Heterozygous gsp Mutation Renders Ion Channels of Human Somatotroph Adenoma Cells Unresponsive to Growth Hormone-Releasing Hormone*

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ABSTRACT

Ionic mechanisms play an important role in the regulation of hormone secretion. The GHRH-induced GH release by human GH-secreting cells is transmitted through protein kinase A (PKA), which activates nonselective cation current (NSCC) and induces membrane depolarization, intracellular Ca$^{2+}$ increase, and GH secretion. To evaluate whether ionic mechanisms have pathophysiological significance in GH oversecretion of GH-secreting pituitary adenomas, we examined four adenomas with constitutively active Gsa mutation (gsp mutation) and compared with three gsp-negative adenomas. In primary-cultured cells of gsp-positive adenomas, GHRH did not increase the NSCC under voltage-clamp experiments. Detailed examination showed that NSCC was maximally activated at the basal level and application of GHRH did not increase the current in these adenomas. Furthermore, by using single-cell RT-PCR method, we demonstrated for the first time at the single cell level that gsp mutation is heterozygous in GH-secreting pituitary adenomas. These indicate that heterozygous gsp mutation fully activates NSCC at the basal level, which may account for the GH oversecretion in gsp-positive GH-secreting pituitary adenomas. (Endocrinology 140: 2018–2026, 1999)

PITUITARY adenomas constitute about 6–18% of brain tumors in adults (1). Among the hormone-secreting pituitary adenomas, GH-secreting adenoma is the second-leading one (2). Hypersecretion of GH from the adenoma not only alters appearance of these patients (acromegalic appearance) but also increases mortality (3). Therefore, it is important to elucidate the mechanisms of GH oversecretion in acromegalic patients.

GHRH is a well-known physiological regulator of GH synthesis and secretion and somatotroph proliferation (4). These actions are mainly mediated by cAMP that is produced by the binding of GHRH to the membrane GHRH receptor, activation of Gs$\alpha$ and adenylyl cyclase (5). Because ionic mechanisms play essential roles in the regulation of hormone secretion from pituitary cells including somatotrophs (6), we have examined the ionic mechanisms of GHRH-induced GH secretion in human GH-secreting adenoma cells (7, 8). In these studies, application of GHRH on GH-secreting adenoma cells increased a nonselective cation current (NSCC) and voltage-gated Ca$^{2+}$ currents (VGCC) (T- and L-type) through protein kinase A (PKA). The NSCC induced membrane depolarization in the GH-secreting adenoma cells and increased action potential frequency. PKA-mediated activation of VGCC, together with the increased action potential frequency, elevated Ca$^{2+}$ influx through the VGCC. This increased Ca$^{2+}$ influx raised the intracellular Ca$^{2+}$ concentration [Ca$^{2+}$], and facilitated GH secretion (5, 7, 8, 9). This GHRH-induced activation of ion channels exists in normal somatotroph cells as well as in adenoma cells and serves as the physiological regulatory mechanism for GH secretion (10–14).

About 40% (15) of GH-secreting adenomas harbor a mutation in Gsa, which renders it constitutively active (gsp mutation) (16). In vitro studies show that constitutively active Gsa mutation induces elevated intracellular cAMP concentration in cells transfected with the mutated Gsa gene. However, the link between the increased cAMP concentration and GH oversecretion has not been clearly demonstrated, especially in GH-secreting human pituitary adenoma cells. Because we have found that the ionic mechanism is essential in GHRH-induced GH secretion, we investigated the responsiveness of ionic currents to GHRH in GH-secreting adenoma cells harboring gsp mutation to understand whether the GHRH-induced electrophysiological responses have pathophysiological significance in GH oversecretion in this subset of GH-secreting adenomas.

This is the first report to elucidate the effect of gsp mutation on ionic currents and to demonstrate that heterozygous gsp mutation is sufficient to render the currents fully active and unresponsive to GHRH at the single cell level.

