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GABA Inhibition of Immortalized Gonadotropin-Releasing Hormone Neuronal Excitability Involves $GABA_A$ Receptors Negatively Coupled to Cyclic Adenosine Monophosphate Formation

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γ-Aminobutyric acid (GABA) has been implicated in the regulation of reproduction, particularly in the developmental modulation of gonadotropin-releasing hormone (GnRH) secretion. GnRH neurons are innervated by GABA-containing processes, and the administration of GABA stimulates and inhibits GnRH secretion in vivo and in vitro. We have previously shown that GABA can exert both of these actions in sequence, by acting directly on immortalized GnRH neurons. While the stimulation is the result of a $GABA_A$ receptor-mediated depolarization of the plasma membrane, the mechanism involved in the delayed inhibition is the subject of the present investigation. GABA (1 n*M*–10 µ*M*) decreased the intracellular concentration of cyclic adenosine monophosphate (cAMP) in a dose- and time-dependent fashion. This effect was blocked by bicuculline and mimicked by muscimol but not by baclofen. To analyze the effect of GABA on cellular excitability, we used fura-2 loaded GT1-7 cells. Activation of voltage-sensitive calcium channels by high K^+-induced depolarization (35 m*M*) increased $[Ca^{2+}]_i$. GABA (10 µ*M*) and muscimol (10 µ*M*) reduced the amplitude of K^+-induced $[Ca^{2+}]_i$ transients. This inhibition was blocked by forskolin (20 µ*M*) or 8-Br-cAMP (1 m*M*). Altogether, these results show that $GABA_A$ receptors mediate a sustained inhibitory effect of GABA on GnRH neurons, and suggest the involvement of the cAMP pathway decreasing cellular excitability.

Key Words: GABA; $GABA_A$-receptors; gonadotropin-releasing hormone; GT1-7 cells; cyclic adenosine monophosphate.

Introduction

The neuropeptide gonadotropin-releasing hormone (GnRH) is at the top of the endocrine axis that controls reproductive function and is released into the hypophyseal portal circulation in a pulsatile fashion. This episodic pattern is necessary for the normal operation of the axis, such as the secretion of preovulatory luteinizing hormone surges and ovulation *(1)*. A variety of experimental approaches have made clear that the synchronous release of GnRH is under the regulatory control of stimulatory and inhibitory neuronal inputs *(2)*. Stimulation and inhibition of synchronous release of GnRH can be induced through various neurotransmitter systems, including γ-aminobutyric acid (GABA).

Morphologic studies in rodents have revealed that GnRH neurons are innervated by GABAergic terminals. Synaptic contacts between GABA-containing processes and GnRH neurons in the medial preoptic area and median eminence of the rat *(3,4)* and expression of three classes ($\alpha_{1/2}$, β_3 and γ_2) of $GABA_A$ receptor subunits in rat GnRH neurons were demonstrated by double-label immunohistochemistry and *in situ* hybridization *(5,6)*. Pharmacological manipulation of GABA-ergic neurons has led to the conclusion that GABA innervation is able to mediate both stimulatory and inhibitory actions on GnRH secretion. GABA stimulates GnRH release in embryonic *(7)* and neonatal *(8)* hypothalamus, although is generally considered inhibitory to hypothalamic GnRH release in adult rats *(9–11)*. $GABA_A$-receptor activation results in two different responses, depending on the developmental stage of the animals. While prepubertal rats respond with an increase in GnRH output, adult animals respond with a reduction in the release of this hormone *(8)*. In contrast to these actions in rodents, a GABAergic inhibitory tone is particularly important as a major restraining influence on GnRH release in prepubertal rhesus monkeys *(12)*, where direct synaptic contacts between GABA and GnRH neurons have not been detected *(13)*.

