Galanin-Stimulated High-Affinity GTPase Activity in Plasma Membranes From RINm5F Cells

G. SKOGLUND, C.R. BLISS, AND G.W.G. SHARP

GTPase activity was studied in plasma membranes purified from the clonal β-cell line RINm5F. GTPase activities were identified as two broad classes with high or low affinity for GTP. The low-affinity GTPase activity had a K_m >60 μM. In contrast, the high-affinity activity had a K_m of 225 nM. Only the high-affinity activity was stimulated by galanin. The stimulated activity had a higher K_m (448 nM) and V_{max} (75 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein) compared with the basal. This does not necessarily reflect a complex mechanism of stimulation. Rather, it may reflect that basal activity most likely results from multiple GTPases, whereas the stimulated activity probably reflects one or two specific GTPases. Galanin stimulated the high-affinity GTPase, over the concentration range in which it inhibits stimulated insulin secretion, to a maximal rate 80% greater than the basal rate. The EC_{50} was 5 nM. Murine and porcine galanin had similar potencies and intrinsic activities on the GTPase. Treatment of the RINm5F cells with PTX before making membranes completely eliminated the stimulatory effect of galanin. Thus, galanin stimulates PTX-sensitive GTPase activity in RINm5F cell membranes in a manner consistent with receptor activation. *Diabetes* 42:74–79, 1993

From the Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York.

Current address: Dr. G. Skoglund, INSERM U 55, Centre de Recherche, Hôpital Saint-Antoine, 184 rue du Faubourg Saint Antoine, F-75571 Paris Cedex 12, France.

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GTPase, guanosine 5′-triphosphatase; P_i, inorganic phosphate; PTX, pertussis toxin; G protein, guanine nucleotide binding protein; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol; cpm, counts per minute; App(NH)p, 5′-adenylimindodiphosphate.

The peptide galanin is an important and potent inhibitor of stimulated insulin secretion (1–3). The intracellular mechanisms underlying the inhibitory effect on insulin secretion are complex and involve several regulatory systems. For example, in the pancreatic β-cell, the ATP-sensitive K^+ channel (4–6), a voltage-dependent Ca^{2+} channel (7,8), adenylyl cyclase (9,10), and a distal mechanism late in stimulus-secretion coupling (11,12) all appear to be affected by galanin. Treatment of the pancreatic β-cell with PTX, which ADP-ribosylates G proteins of G_i and G_o subtypes, abolishes the inhibitory effect of galanin on insulin secretion (9,11,12). Also, the high affinity of the binding site for galanin on the pancreatic β-cell is reduced by PTX treatment (13). These observations indicate that the galanin receptor is a member of the class of plasma membrane receptors, for which the link between the extracellular ligand-binding sites to their intracellular effector(s) is a subset of receptor-activated G proteins, a family of heterotrimeric proteins with α-, β-, and γ-subunits that, on ligand-receptor binding, exchange bound GDP for GTP and dissociate into α- and βγ-subunits (14,15). Five G proteins that are candidates for interaction with the galanin receptor have been identified as G_{i1}, G_{i2}, and G_{i3} and two forms of G_o, presumably G_{o1}, and G_{o2} (16). The α-subunits, which show the greatest diversity among the G protein subunits and which bind and hydrolyze GTP, are major components in signal transmission to the intracellular effector(s) (14,17–19).

PTX treatment is one way to identify whether a receptor is linked to a certain subgroup of G proteins (20). A more direct approach is to study the intrinsic GTPase activity of the G protein α-subunits, because activation of a G protein will result in the hydrolysis of GTP to GDP and P_i formation. One method, which uses γ-^{32}P–labeled GTP, was first used by Cassel and Selinger (21) to show that catecholamines stimulated GTP hydrolysis and has been

used to show G protein linkage to other receptors (22,23). We studied the stimulation of high-affinity GTPase activity by galanin in membranes isolated from RINm5F cells. We found that galanin stimulated high-affinity GTPase activity over the concentration range previously shown to inhibit insulin secretion from this cell line. This stimulation of the high-affinity GTPase activity was blocked by PTX treatment of the cell.

