 mM Hepes/NaOH buffer (pH 7.4), 45 mM glucose, 1 μM tetrodotoxin, and 1 mg of bovine serum albumin per ml with an osmolarity of 340 mosmol·kg^{-1}. The patch pipette solution contained 120 mM CsCl, 11 mM EGTA, 2 mM tetraethylammonium chloride, 2 mM $MgCl_2$, 10 mM Hepes/CsOH buffer (pH 7.4), 4 mM MgATP, 20 mM creatine phosphate, and 50 units of creatine kinase per ml with an osmolarity of 318 mosmol·kg^{-1}. The ATP regenerating system was added to prevent I_{Ca} rundown (35). Nonhydrolyzable GTP analogs, GTP[γS] or p[NH]ppG, were added to the patch pipette solution at the concentrations stated. Some patch pipettes

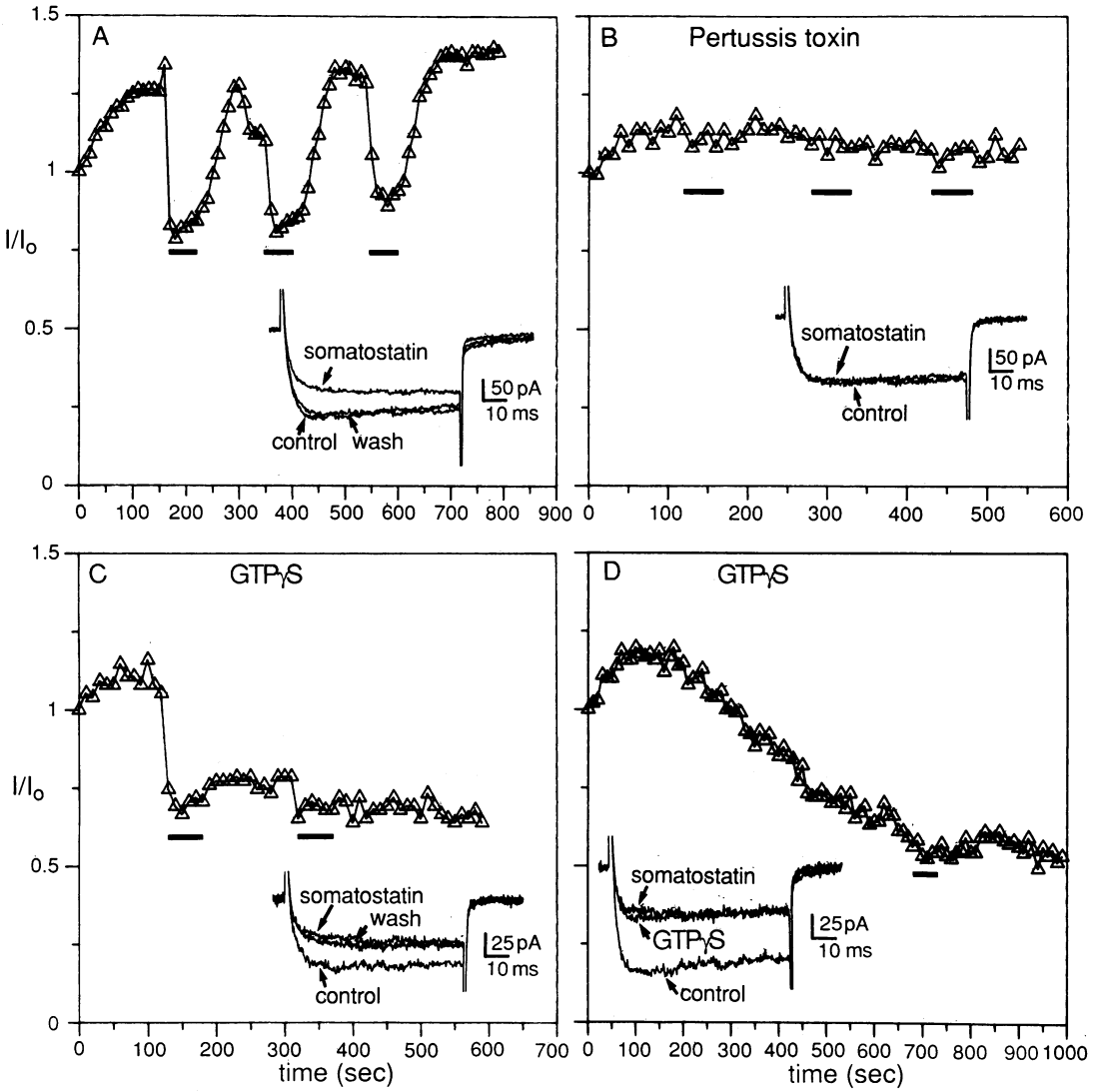

FIG. 1. Effect of pertussis toxin and GTP[γS] on somatostatin-induced inhibition of voltage-dependent Ca^{2+} current (I_{Ca}) using the whole-cell patch-clamp technique. (*A*) Effect of [D-Trp^8]somatostatin on I_{Ca}. [D-Trp^8]Somatostatin (0.1 μM) was applied for three 60-sec periods (bars). Somatostatin application rapidly reduced I_{Ca}, which recovered within 60 sec after termination of somatostatin application. The current at each time point (*I*) is expressed as a ratio with respect to the initial current amplitude (I_o). Current amplitude was measured isochronally (9–15 msec) from the onset of the depolarizing step. An initial increase of I_{Ca} amplitude (run-up) was usually observed under these recording conditions. (*Inset*) Current records for I_{Ca} just prior to somatostatin application 540 sec after beginning patch recording (control), the current 30 sec after beginning somatostatin application (somatostatin), and the current 60 sec after termination of somatostatin application (wash). (*B*) Effect of pertussis toxin on somatostatin-induced inhibition of I_{Ca}. Cells were pretreated with pertussis toxin (100 ng/ml, overnight) or a medium change (control). [D-Trp^8]Somatostatin (10 μM) was applied for three 60-sec periods (bars). Pertussis toxin treatment blocked the effect of somatostatin on I_{Ca}. Somatostatin decreased I_{Ca} in control cells by 29.63% ± 3.29% (n = 4) and in pertussis toxin-treated cells by 1.98% ± 2.66% (n = 4) ($P \leq 0.0005$). (*Inset*) I_{Ca} just prior to somatostatin application 110 sec after beginning patch recording (control) and the current 30 sec after beginning somatostatin application (somatostatin). (*C*) Effect of GTP[γS] on somatostatin-induced inhibition of I_{Ca}. When 100 μM GTP[γS] was in the patch pipette, the first application of 