BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 96–100 (1997)
ARTICLE NO. RC976231

A Point Mutation in the Glucose-Dependent Insulinotropic Peptide Receptor Confers Constitutive Activity

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Received January 16, 1997

The glucose-dependent insulinotropic peptide receptor (GIP-R) is a member of the secretin and parathyroid hormone (PTH) family of seven transmembrane-spanning receptors. Point mutations of a histidine at the junction between the first intracellular loop and the second membrane-spanning domain and a threonine in the sixth membrane-spanning domain of the human PTH-receptor have been reported to be associated with constitutive activation of the PTH receptor in Jansen-type metaphyseal chondrodysplasia. In this study, we explored whether such mutations in the GIP-R might similarly induce constitutive, ligand-independent activation of the receptor. Single amino acid substitutions in the GIP receptor were made by site-directed mutagenesis and receptor binding and cAMP levels were measured in transfected human embryonal kidney cell line (L293). Mutation of the threonine at position 340 in the sixth transmembrane spanning domain to proline (T340P) led to agonist-independent constitutive activity and exhibited a four-fold increase in basal cAMP level as compared to the wild-type GIP-R. The increase in cAMP level in T340P mutant was proportional to the amount of transfected plasmid and corresponded to the receptor number on the cell surface. Despite its high basal cAMP level, the T340P mutant could be further stimulated by GIP, with maximal cAMP generation comparable to the wild-type receptor. The change of amino acid histidine at position 169 to arginine (H169R), however, behaved like the wild type receptor and did not possess constitutive activity. These results illustrate that a point mutation of threonine to proline at position 340 results in constitutive activation of the GIP receptor, without affecting its sensitivity to agonist stimulation.

The glucose-dependent insulinotropic peptide receptor (GIP-R) belongs to a distinct superfamily of G-protein-coupled, seven transmembrane spanning receptors that includes secretin, glucagon, vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), glucagon-like peptide-1 (GLP-1), parathyroid hormone (PTH) and calcitonin receptors. The open reading frame of GIP receptor cDNA encodes a 455-amino acid protein with substantial similarity to other receptors within the family including conserved cysteine, histidine, and threonine residues (1). GIP receptors have been detected by radioligand binding in cell lines derived from rat pancreatic β-cells (2) and hamster insulinoma (3), and in membrane isolates of human insulinoma cells (4). The addition of GIP to insulinoma or islet cells leads to an increase in intracellular cAMP by the stimulation of the enzyme adenylate cyclase and [Ca^{2+}] levels and results in the release of insulin (5-7).

Recent studies in the human PTH receptor have identified a histidine to arginine mutation at the junction of the first intracellular loop and the second membrane-spanning domain responsible for a rare disease Jansen-type metaphyseal chondrodysplasia (8). Expression of this mutated receptor in COS cells resulted in constitutive, ligand-independent cAMP accumulation. An additional mutation of the threonine to proline in the sixth membrane-spanning domain of the human PTH-receptor has also been reported in those patients (9). They concluded that the expression of constitutively active PTH receptors may induce ligand-independent hypercalcemia seen in patients with Jansen-type metaphyseal chondrodysplasia. In this report, we explored whether mutations in those conserved histidine and threonine residuals of the GIP-R might similarly induce constitutive, ligand-independent activation of the receptor.

MATERIALS AND METHODS

Site-directed mutagenesis. The 1.6 kb Hind III/EcoR1 fragment containing the coding sequence of rat GIP-R was subcloned from pcDNA III (Invitrogen, San Diego, CA) into pAlter-1 (Promega, Madi-

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son, WI). Oligonucleotides which encoded 14 complimentary base pairs on either side of the single base mutation were phosphorylated using T4 kinase. Single strand vector was produced in ES1301 *mutS* bacteria and the mutant phosphorylated oligonucleotides and single-stranded template were annealed and double stranded template was synthesized using T4 polymerase. The doubled stranded DNA was transformed into JM109 competent cells and the base pair changes were analyzed by DNA sequencing. Inserts containing the mutations were subcloned into pCDNA III. The mutant at the junction of the first intracellular loop and the second membrane spanning domain is referred to as mutant H169R, while the mutation in the sixth transmembrane domain is mutant T340P.