Experiments with immortalized GnRH neurons of the GT1 lineage, which release GnRH in pulses *(14–16)* at a frequency quite similar to that observed in perfused rat hypothalamic explants *(17,18)*, demonstrated that GABA

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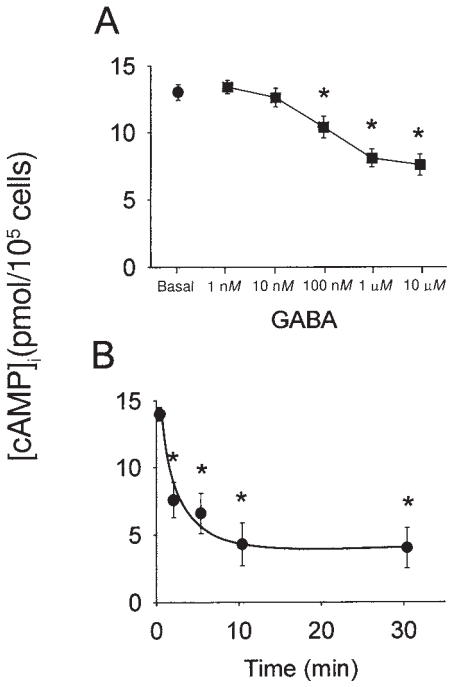

Fig. 1. Effect of GABA on cAMP content in GT1-7 cells. (**A**) Dose-response relationship for GABA-induced inhibition of cAMP accumulation in 30 min in GT1-7 cells, incubated for 90 min in the presence of 1 m*M* IBMX; (**B**) time course of GABA-induced (10 μ*M*) inhibition of cAMP formation. Values represent the mean ± SEM of six independent determinations. *$p < 0.05$ vs basal value before the administration of GABA.

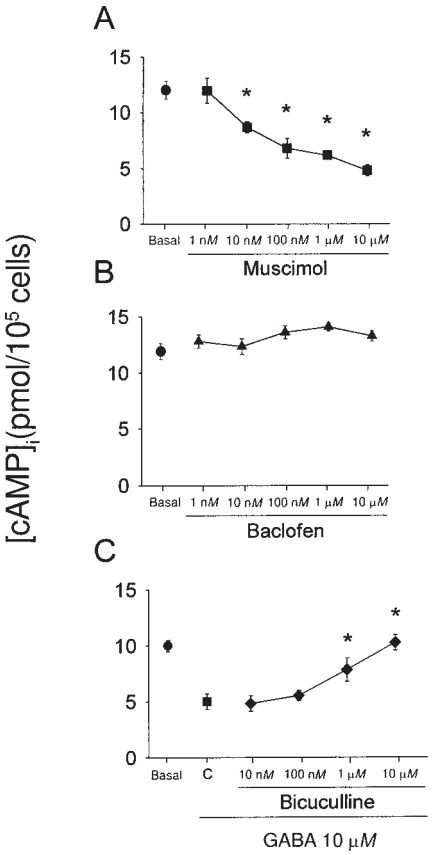

Fig. 2. Effect of muscimol, baclofen, and GABA alone or together with bicuculline on cAMP content in GT1-7 cells. GT1-7 cells (incubated for 90 min in the presence of 1 m*M* IBMX) were challenged for 30 min with increasing concentrations of the GABA$_A$-receptor agonist muscimol (**A**), the GABA$_B$-receptor agonist baclofen (**B**), or with a single concentration of GABA (10 μ*M*) alone or in combination with increasing concentrations of the GABA$_A$-receptor antagonist bicuculline (**C**). Values represent the mean ± SEM of six independent determinations. *$p < 0.05$ vs basal value before the administration of GABA.

can exert both stimulatory and inhibitory actions by acting directly on GnRH neurons *(19,20)*. GT1 neurons express functional GABA$_A$ receptors composed only by α- and β-subunits *(19,21)*, whose activation rapidly leads to chloride efflux, membrane depolarization, increased intracellular calcium concentration ([Ca^{2+}]$_i$), and a single episode of GnRH secretion *(19–22)*. This evanescent stimulatory action of GABA is followed by a long-lasting decrease of GnRH secretion and [Ca^{2+}]$_i$ to levels below basal values and without their characteristic spontaneous oscillations *(20,23)*.