RESEARCH DESIGN AND METHODS

Cells from the insulin-secreting RINm5F cell line of passage nos. 60–65 were cultured in 175 cm^2 flasks (Corning, Medfield, PA) in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin G at 37°C in 95% O_2, 5% CO_2. Plasma membranes were partially purified by the method of Ullrich and Wollheim (24). Briefly, the cells were harvested 5 days after plating, washed three times with homogenization buffer (250 mM sucrose, 5.0 mM HEPES, 0.5 mM EGTA, and PMSF at pH 7.4) and centrifuged at 500 *g* for 5 min. The cell pellet was resuspended in the same buffer (1 g cells/10 ml) and broken by cavitation after being subjected to nitrogen at 9 × 10^5 pascals for 30 min at 4°C in a Parr pressure homogenizer (Parr Instrument, Moline, IL). The resulting broken cell suspension was centrifuged at 700 *g* for 10 min. The supernatant was mixed with 90% Percoll to a final 15% Percoll solution, which was centrifuged at 48,000 *g* for 30 min. The partially purified plasma membrane preparation was collected at a Percoll density of 1.038, as estimated with density marker beads, and diluted three times with the same buffer before the last centrifugation at 70,000 *g* for 90 min. The plasma membrane preparation, which was floating on a Percoll bed, was collected and homogenized with a Teflon-glass homogenizer and diluted with a 15 mM Tris-HCl buffer (pH 7.5) to a concentration of ~4.0 mg protein/ml. The preparation was aliquoted and stored at −70°C until used. In experiments in which the effect of PTX was studied, cells were incubated for 20 h with 30 μg/L PTX before the plasma membrane preparation. A control membrane preparation from the same passage cells not treated with PTX was purified at the same time. Protein concentration was determined by the Bradford technique (25).

GTPase assay. GTPase activity was studied according to the method described by Koski and Klee (26). In short, the buffer contained either 12.5 mM Tris-HCl or 20 mM HEPES buffer (both pH 7.4) supplemented with 100 mM NaCl, 1.0 mM ouabain, 2 mM DTT, 0.1 mM EDTA, 1.0 mM App(NH)p, and an ATP-regenerating system containing 1.0 mM ATP, 10 mM creatine phosphate, and 5.0 U of creatine phosphokinase. [γ-^{32}P]GTP was held constant at 50,000 cpm per incubation and normally mixed with either 0.5 or 50 μM GTP or as indicated in the text. The protein concentration was usually 50 μg/ml. Galanin was added according to the experimental protocol, and the final incubation volume was 100 μl. The experiment was started by adding 50 μg/ml (5.0 μg/test tube) protein at 4°C, and the tubes were transferred at 10-s intervals into a 30°C water bath and incubated, usually for 10 min but

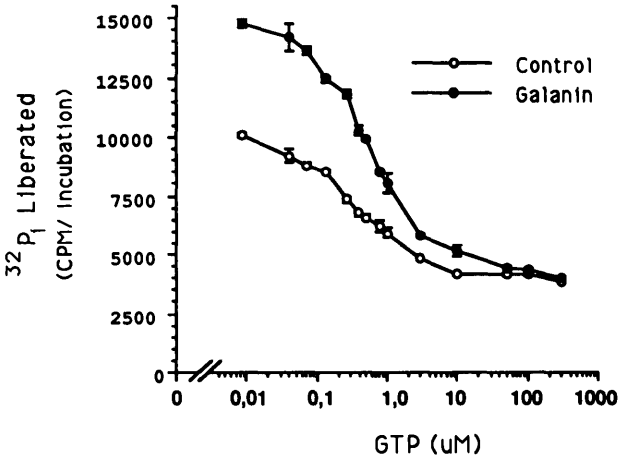

FIG. 1. The liberation of ^{32}P from [γ-^{32}P]GTP by RINm5F cell membranes, at various concentrations of cold GTP, in the absence and presence of 100 nM galanin. Radioactive [γ-^{32}P]GTP concentration was 62,000 cpm per incubation, and the unlabeled GTP concentration was increased from 0.01 to 300 μM. Results are from a typical experiment performed in triplicate. Five such experiments were performed with similar results.

