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Lactogen enhances Nb2 cell GTPase activity after 4 hours incubation

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The lactogen receptor has been suggested to associate with one or more G proteins despite the absence of a 7-transmembrane spanning sequence. These studies were designed to determine whether lactogens acutely increase GTP binding to or GTPase activity in Nb2 cell membrane. Incubation of Nb2 cell membrane with either ovine PRL (10 ng/ml) or diluent for 0–1 h resulted in a decrease in total ^{35}S-GTP binding to both with no difference in GTP binding between PRL- and diluent-treated membranes. There was also no change in ^{35}S-GTP binding to Nb2 cell membrane incubated with increasing oPRL concentrations (0.001–100 ng/ml) for 60 min. α-^{32}P-GTP photoaffinity labelling was used to evaluate changes in GTP binding to specific G proteins. Photoaffinity labelling of α-^{32}P-GTP to no G protein was changed after preincubation with oPRL (10 ng/ml) for 0–60 min or with oPRL (0.01–10 ng/ml) for 60 min. Finally, it was determined whether oPRL had any acute effect on GTPase activity, as determined by release of ^{32}Pi from γ-^{32}P-GTP. When Nb2 cell membrane was preincubated for 0–60 min with oPRL (10 ng/ml) or a range of oPRL concentrations (0–10 ng/ml), no change in GTPase activity was observed. However, when Nb2 cells were incubated with lactogen for 0–7 h, GTPase activity in equal quantities of Nb2 cell membrane prepared from those cells increased over time. Increased GTPase activity (64.9–74.4%; $P < 0.03$ compared to 0 h) was observed after 4–7 h incubation with lactogen.

In summary, addition of lactogen to Nb2 cell membrane did not acutely increase either GTP binding or GTPase activity. Yet when Nb2 cells were incubated with lactogen for 4 h prior to preparation of membrane, GTPase activity was significantly increased. This evidence, in addition to our previous results showing that 4 h incubation with lactogen increased G protein β subunit concentration and pertussis toxin-stimulated ADP-ribosylation of Gi, support a role for delayed lactogen modulation of one or more G proteins in the Nb2 cell, requiring at least 4 h for maximal effect.

Keywords: prolactin; human growth hormone; Nb2 cell, GTP binding protein; beta subunit; GTPase

Introduction

The initiation of the signal transduction cascade by a hormone binding to its receptor in a target cell is mediated in many cases through one or more of a family of GTP binding proteins or G proteins [for review: (Gilman, 1987; Freissmuth *et al.*, 1989; Birnbaumer *et al.*, 1990)]. No 7-transmembrane spanning sequence has been identified in either the prolactin (PRL) or growth hormone (GH) receptor (Boutin *et al.*, 1988; Edery *et al.*, 1989) as is usually present in the amino acid sequence of receptors known to be linked to G proteins. Yet previous studies have suggested an interaction between lactogenic hormones and GTP binding proteins (Barkey *et al.*, 1988; Larsen & Dufau 1988; Too *et al.*, 1989, 1990; Larsen, 1992; Larsen & Burkman, 1994). The following studies were designed to better identify whether lactogen can acutely alter one or more G proteins in the Nb2 cell.

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Results

The effects of acute addition of the lactogen, oPRL, on GTP binding to Nb2 cell membrane was first determined using ^{35}S-GTP. Nb2 cell membranes prepared from cells washed free of lactogen were incubated with oPRL (10 ng/ml) for 0–60 min. ^{35}S-GTP binding to Nb2 cell membrane incubated with oPRL decreased over time (Figure 1). However, a similar decrease in GTP binding was observed in membrane incubated with diluent so there was no significant difference between the two groups (Figure 2). Nb2 membrane was then incubated with a range of concentrations of