0.1 μM [D-Trp^8]somatostatin (bar on left) resulted in an inhibition of I_{Ca} that was not reversed by termination of somatostatin application. (*Inset*) Current records for I_{Ca} just prior to somatostatin application 110 sec after beginning patch recording (control), the current 30 sec after beginning somatostatin application (somatostatin), and the current 60 sec after termination of somatostatin application (wash). A second application of 0.1 μM [D-Trp^8]somatostatin (bar on right) had little effect on I_{Ca}. (*D*) Effect of intracellular GTP[γS] alone. GTP[γS] (100 μM) in the patch pipette caused a decrease in I_{Ca} amplitude that developed slowly over 650 sec. [D-Trp^8]Somatostatin (0.1 μM) when applied at 680 sec (bar) had little effect on I_{Ca}. (*Inset*) Current records for I_{Ca} 190 sec after beginning patch recording with GTP[γS] (control), the current just prior to somatostatin application 670 sec after beginning patch recording with GTP[γS] (GTPγS), and the current 30 sec after beginning somatostatin application (somatostatin).

had an additional 100 μM cAMP and 1 mM IBMX. Patch pipettes had resistances of 3–5 MΩ. Cells were voltage clamped with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, F.R.G.) to a holding potential of −80 mV and stepped to +10 mV, the peak of the current–voltage relationship for I_{Ca} (8). Currents were recorded using a 3-KHz low-pass filter, digitized at 250 μsec per point and stored for analysis using a PDP-11/23 microcomputer. All recording was at room temperature (20°C–22°C). [D-Trp^8]-Somatostatin was diluted from a stock solution of 0.1 mM with external solution to give concentrations of 10 to 0.1 μM. A macropipette was filled with this solution of somatostatin for a length of ≈4 cm, and the tip was lowered into the recording bath near the surface of the cell under study. The weight of the fluid volume in the macropipette was sufficient to generate a constant flow of somatostatin-containing solution onto the cell. The macropipette was withdrawn from the bath to terminate the application of somatostatin.

RESULTS AND DISCUSSION

In a previous report, we described the reduction in I_{Ca} and $[Ca^{2+}]_i$ caused by somatostatin (illustrated in Figs. 1*A* and 2*A*) and discussed the possible causal relationship between the two effects (8). To examine whether these effects are mediated by a GTP-binding protein, we used pertussis toxin and the nonhydrolyzable GTP analogs GTP[γS] and p[NH]ppG.