In the present study we analyzed the nature of the signaling pathways involved in the delayed inhibitory effect of GABA. In particular, we addressed the effect of GABA on the cyclic adenosine monophosphate (cAMP) pathway and on cellular excitability. Our results demonstrate that GABA inhibits the formation of cAMP and the excitability of GT1-7 cells via the activation of GABA$_A$ receptors and suggest that inhibition of this signaling pathway may be responsible for the inhibitory action of GABA on the excitability of these cells.

Results

GABA Inhibition of cAMP Accumulation in GT1–7 Cells

The administration of GABA decreased the accumulation of cAMP in GT1-7 cells in a dose- and time-dependent manner (Fig. 1). Incubation of GT1-7 cells for 30 min with increasing concentrations of GABA in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1 m*M*), resulted in statistically significant inhibitions of the intracellular concentration of cAMP in response to 100 n*M*, 1 μ*M*, and 10 μ*M* GABA (Fig. 1A). cAMP levels gradually decreased in response to 10 μ*M* GABA, becoming statistically lower than the basal value in only 2 min and reaching a minimum after 10 min of treatment (Fig. 1B). Treatment of GT1-7 cells with the selective GABA$_A$-receptor agonist muscimol, but not with the GABA$_B$-receptor agonist baclofen, mimicked the dose-response inhibition of intracellular cAMP accumulation induced by GABA (Fig. 2A,B). Figure 2C shows that the inhibition of cAMP accumulation induced by 10 μ*M* GABA was totally reversed by the coadministration of equimolar concentrations of the selective GABA$_A$-receptor antagonist bicuculline. A lower concentration of bicuculline (1 μ*M*) produced a partial antagonism of the inhibitory effect of GABA on cAMP formation.

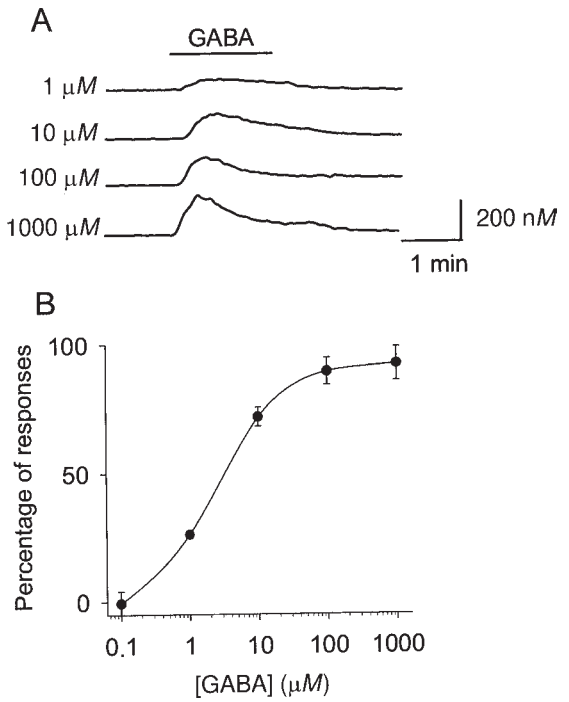

Fig. 3. Dose-response relationship for GABA-evoked $[Ca^{2+}]_i$ transients in GT1-7 cells. (**A**) Traces showing $[Ca^{2+}]_i$ transients evoked by 1, 10, 100, and 1000 μ*M* in fura-2-loaded GT1-7 cell populations; (**B**) dose-response curve for the GABA-evoked $[Ca^{2+}]_i$ transients. Data were collected in seven independent recordings. The $[Ca^{2+}]_i$ transient evoked by 1000 μ*M* GABA was normalized to 100%. The curve is the best fit of data to the logistic equation $Y = E_{max}/[1 + (EC_{50}/C)^n]$ in which *C* is the concentration of GABA, and *Y* is the fraction of the maximum value; EC_{50} was 2.9 μ*M*. The Hill coefficient (*n*) was assumed to be 1.

