An Autocrine Role for Pituitary GABA: Activation of GABA-B Receptors and Regulation of Growth Hormone Levels

Katia Gamel-Didelon[a]   Claudia Corsi[c]   Giancarlo Pepeu[c]   Heike Jung[b]
Manfred Gratzl[a]   Artur Mayerhofer[a]

[a]Anatomisches Institut der Universität München, München, and [b]Lilly Germany, Bad Homburg, Deutschland;
[c]Dipartimento di Farmacologia, Università di Firenze, Firenze, Italia

Abstract

There is increasing evidence suggesting that the neuro-transmitter γ-aminobutyric acid (GABA) is a local factor involved in the regulation of endocrine organs. Examples of such functions are documented in the pancreas, but recent results suggest that GABA may act in a similar way in the pituitary, in which GABA receptors are expressed and pituitary growth hormone (GH) cells provide a source of GABA. We hypothesised that GABA secreted in soma-totropes may act as an autoregulatory signaling mole-cule. To test this hypothesis we first examined the nature of GABA receptors expressed by GH cells. RT-PCR analy-sis demonstrated that GABA-B receptor subunits R1 and R2 are present in the whole rat pituitary. Laser microdis-section of immunostained GH cells, followed by RT-PCR as well as immunoelectron microscopy, showed that GABA-B receptors are expressed on somatotropes. To investigate GABA-B receptor function in somatotropes, we used rat GH3 adenoma cells, which, like pituitary GH cells, express GABA-B R1 and R2 (as assessed by RT-PCR and immunoelectron microscopy) and produce GABA (checked by high performance liquid chromatography). After inhibition of endogenous GABA synthesis, GH pro-duction was stimulated by baclofen, a chromatography).

After inhibition of endogenous GABA synthesis, GH pro-duction was stimulated by baclofen, a GABA-B receptor agonist. By contrast, blocking GABA-B receptors by an antagonist, phaclofen, decreased GH levels. We conclude that in GH-producing cells, GABA acts as an autocrine fac-tor via GABA-B receptors to control GH levels.

Introduction

Production and secretion of hormones in the pituitary is controlled by several hypothalamic factors. Besides the classic releasing and inhibitory factors synthesised by the hypothalamus, the neurotransmitter γ-aminobutyric acid (GABA) regulates the production of pituitary hormones, including corticotropin (ACTH), growth hormone (GH), thyreostimulating hormone (TSH), luteinizing hormone (LH) and prolactin (PRL). The effects of GABA depend on several factors, such as concentration, developmental stage and experimental conditions, and may be mediated either directly or indirectly [1–7]. In the hypothalamus, for instance, GABA acts on neurons secreting releasing factors [8, 9]. In addition, axons of GABAergic hypotha-lamic neurons terminate on endocrine pro-opiomelano-cortin (POMC) cells of the intermediate lobe of the pitu-itary [10, 11]. Moreover, GABAergic neurons extend to the median eminence, where GABA is secreted into portal vessels and reaches the anterior pituitary.

Recently, additional sites of GABA production have been identified in GH cells of the anterior pituitary and POMC cells of the intermediate lobe in rat and monkey pituitaries [12]. The same report showed that GH cells are immunoreactive for a GABA-A receptor subunit (γ2). These findings, together with the fact that all classes of GABA receptors (A, B, C) were found to be present in the anterior lobe [12–18], imply that GABA may exert auto-crine actions within the adenohypophysis. This action appears to include regulation of GH, as seen, for example, in rats [3, 7, 16]. However, neither the mechanisms of action of endogenous GABA nor its consequences for GH cell function are currently known.

In order to define the role of GABA secreted by somato-tropes, we used a rat pituitary adenoma cell line, GH3 [19]. GH3 cells, like GH cells, are able to produce and store GABA [12]. Therefore they represent a valuable model for studying the role of GABA in GH-producing cells. The present work demonstrates that GH3 cells express functional GABA-B receptors involved in the regulation of GH synthesis and secretion. A similar mechanism of regulation of GH synthesis may occur in pituitary GH cells, since we show that they also bear GABA-B receptors.

Methods

Animals

Pituitary glands were obtained from male and female Sprague-Dawley rats that had been bred at the Technische Universität München. According to our animal care guidelines, they were pain-lessly killed under ether anaesthesia and their pituitaries, and in some cases brains, were removed and either processed for paraffin-embedding or electron microscopy, or they were frozen for RNA extraction, as described previously [12].