Materials and Methods

Cell culture

Four adenomas harboring gsp mutation and three adenomas not harboring gsp mutation were used. All the adenomas were resected by transphenoidal surgery. The adenomas resected from patients were used with the patients’ permission. The use of surgically removed tissues for experiments were permitted by The Ethical Committee of Uni-
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university of Tokyo School of Medicine. Methods for primary culture of these tissues were the same as our previous reports (7). Briefly, the adenoma tissues were minced into small pieces (<1 mm) and were digested with 1000 U/ml dispase (Godo Shusei Co., Tokyo, Japan). For electrophysiological experiments, the cells were seeded on 35-mm culture dishes. Cells were cultured in DMEM containing 10% heat-inactivated FCS and kept in humidified air containing 5% CO2 at 37 C. For investigating the hormone release and intracellular cAMP, cells were seeded on 24-well dishes at a density of approximately 1 × 104 cells/dish. However, when the adenomas were small, we had to seed at a lower density (~1 × 103 cells/dish).

RT-coupled RT-PCR

Messenger RNA was extracted from cultured GH-secreting adenoma cells using MicroFastTrack kit (Invitrogen, San Diego, CA). TaKaRa RNA PCR kit Ver. 2 (Takara Biomedicals, Tokyo, Japan) was used for RT-PCR. The RT solution contained 2.5 μm random 9 mers, 0.25 μM reverse transcriptase, and the extracted messenger RNA (mRNA). Primer set used for PCR was 5'-GTG ATC AAG CAG GCT GAC TAT GTG-3' and 5'-CAG GCG GTT GGT CTG GTT-3'. The primers correspond to the sequence in exon 7 and 10 of Gsp gene, respectively. The PCR mixture contained 2.5 μl TaKaRa Tag DNA polymerase and 0.2 μM of each primer. After a denaturing period of 3 min at 94 C, amplification was performed for 40 cycles at 94 C for 1 min, 60 C for 0.5 min, and 72 C for 0.5 min by DNA thermal cycler 480 (Perkin Elmer, Foster City, CA). The final elongation was done at 72 C for 7 min.

Direct sequencing

After confirming the expected size (249 bp) of the PCR product by agarose gel electrophoresis, the PCR product was purified by a PAGE. The band of interest was cut out from the polyacrylamide gel, precipitated by isopropanol, and directly sequenced using PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit and ABI 373A PRISM sequencer (PE Applied Biosystems, Foster City, CA).

Single cell RT-PCR

We mainly followed the methods described by Bochet et al. (17). For this purpose, pipette solution containing (in mM) 150 CsCl, 5 EDTA, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), whose pH was adjusted to 7.2 by CsOH, was used. Pipette solution and geloader tips (Eppendorf, Hamburg, Germany) used for loading pipette solution to the patch pipette were autoclaved at 121 C for 30 min and 80 C for 20 min, respectively. Glass tubes for making patch pipettes were baked in drying oven at 180 C for 30 min. About 5 μl of pipette solution was loaded into each patch pipette. Adenoma cells that were morphologically distinct from fibroblast-like cells were selected for harvesting RNA. The whole-cell condition was obtained by electrophysiological experiments, the cells were seeded on 35-mm dishes. The standard internal solution contained (in mM): 95 K aspartate, 47.5 KCl, 1 MgCl2, 0.1 ethyleneglycolbis(β-aminoethylether)-N,N',N''-tetraacetic acid (EGTA) (lsqb]tetramethylammonium salt), and 10 HEPES (Na salt, pH 7.2). The standard extracellular solution contained (in mM): 128 NaCl, 5 KCl, 1 MgCl2, 2.5 CaCl2, and 10 HEPES (Na salt, pH 7.4) and 1 μM tetrodotoxin. To record the currents through VGCC, Ba2+ was used as a charge carrier. The external solution used to record the Ba2+ current through VGCC was (in mM): 124.3 NaCl, 5 KCl, 1 MgCl2, 5 BaCl2, 10 HEPES (Na salt, pH 7.4) and 1 μM tetrodotoxin. The internal solution to record the Ba2+ current contained Cs1 ions instead of K1+ ions.

