Human Growth Hormone Enhances Pertussis Toxin-stimulated ADP-ribosylation of G_i in Nb2 Cell Membrane*

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Jennifer L. Larsen‡
From the Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68198-3010

The Nb2 node lymphoma cell line has been widely used as a model for investigating lactogenic cellular actions. Both pertussis (PTX) and cholera (CTX) toxins modulate lactogen-stimulated Nb2 cell mitogenesis, suggesting G protein involvement in lactogen signal transduction. The following studies were performed to further investigate this possibility. Both PTX-sensitive (41 kDa) and CTX-sensitive substrates (42 and 45 kDa) were identified in Nb2 cell membrane and recognized by specific anti-G_i and anti-G_o antibodies, respectively. Equal numbers of Nb2 cells were then incubated with the lactogenic human growth hormone (hGH, 10 ng/ml) for 0–72 h. Membrane protein prepared from each time point (50 μg) was compared in toxin-stimulated ADP-ribosylation studies. CTX-stimulated Nb2 cell membrane (in vitro) was augmented by as much as 2.5-fold by prior hGH incubation. PTX-stimulated ADP-ribosylation increased 237 ± 69% (X ± S.E.) compared with 0-h controls (n = 11; p < 0.01) after 4–7 h of hGH incubation then decreased toward 0-h samples by 24 and 72 h. No change in G_o concentration was observed, but β subunit concentration increased (145 ± 14% at 7 h; p < 0.01; n = 3) in a time course that paralleled the changes in PTX-stimulated ADP-ribosylation. In summary, 1) both G_i and G_o were present in Nb2 cell membrane, 2) incubation of cells with a lactogen, hGH, for 4–7 h markedly enhanced PTX-stimulated ADP-ribosylation of G_o, in vitro, whereas CTX-stimulated ADP-ribosylation of G_o was unchanged, and 3) although no change in G_o concentration was observed, β subunit concentration increased in parallel with the increase in PTX-stimulated ADP-ribosylation of G_o. These results suggest that hGH may modify PTX-stimulated ADP-ribosylation of G_o by not changing G_o concentration, perhaps by increasing β subunit concentration, enhancing association of G_o by βγ subunits, which, in turn, is preferentially ADP-ribosylated. This may represent a late signal transduction event and may also have implications for other effectors dependent on G_i-mediated events.

Prolactin was one of the first pituitary hormones to be isolated, yet the cascade of events constituting signal transduction for any lactogenic hormone is not yet well understood. The rat Nb2 node lymphoma cell line has been used extensively to investigate lactogenic hormone actions because it possesses high affinity, specific lactogenic receptors, and, on binding of a lactogenic hormone, the cells grow and divide (1–3). Recently, we have shown that bacterial toxins, pertussis toxin (PTX)1 and cholera toxin (CTX), altered the response of Nb2 cells to lactogen stimulation (4). PTX and CTX are known to alter G proteins associated with adenylate cyclase, G_i and G_o, respectively, as well as other signal transduction mechanisms unassociated with adenylate cyclase (see recent reviews: Refs. 5 and 6). These data suggest that lactogen actions may be coupled to one or more GTP binding proteins. The following studies investigate whether incubation of Nb2 cells with a lactogen alters G proteins in Nb2 cell membrane in order to better understand the relationship between G proteins and lactogenic hormones.

EXPERIMENTAL PROCEDURES

The Nb2 cells were graciously provided by Dr. P. W. Gout (University of Manitoba, Vancouver, Canada). Rabbit anti-G_i, common antibody was generously provided by Dr. David Manning (University of Pennsylvania (7)), and anti-G_i (5) and anti-G_o (8) antibodies were graciously provided by Dr. Suzanne Mumby (University of Texas). Rabbit anti-Gi2/Gi3 (9) and rabbit anti-common β subunit (10) antibodies were purchased from Du Pont-New England Nuclear. Radioiodinated goat anti-rabbit antibody was a gift from Dr. K. Phares (University of Nebraska Medical Center, Omaha, NE). Recombinant human GH (equipotent with National Institutes of Health hGH standard) and 125I-hGH (specific activity 40–70 μCi/μg based on a radioreceptor assay using female rat liver membrane performed in my laboratory) were gifts of Lilly. The following supplies were purchased from the vendors listed: Fischer’s Leukemic Cell Media, antibiotics, and fetal calf serum from GIBCO horse serum, after testing to be lactogen-free, from Flow Laboratories (McLean, VA); electrophoresis-grade materials used in polyacrylamide gels and molecular weight markers from Bio-Rad; polyvinylidene difluoride (PVDF) membrane from Millipore (Bedford, MA); [35S]NAD from New England Nuclear Research Products (24 Ci/mmol); bacterial toxins from List Biologicals Inc. (Campbell, CA); staining reagents for Western blot from Vector Laboratories, Inc. (Burlingame, CA); and β subunit was purchased from Du Pont-New England Nuclear. The remaining chemicals were from Sigma.