Culture Procedures of Rat GH3 Cells

The general culture procedures applied to GH3 cells have been described previously [20]. GH3 cells were grown in F12-DME (Sigma, Deisenhofen, Germany) containing 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria) in 60-mm diameter Petri dishes purchased from Life Technologies (Rockville, Md., USA). They were submitted to treatments with 10^{-4} or 10^{-5} M GABA receptors ago-nists or antagonists (Tocris, Ballwin, Mo., USA) before reaching con-fluency. Unless otherwise stated, the treatments using two different concentrations of chemicals gave similar results and thus were pooled. These results were confirmed by a different handling proce-dure that included a 24-hour starvation step (without FCS) for syn-chronising the cells, followed by an additional day of culture in F12-DME +2.5% FCS. The chemicals were subsequently added to the 2.5% FCS-containing medium for the time of treatment. In some experiments, blocking glutamic acid decarboxylase (GAD) was nec-essary to prevent endogenous GABA synthesis by GH3 cells. To this end, we used 100 μM of γ-acetylenic GABA (Sigma) [21].

Laser Microdissection of Rat Pituitary GH Cells

PALM® Robot-MicroBeam Technology (P.A.L.M. GmbH, Bern-ried, Germany) was used for 'non-contact' laser microdissection of GH-immunostained cells from rat pituitaries. The samples were pre-pared following the guidelines of the manufacturer. Bouin-fixed, par-affin-embedded sections were deparaffinised and immunostained with an anti-GH antiserum (diluted 1:500; courtesy of Dr. Parlow, NHPP, Torrance, Calif., USA) following immunohistochemical methods previously described [12, 22, 23]. From these sections, sam-ples were catapulted directly into the cap of a microfuge tube and stored at –80°C in the RNA*later* buffer (Qiagen, Hilden, Germany) until RNA isolation, reverse transcription (RT) and PCR were per-formed.

RNA Isolation and RT-PCR

RNA isolation from either GH3 cells or rat tissues were per-formed using the RNeasy kit and the QIAshredder columns from Qiagen. The method used for RT (using MMLV, Stratagene, La Jol-la, Calif., USA) and 18-mer polydeoxythymidine primers has been described previously [24–26]. PCR consisted of a 5-min hot start step followed by 40 cycles: 94°C for 30 s, 55°C for 1 min, 72°C for 30–60 s; final extension for 10 min at 72°C.

From laser microdissected samples, RNA was prepared using the Purescript kit (Gentra Systems, Minneapolis, Minn., USA) and was subsequently treated with DNase, precipitated and resuspended in RNase-free water. This isolated RNA was reverse-transcribed using Superscript II (Life Technologies) and random hexamers (MWG Bio-tech, Ebersberg, Germany) according to the manufacturer's instruc-tions. For cDNA amplification, a nested PCR strategy was em-ployed. A first PCR step (PCR1) was performed, the PCR1 product was subsequently purified (MiniElute Kit, Qiagen) and used as tem-plate for a second PCR. For all experiments, the nature of the ampli-fied cDNAs was confirmed by direct sequencing using one of the oligonucleotide primers (Agowa, Berlin, Germany).

Oligonucleotide primers were chosen to encompass exon-intron boundaries to avoid possible genomic DNA contamination. Primers designed for the R1 subunit do not distinguish between the splice variants R1a and R1b. They were purchased from MWG Biotech and their sequences are listed in table 1a, b. In addition a sense prim-er (5′-GTA CGT CTG GTT CCT CAT-3′) was used in combination with nested RI-3′ for the analysis of GH3 and pituitary samples (see fig. 2).