L293 cell transfection. L293 cells were transfected with either wild-type or mutant GIP-R cDNAs by the Lipofectamine method according to the manufacturer's protocol (GIBCO, Gaithersburg, MD). L293 cells were seeded in a 12-well plate (10^5 cell/well) and cultured overnight in the presence of minimal essential medium with 10% fetal bovine serum. For transfection, DNA was diluted into serum-free medium, and lipofectamine (4μl/well) was added and incubated at the room temperature for 15 min to allow DNA-liposome complexes to form. During this 15 min period, L293 cells were rinsed twice with serum-free medium and then incubated with 1 ml of DNA-liposome for 5 h. Following incubation, 1 ml of medium supplemented with 20% fetal bovine serum was added and incubated for an additional 48 h before analysis.

GIP iodination. GIP was iodinated using the Chloramine T method (10). The iodinated peptide was then added to a Waters C-18 Sep Pak cartridge (Waters, Milford, MA) pre-equilibrated with 10 ml acetonitrile (HPLC grade) and 10 ml water both containing 0.1% TFA. The iodinated products were eluted by a 2% stepwise gradient of acetonitrile: water (2 ml) from 30-42%. Each fraction was collected into a tube containing 100 μl aprotinin (Miles Inc, Kankakee, IL) and 100 μl BSA (100 mg/ml, protease free, Sigma). Aliquots were tested for binding to GIP antiserum and the fractions with the highest specific binding were lyophilized and stored at -20°C.

Receptor binding. Binding assay was performed with the intact cells. Forty-eight hours after transfection, cells were detached from plates with trypsin/EDTA, rinsed twice in binding buffer (138 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES, 1% BSA, 10 mM glucose), and resuspended at a concentration of 2×10^6 cells/ml. Aliquots of 0.6×10^6 cells were incubated in Eppendorf tubes with 30 μl (30,000 cpm/tube) ^{125}I-GIP and 300 μl of binding buffer or 3 μl of cold GIP (ranging from 10^{-6} to 10^{-11} M). Tubes were incubated on a rocker at room temperature for 45 min. At the end of incubation, tubes were centrifuged in a Beckman microfuge for 1 min at maximal speed and the cell pellet was washed twice with 0.5 ml of binding buffer containing 4% BSA. The cell pellet was counted in a gamma counter and the specific binding was determined by subtracting the non-specific binding obtained in the presence of 10^{-6} M unlabeled GIP from the total cell associated radioactivity. Receptor binding data was analyzed using the RADLIG program (Biosoft, Cambridge, UK).

Cyclic AMP assay. Transfected L293 cells were grown for an additional 48 h. Cells were washed twice with phosphor-buffered saline (PBS) and then incubated with 500 μl of medium, followed by the appropriate concentrations of GIP. Cells were incubated for 10 min at 37 °C and extracted with 500 μl of cold absolute ethanol, followed by freeze thawing. The lysed cells were collected, and the cAMP levels were measured by radioimmunoassay (Amercham, cAMP assay kit).

Statistics. Results are expressed as means ± SE. Mean changes were compared using Student's t-test. P values less than 0.05 were considered to be statistically significant.

RESULTS

Preparation of mutated GIP receptors. The two GIP receptor mutations were prepared by site-directed mutagenesis. The specific sites of the mutations are demonstrated in figure 1. Mutations on base pair 676 from A to G and base pair 677 from C to A changed a conserved histidine into an arginine residue at amino acid 169 (H169R). Mutation on base pair 1184 from A to C changed a conserved threonine into a proline at amino acid 340 (T340P).