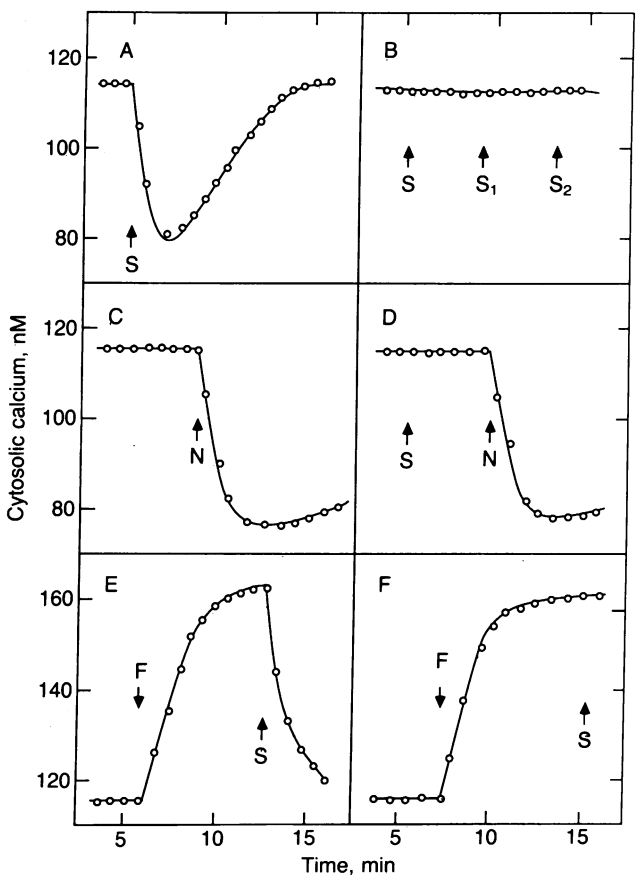

FIG. 2. Effect of pertussis toxin on somatostatin-, nifedipine-, and forskolin-induced alterations of cytosolic Ca^{2+} concentration. Effect of 0.1 μM somatostatin-14 (S), 0.1 μM nifedipine (N), and 10 μM forskolin (F) in control cells (*A*, *C*, and *E*), and in cells pretreated with pertussis toxin (50 or 100 ng/ml, overnight) (*B*, *D*, and *F*). S_1 and S_2, 1 μM and 10 μM somatostatin-14, respectively. Values are means from four to six experiments carried out using at least three different cell preparations. Standard deviations did not exceed 15% of the means.

Pertussis Toxin Blocks the Somatostatin-Induced Inhibition. Pertussis toxin selectively ADP ribosylates G_i and G_o (24–27) and inhibits the ability of G_i to couple inhibitory receptors to adenylate cyclase (28). ADP ribosylation of a 40-kDa protein (presumably G_i or G_o or both) by pertussis toxin has been demonstrated in membranes from AtT-20 cells (9). We have found that pretreatment of AtT-20 cells with pertussis toxin (50 or 100 ng/ml, overnight) abolished both the inhibition of I_{Ca} (Fig. 1*B*) and the decrease in $[Ca^{2+}]_i$ (Fig. 2*B*) induced by somatostatin. The affinity of receptors coupled to G_i for their agonistic ligands (including somatostatin) can be decreased in membranes from pertussis toxin-treated cells (10, 29, 36, 37) probably due to loss of G protein–receptor interaction. This raises the question of whether the suppression of somatostatin's effect on I_{Ca} and $[Ca^{2+}]_i$ caused by pertussis toxin could be due to a change in affinity of the somatostatin receptor or to a loss of coupling between receptors and Ca^{2+} channels. This possibility appears to be unlikely for the following reasons. First, pertussis toxin has been reported to decrease receptor affinity for somatostatin-14 and for [D-Trp^8]somatostatin in membranes from AtT-20 cells by a factor of 400 and 1100, respectively (10). If the loss of somatostatin's activity is due to a decrease in receptor affinity, then increasing the concentration of the peptide should restore its effectiveness. We found, however, that concentrations of somatostatin 10,000-fold higher than those active in control cells did not overcome the effect of pertussis toxin (Figs. 1*B*, 2*B*, and 3). Second, the effect of pertussis toxin on ligand receptor affinity has so far been demonstrated only in isolated membrane preparations (10, 29, 36, 37). In a binding study on intact cells, pertussis toxin did not change the binding of somatostatin (7). To rule out the possibility that pertussis toxin might nonspecifically disrupt cellular Ca^{2+} homeostasis, we used agents known to affect cellular Ca^{2+} by mechanisms of action not believed to involve G_i. AtT-20 cells are normally spontaneously active (38, 39). Nifedipine, a specific blocker of Ca^{2+} channels, should inhibit Ca^{2+} flux through Ca^{2+} channels opened by these spontaneous action potentials. With the patch-clamp technique in the whole-cell voltage-clamp mode, nifedipine (10 μM) decreased I_{Ca} by 33.44% ± 4.65% (n = 3). In quin-2 experiments, nifedipine