it depended on the experiment. The reaction was stopped by addition of 900 μl of 20 mM H_3PO_4 containing 5% charcoal (pH 2.3) and centrifuged at 2500 *g* for 12 min. The liberated $^{32}P_i$ was quantitated by liquid scintillation spectrometry of 200 μl of the supernatant. Normally, <15% of the added [γ^{32}P]GTP was hydrolyzed.

In most experiments, porcine galanin from Peninsula (Belmont, CA) was used. In one series of experiments, murine galanin from Sigma (St. Louis, MO) was compared with the porcine galanin. ATP, creatine phosphokinase, creatine phosphate, App(NH)p, GTP, PTX, ouabain, DTT, EDTA, Tris, H_3PO_4, and washed charcoal was from Sigma. [γ-^{32}P]GTP, 30 Ci/mmol, was from Du Pont–NEN (Boston, MA). All other chemicals were analytical grade and from Mallinckrodt (Paris, KY).

Statistical analysis. Data are from experiments performed on two or more independent membrane preparations except for the PTX-treated membranes. Data are means ± SE. Paired Student's *t* test or variance analyses were used for statistical analysis.

RESULTS

Characterization of high-affinity GTPase activity in an RINm5F cell plasma membrane preparation. The hydrolysis of GTP, shown as liberation of $^{32}P_i$ from [γ-^{32}P]GTP, in partially purified plasma membranes from RINm5F cells is shown in Fig. 1. The radioactive [γ-^{32}P]GTP concentration was held constant at ~50,000 cpm per incubation (8–25 nM, depending on the specific activity of the [γ-^{32}P]GTP), and the nonradioactive GTP concentration was varied from 0.01 to 300 μM GTP. It can be seen from the data in Fig. 1 that 100 nM galanin increased GTP hydrolysis at GTP concentrations up to 10 μM GTP compared with the GTP hydrolysis in the absence of galanin.

The results show that the RINm5F cell membrane contains at least two different classes of GTPase activities, one of high affinity, which is stimulated by galanin,

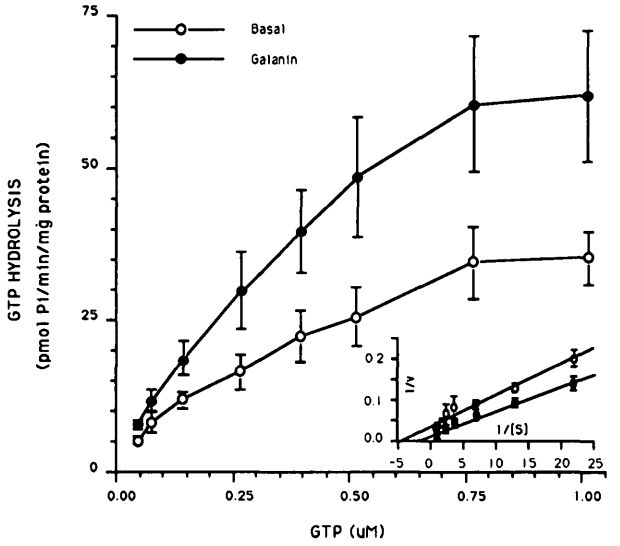

FIG. 2. High-affinity GTPase activity studies over the range of 0.1–1 μM GTP in RINm5F cell membranes. Data were obtained after subtraction of the low-affinity GTPase activity, as described in text, and are derived from 5 experiments performed in triplicate. A Lineweaver-Burke plot of data is in the insert. Galanin stimulated high-affinity GTPase activity at all concentrations tested.

and the low-affinity GTPase activity, which is not affected by galanin. Lineweaver-Burke analysis suggested that the low-affinity GTPase activity had a K_m of >60 μM. Because the low-affinity GTPase activity was not stimulated by galanin, it was not characterized further.