During the experiments, the external solution was continuously perfused by a peristaltic pump. Various agents were applied by changing the perfusion solution. An L/M EPC-7 amplifier (List Medical, Darmstadt, Germany) was used for recording the membrane currents. All the experiments were performed at room temperature (22–25 C). Glass capillaries of 1.5 mm in diameter with a filament were used to make patch electrodes. The resistance of the patch electrodes was between 5 and 8 MΩ. The methods for the perforated whole cell clamp technique have been described elsewhere (19). In brief, a fresh stock solution of nystatin (Sigma Chemical Co., St. Louis, MO) was made daily in dimethylsulfoxide (50 mg/ml). Shortly before recording, the stock solution was diluted with the patch electrode solution (final nystatin concentration, 200 μg/ml). Voltage clamp recordings were made after the series resistance fell below 20 MΩ. The experiments were controlled by an IBM AT clone computer (Gateway, North Sioux City, SD) using TL-1–125 interface and pCLAMP programs (Axon Instruments Inc., Foster City, CA).

Hormone release study

Hormone release studies were carried out using cells cultured in 24-well dishes. Cells were washed twice with serum-free DMEM containing 0.1% BSA. They were incubated in 1 ml of DMEM containing 0.1% BSA with or without GHRH (10 nm) for 2 h at 37 C. After incubation the medium was collected and stored at −20 C until the hormonal assay. GH was assayed by using a radioimmunoassay kit (Daiichi Radioisotope Laboratories, Tokyo, Japan).

cAMP assay

Cells cultured in 24-well dishes were washed twice with serum-free DMEM containing 0.1% BSA. They were preincubated in DMEM containing 0.1% BSA with or without GHRH (10 nm) for 15 min at 37 C. The preincubation medium was replaced with incubation medium of DMEM containing 0.1% BSA with 0.1 mM IBMX with or without GHRH (10 nm) and were incubated for 15 min at 37 C. Following incubation, the medium was discarded and replaced with 1.75% perchloric acid. After freezing and thawing once, the medium was collected in 1.5 ml microtube and was neutralized by 100 μl of 1.2 M KHCO3. The microtubes were then centrifuged at 1500 rpm for 10 min. The supernatant was collected in new microtubes and stored at −70 C until the assay. RIA kit for measuring cAMP (Yamasu Corp., Chiba, Japan) was used for the assay.

Statistical analysis

One-way ANOVA followed by Bonferroni’s multiple comparison test or Student’s unpaired t test were used as appropriate. These analyses were performed by GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Drugs

Human GH-releasing hormone (GHRH), 8-bromoadenosine 3',5'-cyclic monophosphate (8Br-cAMP), forskolin, adenosine 3',5'-monophosphothioate (Rp-cAMPS) and 1-methyl-3-isobutylxanthine (IBMX) were purchased from Sigma Chemical Co.. Cholera toxin was purchased from List Biological Laboratories (Campbell, CA).
Results

Gsp mutation in the adenomas examined in this study

Seven adenomas, three without gsp mutation (adenoma 1, 2, and 3) and four with gsp mutation (adenoma 4, 5, 6, and 7) were used in this study. Two of the gsp mutations were Arg-to-Cys mutation at codon 201 (Arg201Cys) (adenomas 5 and 6), one was Gln227Leu (adenoma 4), and another one was Arg201Ser (adenoma 7). All the mutations have been reported (16, 20).

GHRH-induced electrophysiological responses in GH-secreting adenoma cells with or without gsp mutation

Figure 1A shows the membrane currents under the voltage clamp obtained from an adenoma (adenoma 1) cell that did not have gsp mutation. Both the extracellular and intracellular solution were the standard solutions, and the holding potential was −48 mV. Application of GHRH (10 nM) induced an inward current whose reversal potential was between the equilibrium potential of K⁺ and Na⁺. This response was essentially the same as the response seen in our previous study using different adenomas (7), which was characterized to be an increase in nonselective cation current (NSCC). In all the cells measured in this adenoma, GHRH application activated NSCC (n = 10). GHRH (10 nM) induced NSCC in the other two adenomas (adenoma 2 and 3) without gsp mutation in all the cells examined (n = 6 in each adenoma).

