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γ-Aminobutyric acid (GABA)-C receptor stimulation increases prolactin (PRL) secretion in cultured rat anterior pituitary cells

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ARTICLE INFO

Article history:
Received 8 February 2006
Accepted 14 March 2006

Keywords:
GABA
GABA-C receptor
Prolactin
Pituitary

Abbreviations:
GABA, γ-aminobutyric acid
CACA, *cis*-aminocrotonic acid
PRL, prolactin
TPMPA, 1,2,5,
6-tetrahydropyridin-4-yl
methylphosphinic acid
GAD, glutamic acid decarboxylase

ABSTRACT

γ-Aminobutyric acid (GABA) reportedly inhibits secretion of anterior pituitary hormones by directly acting on GABA-A and GABA-B receptors on anterior pituitary cells, but the roles of GABA-C receptors are little known. In this study, involvement of GABA-C receptors in the secretion of prolactin (PRL) was examined using cultured rat anterior pituitary cells. GABA-C receptor agonist, *cis*-4-aminocrotonic acid (CACA, 0.1–1 mM) increased PRL secretion dose-dependently, while GABA-A receptor agonist, 100 μM muscimol, but not GABA-B receptor agonist, 100 μM baclofen, decreased the secretion. GABA-C receptor antagonist, 15 μM (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA), and GABA-A receptor antagonist, 100 μM bicuculline, not only reversed such an agonist-induced increase or decrease in PRL secretion, but also suppressed or enhanced spontaneous PRL secretion, raising a possibility of GABA-C or GABA-A receptor stimulation by intrinsic pituitary-derived GABA. GABA-C receptor subunits (ρ1, ρ2, ρ3) and GABA synthesizing enzymes (GAD 65 and GAD 67) were shown to be expressed as assayed by RT-PCR, and GABA-C receptor stimulation by CACA obviously increased intracellular Ca^{2+} concentration in the anterior pituitary cells. Thus, PRL secretion from anterior pituitary cells appears to be enhanced via direct GABA-C receptor stimulation by GABA originating from the anterior pituitary cells besides well-known hypothalamic GABA.

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1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, and activates three pharmacologically and structurally different classes of GABA receptors: GABA-A, GABA-B and GABA-C receptors [1]. GABA-A receptors are ligand gated Cl^- channels activated by muscimol and inhibited by bicuculline. GABA-B receptors are coupled to G proteins, and activated by baclofen and inhibited by saclofen. GABA-C receptors are ligand gated Cl^- channels like GABA-A receptors, and stimulated by *cis*-4-aminocrotonic acid (CACA) and antagonized by (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA). GABA-A and GABA-B receptors are widely distributed in central nervous system (CNS), while GABA-C receptors were believed to be exclusively expressed in retina. However, several studies have demonstrated that GABA-C receptors are also widely expressed in CNS such as cortex, thalamus, hippocampus, superior colliculus, cerebellum and spinal cord [2,3].

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doi:10.1016/j.bcp.2006.03.014

Accumulating evidence has demonstrated that GABA regulates or modulates the secretion of several anterior pituitary hormones through either altering the dopaminergic tone or directly acting on anterior pituitary cells [4–7]. GABA-A and GABA-B receptors are shown to exist in all the endocrine cell types in the anterior pituitary gland [8], and they transmit mainly inhibitory signals upon their stimulation. In contrast to the accumulating reports on GABA-A and GABA-B receptor stimulations [5–7], such information about GABA-C receptors is limited. In the present study, we examined the regulation of prolactin (PRL) secretion by direct stimulation of GABA-C receptors in cultured rat anterior pituitary cells.

2. Materials and methods

2.1. Animals and culture procedures

Male Wistar rats (180–200 g) were killed by decapitation and the pituitary glands were rapidly removed from animals. The anterior lobes were carefully dissected from the posterior and intermediate lobes on ice. The animal treatment and the experimental procedures were all based on the Guidelines for Animal Care and Use Committee at Kansai Medical University. The anterior lobes of the pituitary glands were enzymatically dispersed by the method of Watanabe et al. [9] with slight modifications. Briefly, the anterior pituitary cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin G potassium, 1 mg/mL streptomycin sulfate and 10% fetal calf serum. The cells were cultured in a humidified incubator at 37 °C in 5% CO_2 and 95% air for 7 days, and used for the following experiments. On day 7, the viability of the anterior pituitary cells on plates was almost 100% as judged by trypan blue cell exclusion method.