RESULTS

Effect of Incubation of Cells with hGH (10 ng/ml) Prior to CTX- and PTX-stimulated ADP-ribosylation of Nb2 Cell Membrane—Once both CTX and PTX substrates were identified and recognized by specific G_i and G_o antisera (see Supplemental Material, Figs. 1–4), Nb2 cells were incubated with hGH for 0–24 h prior to membrane preparation. There

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† To whom correspondence and reprint requests should be addressed: Dep. of Internal Medicine, University of Nebraska Medical Center, 42nd and Dewey, Omaha, NE 68198-3010.

1 The abbreviations used are: PTX, pertussis toxin; CTX, cholera toxin; GH, growth hormone; hGH, human growth hormone; PVDF, polyvinylidene difluoride.
2 Figs. 1–4 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
was no demonstrable effect of prior hGH incubation on subsequent CTX-stimulated ADP-ribosylation of the 42- and 45-kDa substrates in vitro (Fig. 5), but PTX-stimulated ADP-ribosylation did change over time (Fig. 6). The change in quantity of ADP-ribosylated 41-kDa protein occurred after a 4-7 h incubation with hGH, an increase of 237 ± 69% over 0-h controls (X ± S.E.; n = 11; Fig. 7). After 24-72 h incubation with hGH, ADP-ribosylation of G was observed to decrease.

**Western Blots of Time Course Samples—**G concentration was determined by Western blots (for method, see Supplemental Materials, Figs. 3 and 4) using either anti-G, common or anti-G, antibodies (Fig. 8). No change in G concentration was detected over this period (0-24 h) using either antibody. However, β subunit concentration as determined by immunoblot did change over time (Figs. 9 and 10). Two proteins (36 and 27 kDa) were recognized by this anti-common β subunit antibody. The 36-kDa protein was significantly increased by 4 h and remained elevated in the 7-h sample but decreased by 24 h (Figs. 9 and 10), whereas the 27-kDa protein steadily increased over this time period from 0 to 24 h. Recognition of both bands was markedly diminished if the first antibody (anti-β subunit) was preincubated with excess β subunit peptide prior to performance of Western blotting (Fig. 11, right lane). Thus, although β subunit is a 36-kDa protein, the lower 27-kDa band is also specifically recognized by the anti-β subunit antibody.

**DISCUSSION**

What events direct lactogenic hormone cellular actions following receptor binding are still largely unknown. GTP binding proteins have been found to mediate many different hormones' actions. Although G proteins associated with adenylate cyclase have been best characterized, it is now clear that G proteins mediate many other mechanisms of signal transduction, including cGMP phosphodiesterase, inositol triphosphate, and ion channels (for review see Refs. 5, 6, and 11). Because bacterial toxins can alter G protein functions by stimulating the transfer of ADP-ribose to specific acceptor amino acids on the α subunits of sensitive G proteins, they are often used as probes to determine possible involvement of

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**FIG. 5:** Effect of incubation with hGH on CTX-stimulated ADP-ribosylation. The cell were maintained as described previously (4). Nb2 cell membrane was prepared by washing the cells free of fetal calf serum containing media, and after hGH (10 ng/ml) was added for a specified incubation time (0-24 h), cells were homogenized, the membranes were pelleted at 20,000 × g, and equal amounts of membrane protein (50 μg) as determined by the method of Lowry (28) were subjected to CTX-stimulated ADP-ribosylation. ADP-ribosylation mixture is outlined in Fig. 1, modified slightly from Ref. 29. ADP-ribosylated bands were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30). Cumulative results (n = 4) show no change in ADP-ribosylation of G, following hGH incubation. [32P]ADP-ribose G; total protein.

**FIG. 6:** Effect of incubation with hGH on PTX-stimulated ADP-ribosylation. Membrane was prepared from Nb2 cells following different times of incubation with hGH (10 ng/ml): 0-72 h. Equal amounts of membrane protein (50 μg) from each membrane preparation was subjected to PTX-stimulated ADP-ribosylation in vitro. These samples were then applied to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being electrophoretically transferred to PVDF membrane (31). An autoradiogram prepared from this PVDF membrane is shown. Lanes 1-8 represent ADP-ribosylation of a 4-kDa protein after 0, 2, 3, 4, 5.5, 7, 24, and 72 h, respectively. A consistent increase in ADP-ribosylation of G was observed in membrane prepared from cells incubated with hGH for 4-24 h (lanes 4-8).