The high-affinity GTPase activity was estimated by the method of Cassel and Selinger (21). Briefly, the P_i (in cpm) liberated from [γ-^{32}P]GTP at 50 μM GTP was subtracted from the P_i (in cpm) liberated at lower GTP concentrations. We had two reasons to use 50 μM GTP for the subtraction: galanin did not affect GTP hydrolysis at this GTP concentration, and the liberation of P_i (in cpm) reached a plateau at this GTP concentration. This method of calculation was used for subsequent studies, such as those illustrated in Fig. 2, which shows the rates of GTP hydrolysis by the high-affinity GTPases in the presence and absence of galanin. From these results, it can be seen that 100 nM galanin increased the rate of GTP hydrolysis at all GTP concentrations tested to almost twice the rate of hydrolysis at concentrations >0.25 μM. The Lineweaver-Burke plot (insert in Fig. 2) shows that the apparent K_m was 226 ± 16 nM GTP for basal GTPase activity and 448 ± 56 nM GTP for galanin-stimulated activity ($P < 0.01$). The V_{max} was 29 ± 11 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein for basal activity and 75 ± 16 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ for galanin-stimulated activity ($P < 0.01$).

These experiments showed clearly that 100 nM galanin stimulated high-affinity GTPase activity in the RINm5F cell-membrane preparation. From the results of these experiments, two GTP concentrations were chosen for the further characterization of the galanin-stimulated high-affinity GTPase activity: 0.5 μM GTP, which gave a good separation between basal and galanin-induced GTP hydrolysis, and 50 μM GTP, to separate by subtraction the low-affinity GTPase activity from the high-affinity GTPase activity.