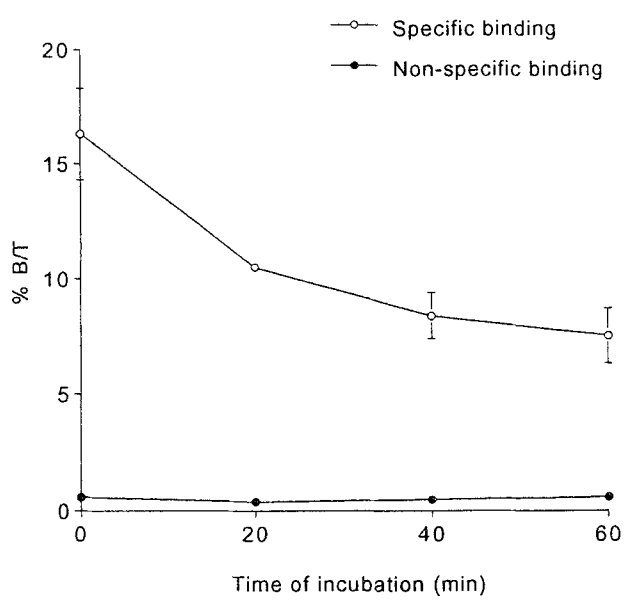

Figure 1 Effect of PRL on ^{35}S-GTP binding over time. Equal quantities of Nb2 cell membrane were incubated with oPRL (10 ng/ml) for 0–60 min at 37°C prior to performing ^{35}S-GTP binding. Each value represents the mean ± SEM of triplicate samples in one experiment. Similar results were obtained with a repeat experiment

oPRL (0.001–100 ng/ml) for 60 min. There was no change in GTP binding with changes in PRL concentration (Figure 3).

Photoaffinity labelling with α-^{32}P-GTP was performed to determine if a small change in GTP binding to one G protein was present that might not be visualized by a change in total GTP binding. GTP labelling of multiple proteins was observed but the predominant protein labelled by α-^{32}P-GTP was a 45 kDa determined to be Gs by immunoprecipitation (Larsen and Burkman 1994). Using Nb2 cell membrane prepared from cells washed free of lactogen for 24 h, membrane was then incubated with oPRL (10 ng/ml), or diluent for 0–60 min at 37°C prior to photoaffinity labelling. No significant change in GTP binding to any protein including Gs was observed over time with PRL or diluent (Figure 4). Incubation of Nb2 cell membrane with a range of oPRL concentrations (0–10 ng/ml) also had no change on GTP photoaffinity binding to any proteins (Figure 5).

A further test of whether the signal transduction pathway of a hormone is mediated through a heterotrimeric G protein is the demonstration of enhanced GTPase activity after hormone-receptor binding. Nb2 cell membrane, again prepared from cells washed free of lactogen, was incubated with oPRL (10 ng/ml) for 0–60 min at 37°C. GTPase activity was assumed to be proportional to the amount of ^{32}Pi released from γ^{32}P-GTP. When Nb2 cell membrane was incubated with oPRL or diluent, no change in ^{32}Pi was observed (Figure 6). Further experiments were done to evaluate changes in GTPase activity with a range of oPRL concentrations. There was again no change in ^{32}Pi released when Nb2 cell membrane was incubated with increasing concentrations of PRL (0.01–100 ng) for 60 min at 37°C prior to quantitation of ^{32}Pi (data not shown).

GTPase activity was then evaluated in Nb2 cell membrane prepared from cells previously incubated with lactogen for 0–7 h. In these experiments the lactogen hGH (10 ng/ml) was used, equipotent (mg/mg) with oPRL in stimulating both mitogenesis and other biochemical changes in the Nb2 cell. Equal quantities of membrane protein prepared from those cells were then assessed for GTPase activity. GTPase activity progressively increased with increasing time of Nb2 cell incubation with hGH (Figure 7). GTPase activity was

