Gonadotropin-releasing hormone initiates multiple signaling pathways in human GH-secreting adenomas

A. Lania¹, G. Mantovani¹, E. Ferrante², L.M. Zavanone², M. Locatelli², S. Corbetta¹, P. Beck-Peccoz¹, and A. Spada²

¹Institute of Endocrine Sciences; ²Department of Neurosurgery, University of Milan, Ospedale Maggiore IRCCS, Milan, Italy

ABSTRACT. Abnormal GH responses to GnRH test, observed in about 15% of patients with acromegaly, have been reported exclusively in patients bearing tumors without gsp mutation. The absence of responsiveness to GnRH in gsp+ tumors was not predicted on the basis of the mechanism of GnRH action that mainly involves the activation of calcium and protein kinase C dependent pathways. The aim of the present study was to investigate in detail the transduction of GnRH signaling in these tumors. GH-secreting adenomas removed from patients in vivo responsive to GnRH test were studied. Tumor DNA was screened for Gsα and GnRH receptor gene sequences. Intracellular calcium ([Ca²⁺]) and cAMP levels were measured in dispersed cells and adenyl cyclase (AC) activity in membrane preparations. DNA analysis showed wild sequence of both Gsα and GnRH receptor genes. GnRH caused a significant increase in intracellular Ca²⁺ that was associated with a significant stimulation of cAMP accumulation. In these cells neither TRH nor GHRP-6 were effective in causing significant modifications of cAMP levels, despite their ability to increase [Ca²⁺]. Finally, GnRH was able to directly stimulate AC from 11.1±3.3 pmol/mg prot/min to 26.9±5.4 (p<0.005). We report that GnRH was effective in increasing both [Ca²⁺] and AC in GH-secreting adenomas removed from responsive patients. The ability of GnRH to signal through Gsα protein may account for the lack of GH responses to GnRH observed in acromegalic patients with tumors carrying gsp mutation.


INTRODUCTION

Patients with acromegaly frequently present abnormal GH responses to various hypothalamic agents. In particular, serum GH increases after TRH and GnRH injection have been reported in about 60 and 15% of patients, respectively (1). The presence of abnormal responses has been mainly used as a disease marker, particularly after transphenoidal surgery, since no difference in clinical and biochemical characteristics, tumor size and outcome between patients with and without these alterations have been observed (2). However, considering the subset (30-45%) of patients with GH secreting adenomas (GH-omas) expressing activating mutations of Gsα gene (gsp oncogene), these patients share a particular and partially unexicted pattern of responsiveness to hypothalamic agents (3-5). In fact, in gsp-positive patients previous studies reported poor or absent GH responses to GHRH injection, likely to be due to the maximal activation of adenyl cyclase (AC) by the mutant Gsα not further stimulated by GHRH, positive responses to TRH, that is known to operate via calcium- and protein kinase C-dependent pathways and no response to GnRH (3, 5). The absence of responsiveness to GnRH in the context of tumors with constitutive activation of cAMP pathway is at least in part in contrast with the putative mechanism of GnRH action. Classically, by coupling to Gq/11 protein, GnRH induces phospholipase C activation resulting in inositol phosphate production and calcium mobilization (6-9). However, considering the number of GnRH actions on the central and peripheral nervous system as well as on extraneural and neoplastic tissues, an inherited ability of the GnRH receptor to couple to multiple G proteins and to activate multiple signal transduction pathways has been postulated (10). Since data available for the pituitary are consistent with either an exclusive coupling to Gq/11 or multiple coupling

Key-words: GnRH, adenylate cyclase, cAMP, G protein.
Correspondence: A. Spada, MD, Institute of Endocrine Sciences, Pad. Granelli, Via F. Sforza 35, 20122 Milan, Italy.
E-mail: anna.spada@unimi.it
Accepted November 14, 2003.
to both Gq/11 and Gs proteins, depending on the cell line studied (9-13), we set out to investigate the intracellular effectors generated by GnRH in human GH-secreting adenomas (GH-omas). We provide evidence that in this particular cell environment activation of the normal GnRH receptor stimulated adenylate cyclase activity, and this phenomenon probably accounts for the lack of responses to GnRH in acromegalic patients with gsp positive tumors.

MATERIALS AND METHODS

Tumors

The study was carried out on 4 GH-secreting adenomas (GH-omas) surgically removed from acromegalic patients selected on the basis of the presence of abnormal GH response to GnRH test. Acromegaly was diagnosed on the basis of clinical features, elevated IGF-I plasma levels, and elevated GH levels not suppressible during oral glucose tolerance test. Preoperatively, patients received 100 μg GnRH (Serono, Milan, Italy) as an iv bolus and blood samples were collected at 15 min intervals for 2 h. An abnormal GH response was considered present when a serum GH increase of at least 50% and higher than 6 μg/l above basal levels was observed, according to previously reported criteria (2). Of the removed tumors, small adenoma fragments were fixed for light microscopy, to check the adenomatous nature of the material, as previously described (14). Part of the tissues was quickly frozen for the analysis of Gsα and GnRH receptor genes and AC assay and part was placed in sterile culture medium: the cells were enzymatically dispersed for intracellular calcium ([Ca2+]i) and cAMP measurements. Local ethical approval was obtained for all studies.