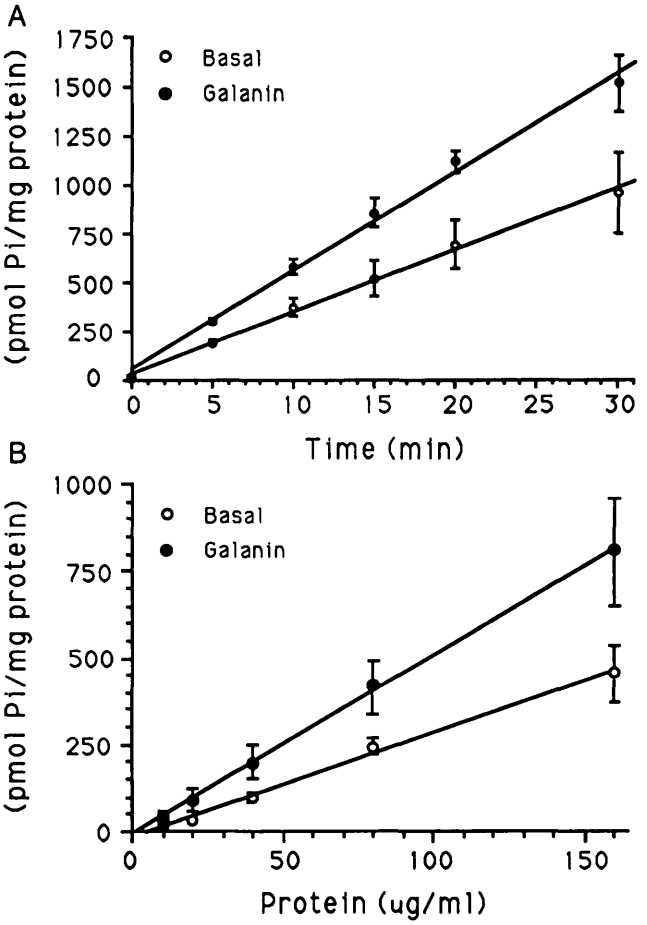

FIG. 3. High-affinity GTPase activity in the absence and presence of 100 nM galanin as a function of time and protein concentration. Rates of GTP hydrolysis with 50 μg/ml membrane protein over a 30-min period (*A*). Rates of GTP hydrolysis over a 10-min period where protein concentration was varied from 10 to 160 μg/ml (*B*). Experiments were performed in triplicate and repeated 4 and 3 times, respectively.

To define the assay as a measure of the high-affinity GTPase activity, GTP hydrolysis was studied over different times up to 30 min and over a wide range of protein concentrations. The results are shown in Fig. 3*A* and B. The rates of both basal and galanin-stimulated high-affinity GTPase activities were linear over time up to at least 30 min and proportional with protein concentration up to at least 160 μg/ml. In both studies, 100 nM galanin increased the rate of GTP hydrolysis to almost twice that of the basal activity. The results in Fig. 3*A*, where GTP hydrolysis was measured over a 30-min incubation period, show a basal rate of GTP hydrolysis of 33.6 ± 6.6 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein and during stimulation with 100 nM galanin a rate of 52.8 ± 2.6 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein ($P < 0.05$). From the slope of the lines shown in Fig. 3*B*, it can be derived that the basal rate of hydrolysis of 26.2 ± 2.3 pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein was increased in the presence of 100 nM galanin to the rate of 45.3 ± 5.7 ($P < 0.05$).

Galanin stimulation of high-affinity GTPase activity. After defining the assay, we studied the effects of various concentrations of galanin on GTPase activity in RINm5F

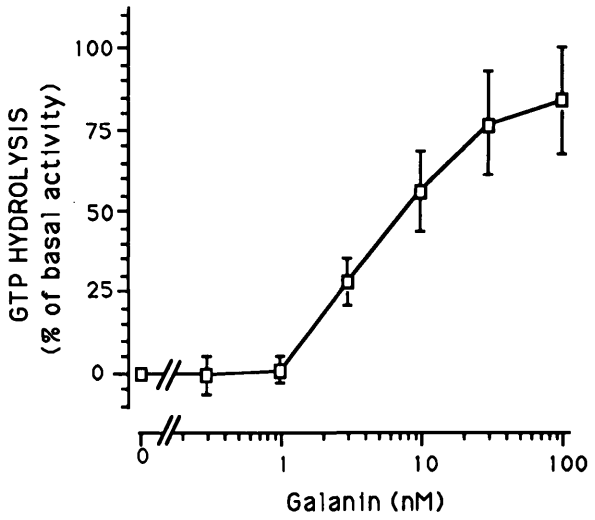

FIG. 4. Effect of various concentrations of galanin from 0.3 to 100 nM on high-affinity GTPase activity. Basal activity was 38 ± 5 pmol · min^{-1} · mg^{-1} protein, and data are derived from 5 experiments performed in triplicate.

cell membranes. From the results, which are presented in Fig. 4, it can be seen that galanin, at concentrations increasing from 1 to 100 nM, stimulated high-affinity GTPase activity in a concentration-dependent manner. The maximal response of ±80% over the basal rate of GTP hydrolysis was reached at 50 ± 5 nM. The half maximally effective concentration was 5 ± 2 nM galanin.