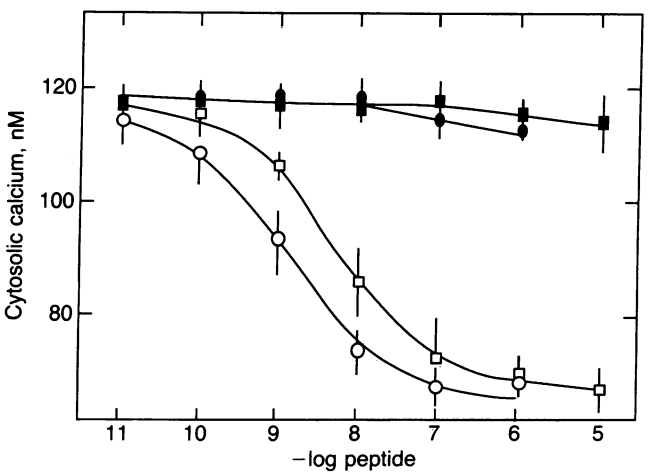

FIG. 3. Concentration dependence of the effects of somatostatin-14 and [D-Trp^8]somatostatin on cytosolic Ca^{2+} concentration. The cytosolic concentrations of Ca^{2+} at the time at which somatostatin-14 (□) and [D-Trp^8]somatostatin (○) produced their maximal effect (≈90 sec after application) are shown as a function of the concentrations of the peptides. In cells pretreated with pertussis toxin, neither somatostatin-14 (■) nor [D-Trp^8]somatostatin (●) significantly modified the cytosolic Ca^{2+} level. Values are means from four to six experiments carried out using at least four different cell preparations. Standard deviations are shown.

(0.1 μM) decreased $[Ca^{2+}]_i$ similarly in control (Fig. 2*C*) and pertussis toxin-treated cells (Fig. 2*D*). Forskolin increases $[Ca^{2+}]_i$ in AtT-20 cells (31) (Fig. 2*E*), and the effect of forskolin on $[Ca^{2+}]_i$ was not modified by pertussis toxin (Fig. 2*F*). In addition, basal $[Ca^{2+}]_i$ levels and the current–voltage characteristics of I_{Ca} were similar in control and toxin-treated cells.

GTP[γS] Inhibits the Ca^{2+} Current. The nonhydrolyzable GTP analog, GTP[γS], was also used to test whether a G protein is involved as a coupler between somatostatin receptors and Ca^{2+} channels. GTP activates the transition of G_i from the inactive to the active form, which involves dissociation of the heterotrimeric G protein into its α and $\beta\gamma$ subunits (25). Activation of G_i involves displacement of bound GDP (inactive, undissociated state) followed by binding of GTP (active, dissociated state). Inactivation is then brought about by the hydrolysis of GTP to GDP, which is catalyzed by the G protein (29). The binding to G_i of the nonhydrolyzable GTP analog, GTP[γS], therefore makes the activation of G_i virtually irreversible, so that even removal of the hormone from the receptor does not terminate its action (30). In control cells, the effect of somatostatin was readily reversible and could be reproduced by repeated applications of the peptide (Fig. 1*A*). When GTP[γS] was applied to the cell interior via the patch pipette, somatostatin still produced a rapid inhibition of I_{Ca}, but the inhibition persisted after removal of the peptide (Fig. 1*C*) in 9 of 12 cells. Moreover, a second application of somatostatin had little or no effect on the already inhibited I_{Ca} (Fig. 1*C*). Intracellular application of p[NH]ppG (100 μM) had very similar effects in 5 of 7 cells (data not shown). When GTP[γS] was in the pipette and somatostatin was not applied, an inhibition of I_{Ca} developed, very slowly reaching a maximum in 500 or more sec after establishment of the whole-cell patch (Fig. 1*D*). Control experiments without GTP[γS] in the pipette showed little or no decline of I_{Ca} over 1000 sec. After GTP[γS] had produced its effect, the application of somatostatin produced little or no additional inhibition of I_{Ca} (Fig. 1*D*). These results appear to be analogous to observations made in cyc^- S49 lymphoma membranes where GTP[γS] causes an irreversible dissociation of G_i, and somatostatin facilitates this dissociation (40).