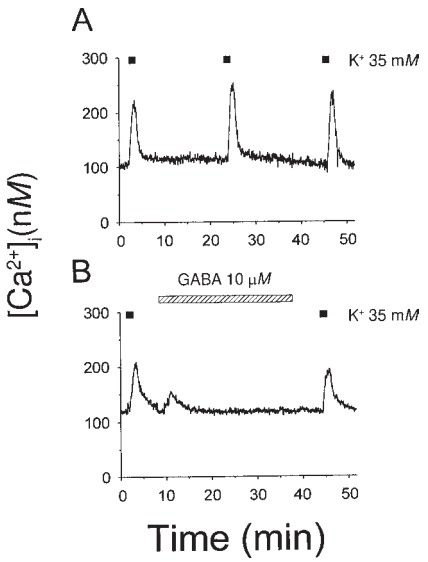

Fig. 4. Effect of 35 m*M* $[K^+]_o$ and GABA on $[Ca^{2+}]_i$ in GT1-7 cells. $[Ca^{2+}]_i$ was measured in fura-2-loaded, cover slip–attached GT1-7 cell populations by spectrofluoromety. The horizontal bars above each trace indicate the time period during which external conditions were changed. (**A**) Effect of repeated exogenous challenges (120 s) with 35 m*M* $[K^+]_o$; (**B**) effect of long-term (25 min) administration of GABA (10 μ*M*). Traces represent typical responses obtained in at least 10 independent experiments performed for each condition.

GABA Inhibition of K$^+$-Induced $[Ca^{2+}]_i$ Transients in GT1-7 Cells

By using fura-2 fluorometric recordings, we determined the effects of GABA on the resting level of $[Ca^{2+}]_i$ and on the amplitude of K^+-induced $[Ca^{2+}]_i$ transients in populations of GT1-7 cells. Under the present experimental conditions, GT1-7 cells showed stable basal $[Ca^{2+}]_i$ (108 ± 10.3 n*M*; *n* = 45). The administration of GABA resulted in concentration-dependent $[Ca^{2+}]_i$ transient responses (Fig. 3). We examined seven cultures to generate a dose-response curve for GABA. None of the cultures responded to 0.1 μ*M* GABA. In all of them, 1 μ*M* GABA infused for 120 s induced a measurable rapid and transient $[Ca^{2+}]_i$ response. At concentrations of 10–100 μ*M*, GABA reliably evoked responses of progressively higher amplitude. The effect induced by 1000 μ*M* was similar to that induced by 100 μ*M* GABA. The concentration at which GABA evoked a half-maximal response (EC_{50}) was 4.5 μ*M* (Fig. 3B). These responses were blocked by the $GABA_A$-receptor antagonist picrotoxin (data not shown).

A typical series of $[Ca^{2+}]_i$ responses to repeated (20 min apart) short-term (120 s) infusions of high K^+ concentrations (35 m*M*) are shown in Fig. 4. These responses were highly reproducible within each culture (Fig. 4A), reaching an average amplitude of 210 ± 23 n*M* (*n* = 10). The sustained infusion of GABA (10 μ*M*) for 30 min induced a rapid and transient rise in $[Ca^{2+}]_i$ (Fig. 4B). $[Ca^{2+}]_i$ rapidly increased to peak at average values of 150 ± 15 n*M* and then decreased to return to basal levels between 5 and 8 min after the onset of GABA infusion. The administration of high K^+, 5 min after the suspension of GABA infusion, produced $[Ca^{2+}]_i$ responses of similar amplitude to those induced by K^+ just before the infusion of GABA.

However, $[Ca^{2+}]_i$ responses triggered by high K^+ were substantially and reversibly affected during long-term infusions of GABA (Fig. 5A). The amplitude of the K^+-induced $[Ca^{2+}]_i$ transient responses during GABA infusion reached a peak value roughly one-half of the value reached just before the infusion of GABA. The amplitude of the transient responses triggered by K^+ infused right after the end of the infusion of GABA showed a partial recovery. Figure 6A shows the average amplitude of 10 $[Ca^{2+}]_i$ responses triggered by K^+ infused before (1st column) and during (15 min after the onset, 2nd column) the administration of GABA. GABA decreased the amplitude of the K^+-induced $[Ca^{2+}]_i$ transients to 157 ± 15 n*M* (i.e., 50% lower than the corresponding controls). Coadministration of the $GABA_A$-receptor antagonist picrotoxin (100 μ*M*) completely blocked both of the effects of GABA (10 μ*M*), that is, the rapid and transient stimulation of $[Ca^{2+}]_i$ and the inhibition of the amplitude of K^+-triggered $[Ca^{2+}]_i$ transients (Fig. 5B).