Characterization of GIP receptor binding in transfected L293 cells. Cells were transfected with either the wild type or mutant receptor cDNAs constructed in the pCDNA III vector. Scatchard analysis of the competitive binding of ^{125}I-GIP to wild type receptor with increasing concentrations of unlabeled GIP showed a *Kd* of $7.7 \pm 1.0 \times 10^{-8}$ M and a B_{max} of 681 ± 34 fmol/mg protein (Fig. 2). H169R and T340P mutants exhibited similar binding affinity and receptor numbers with a *Kd* of $6.9 \pm 0.6 \times 10^{-8}$ M, and a B_{max} of 630 ± 23 fmol/mg protein for H169R and a *Kd* of $7.3 \pm 0.8 \times 10^{-8}$ M, and a B_{max} of 560 ± 76 fmol/mg protein for T340P respectively.

Effects of GIP-R mutations on cAMP production. The ability of the GIP receptors to couple to adenylate cyclase was determined by measuring cellular cAMP levels in response to GIP at 10 min when maximal stimulation was detected (data not shown). Ligand-independent constitutive accumulation of cAMP occurred only in L293 cells expressing T340P but not in cells expressing either wild-type GIP-R or H169R mutant (Fig. 3). The basal cAMP level was 4-fold higher in T340P then wild-type GIP receptors (1.25 ± 0.22 vs. 0.28 ± 0.11 pmol per well; Fig. 3, insert). GIP-stimulated cAMP response was, however, similar in these three receptors with maximal response occurring at 10^{-8} M (Fig. 3). To assess the effect of receptor number on the basal cAMP generation, L293 cells were transfected with different concentrations of GIP receptor cDNAs. As shown in figure 4A, the expression of surface receptor was similar in both wild-type and T340P receptors and correspond to the amount of DNA transfected. Transfection with increasing amounts of T340P led to a corresponding increase in basal cAMP accumulation that reached maximal at about 750 ng per well (Fig. 4B). In contrast, cells that expressed increasing amount of wild type GIP receptor showed no change in basal cAMP level (Fig. 4B), which was indistinguishable from that of cells transfected with vector alone (data not shown).

DISCUSSION

The results of this study demonstrate that a point mutation of the threonine to proline in the sixth transmembrane-spanning domain of the GIP receptor results in constitutive, ligand-independent accumulation of cAMP without having direct effects on GIP binding