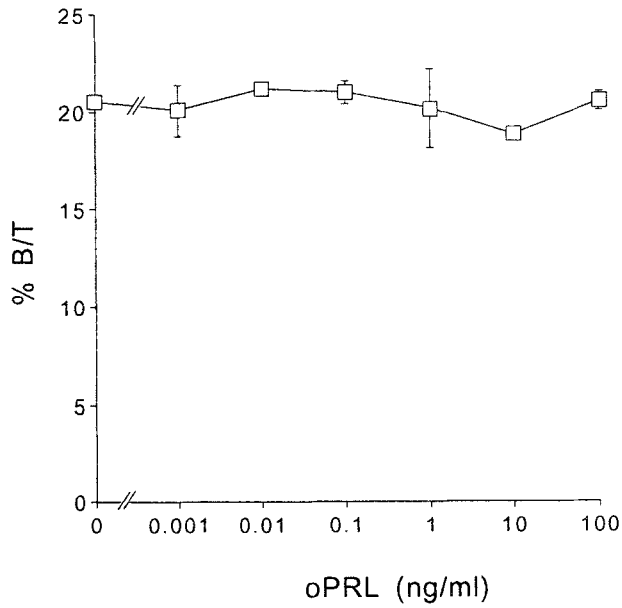

Figure 3 Effect of a range of concentrations of PRL on ^{35}S-GTP binding. ^{35}S-GTP binding to Nb2 cell membrane was determined after incubation with oPRL (0.001–100 ng/ml). Triplicate samples of Nb2 cell membrane in a single experiment are shown (X ± SEM). A repeat experiment gave similar results

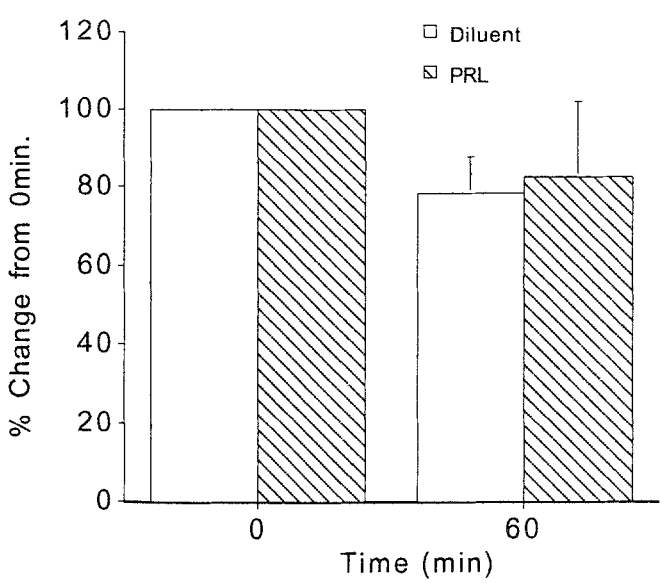

Figure 2 Effect of PRL or diluent on ^{35}S-GTP binding at 60 min: cumulative results. ^{35}S-GTP binding to Nb2 cell membrane after incubation with oPRL (10 ng/ml) or diluent for 60 min at 37°C. The results represent the mean of three separate experiments (± SEM) after converting the data to % change from start 0 time values, where 0 time represent 100%

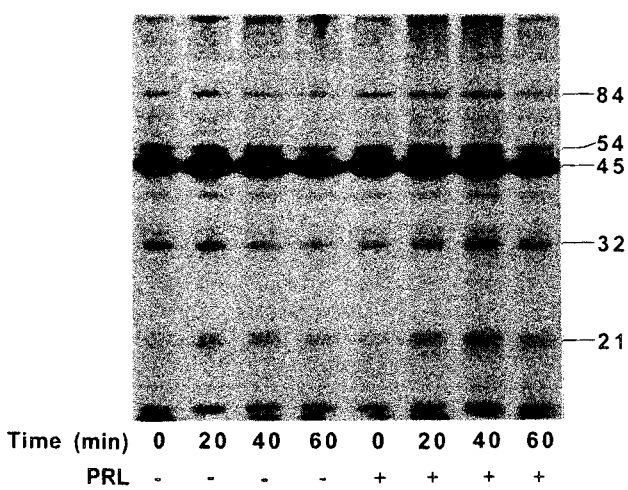

Figure 4 Effect of time of incubation with PRL on photoaffinity binding of α-^{32}P-GTP to Nb2 cell membrane. Equal quantities of Nb2 cell membrane prepared from cells previously washed free of lactogen were incubated with PRL for 0–60 min at 37°C prior to performing photoaffinity binding with α-^{32}P-GTP. These samples were applied to SDS–PAGE, and the resulting gel was dried and autoradiography was performed. The resulting autoradiogram of GTP labelled proteins is shown. Molecular weights of GTP-labelled proteins are shown on the right. Time of incubation with PRL prior to photoaffinity binding is shown below