**FIG. 7:** Cumulative results of prior hGH incubation on PTX-stimulated ADP-ribosylation of G. The percent change of ADP-ribosylation of G was determined by Western blots (for method, see Supplemental Materials, Figs. 3 and 4) using either anti-G, common antibody (1:500) in equal quantities of membrane protein prepared from Nb2 cells incubated for 0, 2, 3, 4, 5.5, 7, 24, and 72 h (lanes 1-8, respectively) with hGH (10 ng/ml) was determined. There was no significant change in G concentration over this time interval despite a significant change in ADP-ribosylation of G, as noted in Fig. 6, which represents an autoradiogram of the [32P]ADP-ribose-G, identified in the same blot.

**FIG. 8:** Quantification of G, in membrane by immunoblot following hGH incubation. The quantity of 41-kDa protein identified by a specific anti-G, common antibody (1:500) in equal quantities of membrane protein prepared from Nb2 cells incubated for 0, 2, 3, 4, 5.5, 7, 24, or 72 h (lanes 1-8, respectively) with hGH (10 ng/ml) was determined. There was no significant change in G, concentration over this time interval despite a significant change in ADP-ribosylation of G, as noted in Fig. 6, which represents an autoradiogram of the [32P]ADP-ribose-G, identified in the same blot.

**FIG. 9:** Identification of β subunit concentration in Nb2 membrane. Western blot of Nb2 membrane prepared from cells following hGH (10 ng/ml) for 0, 2, 4, 7, and 24 h was performed using an anti-β subunit antibody (1:1000). An increase in both a 36-kDa and a 26.6-kDa protein after a 4-h incubation with hGH was observed. The 26.6-kDa protein continued to increase from 4 to 24 h, whereas the 36-kDa protein concentration plateaued and then decreased by 24 h. The higher molecular mass band was stained in the absence of specific antibody, so it is a nonspecific contaminant.

**FIG. 10:** A consistent increase in ADP-ribosylation of G was observed in membrane prepared from cells incubated with hGH for 4-24 h (lanes 4-8).
G proteins in effector actions. Thus, when CTX and PTX were found to modulate lactogenic actions in the Nb2 cell (4), the results suggested that one or more G proteins could be involved in lactogenic hormone signal transduction even though the second messenger for any lactogen is currently unknown.

The molecular weight of the PTX-sensitive substrate, 41 kDa, was the same as previously described for G, and was recognized by specific anti-G, antibodies. Too and co-workers (12) have reported a similar molecular mass (41.5 kDa) for a PTX substrate in Nb2 cell membrane and demonstrated expression of G2 and G3 mRNA in Nb2 cell (12). However, Barkey and co-investigators (13) identified a much smaller PTX substrate (38 kDa).

The CTX-sensitive substrates, 42 and 45 kDa, were consistent with the molecular masses of G, observed by other investigators due to differential splicing (6), as well as those reported by Too and colleagues in Nb2 membrane (42 and 45 kDa (12)). The molecular mass of the CTX substrate described by Barkey and co-investigators (13) was again lower than either of those seen in these studies (41 kDa). The 42- and 45-kDa proteins were not identified with horseradish peroxidase staining, but the 42-kDa protein was recognized by an anti-G, antibody using a more sensitive detection method (data not shown). This suggests G, was present at a much lower concentration in the Nb2 cell membrane as has been described in other cell membranes.

Incubation of Nb2 cells with hGH for 0–24 h prior to preparing membrane altered PTX-stimulated ADP-ribosylation in vitro, whereas CTX-stimulated ADP-ribosylation was unchanged. The change in PTX-stimulated ADP-ribosylation was not explained by a change in G, subunit concentration. Other hormones have been reported to alter the ability of a toxin to stimulate ADP-ribosylation of a G protein (14–18). In all cases, it was assumed that if a hormone can alter ADP-ribosylation of a G protein, it must in some way alter its conformation or concentration, although the exact mechanism in any of these cases has not yet been identified.

Although there was no change in G, subunit concentration (36 kDa) did change over time, paralleling the changes observed in PTX-stimulated ADP-ribosylation. Intact G protein heterotrimer is required for PTX-catalyzed ADP-ribosylation of the α subunit (19). Thus, even though G, concentration does not change, the fact that more β subunit is available, in addition to the described increase in PTX-stimulated ADP-ribosylation, suggests that a greater proportion of G, may be bound to βγ in the inactive trimeric form. Regulation of G protein β subunit has been proposed as a mechanism of thyroid hormone action (20, 21), but the significance of a change in β subunit to signal transduction in intact cells is not yet well understood. In addition, a second protein, 27 kDa, was also specifically recognized by the anti-β subunit antibody. The concentration of the 27-kDa protein increased steadily over the period of hGH incubation (0–24 h), so it could represent a degraded fragment of β subunit. A tryptic digest of β subunit with a molecular mass 27 kDa has been identified by other investigators (22), but the significance of this second band is otherwise not known.