We examined the effect of 8Br-cAMP on the membrane current in gsp-negative adenomas. Application of 8Br-cAMP activated a current whose reversal potential was almost the same as that induced by GHRH (n = 5 in adenoma 1, n = 6 in adenoma 2, n = 4 in adenoma 3). Figure 1B shows the membrane currents before (control) and after (8Br-cAMP) the application of 8Br-cAMP (100 µM). This result is basically the same observed in our previous study (7). The effect of GHRH on the membrane current was abolished by pretreat-
ing the cell with Rp-cAMPS (10 μM) for 10 min (Fig. 1C). These data indicate that the GHRH-induced activation of the nonselective cation current is mediated by PKA, which is consistent with the results of our previous paper (7).

Figure 1D is a trace obtained from a cell in an adenoma (adenoma 4) that harbored gsp mutation. The mutation was Gln227Leu. In this cell, application of GHRH (10 nm) did not induce any change of the membrane current. Application of higher concentration of GHRH (1 μM) did not induce any current either. We further investigated the mechanism of this unresponsiveness. Application of 8Br-cAMP (100 μM), a cell-permeable cAMP analog, did not increase the membrane current, which is shown in Fig. 1D as well. Forskolin, which activates adenyl cyclase directly, did not increase the current, either (data not shown) (n = 10). Similar results were obtained in the other three adenomas with gsp mutation (adenomas 5, 6, and 7, n = 6 in each adenoma).

To examine whether these unresponsiveness to GHRH is mimicked in cells whose Gsα was activated, the effect of cholera toxin treatment (1 μg/ml, 4 h) on the GHRH-induced activation of the nonselective cation current were examined in gsp-negative adenoma cells. Figure 1E shows the membrane current from a cell of adenoma 1 pretreated with cholera toxin before and after the application of GHRH (10 nm). The application of GHRH did not induce any current in this cholera toxin-pretreated cell. The application of GHRH did not induce any current in this cholera toxin-pretreated cell, thereby mimicking the response of gsp-positive cells. Similar results were obtained in four other cells in adenoma 1 and 5 cells in adenoma 2.

Figure 1F summarizes the data of GHRH-induced conductances. The P value of one-way ANOVA was less than 0.0001, suggesting that the mean GHRH-induced conductances among the adenomas were significantly different statistically. Post test (Bonferroni’s multiple comparison test) showed that P values for every pair of adenoma within the gsp-negative or gsp-positive group were larger than 0.05. On the other hand, any combination of pairs between gsp-negative and gsp-positive adenomas had P values less than 0.001. These suggest that while GHRH-induced conductances are similar within gsp-negative or gsp-positive adenomas, the conductances of gsp-negative adenomas were significantly higher than those of the gsp-positive adenomas.

We analyzed the mean basal slope conductance of gsp-negative and gsp-positive cells, and the mean membrane conductance after GHRH (10 nm) application in gsp-negative cells. The slope conductance was calculated by dividing the difference of the membrane current at ~58 mV and ~88 mV by the potential difference (30 mV). Six measurements were used for each adenoma (adenoma 1 through 7). The results were, 302 ± 62 pS (n = 18) for gsp-negative cells, 416 ± 67 pS (n = 18) for gsp-negative cells after GHRH application, and 409 ± 64 pS (n = 24) for gsp-positive cells (expressed as mean ± sd). The P value of one-way ANOVA was less than 0.01, suggesting that the mean basal slope conductance among adenomas were significantly different. Post test (Bonferroni’s multiple comparison test) showed that the basal membrane conductance is similar within gsp-positive and gsp-negative adenomas, whereas the conductance of gsp-positive adenomas is significantly higher than that of the gsp-negative adenomas. When we compared the basal slope conductance of the gsp-positive adenomas with the slope conductance after the application of GHRH (10 nm), these were similar in amplitude.