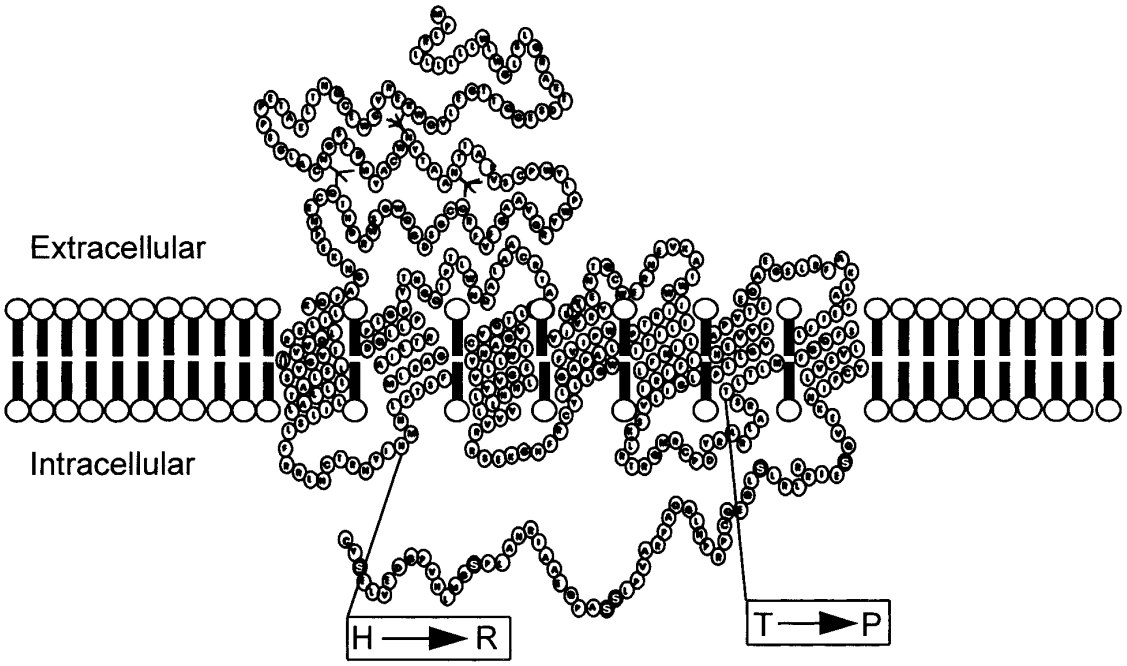

FIG. 1. Schematic representation of the rat GIP receptor structure. Amino acids are denoted by the one letter code. Rectangular areas identify amino acids in which mutations were inserted. Histidine 169 at the junction of the first intracellular loop and the second transmembrane-spanning domain was changed to a arginine. Threonine 340 in the sixth transmembrane spanning domain was changed to a proline.

affinity or ligand-stimulated cAMP response. However, mutation of the histidine to arginine (H169R) mutation in the GIP receptor was found to have no effect on basal cAMP production. These data are consistent with the previous report demonstrating that the threonine to proline (T410P) mutation in the PTH receptor leads to constitutive activation of the receptor and is responsible for Jansen-type metaphyseal chondrodysplasia (9). In agree with our finding, COS cells expressing the mutant PTH receptor T410P accumulated about four times more cAMP than cells expressing the wild type receptor (9). This degree of ligand-independent, constitutive activation of cAMP was, however, lower than that caused by another mutant H223R (histidine to arginine) in the PTH receptor, reported by the same investigators (8). In contrast to the PTH receptor, the

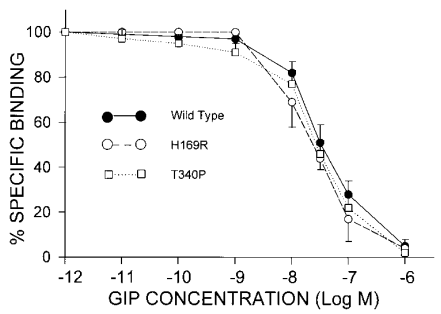

FIG. 2. Displacement binding of ^{125}I-GIP to L293 cells transiently transfected with wild type receptor or mutant H169R and T340P receptors. ^{125}I-GIP was used as a radioligand for the analysis of receptor binding. Data are represented as a percent of specific binding and are the mean of five experiments ± SE, with assay performed in duplicate.

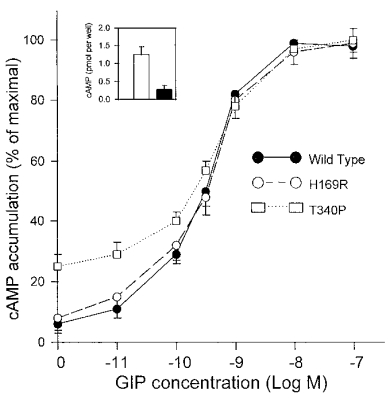

FIG. 3. Dose-dependent cAMP accumulation in L293 cells transiently transfected with wild type or mutant H169R and T340P receptors. Cells were treated with GIP for 10 min and cellular cAMP was measured by RIA. Data represent the average of triplicate wells repeated in five separate experiments. Basal cAMP accumulation in L293 cells expressing wild type (solid bar) and T340P mutant (open bar) was shown in the insert.