2.2. Prolactin secretion from the cultured anterior pituitary cells

After 7 days of culture, confluent cells (1.3–1.4 $\times$ 10^6 cells/well) were washed with an incubation medium (DMEM supplemented with 10 nM bromocriptine, 0.1% BSA and 20 mM HEPES, pH 7.3), and incubated further for 24 h in 1 mL of the same medium containing GABA, GABA receptor agonists (muscimol for GABA-A, baclofen for GABA-B, and CACA for GABA-C), or GABA receptor antagonists (bicuculline for GABA-A, saclofen for GABA-B and TPMPA for GABA-C). At the end of incubation, the medium was collected and stored at −20 °C. PRL concentrations in the medium were measured with enzyme-linked immunosorbent assay (ELISA).

2.3. Enzyme-linked immunosorbent assay

ELISA kit for rat PRL was purchased from Seikagakukogyo Co. (Tokyo, Japan) and used according to the instructions. The detection limit was 3.12 μg/L, and intra- and inter-assay coefficients of variations were both less than 10%.

2.4. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from rat anterior pituitary cells, retina and hippocampus using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco-BRL, Rockville, MD, USA), followed by extraction and precipitation with isopropyl alcohol. The amounts of RNA were quantitated using UV spectroscopy (absorption at 260 nm). Reverse transcription was performed with superscript III reverse transcriptase and polymerase chain reaction (PCR) was performed with high fidelity Taq DNA polymerase (Invitrogen, Grand Island, NY, USA). The sets of oligonucleotide primers for GABA-C ρ subunits and glutamic acid decarboxylase (GAD 65 and GAD 67) were designed to sandwich at least one intron to discriminate the products derived from mRNA and genomic DNA (Table 1).

2.5. Measurement of $[Ca^{2+}]_i$

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using calcium-sensitive fluorescent dye, fura 2-AM, as previously descried [10]. Briefly, cultured anterior pituitary cells

Table 1 – Oligonucleotide primers used for PCR

Name	Sequence	Position	Accession number
ρ1 forward	5′-tgaaatttggcatattcctt-3′	130–150	X95579
ρ1 reverse	5′-ctaagagaaaattgaccagta-3′	1554–1534	
ρ2 forward	5′-atgccttattttatgagactc-3′	169–189	D38494
ρ2 reverse	5′-ctaggaaaacactgaccaata-3′	1566–1546	
ρ3 forward	5′-atggtcctggctttttggttg-3′	196–216	D50671
ρ3 reverse	5′-tcacacatatataccccagta-3′	1590–1570	
GAD 65 forward	5′-tcttttctcctggtggtgcc-3′	787–806	M72422
GAD 65 reverse	5′-ccccaagcagcatccacat-3′	1177–1159	
GAD 67 forward	5′-tacggggttcgcacaggtc-3′	713–731	M76177
GAD 67 reverse	5′-ccccaggcagcatccacat-3′	1311–1293	

All primer pairs were designed to include at least one intron between them to discriminate the PCR products of mRNA from those of genomic DNA. We confirmed that each PCR product derived from mRNA for each peptide by digestion with restriction enzymes. As expected from the nucleic acid sequences of cDNAs, ρ1 PCR product (1425 bp) was digested with *Eco*RI to 739 and 686 bp, and with *Xho*I to 992, 265 and 168 bp; ρ2 (1398 bp) with *Xho*I to 947 and 451 bp, and with *Bgl*II to 733, 395 and 270 bp; ρ3 (1395 bp) with *Eco*RI to 677, 420 and 298 bp, and with *Xba*I to 742, 588 and 65 bp; GAD 65 (391 bp) with *Bgl*II to 361 and 30 bp, and with *Pst*I to 233 and 158 bp; GAD 67 (599 bp) with *Eco*RI to 542 and 57 bp, and with *Dde*I to 327 and 272 bp.