Electron Microscopy

GH3 cells and rat pituitaries were fixed on ice for 1 h with 2% paraformaldehyde and 0.5% glutaraldehyde in 10 mM PBS, pH 7.4. They were dehydrated with ethanol (30% at 0°C, 50% at –10°C, 70% at –30°C) and infiltrated at –30°C with the hydrophilic resin Lowicryl K4M (Plano, Wetzlar, Germany). Polymerisation was achieved by exposure to ultraviolet light for 2 days at –30°C and for 3 days at room temperature, using an EM AFS apparatus from Leica (Vienna, Austria). Following ultra thin sectioning, immunocyto-chemistry was performed as described previously [27]. For immuno-localisation of GABA-B R2, the primary antiserum was raised in rab-bits and diluted 1:1,000 (courtesy of Graham Disney and Fiona Mar-shall, GlaxoWellcome Research and Development, Stevenage, UK). A secondary goat anti-rabbit antiserum was labelled either with 10-nm (immunostaining on GH3 cells) or 6-nm (double immuno-staining on rat pituitaries) gold particles (diluted 1:20; Aurion, Wage-ningen, The Netherlands). For immunodetection of GH, the first

a Rat GABA-B R1a+b (Genbank accession No. Y10369 and Y10370) and positions of primers

R1–5′	5′ CTGGCCTATGATGCTATC 3′	1402–1419 (R1a)/1054–1071 (R1b)
R1–3′	5′ CGGAATGTCTTGATGACC 3′	1751–1734 (R1a)/1403–1386 (R1b)
Nested R1–5′	5′ AACTACAACAACCAGACC 3′	1492–1509 (R1a)/1144–1161 (R1b)
Nested R1–3′	5′ AGATCATCCTTGGTGCTG 3′	1676–1659 (R1a)/1328–1311 (R1b)

b Rat GABA-B R2 (accession No. AF058795) and positions of primers

R2–5′	5′ CATCATCTTCTGCAGCAC 3′	2328–2345
R2–3′	5′ TCTGTGAAGTTGCCCAAG 3′	2681–2664
Nested R2–5′	5′ TCCAGTTCACACAGAACC 3′	2426–2443
Nested R2–3′	5′ CCAGCTCTGTGATCTTCA 3′	2563–2546

antibody was raised in monkeys (diluted 1:500; courtesy of Dr. Parlow, Torrance, Calif., USA) and the second antibody was a 10 nm gold-labelled anti-human antibody (diluted 1:20; Aurion). Sections were examined with the Zeiss electron microscope EM10.

Western Blotting

Western blotting was performed as described previously [28, 29]. In order to control small loading differences, Ponceau stainings of separated proteins were performed. In addition, for some experiments, β-actin was detected and used as an additional reference protein. To this end, images were digitalised and optical densities of Western blot results were evaluated using NIH Image, as described previously [25].

For detection of GH, a monkey anti-GH primary antiserum (diluted 1:500) and peroxidase-conjugated goat anti-human immunoglobulins were used (diluted 1:5,000; Jacksons Immunoresearch Laboratories, West Grove, Pa., USA). Immunodetection of β-actin was achieved using a mouse anti-β actin antibody purchased from Sigma (diluted 1:5,000) and peroxidase-conjugated goat anti-mouse immunoglobulins (diluted 1:5,000; Jacksons Immunoresearch Laboratories). Results were visualised using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK).

Enzyme Immunoassay

After treatment, the supernatants from confluent GH3 cells (4–10 × 10⁶ cells) were harvested and stored at –20°C until use. In order to measure the quantity of GH secreted by GH3 cells, a commercial enzyme immunoassay (EIA) test was used (Biotrak system, Amersham). GH levels were determined after reading the optical density at 450 nm with a spectrophotometric plate reader (Dynex Technologies, Chantilly, Va., USA). The values were finally expressed as ng GH. The sensitivity of the commercial test used was 1.7 ng/ml of GH.

High Performance Liquid Chromatography

As for the EIA, the supernatants from the GH3 cell cultures were harvested after 24 h of treatment and stored at –20°C until use. The culture medium was used as a reference. GABA analysis was carried out by a high performance liquid chromatography (HPLC) method

with fluorimetric detection [30]. Briefly, amino acids were derivatized with mercaptoethanol and o-phtalaldehyde (OPA). The OPA derivatives were then separated on a 5-μM reverse-phase Nucleosil C18 column (250 × 4 mm; Macherey-Nagel, Duren, Germany), kept at room temperature, using a mobile phase consisting of methanol and potassium acetate (0.1 M, pH adjusted to 5.52 with glacial acetic acid) at a flow rate of 1.0 ml/min in a three-linear step gradient (from 25 to 90% methanol). This method detects GABA quantities as low as 100 fmol per volume injected. In the samples, GABA levels were given as pmol/10⁶ cells.