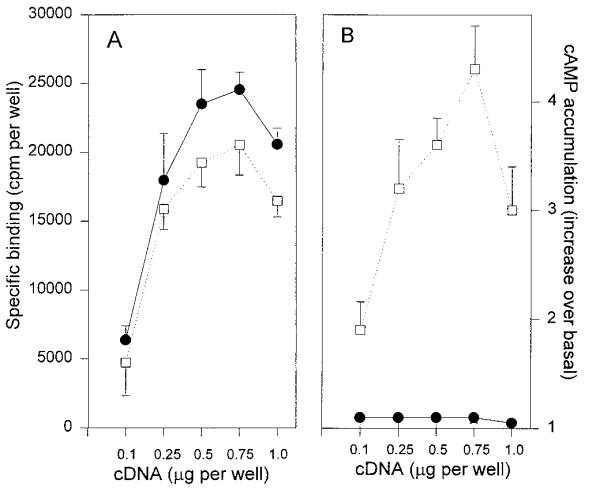

FIG. 4. (A) Assessment of cell-surface receptor expression. L293 cells were transfected with increasing concentration of the plasmid DNA encoding wild type (solid circle) or with T340P mutant (open square). Cell surface receptor expression was determined with the use of ^{125}I-GIP. Data were the mean ± SE of at least three independent experiments each done in duplicate. (B) cAMP accumulation. L293 cells were transfected with different concentration of the plasmid as described in (A) and treated with 10^{-8} M of GIP for 10 min. Cellular cAMP was measured by RIA. Data were the mean ± SE of three independent experiments each done in triplicate.

histidine to arginine (H169R) mutation in the GIP receptor does not confer constitutive signaling activity. Our findings were, nevertheless, similar to a recent report in which the mutation of the histidine residual in the first intracellular loop of the GLP-1 receptor does not affect the basal cAMP level (11).

Activating receptor mutations have been implicated in human diseases. In addition to the PTH receptor, mutation of the aspartic acid to glycine, and the alanine to isoleucine in the third cytoplasmic loop of the thyrotropic receptor confer constitutive activation of the cAMP signaling pathway and result in the development of hyperfunctioning thyroid adenoma (12). A single aspartate to glycine mutation in the sixth transmembrane-spanning domain of the luteinizing hormone (LH) receptor causes constitutive activation of the LH receptor and is responsible for gonadotropin-independent male precocious puberty (13). Similarly, mutation in the calcium-sensing receptor has been implicated as a cause of hypoparathyroidism (14).

Although GIP was originally named for its ability to inhibit gastric acid secretion, it is now apparent that another important physiological function of GIP is its insulinotropic effect. As stated above, GIP receptor have been detected by radioligand binding in cell lines derived from rat pancreatic β-cells, hamster insulinoma, and human insulinoma cells and the addition of GIP to islet cells leads to insulin release. The contribution of GIP in the pathogenesis of diabetes mellitus is currently unclear; nevertheless, hypersecretion of GIP has been detected in diabetic animals and in humans (15,16). Our laboratory has recently demonstrated that high serum GIP level in diabetic animals induced chronic desensitization of the GIP receptor and that this mechanism might play a significant role in decreasing insulin release in those animals (17). No mutation in the GIP receptor has yet been identified in normal or diabetic populations. Whether mutations reported in this study occur in human GIP-R and lead to human pathology warrants further investigation. A recent genetic study in a French and Sardinian population has demonstrated a strong relationship between mutation of the glucagon receptor and the development of non-insulin dependent diabetes mellitus (NIDDM) (18).

In summary, a mutation of threonine residual in the sixth transmembrane-spanning domain of the GIP receptor induces receptor activation that is independent of the ligand. In contrast to the PTH receptor, mutation of the histidine residual at the junction of the first intracellular loop and the second membrane-spanning domain has no effect on basal cAMP accumulation. Both the histidine and threonine residues are conserved in all members of this receptor family; hence, they may play an important role in the function of these G protein-coupled receptors.

ACKNOWLEDGMENTS

The authors thank Dr. M. Michael Wolfe for his thoughtful suggestion and support during the course of this study. This work was supported by United States Public Health Service Grants DK-02277 to C.C.Tseng and RO1 DK-48042 to M.M. Wolfe.

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