on glass coverslips were washed on culture day 7 and loaded with 2.5 μM fura 2-AM for 40 min in Krebs-HEPES solution (120 mM NaCl, 5.4 mM KCl, 1.5 mM $MgCl_2$, 1 mM $NaHPO_4$, 20 mM HEPES, 10 mM glucose; pH 7.2). GABA-C receptor agonist, CACA (200 μM), was administered with or without pretreatment of GABA-C receptor antagonist, TPMPA (15 μM) for 15 min. $[Ca^{2+}]_i$ were measured in an inverted microscope attached to a cooled CCD camera and fluorescence image analysis system (Argus Hisca Imaging System, Hamamatsu Photonics, Hamamatsu, Japan).

2.6. Statistical analyses

All data are expressed as the means ± S.E.M. The data were analyzed by one-way analysis of variance. Changes between the mean values with $P < 0.05$ were considered significant.

3. Results

3.1. Effects of exogenous GABA receptor agonists on PRL secretion from anterior pituitary cells

As shown in Fig. 1, exposure of cultured rat anterior pituitary cells to GABA-C receptor selective agonist, CACA (0.1–1 mM), dose dependently increased PRL secretion and reached a plateau, while GABA (100 μM) or GABA-A receptor selective agonist, muscimol (100 μM), decreased the secretion. GABA-B receptor selective agonist, baclofen (100 μM), did not show any significant effects on PRL secretion. Such an increase or a decrease in the secretion by CACA or muscimol was completely inhibited respectively by GABA-C receptor antagonist (TPMPA) or GABA-A receptor antagonist (bicuculline), suggesting that in contrast to the inhibitory effects of GABA-A receptor stimulation, GABA-C receptor stimulation enhances PRL secretion from the anterior pituitary cells.

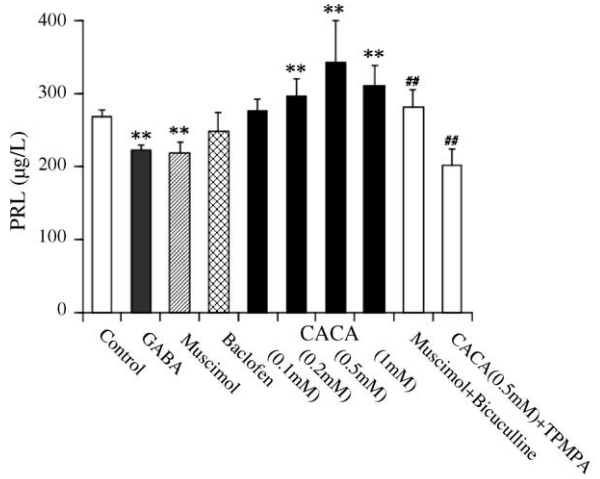

Fig. 1 – Effects of GABA receptor agonists on PRL secretion in cultured rat anterior pituitary cells. The anterior pituitary cells were incubated for 24 h in DMEM supplemented with 10 nM bromocriptine, 0.1% BSA and 20 mM HEPES, pH 7.3 with or without GABA, GABA receptor agonists (100 μM muscimol for GABA-A, 100 μM baclofen for GABA-B, and 0.1–1 mM CACA for GABA-C receptors), and GABA receptor antagonists (100 μM bicuculline for GABA-A and 15 μM TPMPA for GABA-C receptors). $^{**}P < 0.001$ compared with control. $^{\#\#}P < 0.001$ compared with agonist alone (n = 6–20). Each bar represents the mean ± S.E.M.