Because murine and porcine galanin have a difference of three amino acids in the COOH-terminal portion of the molecule (1,27) and because the RINm5F cells are derived from a murine insulinoma, it was considered possible that an affinity difference might exist between these two galanin molecules and the murine receptor. Therefore, the effects of murine and porcine galanin on GTPase activity were compared. In Fig. 5, the concentration-response curves for these two forms of galanin are presented. It can be seen that no significant differences

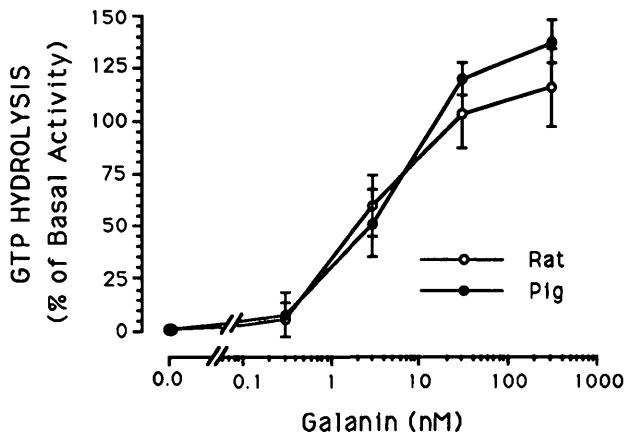

FIG. 5. Comparison of effects of rat and porcine galanin on high-affinity GTPase activity. Rat and porcine galanin at concentrations from 0.3 to 300 nM were tested. Data are from 3 experiments performed in triplicate.

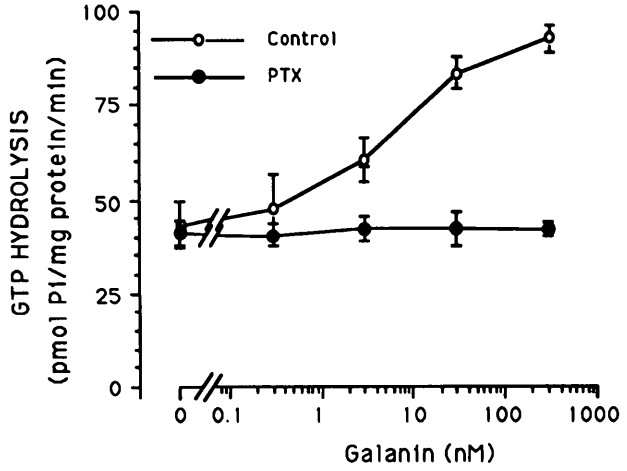

FIG. 6. Effect of 0.3 to 300 nM galanin on high-affinity GTPase activity of membranes prepared from RINm5F cells treated with or without PTX, as described in METHODS. Experiments were performed in triplicate and repeated twice.

were observed in the stimulation of GTP hydrolysis caused by murine and porcine galanin.

To determine that the galanin-stimulated GTP hydrolysis was caused by activation of a PTX-sensitive G protein, the effect of galanin on GTP hydrolysis was studied on cell membranes prepared from PTX-treated RINm5F cells. The results in Fig. 6 show GTP hydrolysis by membranes from cells that had been treated with 30 μg/L PTX for 20 h before membrane preparation, and from simultaneously purified plasma membranes of control cells (not treated with PTX). As expected, galanin stimulated GTP hydrolysis in the control membranes in a concentration-dependent manner. However, galanin failed to stimulate GTP hydrolysis in the membrane preparation from PTX-treated cells. PTX treatment did not significantly affect the basal rate of GTP hydrolysis, which was 43 ± 6 pmol P_i · min^{-1} · mg^{-1} for the control and 41 ± 3 for the PTX-treated membranes. These data demonstrate that the high-affinity GTPase activity stimulated by galanin is attributable to a PTX-sensitive G protein.