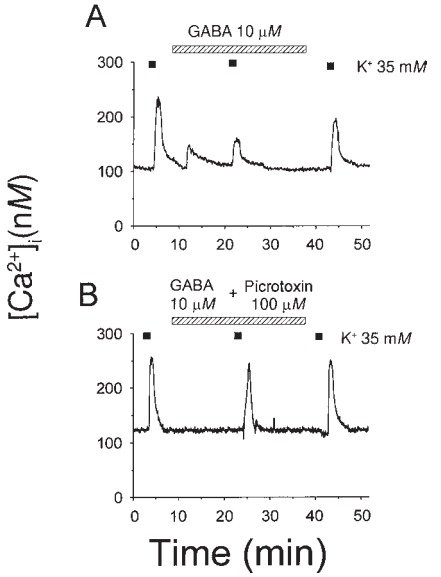

Fig. 5. Effect of GABA or GABA plus picrotoxin on K^+-evoked $[Ca^{2+}]_i$ transients in GT1-7 cells. (**A**) Effect of GABA (10 μ*M*) on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$; (**B**) effect of the combined administration of 10 μ*M* GABA with the $GABA_A$-receptor antagonist picrotoxin (100 μ*M*) on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$. Traces represent typical responses obtained in 10 independent experiments performed for each condition.

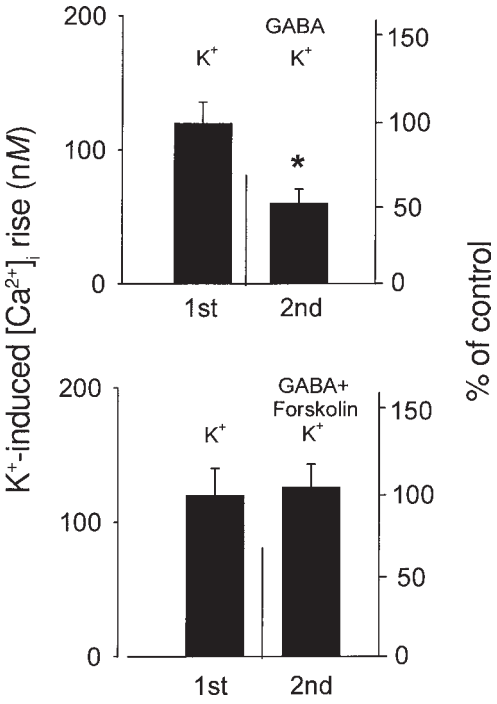

Fig. 6. Summary of the effects of GABA alone or in combination with forskolin on K^+-evoked $[Ca^{2+}]_i$ rises in GT1-7 cells. GABA inhibited the $[Ca^{2+}]_i$ rises evoked by K^+ to about 50% of control (**A**). Coadministration of forskolin (20 μ*M*) reverted the effect of GABA (**B**). 1st, K^+-evoked $[Ca^{2+}]_i$ rise before the administration of GABA or GABA plus forskolin; 2nd, K^+-evoked $[Ca^{2+}]_i$ rises during GABA or GABA plus forskolin. *$p < 0.05$ vs 1st value.