In our previous studies, we have shown that the activation of VGCC and NSCC by GHRH in GH-secreting adenoma cells are mediated by similar signal transduction mechanism (7, 8). Therefore, we evaluated the response of VGCC to GHRH in the unresponsive adenoma (adenoma 4) to examine whether the unresponsiveness to GHRH is restricted to the NSCC response. Figure 2A is an example of GHRH-induced activation of VGCC in a cell of the adenoma that did not have gsp mutation (adenoma 1), which was essentially the same in our previous report (8). For this experiment, Ba²⁺-containing extracellular solution and Cs⁺-containing intracellular solution were used, and the holding potential was ~76 mV and voltage step to ~16 mV was applied to the cell. Similar results were obtained in all the five other cells of adenoma 1 examined, and five cells each from adenomas 2 and 3, respectively. We examined the effect of 8Br-cAMP on the voltage-gated Ca²⁺ current in gsp-negative adenomas. Application of 8Br-cAMP activated voltage-gated the Ca²⁺ current in these adenoma cells (n = 5 in adenoma 1, n = 5 in adenoma 2, n = 5 in adenoma 3). Figure 2B shows the membrane currents before (control) and after (8Br-cAMP) the application of 8Br-cAMP (100 μM). This result is basically the same observed in our previous study (8).

In the adenoma cell that harbored gsp mutation, the application of neither GHRH (n = 6) nor 8Br-cAMP (n = 6) induced any change of the membrane current (Fig. 2C). We quantified the percent increase of the voltage-gated Ca²⁺ current in gsp-negative and -positive adenomas. The per-
The percentage increase of the voltage-gated Ca\(^{2+}\) current was 25 ± 9\%, 24 ± 7\%, 27 ± 6\%, -2 ± 5\%, -0.4 ± 9\%, and 2.8 ± 5\% in adenomas 1 through 7, respectively (mean ± sd, n = 5 each). The P value of one-way ANOVA was less than 0.001, suggesting that the percentage increase of the voltage-gated Ca\(^{2+}\) current among adenomas were significantly different. Post test (Bonferroni’s multiple comparison test) showed that the percentage increase was similar within gsp-positive and gsp-negative adenomas (P > 0.05 in all the combination), whereas those of gsp-positive adenomas was significantly higher than that of the gsp-negative adenomas (P < 0.001 in all the combination).

These data indicate that the unresponsiveness of the membrane current in the adenoma cells with gsp mutation is not restricted in the NSCC response but also shared by the VGCC response to GHRH. The unresponsiveness in both kinds of the currents not only to GHRH but also to activators of PKA suggests at least two possibilities: first, the unresponsiveness is due to the lack of the component(s) in the signal transduction system distal to PKA; second, the unresponsiveness is due to the constitutively activated Gso, which rendered the mechanism unresponsive to further stimuli such as application of GHRH or 8Br-cAMP.

**Effect of PKA inhibitor on the basal current in adenoma cells with or without gsp mutation**

To discriminate between the two possibilities listed above, we investigated the effect of PKA inhibitor on the basal current of the adenoma cells without gsp mutation (adenoma 2 and 3) or with gsp mutation (adenoma 5 and 6). When a cell of adenoma 2 (gsp mutation negative) was applied with a cell permeable PKA inhibitor, Rp-cAMPS (10 \(\mu\)M), the basal current was not changed (Fig. 3A, n = 6). Similar results were obtained in adenoma 3 (n = 6). When Rp-cAMPS (10 \(\mu\)M) was applied to cells of adenoma 5 (gsp mutation positive), the basal current was decreased. The reversal potential and the I-V relationship of the inhibited current obtained by subtracting the current after Rp-cAMPS application from the control current was similar to the current activated by GHRH in cells without gsp mutation (Fig. 3B, n = 6), suggesting that an NSCC is inhibited. Similar results were observed in adenoma 6 (n = 6). Figure 3C summarizes the data. The P value of one-way ANOVA was less than 0.0001, suggesting that the mean Rp-cAMPS-suppressed conductances among the adenomas were significantly different. Post test (Bonferroni’s multiple comparison test) showed that Rp-cAMPS-suppressed conductances are similar (P < 0.001) within gsp-negative (adenoma 2 and 3) or gsp-positive (adenoma 5 and 6) adenomas, whereas the conductances of gsp-positive adenomas were significantly higher than those of the gsp-negative adenomas (P values between adenomas 2 and 5, 2 and 6, 3 and 5, 3 and 6 were less than 0.001, respectively).