significantly increased at 4 h (X ± SEM: 64.9 ± 16.2%; $n = 0.027$ compared to 0 h). GTPase activity remained elevated in Nb2 membrane prepared from cells incubated with lactogen for 7 h (74.4 ± 40.6%; $n = 4$; $P = 0.0131$ compared to 0 h). In membrane prepared from cells exposed to lactogen for 24 h, GTPase activity had begun to decrease ($P = 0.0567$ compared to 0 h). The lactogen-stimulated increase in GTPase activity appeared to depend on new protein synthesis as no increase in GTPase activity was observed when Nb2 cells were incubated with hGH (10 ng/ml) and cyclohexamide for 4 h (−0.02 ± 0.16% change compared to 0 h). Incubation of Nb2 cells with diluent alone (−0.041 ± 0.43) or cyclohexamide alone (10.3 ± 16.6%) for 4 h also resulted in no significant change in Nb2 cell membrane GTPase activity compared to 0 h membrane. A significant increase in GTPase activity was also observed after 4 h incubation of Nb2 cells with ovine prolactin compared to 0 h values.

Discussion

Multiple methods have been used to implicate GTP binding protein involvement in the signal transduction of a hormone receptor. When the second messenger of a hormone signal transduction cascade is known, a G protein can be implicated in its generation if GTP can stimulate the release of the second messenger in question. However, no single messenger has been identified to mediate lactogen actions even though PRL receptor binding has recently been demonstrated to activate tyrosine kinase activity. As most effectors found to associate with a G protein have a 7-transmembrane spanning sequence, knowing the receptor amino acid sequence is helpful itself in implicating a G protein. The amino acid sequences of multiple forms of the lactogen receptor have been established [rev: Kelly *et al.*, 1992] and they do not have the 7-transmembrane spanning sequence. Yet lacking the 7-transmembrane spanning sequence has not excluded the possibility of G protein involvement as some have been shown to associate with one or more G proteins, including EGF (Church & Buick, 1988; Liang & Garrison, 1991), insulin (Burdett *et al.*, 1990; Moises & Heidenreich

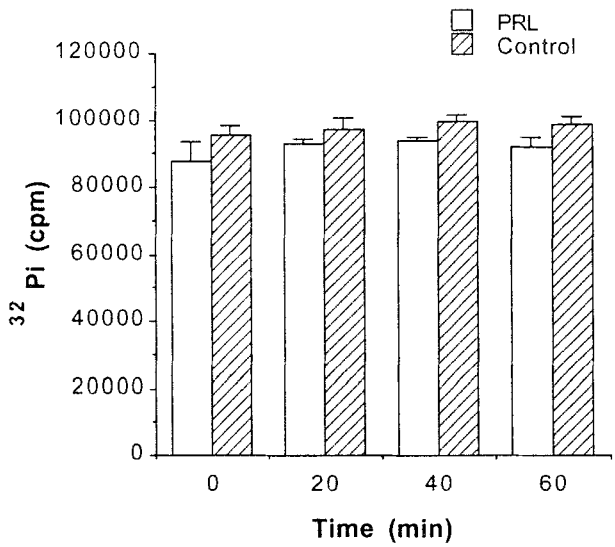

Figure 6 Effects of acute administration of PRL on GTPase activity. Equal quantities of Nb2 cell membrane were preincubated with either PRL (10 ng/ml) or diluent for 0–60 min prior to demonstration of GTPase activity, shown as the quantity of released ^{32}Pi after pre-incubation. Mean ± SEM of triplicate samples in one experiment are shown. Repeat experiment gave similar results

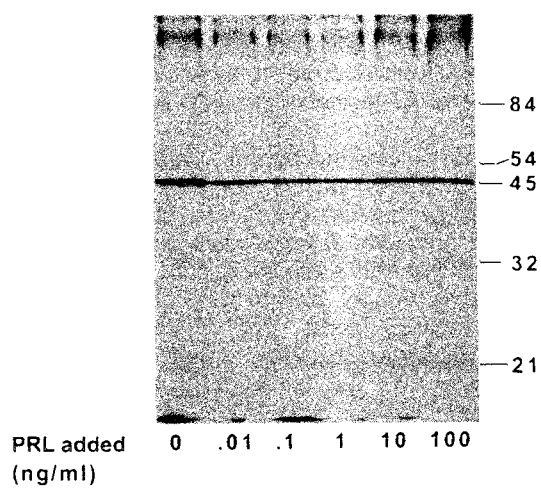

Figure 5 Effect of PRL concentration on photoaffinity binding of α-^{32}P-GTP to Nb2 cell membrane. Equal quantities of Nb2 cell membrane prepared from cells previously washed free of lactogen were incubated with oPRL (100 ng/ml) for 0–60 min prior to performing photoaffinity binding with α-^{32}P-GTP. The samples were applied to SDS–PAGE, and the resulting gel was dried and autoradiography was performed. A representative autoradiogram of GTP labelled proteins is shown. Molecular weights of GTP-labelled proteins are shown on the right. Concentration of PRL used during preincubation is also shown