Statistical Analyses

For statistical data analyses, ANOVA and the Student-Newman-Keuls multiple comparison test (Western blot results), or the Student paired t test (HPLC and EIA data) were employed.

Results

GABA-B Receptor Subunits Are Expressed in GH-Producing Cells of the Rat Pituitary and in GH3 Cells

Immunoelectron microscopy experiments, using double immunostaining, identified GH-immunoreactive cells to possess GABA-B R2 subunits at their membrane (fig. 1a). The expression of R2 by GH cells was confirmed at the mRNA level. To this end, rat pituitary sections immunostained for GH were submitted to laser microdissection. RNA was extracted, RT-PCR experiments performed and, using a nested PCR approach, the GABA-B R2 subunit was detected in GH-immunoreactive cells. GABA-B R2 was absent in samples in which GH-immunoreactive cells had been destroyed by a laser beam (fig. 1b).

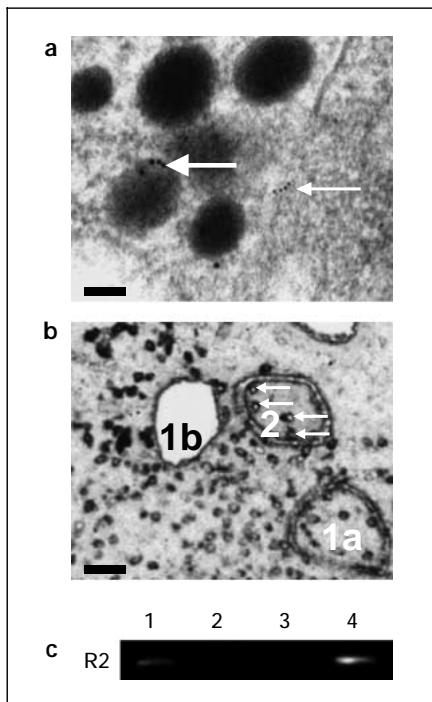Both R1 and R2 subunits of the GABA-B receptor are also expressed in cultured GH3 cells, as shown by RT-

Fig. 1. GH cells express GABA-B R2 subunit. **a** Immunoelectron microscopy performed on an ultra thin section of a rat pituitary. The nature of the cell shown was clearly identified by immunostaining of GH-containing secretory granules (10-nm gold; bold arrow). At the membrane of the GH cell, 6-nm gold labels indicate the presence of GABA-B R2 subunit proteins (narrow arrow). Bar equivalent to 100 nm. **b** Section of a rat pituitary in which GH cells were identified by immunohistochemistry and submitted to laser microdissection (upper panel). Groups of GH-positive cells were separated from the surrounding tissue by a laser beam (1a) and subsequently catapulted (1b) inside the cap of a microfuge tube. To control false positives, all GH cells (arrows) were destroyed in another area by laser shots before laser excision (2). Bar equivalent to 80 μm. **c** RNA extracted from laser-dissected pituitary GH cells was subsequently reverse transcribed and submitted to nested PCR amplification. GH-producing cells express GABA-B R2 (lane 1). No GABA-B R2 product is detected after destroying GH cells in the surrounding tissue (lane 2). Lane 3 is a control (PCR reaction without template). Lane 4 shows the expression of R2 in the whole brain of a 4-week-old rat (positive control). Sequencing of RT-PCR products confirmed their identity.

Fig. 2. GABA-B receptor subunits in GH3 cells. **a** RNA samples extracted from GH3 cells and rat pituitary were reverse transcribed and used for PCRs using specific primers for GABA-B receptors R1 and R2 subunits. Both subunits are expressed in GH3 cells and in whole rat pituitary (Pit). Control experiments without template were negative (not shown). Sequencing of RT-PCR products confirmed their identity. **b** Immunoelectron microscopy experiments localised the GABA-B R2 subunit protein at GH3 cell membrane (10-nm gold particles, arrow). Bar equivalent to 50 nm.

HPLC measurements were now performed in order to quantify GABA secretion by GH3 cells. The amount of GABA released (± SEM) after 24 h was 1.72 ± 0.54 nmol/10⁶ cells (3 series of experiments, n = 5).