The same was the case about basal VGCC. When a cell of adenoma 2 (gsp mutation negative) was applied with Rp-cAMPS (10 \(\mu\)M), the basal current was not changed (Fig. 4A, n = 5). When Rp-cAMPS (10 \(\mu\)M) was applied to cells of adenoma 5 (gsp mutation positive) the basal current was decreased (Fig. 4B, n = 5). The percentage decrease of the voltage-gated Ca\(^{2+}\) current was 0.4 ± 2\% in adenoma 2 and 19 ± 4\% in adenoma 5, respectively (mean ± sd, n = 5 each). The P value was less than 0.01 by Student’s unpaired t test.

The above findings indicate that the unresponsiveness of both the NSCC and VGCC is the consequence of the full activation of these currents by constitutively activated signal transduction mechanism induced by the constitutively active Gso.

**In vitro cAMP and GH measurements**

To evaluate how these observations relate to intracellular cAMP and GH release by GHRH, we examined them in two
of the \( gsp \)-positive adenomas and all of the \( gsp \)-negative adenomas (Tables 1 and 2). Data were obtained in quadruplicate. Because the concentration of cells plated in the 24-well dishes varied slightly depending on the availability of the cell, the data for GH release are expressed as percent of control GH levels (mean ± SD). For cAMP measurements, the basal intracellular cAMP concentration could not be used for standardizing because most of them were below the detection limit in \( gsp \)-negative adenomas. The raw data are presented, and the basal and GHRH-stimulated cAMP levels are put in parentheses to denote that cell concentration slightly varied among the adenomas. We observed the tendency that basal cAMP levels were high in \( gsp \)-positive adenomas, whereas basal cAMP levels of \( gsp \)-negative adenomas were mostly below the detection limit. In every adenoma examined, both \( gsp \)-negative and -positive, the intracellular cAMP levels increased by GHRH application compared with control with a statistical significance. When we looked at fold-increase of cAMP levels by GHRH, the \( gsp \)-positive adenomas were 2.84- and 4.29-fold, which were about the same or less pronounced compared with those of the \( gsp \)-negative adenomas (over ×18.00, over ×4.20, and over ×7.50, respectively).

The response of GH release to GHRH was either increased or not changed in the adenomas examined. Both types of responses were found in \( gsp \)-negative and \( gsp \)-positive adenomas. The GH was increased in one from each group of adenomas, and GH release did not differ in the rest of the adenomas.

Single cell RT-PCR of the adenoma cell with \( gsp \) mutation

\( Gsp \) mutation is considered to be heterozygous in \( gsp \)-secreting pituitary adenomas because of its activating nature. However, it has not been proven because cell mixtures or tissue fragments used for extracting mRNA or genomic DNA unavoidably contain nonadenoma cells including vascular cells and fibroblast-like cells. It is also unclear so far whether one (heterozygote) or two \( gsp \) alleles (homozygote) were required for the maximal basal PKA-dependent activation of the two classes of currents (NSCC and VGCC) in adenoma cells. To clarify these ambiguities, we employed single cell RT-PCR that utilizes cytosol of a single cell for collecting mRNA.

When the whole-cell clamp conditions were obtained between patch electrode and the cells, we applied a depolarizing pulse from the holding potential of −68 mV to +2 mV and checked for the presence of overt inward currents. The inward currents seen in this condition were mainly VGCC, which is abundant only in excitable cells and not in mesenchymal cells including fibroblasts. Only the cells with overt inward currents were selected to collect cytosol. Messenger RNA was extracted, reverse-transcribed, and amplified by RT-PCR using primers for \( Gs \)α. Sequencing revealed that the wild-type sequence was present together with the corresponding mutant sequence (CAG(Gln)227CTG(Leu) for adenoma 4, CGT(Arg)201TGT(Cys) for adenoma 5, and CGT(Arg)201AGT(Ser) for adenoma 7) in all of the three \( gsp \)-positive adenomas examined. These data strongly suggest that \( gsp \) mutation is heterozygous in \( gsp \)-secreting pituitary adenomas (Fig. 5). \( Gsp \) mutation was not detected in single-cell RT-PCR from an adenoma, which was determined to be \( gsp \)-negative by cell-mixture RT-PCR.