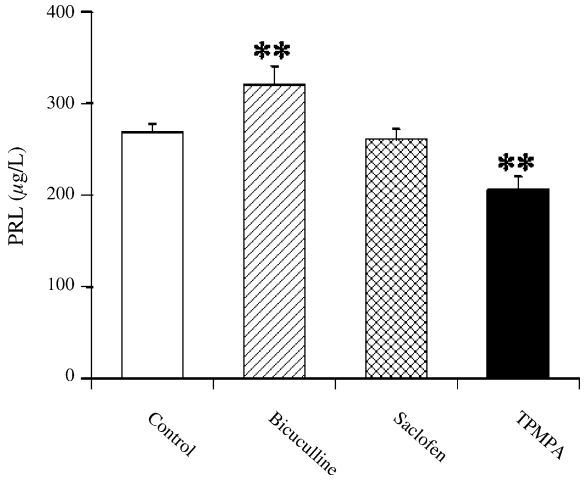

Fig. 2 – Effects of GABA receptor antagonists on spontaneous PRL secretion in cultured rat anterior pituitary cells. The anterior pituitary cells were incubated for 24 h in DMEM supplemented with 10 nM bromocriptine, 0.1% BSA and 20 mM HEPES, pH 7.3 with or without GABA receptor antagonists: bicuculline (100 μM) for GABA-A, saclofen (100 μM) for GABA-B and TPMPA (15 μM) for GABA-C receptors. $^{**}P < 0.001$ compared with control (n = 6–20). Each bar represents the mean ± S.E.M.

3.2. Effects of GABA receptor antagonists on spontaneous PRL secretion

Since, in the above-mentioned experiments, GABA-C and GABA-A receptor antagonists reversed the effects of exogenously administered agonists beyond the control level, spontaneous PRL secretion may be regulated by intrinsic GABA. To test this possibility, the effects of GABA receptor antagonists on the spontaneous (control) PRL secretions without exogenous GABA were examined. As shown in Fig. 2, GABA-C receptor antagonist, TPMPA (15 μM), significantly decreased spontaneous PRL secretion, while GABA-A receptor antagonist, bicuculline (100 μM), increased the secretion, and GABA-B receptor antagonist, saclofen (100 μM), showed no effects. Thus, spontaneous PRL secretion also appeared to be stimulated or inhibited respectively by GABA-C or GABA-A receptor stimulation probably by pituitary cell-derived GABA.

3.3. mRNA expression of GABA-C ρ subunits and GABA synthesizing enzymes in anterior pituitary cells

To test whether the cultured cells express GABA-C receptors and are able to synthesize GABA, mRNAs of GABA-C receptor subunits ρ1, ρ2 and ρ3, and GABA synthesizing enzymes, glutamic acid decarboxylase (GAD) 65 and GAD 67, were examined using RT-PCR technique. As shown in Fig. 3a, all the

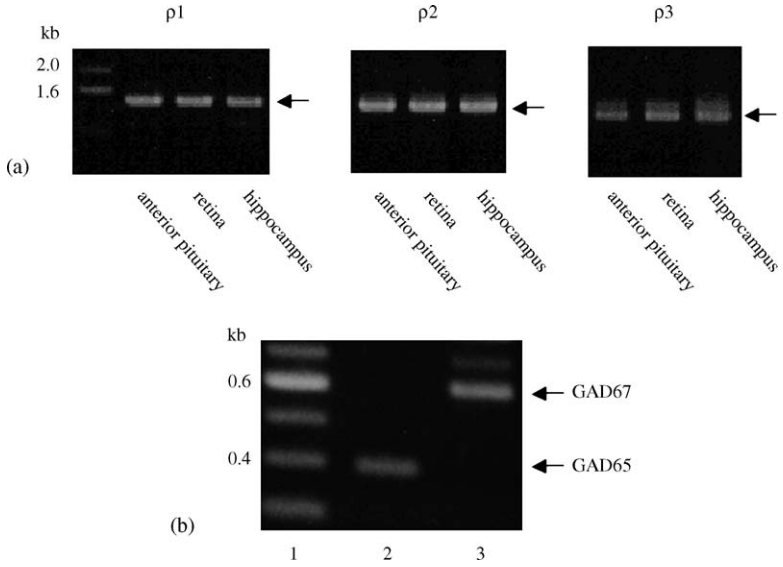

Fig. 3 – Expression of mRNAs for GABA-C receptors and GABA synthesizing enzymes (GAD 65 and GAD 67) in cultured rat anterior pituitary cells. (a) mRNA expression of GABA-C receptor ρ subunits in cultured rat anterior pituitary cells, retina and hippocampus. RNA samples were reverse transcribed and used for PCR using primer sets for full length of ρ1, ρ2 and ρ3 subunits of GABA-C receptors. (b) mRNA expression of GABA synthesizing enzymes (GAD 65 and GAD 67) in cultured rat anterior pituitary cells. RNA samples were reverse transcribed and used for PCR using primer sets for GAD 65 and GAD 67. Lane 1: 100 bp DNA ladder. Lane 2: GAD 65. Lane 3: GAD 67.