DISCUSSION

The mechanisms underlying the inhibitory effects of the neuropeptide galanin on insulin secretion are still not fully understood. That PTX treatment of pancreatic β-cells blocks the inhibitory effect of galanin on insulin secretion and mediation through activation of PTX-sensitive G proteins can be inferred. In this study, the galanin-stimulated G-proteins have been characterized by assaying the intrinsic GTPase activity of the galanin-activated α-subunits of the G proteins. We found that the insulin secretory β-cell line RINm5F contained two distinct classes of GTPases, one characterized by low-affinity activity with a K_m >60 μM GTP, and one by high-affinity activity with a basal K_m of ~200 nM GTP. These findings are similar to those in other tissues (22,26). Similarly, the observation that only the high-affinity GTPase activity is stimulated by the receptor ligand galanin and that the low-affinity activity is not is in accord with previous observations with other hormones

and transmitters in other tissues (22–23,26). It should be emphasized here that the low-affinity GTPase activity detected will be attributable to multiple GTPases in the cell-membrane preparation used. Similarly, the basal high-affinity GTPase activity may be composed of several GTPase activities. The GTPase activity stimulated by galanin also may be caused by more than one GTPase; in this case, the GTPase activities would be the α-subunits of the G proteins activated by galanin. It is not known yet whether the four known effects of galanin (activation of the K^+-ATP channel, inactivation of an L-type Ca^{2+} channel, inhibition of adenylyl cyclase, and inhibition at the distal site in stimulus secretion coupling) are all mediated by activation of one G protein or by activation of more than one. Nevertheless, it is clear that the galanin-stimulated GTPase activity cannot be thought of as a stimulation of the basal activity, because different enzyme activities are present in the two measurements. This most likely explains why galanin almost doubled both the apparent K_m and the V_{max} over basal GTPase activity, i.e., the basal GTPase activity in the RINm5F cell membranes is a mixture of several different high-affinity GTPase activities, for example, different forms of heterotrimeric G proteins, low-M_r G proteins, and other GTP-hydrolyzing enzymes, whereas the galanin-stimulated high-affinity GTPase activity is caused by activation of only one or a few of the heterotrimeric G proteins that are specific for the galanin receptor.

That the galanin-stimulated high-affinity GTPase activity is the receptor-activated G protein GTPase is indicated by the observation that the concentration-response curve for galanin-stimulated GTP hydrolysis correlates closely with that for inhibition of stimulated insulin secretion by galanin. The EC_{50} for inhibition of stimulated insulin secretion by galanin is in the range of 1–10 nM, and the maximal inhibitory effect is reached at 30–100 nM galanin (9–12). The concentration of galanin required for inhibition of adenylyl cyclase activity in RINm5F cell membranes and the high-affinity binding of galanin to its receptor on RINm5F plasma membranes (9,10,13) also correlate with this concentration range. Murine galanin, which differs from porcine galanin by three different amino acids in the COOH-terminal part of the molecule (1,28), also stimulated the high-affinity GTPase activity. The activity of murine and porcine galanins were similar with respect to potency and intrinsic activity. This observation is in line with previous studies with COOH- and NH_2-terminal fragments of galanin showing that the COOH-terminal end of the molecule plays only a minor role in the galanin-receptor interaction as judged by inhibition of adenylyl cyclase and insulin secretion (10,28). An intact NH_2-terminal is essential for galanin binding and mediation of receptor function.

PTX treatment, which ADP-ribosylates the α-subunits of G proteins of the G_i and G_o subtypes, abolishes the receptor activation of these G proteins (20) and, as shown in this study, the galanin-stimulated GTPase activity. The effects of galanin on insulin secretion and adenylyl cyclase also are abolished by PTX treatment (9,11), and the high-affinity binding site is converted to a lower-affinity site. The observation that the stimulatory effect of galanin on GTP hydrolysis was completely abolished by PTX pretreatment of the RINm5F cells strongly suggests that all of the galanin-stimulated GTP hydrolysis is attributable to the intrinsic GTPase activity of the α-subunit(s) of G_i and/or G_o proteins.

A previous study on RINm5F cells revealed that this cell line contains at least five different PTX-sensitive α-subunits (16). Because galanin's effect on the high-affinity GTPase activity was abolished by PTX treatment, all of these α-subunits are candidates for the stimulated GTPase activity seen with galanin. Further studies are required to identify the specific G proteins that interact with the galanin receptor.

ACKNOWLEDGMENTS

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