GABA-B Receptors Are Involved in the Control of GH Production and Secretion by GH3 Cells

In order to examine the function of the GABA-B receptors present in GH3 cells, the cultures were treated with the GABA-B receptor antagonist phaclofen (pilot experiments were run in order to identify efficient phaclofen concentrations and the appropriate duration of treatment). The cellular and extracellular GH levels in the samples treated with phaclofen (10 or 100 μ*M* for 24 h) were compared with the ones of untreated controls. Cellular GH levels were analysed by SDS-PAGE and immunoblotting. The accuracy of the protein loading for the SDS-PAGE was controlled by Ponceau staining of the mem-

PCR and sequencing (fig. 2a). Furthermore, immunoelectron microscopy showed localisation of the R2 subunit at GH3 cell membranes (fig. 2b).

GABA Is Produced in GH3 Cells

Since GH3 cells have previously been shown to express functional glutamic acid decarboxylase (GAD) and the vesicular inhibitory amino acid transporter/vesicular γ-aminobutyric acid transporter (VIAAT/VGAT) [12],
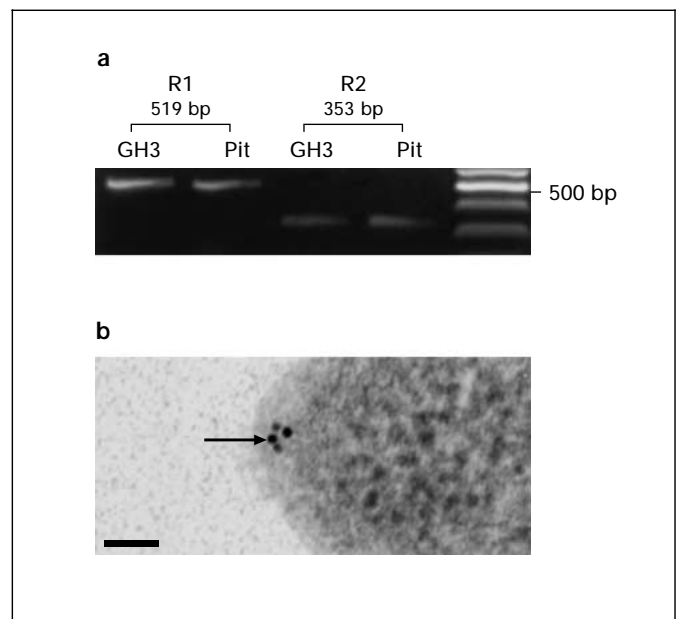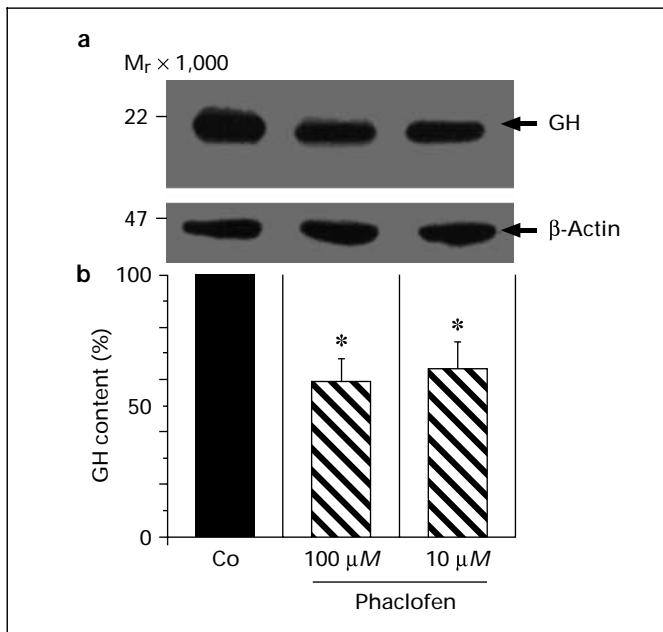
Fig. 3. Blockade of GABA-B receptors by phaclofen reduces GH production in GH3 cells. In order to block GABA-B receptors, GH3 cells were treated with phaclofen for 24 h. After treatment, proteins were extracted and Western blots were performed to determine GH content. **a** A representative experiment is shown. The blockade of GABA-B receptors resulted in a decrease of the GH content by phaclofen used at 10 or 100 μM (upper panel); β-actin levels detected in the same experiment were unchanged (lower panel), indicating equal loading of the gel. **b** A graphical representation of 3 independent experiments is depicted and shows a decrease in the GH content of about 40%. Results are expressed as percent of untreated controls (Co). Values are means + SEM. Differences between untreated and treated samples were evaluated using ANOVA and Student-Newman-Keuls tests; * p < 0.05.