GABA-C ρ subunits were detected in the cultured rat anterior pituitary cells as products for full length of the coding regions of ρ1, ρ2 and ρ3 subunits. Sizes of the PCR products were the same as those in the rat retina and hippocampus. Expression of GABA synthesizing enzymes, GAD 65 and GAD 67, was also demonstrated (Fig. 3b), suggesting that anterior pituitary cells synthesize and probably spontaneously release GABA.

3.4. $[Ca^{2+}]_i$ response to GABA-C receptor stimulation

Since PRL release is regulated by intracellular Ca^{2+} signaling [11], $[Ca^{2+}]_i$ response to GABA-C receptor stimulation was examined. As shown in Fig. 4, GABA-C receptor agonist, CACA (200 μM), induced $[Ca^{2+}]_i$ transient within 15 s after the administration, and $[Ca^{2+}]_i$ reached around 200 nM within 1 min. Pretreatment of the cells with GABA-C receptor antagonist, TPMPA (15 μM), significantly decreased the response, showing that GABA-C receptor functioned to increase $[Ca^{2+}]_i$.

4. Discussion

The PRL secretion in mammals is tonically inhibited by dopamine and stimulated by PRL releasing factors such as thyrotropin releasing hormone and oxytocin [12]. Although GABA reportedly inhibits PRL secretion in rat cultured anterior pituitary cells [13], intracerebroventricularly administered GABA stimulates the secretion [14]. Such discrepancy can be explained as follows: centrally administered GABA inhibits

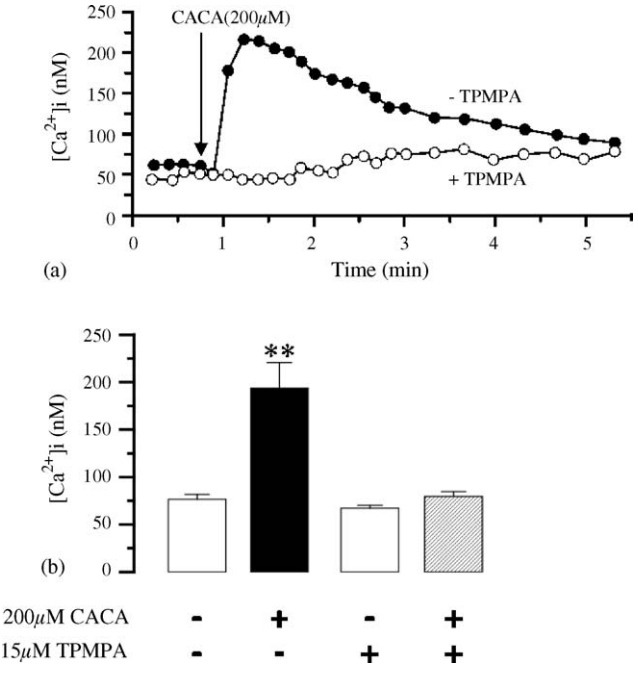

Fig. 4 – $[Ca^{2+}]_i$ response to GABA-C receptor stimulation in rat anterior pituitary cells. (a) Typical recording of changes in $[Ca^{2+}]_i$ by CACA (200 μM) with or without pretreatment with GABA-C receptor antagonist, TPMPA (15 μM). (b) Summaries of changes in $[Ca^{2+}]_i$ induced by CACA (200 μM) with or without TPMPA pretreatment. Each bar represents the mean ± S.E.M. for 27 (without TPMPA) and 29 (with TPMPA) cells. $^{**}P < 0.001$ compared with pre-application of CACA.

tuberoinfundibular dopaminergic activity leading to the increase in PRL secretion. GABA directly administered to anterior pituitary cells may decrease PRL secretion via different GABA receptors with different pharmacological properties [15]. In this study, using specific agonists and antagonists for GABA receptor subtypes, we demonstrated the direct effects of GABA on PRL secretion via different types of GABA receptors on anterior pituitary cells, especially focusing on GABA-C receptors.