As the genes for the prolactin and hGH receptor have now been cloned (23, 24), it is known that these receptors do not have the seven trans-membrane receptor spanning regions commonly associated with effectors that interact with GTP binding proteins. Yet there is growing evidence to suggest that lactogenic hormones may interact with G proteins. As already discussed, bacterial toxins inhibit both lactogenic hormone-stimulated DNA synthesis and mitogenesis (4, 12), guanine nucleotides inhibit the binding of 125I-human growth hormone to the lactogen receptor (12), and the lactogenic receptor has been cross-linked to G proteins using a long molecular length (16.1 Å), suggesting some proximity between the two, although shorter lengths were ineffective (25). Classically, signal transduction events are thought to occur within minutes of receptor binding. Yet a prolonged signal may be required for lactogen action in the Nb2 cell as suggested by the fact that stimulation of ornithine decarboxylase is not required for lactogen action in the Nb2 cell as suggested by the fact that stimulation of ornithine decarboxylase is not maximal until 8 h (26), and mitogenesis is prevented if hGH is removed after 4 h, even though maximal expression of the proto-oncogene c-myc is achieved by 3 h (27). Even if this late event is not important to signal transduction, it could still represent a change in G, function that could alter the actions of other effectors dependent on this G protein.

In summary, 1) G3, G13, and G, were present in Nb2 cell
membrane. 2) PTX-stimulated ADP-ribosylation of \( G_\alpha \) prepared from cells incubated with hGH for 4-7 h was increased by over 200%. 3) This change in PTX-stimulated ADP-ribosylation of \( G_\alpha \) was not explained by a change in \( G_\alpha \) concentration, and 4) \( \beta \) subunit concentration increased in a parallel fashion to the changes observed in PTX-stimulated ADP-ribosylation. Therefore, the change in observed PTX-stimulated ADP-ribosylation of \( G_\alpha \) may reflect a change in proportion of \( G_\alpha \), in the inactive heterotrimeric form compared with the active, separated form. It is currently unknown how or if this modification alters \( G_i \) actions as the signal transduction pathway(s) associated with \( G_i \) in this cell system have not yet been determined. It is hoped that these studies may also provide insight into why a prolonged lactogenic signal is required for commitment of the Nb2 cell to mitogenesis.

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hGH Enhances PTX-stimulated ADP-ribosylation of G

Supplemental material to Human Growth Hormone Enhances Pertussis Toxin-Stimulated ADP-Ribosylation of G, in Nb2 Cell membrane
Jennifer Larese-M.D.

Figure 1. CTX-stimulated ADP-ribosylation of Nb2 membrane.

Figure 2. PTK-stimulated ADP-ribosylation of Nb2 membrane.

Figure 3. Western blot of Nb2 membrane using a specific anti-Gi common antibody and horseradish peroxidase staining.

Figure 4. Western blot of Nb2 membrane following sH-Phos using anti-Go and anti-Gi antibodies.

Using the methods described in Figure 1, activated PT (10ug/100ul; f.d.) was used in the ADP-ribosylation mix with the only difference being a substitution of 150 mM Tris for 150mM GppNHp. Autoradiogram following SDS-PAGE of PTK-stimulated ADP-ribosylation of Nb2 membrane demonstrated 2 labeled proteins of 41 and 100kD. Lane 1, when membrane was prepared from Nb2 cells incubated with PTX (10nM) for 24h, the higher molecular weight species (100kD) was not down-regulated so was secondary to non-specific membrane ribosyl-transferase activity.

Using the methods described in Figure 2, activated PT (10ug/100ul; f.d.) was used in the ADP-ribosylation mix with the only difference being a substitution of 150 mM Tris for 150mM GppNHp. Autoradiogram following SDS-PAGE of PTK-stimulated ADP-ribosylation of Nb2 membrane demonstrated 2 labeled proteins of 41 and 100kD. Lane 1, when membrane was prepared from Nb2 cells incubated with PTX (10nM) for 24h, the higher molecular weight species (100kD) was not down-regulated so was secondary to non-specific membrane ribosyl-transferase activity.

Using the methods described in Figure 3, lanes 1 and 2 but not in lane 3 but the 100kD protein was observed in all 3 conditions, suggesting that the 100kD band is nonspecifically labeled band. Anti-Go antibody did not label the 41kD protein but did identify the 41kD protein when a radiolabeled asow antibody was used (data not shown).