cAMP Independence. Previous reports indicate that the somatostatin receptor in AtT-20 cells, like several other inhibitory receptors, is coupled to G_i, which mediates an inhibitory action on adenylate cyclase (9, 10). Since I_{Ca} and $[Ca^{2+}]_i$ can be regulated by cAMP in these cells (31), it is likely that under conditions of hormonal stimulation of adenylate cyclase, a component of somatostatin's effect on calcium movement is due to inhibition of cyclase stimulation. It is thus theoretically possible that GTP[γS] could reduce I_{Ca} by inhibiting adenylate cyclase via activation of G_i and thus reduce cAMP levels, as was shown in membranes from cyc^- S49 lymphoma cells (41). Since I_{Ca} can be regulated by cAMP in AtT-20 cells (31), we examined whether the effect of GTP[γS] on I_{Ca} occurred when cAMP-dependent protein kinase was maximally activated. We found that 100 μM cAMP, which is a concentration 50- to 100-fold greater than that needed to saturate cAMP-dependent protein kinase in AtT-20 cells (42), and 1 mM of the phosphodiesterase inhibitor IBMX in the patch pipette had no effect on either the action of somatostatin on I_{Ca} or the effect of GTP[γS] on the inhibition of I_{Ca} by somatostatin. The mean value of the I_{Ca} amplitudes prior to somatostatin application for GTP[γS], cAMP, and IBMX loaded cells was 131 $\pm$ 25 pA (n = 4). For non-cAMP-loaded cells with GTP[γS] only in the patch pipette, the I_{Ca} amplitude was 106 $\pm$ 18 pA (n = 6). Somatostatin (0.1 μM) reduced I_{Ca} by 34.06% $\pm$ 3.58% (n = 4) in cells loaded with GTP[γS], cAMP, and IBMX, and by 39.10% $\pm$ 4.45% (n = 6) in cells loaded with GTP[γS] only. There was no significant difference between these two groups in the fractional reduction of I_{Ca} ($P > 0.1$). This is consistent with previous observations in these cells that somatostatin can inhibit I_{Ca} and reduce $[Ca^{2+}]_i$ by a cAMP-independent mechanism (8).

Several recent studies have suggested that G proteins may mediate cellular processes that are independent of adenylate cyclase. In mast cells, the intracellular application of nonhydrolyzable GTP analogs induces histamine secretion (12, 13). Moreover, histamine release in mast cells is pertussis toxin sensitive (19). In several cell types, the breakdown of phosphatidylinositols is stimulated by GTP analogs and/or inhibited by pertussis toxin (15–18). The receptor-mediated release of arachidonic acid is blocked by pertussis toxin in neutrophils (14) and 3T3 fibroblasts (20). In GH_4C_1 cells, the decrease in basal $[Ca^{2+}]_i$ induced by somatostatin is abolished by pertussis toxin (7). Since the toxin does not modify the binding characteristics of somatostatin in these cells, this response presumably involves G_i (7). The stimulation of the K^+ conductance by muscarinic receptors in cardiac cells (21, 22) and the reduction of calcium current by norepinephrine and γ-aminobutyric acid in dorsal root ganglion cells (43) have recently been found to be sensitive to pertussis toxin and guanine nucleotide analogues.

Our results suggest that a G protein is directly involved in a cAMP-independent receptor-mediated inhibition of voltage-dependent Ca^{2+} channels in an endocrine cell. Our data do not distinguish, however, whether the regulation of Ca^{2+} channels involves G_i or G_o. Conceivably, G_i could mediate somatostatin's inhibition of both Ca^{2+} channels and adenylate cyclase. On the other hand, somatostatin receptors could be coupled to both G_i and G_o; G_i could inhibit adenylate cyclase and G_o could inhibit the Ca^{2+} channel. It is also possible that somatostatin might be coupled to another class of GTP-binding proteins. The molecular mechanisms by which G proteins inhibit voltage-dependent Ca^{2+} channels remain to be explored. It is possible, for example, that the G protein may directly induce a conformational change in the Ca^{2+} channel that inhibits its function. Alternatively, G proteins could control an enzymatic mechanism that, in turn, affects the function of the Ca^{2+} channel. For example, increased activity of Ca^{2+} channels in AtT-20 cells may be associated with protein phosphorylation, possibly of a component of the channel itself. In such a scheme, activation of a phosphatase by a GTP-binding protein could result in a dephosphorylation that would lead to Ca^{2+}-channel inactivation.

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