Fig. 4. Activation of GABA-B receptors by baclofen stimulates GH production in GH3 cells. An inhibitor of GABA synthesis (100 μM of γ-acetylenic GABA) was added to the cell culture medium 2 h before the supplement of baclofen (24 h). After treatment, proteins were extracted and Western blots performed to analyse GH production. Four independent experiments were performed. **a** As shown in the upper panel, use of baclofen increased GH production. This was observed in 3 of 4 experiments (no change was detected in 1 experiment). β-Actin levels were unchanged (lower panel), indicating equal loading of the gel. **b** Activating GABA-B receptors led to a 29–47% increase of GH content. A graphical representation of 3 experiments showing similar results is given. GH contents are expressed as percent of untreated control samples (Co). Values are means + SEM. Differences between untreated and treated samples were evaluated using ANOVA and Student-Newman-Keuls tests; * p < 0.05.

branes. Blocking of GABA-B receptors resulted in decreased GH content compared with untreated control cells (n = 8). In some cases, GH values were normalised to levels of a reference protein, β-actin (n = 3). The values obtained after optical density analysis of GH levels revealed statistically significant decreases of about 40% (fig. 3).

GH secretion was also modified by phaclofen treatment, as shown by the results of the GH EIA. Supernatants of untreated cells of two different series of experiments contained 27.39 ± 3.01 ng GH (± SEM). Following the blockade of GABA-B receptors by phaclofen, GH levels were reduced by 60% (10.58 ± 0.75 ng GH; p = 0.0023).
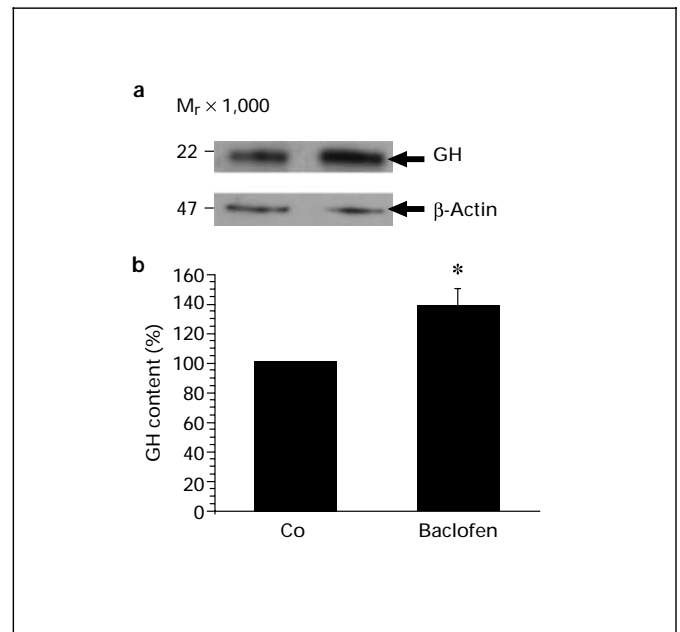
The samples used for the EIA and one additional series of samples were also analysed by HPLC in order to determine GABA levels. The average amount of GABA released (± SEM) by untreated GH3 cells was 1.72 ± 0.54 nmol/10^6 cells/24 h (n = 5). Like for GH, we observed that GABA levels in the medium were decreased by 20–80% in all samples treated with phaclofen (0.87 ± 0.56 nmol GABA; p = 0.0022).

In 3 different experiments, consisting each of 2 control samples and 2 samples treated with 100 μM of the GABA-B receptor agonist baclofen, the cellular levels of GH were analysed by SDS-PAGE and immunoblotting. The analysis showed that direct activation of GABA-B receptors increased GH levels. The accuracy of the protein loading for the SDS-PAGE was controlled by Ponceau staining of

Gamel-Didelon/Corsi/Pepeu/Jung/Gratzl/
Mayerhofer

the membranes and, in some cases, by immunoblotting for a reference protein, β-actin. The values obtained after optical density analysis of GH levels were expressed as percent of untreated control samples and showed statistically significant increases of GH levels, ranging from 29 to 47% (fig. 4). In order to avoid interference of endogenous GABA produced by GH3 cells, a blocker of GABA synthesis (100 μM of γ-acetylenic GABA) was added to the culture medium 2 h before addition of baclofen for 24 h.