We confirmed the presence of GABA-C receptors in cultured anterior pituitary cells. The mRNAs of all GABA-C receptor subunits (ρ1, ρ2 and ρ3) were expressed and the size of each PCR product was the same as in retina and hippocampus. Boue-Grabot et al. reported that mRNAs of GABA-C receptor subunits, ρ1 and ρ2, in the anterior pituitary were shorter in size than those expressed in the retina using Northern blot analysis [16]. Since we used a set of primers, which produced a full length of the coding region of each subunit, the discrepancy may be explained by alternative polyadenylation which may alter the size of mRNA on Northern blotting. Besides Northern blot analysis, the existence of GABA-C receptors in the anterior pituitary cells has been demonstrated by RT-PCR, patch clamp, or immunohistochemistry techniques in rat and guinea pig TSH secreting cells, rat GH secreting cells and GH adenoma cell line, GH3 [16,17]. They also showed that the application of GABA or CACA led to increased $[Ca^{2+}]_i$ in GH3 cells. However, neither study demonstrated the changes in the hormone secretion via GABA-C receptor stimulation. The present study first showed that GABA-C receptor specific agonist, CACA, increased PRL secretion from cultured rat anterior pituitary cells, and that GABA-C receptor specific antagonist, TPMPA, blocked this response, suggesting that stimulation of GABA-C receptors facilitates PRL secretion from anterior pituitary cells. This is also supported by the finding that CACA increased $[Ca^{2+}]_i$ in anterior pituitary cells because PRL secretion is well known to require $[Ca^{2+}]_i$ increase [11].

There are several reports on the regulation of PRL secretion via GABA-A and GABA-B receptors. GABA inhibited PRL secretion in vitro, the inhibition being reversed by GABA-A receptor antagonists, bicuculline and picrotoxin, and further GABA-A receptor agonist, muscimol, decreased PRL secretion [13,18]. In agreement with these reports, the present data showed bicuculline-sensitive muscimol-induced inhibition of PRL release. On the other hand, in contrast to the previous report showing GABA-B receptor activation-induced decrease in vitro PRL secretion [6], GABA-B receptor agonist, baclofen, did not show any significant effects on the secretion in the present study. This discrepancy may be due to the different experimental conditions: we used cultured anterior pituitary cells from Wistar male rats, while they used those from female proestrus rats.

GABA-C receptor specific antagonist, TPMPA, decreased not only CACA-induced PRL secretion, but also spontaneous (control) secretion, which prompted us to examine the possibility whether, besides hypothalamic GABA, anterior pituitary-derived GABA may be involved in GABA-mediated regulation of PRL secretion. GABA in the anterior pituitary has been assumed to originate from GABAergic hypothalamic neurons, which secrete GABA into portal blood [19]. However, there are some reports suggesting that GABA is produced and secreted in the anterior pituitary, as an autocrine and/or paracrine hormone [8,20]. We confirmed the expressions of GABA synthesizing enzymes, GAD 65 and GAD 67, in the cultured rat anterior pituitary cells. Our findings that GABA-A receptor antagonist increased and GABA-C receptor antagonist decreased PRL secretion suggest that pituitary-derived GABA suppresses and facilitates PRL secretion via GABA-A and GABA-C receptors, respectively, in an autocrine/paracrine manner.

PRL secretion is mainly under the inhibitory control by dopamine, and the factors to stimulate PRL secretion at the pituitary level are limited. The present study first demonstrated that GABA which was believed to inhibit PRL secretion could enhance it via GABA-C receptor stimulation. It is well known that GABA-C receptors are ten times more sensitive to GABA than GABA-A receptors and that the desensitization to agonist binding is less than GABA-A receptors [15]. Therefore, it is likely that certain levels of intrinsic GABA activates mainly GABA-C receptors and facilitates PRL secretion from the anterior pituitary cells.

In conclusion, GABA originating from the anterior pituitary cells appears to enhance PRL secretion via GABA-C receptors, while it suppresses the secretion via GABA-A receptors.

Acknowledgements

This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Japanese Private School Promotion Foundation; and Salt Science Research Foundation, Japan. We thank Kansai Medical University Laboratory Center for providing experimental instruments.

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