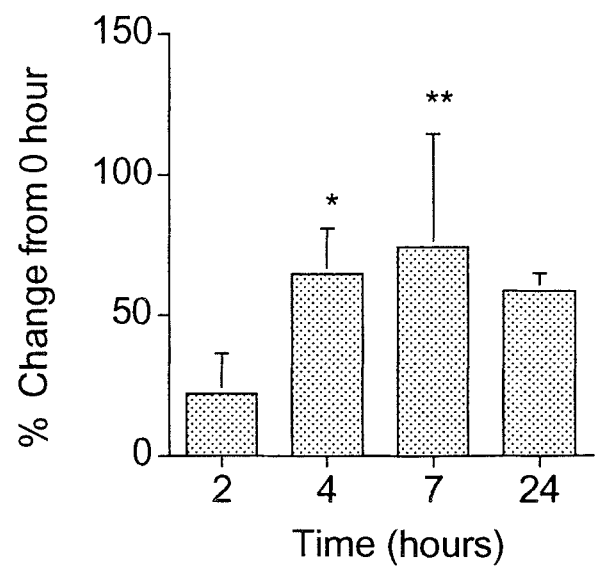

Figure 7 GTPase activity in Nb2 cell membrane prepared from cells incubated previously with PRL. Nb2 cell membrane was prepared from equal numbers of cells incubated with the lactogen human growth hormone (10 ng/ml) for 0–7 h. Equal quantities of protein from each group of cells were then used for determination of GTPase activity as demonstrated by quantity of released ^{32}Pi as shown. These results represent the mean ± SEM of three separate experiments. $*P = 0.027$ and $**P = 0.0131$ compared to 0 h by ANOVA

1990; Sriuvastava & Singh, 1990; Galdin *et al.*, 1991), and IGF-II (Nishimoto *et al.*, 1989; Okamoto *et al.*, 1990).

Two studies specifically support a role for G proteins in PRL receptor action. In one study the nonhydrolyzable GTP analog guanylylimidiphosphate (GppNHp) decreased Nb2 cell PRL receptor binding as can occur when a hormone is mediated through a GTP binding protein. Both cholera and pertussis toxins alter G protein functions, so these bacterial toxins have often been used as probes for involvement of G proteins in hormone actions [rev: Freissmuth *et al.*, 1989]. We have previously shown that both cholera and pertussis toxins inhibited PRL-stimulated Nb2 cell mitogenesis (Larsen & Dufau 1988).

With some evidence to support a role for G proteins, these studies were designed to further determine whether there was a role for one or more G proteins in immediate lactogen action. Activation of a heterotrimeric G protein results in the exchange of GTP for GDP, followed by a stimulation of GTPase activity to return the activated G protein to its GDP-bound state [for review: Gilman, 1987; Freissmuth, 1989; Birnbaumer *et al.*, 1990)]. Thus, addition of a hormone that mediates its signal transduction pathway through a G protein should result in an immediate increase in GTP binding to the target cell membrane and increase membrane GTPase activity. We specifically evaluated whether or not PRL could affect GTP binding to Nb2 cell membrane with timed incubation studies, concentration studies and two different label systems, ^{35}S-GTP for total GTP binding and ^{32}P-GTP for photoaffinity labelling of specific G proteins. None of these studies demonstrated an increase in GTP binding immediately following the administration of PRL to Nb2 cell membrane. Likewise, additional timed incubation and dose-response studies demonstrated no effect of acute administration of PRL on the acute release of ^{32}Pi from γ-^{32}P-GTP suggesting no immediate activation of GTPase activity.