Discussion

The results of our study show that GH/GH3 cells produce GABA and express GABA-B receptors. We describe that GABA, via GABA-B receptors, is involved in the autocrine control of the GH cell phenotype.

GABA plays a role in the physiological regulation of pituitary functions [1–7]. It is well established that GABA is one of several signaling molecules involved in the control of GH production [3, 7, 31, 32]. GABA-affecting hormone secretion by pituitary endocrine cells is produced in the hypothalamus [8–11], but, as shown recently, also within the pituitary itself, namely in POMC and in GH cells [12]. GH3 cells are somatotropic cells derived from a rat GH pituitary adenoma. They provide a well established, widely used model for the study of the regulation of GH and PRL production [19, 33]. Since our previous results showed that GH3 cells produce GABA, we used this cell line to investigate whether GABA derived from GH-producing cells may contribute to the regulation of GH synthesis and release. This mechanism of regulation may involve all three different types of GABA receptors, since evidence of their existence in the pituitary has been provided [12–18, 34].

In the current study, we focussed on GABA-B receptors. Their presence was recently confirmed at the molecular level in the anterior lobe of rat pituitaries and in cells derived from the mouse intermediate lobe [35, 36]. Results of the present study show that GABA-B receptors are also expressed in a particular cell type of the anterior lobe of the pituitary, the GH cell. Using laser microdissection [37–39] of rat pituitary GH cells followed by RT-PCR, we found that GH cells possess mRNA for GABA-B receptors. This result was confirmed by immunoelectron microscopy.

Likewise, GH3 adenoma cells express GABA-B receptor subunits, as shown in the present study, and were therefore used to examine GABA-B receptor-mediated events. To this end we blocked the action of GABA by treating GH3 cells with the GABA-B antagonist phaclofen. Phaclofen treatment led to a decreased GH content. A reduction in GH levels in the medium, and thus presumably in GH release, was also measured. Results were obtained using concentrations determined in pilot studies. We chose not to study dose-response relations, since they appear questionable in a system in which GABA is produced endogenously. Taken together, our results imply that levels of GH inside the cells and in the medium were decreasing in parallel. We therefore hypothesise that a role for GABA may be to maintain or increase GH production via GABA-B receptors.

To examine this possibility further, endogenous GABA synthesis was blocked by preventing the decarboxylation of L-glutamate, using γ-acetylenic GABA [21], before activation of GABA-B receptors by a GABA-B receptor agonist, baclofen. Indeed, baclofen increased GH content after 24 h of treatment, supporting our conclusion that GABA regulates GH via GABA-B receptors.

These results obtained in a cell culture system are in line with in vivo results showing an increase in GH secretion following baclofen administration in humans [40, 41], or following GABA treatment in newborn rats [3, 31]. Therefore our results from GH3 cell and the localisation of GABA-B receptors at the membrane of pituitary GH cells are probably of relevance to mammalian species, including humans, and highlight a novel aspect of GH cell regulation.

Previously, we reported that VIAAT and GAD are colocalised to secretory granules of GH cells [12], a result suggesting corelease. Indeed, in the present study we provide evidence for cosecretion of GH and GABA, using GH3 cells. Provided that pituitary GABA is released together with GH, this may imply that GABA exerts an autocrine positive feedback on GH cells. By acting on GABA-B receptors, it may thus stimulate both GH production as well as its own secretion.

GABA is mainly known for its synaptic role as a major inhibitory neurotransmitter of the central nervous system. That it can be produced and act in other, nonneuronal tissues is an emerging field. A role for GABA in endocrine tissues has been shown for the pancreas. There GABA is cosecreted with insulin from β-cells and involved in the inhibitory action of glucose on glucagon release by activating GABA-A receptors present in α-cells [42]. Our work in the pituitary and GH3 cells provides a new example for an endocrine role of GABA acting as a stimulatory autocrine regulator of GH production.

Acknowledgments

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