Analysis of Gsα and GnRH receptor genes

DNA and RNA were extracted from all samples with standard methods. The hotspots of Gsα gene were amplified using intronic oligonucleotide primers, as previously described (15). The GnRH receptor gene (exons 1-3), including each bordering intron region, was amplified by PCR using the following set of oligonucleotide primers: 1-2: 5′-ACAGAGCAC111AGAA11CAGG111ACAA-3′ and 1-1: 5′-AGAC11111-TAGATAACTGAACCTTG-3′ (Ta 58); 2-2: 5′-AAAACATCTGAAGCCACCTG-3′ and 2-1: 5′-CAAGTAAACAGAACAGGCAA-GAA-3′ (Ta 62); 3-1: 5′-AGGAGCTTAGAAATTGCTATTAGT-3′ and 3-3: 5′-GCTATTAAAACACTGCCCCACAA-3′ (Ta 58); 3-4: 5′-GCTGCTGCTCCTTTTGTCCACTTTG-3′ and 4-3: 5′-ATTCTATTACCTACCCCTCTTTCATA-3′ (Ta 58). RT-PCR of the entire GnRH receptor gene cDNA was performed using primers 1-3 and 4-3. Direct sequencing of the amplified fragments was then performed using the AmpliTaq BigDye Terminator kit and 310 Genetic Analyzer (Perkin Elmer Corp., Applied Biosystems, Foster City, CA).

Measurements of [Ca2+]i

After 24 h culture in Dulbecco modified eagle’s medium (DMEM), tumoral somatotrophs were collected, washed and resuspended at 4.5 x 104 cells/ml in Krebs-Ringer-HEPES incubation medium (KRM) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 25 mM HEPES-NaOH (pH 7.4) and 6 mM glucose, as previously described (16). Cells were loaded with the Ca2+ indicator fura-2 by incubating the cells with 5 μM fura-2-acetoxy methyl ester (AM) for 30 min at 37 C. Thereafter, fluorescence recordings were carried out with a cell concentration of 3-4 x 104 cells/ml in KRM using a Perkin-Elmer LS5 spectrofluorimeter (Perkin-Elmer, Norwalk, CT) at 345 nm excitation and 490 nm emission with slits of 5 and 10 nm, respectively, [Ca2+]i was calculated as previously described (16).

Intracellular cAMP levels

The dispersed cells were plated at the density of 2 x 105 cells /ml in 24-well plastic cluster dishes and cultured in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics at 37 C in an atmosphere of 95% air-5% CO2, in a humidified incubator. After 2 days cell monolayers were washed and incubated at 37 C for 1 h in serum free DMEM containing 2 mM 3-isobutyl-1-methylxanthine (IBMX) and the different substances to be tested in triplicate, as previously described (17). Thereafter, the medium was removed and intracellular cAMP levels were measured in supernatants obtained from cells maintained in 80% ice-cold ethanol at -20 C overnight, using an enzyme immunoassay commercial kit (Amersham) as previously described (17).

AC assay

AC activity was evaluated in membrane obtained from the tumors of this series and from 2 gsp-positive tumors included in a previous series, for comparison (17). Membrane preparations were sedimented from tumor homogenates by centrifugation at 20,000 x g for 10 min. and AC assay was performed, as previously described (18). Briefly, the assay mixture contained 25 mM tris HCI pH 7.4, 10 mM theophylline, 1 mM cAMP, 0.2 mM ethyleneglycol-bis-(-aminoethyl)-ether-; N, N’, N” tetraacetic acid (EGTA), 0.15 mM [8-14C] ATP (40 dpm/pmol), 7 mM phosphocreatine and creatinphosphokinase (20 U/ml). The reaction was initiated by the addition of membranes (0.5 mg protein/ ml) and incubated at 30 C for 8 min. Isolation and estimation of the amount of [8-14C] cAMP were performed as described (18).

Reagents

ATP, CAMP, creatine phosphate, creatin phosphokinase, TRH, GHRH, GnRH, GHRP-6, trypsin, soybean trypsin inhibitor, were purchased from Sigma Chemical Co. (St. Louis, MO). [8-14C] ATP and [8-14C] cAMP were purchased from Amersham (United Kingdom). Culture media were supplied by Flow Laboratories (Mackenheim, Germany). Fura-2/AM was purchased from Molecular Probes, Inc. (Junction City, OR). All other reagents were reagent grade.

Statistical analyses

The results are expressed as the mean±SD. A paired or unpaired two-tailed Student’s t test was used to detect the significance between two series of data. p<0.05 was accepted as statistically significant.

RESULTS

Molecular analysis of Gsα and GnRH receptor genes

No mutation in the hotspots of Gsα gene was detected in this series of tumors removed from patients responsive to GnRH. Analysis of the GnRH receptor gene performed in 3 tumors showed a wild type coding sequence. The RT-PCR of the entire GnRH receptor cDNA revealed the amplification of a single band of the expected size, thus excluding the presence of splicing alterations in the samples.