Discussion

The selectivity of the PKA inhibitor we used in the present study was confirmed by examining the effect of this com-

![Fig. 4. Effect of Rp-cAMPS on the basal voltage-gated Ca\(^{2+}\) current. A, Application of Rp-cAMPS (10 \( \mu \)M) did not change the VGCC in a cell from adenoma 2 without \( gsp \) mutation. B, VGCC in a cell from adenoma 5 decreased from the basal level by the application of Rp-cAMPS (10 \( \mu \)M).](https://academic.oup.com/endo/article-abstract/140/5/2018/2990322/fig4)

### Table 1. Intracellular cAMP response to GHRH stimulation

| Adenoma No. | \( gsp \) status | Basal cAMP (pmol/µl) | GHRH stim. cAMP (pmol/µl) | Fold-increase Significance |
|-------------|-----------------|------------------------|----------------------------|---------------------------|
| 1           | (−)             | (1.0)                  | (18.0 ± 2.2)               | over ×18.00 ***           |
| 2           | (−)             | (<1.0)                 | (5.25 ± 0.96)             | over ×4.20 ***           |
| 3           | (−)             | (−<1.0)                | (7.5 ± 1.7)               | over ×7.50 ***           |
| 5           | (+)             | (25.5 ± 3.1)           | (72.3 ± 6.7)              | −2.84 ***                |
| 7           | (+)             | (10.8 ± 1.3)           | (46.3 ± 5.1)              | ×4.29 ***                |

Data are collected in quadruplicate. Values are expressed as mean ± SD. In adenomas 1, 2, and 3, most of the basal cAMP concentrations were below the sensitivity of the cAMP assay, which was 1.0 pmol/µl. In such cases the value is expressed as "< (below) mean," and fold-increase in response to GHRH is expressed as "over-fold" for best approximation. The basal and GHRH-stimulated cAMP levels are put in parenthesis to denote that cell concentration slightly varied among the adenomas. Significance describes the \( P \) value summary of Student’s \( t \) test: ***, \( P < 0.001 \).
pound on GHRH-induced activation of the NSCC (Fig. 1C). We also confirmed this by examining the effect of this compound on CRH-induced activation of the NSCC. We have previously demonstrated that CRH-induced activation of an NSCC is mediated by PKA, by using specific PKA inhibitor peptide (21). Pretreatment with Rp-cAMPS (10 μm) abolished the CRH-induced activation of the NSCC (data not shown). There are also data on the specificity of Rp-cAMPS on PKA inhibition in the literature (22).

We found that gsp mutation renders both the NSCC and VGCC unresponsive to GHRH by fully activating these currents at the basal state. This is the first report to show the pathophysiological significance of ion channels in GH over-secretion. Because the analyses of the gsp mutation revealed that it is heterozygous, heterozygote mutation is enough to render the ion currents fully active and unresponsive to GHRH.

The present paper referred to the mechanism of GH over-secretion in gsp-positive adenomas in comparison with gsp-negative adenomas. However, we have seen that the same NSCC is induced by GHRH in normal human somatotrophs (7) as in gsp-negative GH-secreting adenomas. It is also reported that normal rat somatotrophs show an increase in NSCC in response to GHRH through the same signal-transduction mechanism as in human gsp-negative GH-secreting pituitary adenomas presented in this study (12, 13). These allow us to speculate that the findings about ionic currents from comparing gsp-positive and -negative adenomas apply to comparing gsp-positive adenomas and normal somatotroph.

It has been thought that gsp mutation is heterozygous in gsp-positive adenomas because the mutant allele is always detected together with the wild-type allele, and the activating nature of gsp mutation presumably requires only one mutant allele to affect the phenotype. However, all the analyses of gsp mutation used tumor tissue for extracting nucleic acids. The tumor tissue consists of the mutated GH-secreting cells and nonadenoma cells including blood cells, fibroblasts, and vascular cells. Therefore, strictly speaking, it is not possible to say that the mutation of the adenoma cell is heterozygous at the single adenoma cell level, as is discussed in Ref. 18. To solve this problem, it is necessary to investigate the heterozygosity of gsp mutation in a single adenoma cell. We used single cell RT-PCR technique to answer this question. With this method, the wild-type sequence was detected together with the corresponding mutant sequence in all of

| TABLE 2. GH release by GHRH stimulation |
|------------------------------------------|
| Adenoma No. | gsp status | GH release (% of control GH) | Significance |
|--------------|------------|-----------------------------|--------------|
| 1(+)         | (−)        | 100 ± 39.3                  | 115.6 ± 16.2 | n.s.       |
| 2(-)         | 100 ± 20.8 | 298.1 ± 30.7 D              | 12.2 ± 12.4  | n.s.       |
| 3(-)         | 100 ± 13.4 | 132.1 ± 16.3               | 12.4         | **         |
| 5 (+)        | 100 ± 14.7 | 94.8 ± 19.8 D              | n.s.         |