However, the last studies suggest that an interaction between lactogen and G proteins, while not immediate, may still be present. Incubation of Nb2 cells with lactogen for 0–7 h prior to preparation of Nb2 cell membrane resulted in a progressive stimulation of GTPase activity over time, maximal after 4–7 h. This time course parallels the time course of other lactogen effects of G protein functions, specifically lactogen-stimulated β subunit concentration and lactogen-enhancement of pertussis toxin-stimulated ADP-ribosylation of Gi (Larsen, 1992). Neer has demonstrated that more effective ADP-ribosylation of Giα occurs when it is bound to $\beta\gamma$ as a heterotrimeric protein (Neer *et al.*, 1984). Thus, as we identified no change in Giα concentration over the same time interval, the change in susceptibility to pertussis toxin-stimulated ADP-ribosylation suggests that a greater proportion of Giα may be $\beta\gamma$ bound. A parallel increase in available $\beta\gamma$ subunits, represented by an increased concentration of β subunit, supports this premise (Larsen, 1992). However, if a greater proportion of Gi is $\beta\gamma$ bound, Gi would also be expected to be less active, and not the source for the increase in GTPase activity observed in these studies. Membrane was prepared from Nb2 cells previously exposed to lactogen for 0–7 h to determine whether chronic lactogen exposure altered GTP binding by photoaffinity labelling. However, no significant photoaffinity labelling of Gi was observed in these studies although GTP binding to Gs was decreased over the same time period (maximal 4–7 h after the addition of lactogen (Larsen & Burkman, 1994).

$\beta\gamma$ subunits themselves can directly alter multiple signal transduction pathways, including those linked to specific Gα subunits. These include inhibition of phospholipase C (Moriarty *et al.*, 1988), adenylate cyclase activity (Bokoch, 1987; Tang & Gilman 1991), and basal Ca^{2+} pump activity (Lotersztajn *et al.*, 1992). They have also been linked to stimulation of K^+ channel activity by phospholipase A_2 (Kim *et al.*, 1989), and Type II adenylate cyclase activity (Tang & Gilman 1991). Thus, although Gi activation is unlikely to be the source of enhanced GTPase activity, the increase in $\beta\gamma$ subunits may still be the mechanism by which PRL enhances the activation of another G protein. In support of this hypothesis is the evidence that coincubation of Nb2 cells with lactogen and cyclohexamide, previously shown to also prevent lactogen-stimulated increases in β subunit concentration (Larsen, 1992), also prevents the increase in lactogen-stimulated GTPase activity. Cyclohexamide blockade of protein synthesis also prevented lactogen-stimulated decreased in photoaffinity labelling of Gs by GTP (Larsen & Burkman, 1994).

Many would question whether activation of a G protein 4 h after hormone receptor binding could still represent a late event in the lactogen signal transduction pathway. However, there is some evidence to suggest that PRL needs to be present for 4 h in order to complete PRL-stimulated mitogenesis. In the Nb2 cell, maximal c-*myc* transcription occurs 3 h after PRL addition. However, when lactogen was removed after 4 h, Nb2 cell mitogenesis did not proceed (Fleming *et al.*, 1985). Thus, later actions following lactogen receptor binding may be required for commitment of the cell to mitogenesis. At the very least, demonstration that lactogen stimulates the activation of a G protein even 4 h after receptor binding suggests lactogen actions in a target cell may result from, in part, the modulation of one or more G proteins on which other effectors depend.

In summary, acute incubation of Nb2 cell membrane with PRL did not alter either GTP binding to or GTPase activity in that membrane. However, GTPase activity was increased in membrane prepared from cells previously incubated with lactogen for 4–7 h suggesting the activation of one or more G proteins. The lactogen-mediated increase in GTPase activity was prevented by cyclohexamide-blockade of protein synthesis. These data are consistent with our previous studies suggesting that one delayed action of lactogen is the modulation of one or more G proteins.