Data are collected in quadruplicate. Values are expressed as percent of control GH (mean ± sd%). Significance describes the P value summary of Student’s unpaired t test: n.s., P ≥ 0.05; **, P < 0.01; ***, P < 0.001.
the three gsp-positive adenomas examined. This is not likely to be a result of amplification error of Taq polymerase because it would require the error to occur at the specific position to the specific nucleotide in both cell-mixture and single-cell RT-PCR in all of the three adenomas. These indicate that the gsp mutations are heterozygous in single cells from all the three gsp-positive adenomas examined. Because human GH-secreting adenoma is known to be monoclonal (23), the findings from a single adenoma cell can be applied to other adenoma cells. This is the first direct evidence that gsp mutation is heterozygous in GH-secreting pituitary adenomas. The fact that this mutation is heterozygous supports that this mutation is an activating (gain of function) mutation. In these adenomas, one gsp allele was enough to fully activate the PKA-dependent activation of the two classes of currents (NSCC and VGCC). Full activation of these currents at the basal state likely accounts for GH oversecretion in gsp-positive adenomas.

For intracellular cAMP response, all the adenomas examined responded to GHRH. Yet only the gsp-negative adenomas showed NSCC increase by GHRH, which is one of the responses downstream of cAMP increase. It is reported in normal pituitary rat cultures that GHRH application induces intracellular cAMP increase by 6- to 57-fold of control depending on the concentration of GHRH and incubation time (24, 25). It is also shown in the reports that stimulation of cAMP accumulation occurs over a GHRH concentration range that is approximately one order of magnitude higher than required for dose-related GH release (24, 25). The finding that basal cAMP levels for gsp-positive adenomas were well within the detection range compared with mostly undetectable gsp-negative adenomas make it attractive to consider that gsp-positive adenomas have high basal cAMP levels. These may suggest that, even though CAMP increases by GHRH in gsp-positive adenomas, NSCC is fully activated by high basal CAMP levels and GHRH application have no further effect on the current. In other words, maximal NSCC activation is achieved at a submaximal concentration of CAMP. The response of GH release by GHRH did not show clear segregation between gsp-negative and -positive adenomas. This finding has been known (26, 27). Our model does not offer explanation to this. This suggests that there are other factors that promote or inhibit GHRH-induced GH release, both of which requiring further investigation.

GH-secreting adenomas is the second largest subgroup in occurrence among pituitary adenomas. Gsp mutation accounts for about half of the GH-secreting adenomas. However, the etiology of tumorigenesis or hormone oversecretion in the remaining half is not yet clear. There are other kinds of pituitary adenomas such as PRL-secreting adenomas, ACTH-secreting adenomas, and gonadotropin-secreting adenomas. Gsp mutation is demonstrated in some of these adenomas, for example, ACTH-secreting adenomas. ACTH-secreting adenomas share in common with GH-secreting adenomas that the hormone secretion is regulated through Gαs by corresponding regulatory hormones (CRH and GHRH, respectively). Therefore, it is worthwhile investigating whether aberrant electrophysiological responses to agonists that act through Gαs have pathophysiological significance in hormone oversecretion in these adenomas.

Electrophysiological studies have contributed profoundly to the understanding of the physiology of endocrine cells. In the case of anterior pituitary cells, the physiological responses of these cells to hypothalamic hormones and its signal transduction mechanisms have been revealed by many electrophysiological studies (28). Investigation with electrophysiological technique in combination with molecular biological methods of this area may facilitate the discovery of novel etiology of hormone oversecretion or tumorigenesis.

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