Materials and methods

Materials

The Nb2 cells were graciously provided by Dr P.W. Gout (U Mannitoba, Vancouver, Canada). Rabbit anti-common β subunit, Giα common and Gs antibodies were purchased from Dupont/New England Nuclear (Boston, MA; Goldsmith *et al.*, 1988). Ovine PRL (oPRL) was obtained from

the NIH hormone administration program (NIH oPRL-18) and recombinant human GH (equipotent with NIH hGH standard) was a gift of Ronald Chance, Lilly Research Laboratories (Indianapolis, IN). The following supplies were purchased from the vendors listed: Fischer's Leukemic Cell media, antibiotics and fetal calf serum (FCS) from GIBCO (Grand Island, NY); horse serum (HS), after testing to be lactogen free, from Flow Laboratories (McLean, VA); electrophoresis-grade materials used in polyacrylamide gels and molecular weight markers from BioRad Laboratories (Richmond, CA); polyvinylidene fluoride (PVDF) membrane from Millipore (Bedford, Mass); ^{32}P-NAD from New England Nuclear Research Products (24 Ci/mmol; Boston, Mass), bacterial toxins from List Biologicals Inc. (Campbell, CA); staining reagents for Western blot from Vector Laboratories, Inc. (Burlingame, CA); ^{35}S-GTP (1200 Ci/mmol) and α-^{32}P-GTP (3000 Ci/mmol) from New England Nuclear (Boston, MA), γ-^{32}P-GTP from Amersham (Arlington Heights, IL; 5000 Ci/mmol); and the remaining chemicals from Sigma Chemical Co. (St. Louis, MO).

Methods

Nb2 cells were maintained and arrested as previously described (Larsen & Dufau, 1988). The cells were washed free of lactogen-containing serum overnight, and the following morning washed again prior to preparing membrane as previously described. Membrane was prepared from these cells as previously described (10) and assayed by the method of Lowry *et al.* (1951). Additional cells were incubated (10^6 cells/ml) with hGH for 0, 2, 4 or 7 h prior to membrane preparation.

^{35}S-GTP binding was performed by incubation of 100 μg membrane protein with 50 nM ^{35}S-GTP in 0.01 M PBS/0.5% BSA pH 7.4 in the presence or absence of 100 μM GppNHp (as non-specific binding) for 1 h at 37°C. Samples were placed on ice and GTP-bound membrane was harvested on vacuum manifold (0.45 μ nitrocellulose membrane). Filters were placed in scintillation fluid and counted in a beta counter.

Photoaffinity binding were performed using previously described techniques (Im & Graham, 1990). Briefly, equal concentrations of Nb2 membrane (200 μg) were incubated for 10 min at 37°C in the dark with the following constituents (final concentration): 0.2 mM App(NH)p, 5 mM $MgCl_2$, 1 mM dithiothreitol, and α-^{32}P-GTP (5 μCi; 3000 Ci/mmol) in a final volume of 200 μl of 50 mM Tris, pH 7.5. The samples were then irradiated with u.v. light (254 nm) at a distance of 6 cm for 5 min. One ml of ice-cold Tris buffer was added to each tube to stop the reaction, and the sample was centrifuged at 12 000 *g* for 10 min. The pellet was re-suspended in sample buffer, and applied to a 10% SDS-polyacrylamide gel. Quantitation of radioactivity was performed using betagen scanning (Betascope: Betagen Corp. Waltham, MA) or by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA) of XAR film (Kodak, Rochester, NY).

GTPase activity was quantitated similar to the method of Cassel and Selinger (1976). Acute changes in GTPase activity were determined using equal quantities of membrane protein (20 μg) prepared from cells previously washed free of lactogen. These membrane samples were incubated with γ^{32}P-GTP in 50 mM imidazole-HCl buffer, pH 6.7 for 0–60 min at 37°C. The samples were then applied to a column previously prepared of celite overlaid with activated charcoal mixed with celite. The tubes were washed once and a total of 3 ml buffer were applied directly to each column. Scintillation fluid was added to the combined effluent and ^{32}Pi eluted from each sample was quantified in a beta counter over 5 min. Contaminating ^{32}Pi in the radiolabel was quantitated by applying an equal volume of γ^{32}P-GTP without membrane to a column, and counting the effluent, representing ^{32}Pi, in a beta counter with the other samples. This value was then subtracted as background from all tubes and represented 2.5 ± 0.5% (X ± SEM) of total counts in each tube. GTPase activity was assumed to represent any increase in ^{32}Pi released in samples exposed to PRL over ^{32}Pi in control samples. The results were calculated from triplicate values.

Statistical analysis

ANOVA was used to compare multiple groups and Fisher's LSD was used to determine significant changes between groups. $P < 0.05$ was considered significant.

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