Figure 7 shows typical traces of K^+-induced $[Ca^{2+}]_i$ responses in GT1-7 cell populations infused with muscimol or baclofen. Infusion of GT1-7 cell populations with the selective $GABA_A$-receptor agonist muscimol (10 μ*M*) mimicked both of the effects elicited by GABA, that is, a rapid and transient stimulation of $[Ca^{2+}]_i$, followed by the inhibition of $[Ca^{2+}]_i$ transients triggered by high concentrations of K^+ (Fig. 7A). By contrast, the infusion of GT1-7 cell populations with the selective $GABA_B$-receptor agonist baclofen (100 μ*M*) did not affect the amplitude of K^+-triggered $[Ca^{2+}]_i$ transients nor induce any early deflection on the basal level of $[Ca^{2+}]_i$ (Fig. 7B).

Blockade of GABA Inhibition on K$^+$-Induced [Ca^{2+}]$_i$ Transients by cAMP

Co-infusion of GT1-7 cell populations with GABA (10 μ*M*), plus the direct-activator of adenylyl cyclase forskolin (20 μ*M*), resulted in the blockade of the inhibitory response of GABA on the amplitude of K^+-triggered $[Ca^{2+}]_i$ transients (Figs. 6 and 8A). The infusion of forskolin had no effect on the rapid stimulatory action of GABA on $[Ca^{2+}]_i$ (Fig. 8A). As shown in Figs. 6B and 8A, the amplitude of K^+-induced $[Ca^{2+}]_i$ transients was not modified in 14 GT1-7 cell cultures coinfused simultaneously with GABA and forskolin. A similar result was obtained with the cAMP analog 8-Br-cAMP. Coinfusion of GT1-7 cell populations with 8-Br-cAMP (1 m*M*) prevented the inhibition of K^+-triggered $[Ca^{2+}]_i$ transients induced by GABA (10 μ*M*), while leaving the rapid and transient stimulatory action of GABA on $[Ca^{2+}]_i$ unaffected (Fig. 8B).

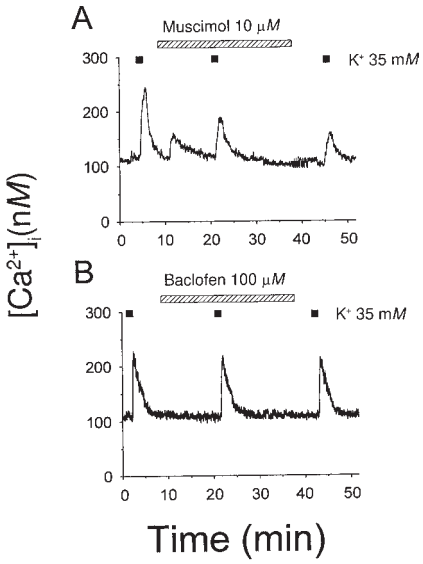

Fig. 7. Effect of muscimol or baclofen on K^+-evoked $[Ca^{2+}]_i$ rises in GT1-7 cells. (**A**) Effect of administration of the $GABA_A$-receptor agonist muscimol (10 μ*M*) on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$; (**B**) effect of administration of the $GABA_B$-receptor agonist baclofen (100 μ*M*) on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$. Traces represent typical responses in five independent experiments performed for each condition.

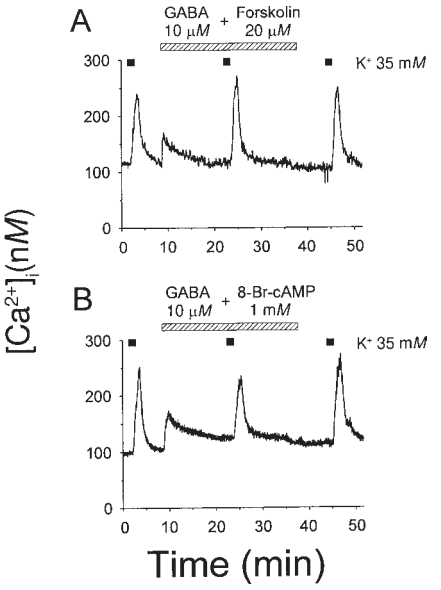

Fig. 8. Effect of GABA in combination with a cAMP analog or an activator of adenylyl cyclase on K^+-evoked $[Ca^{2+}]_i$ rises in GT1-7 cells. (**A**) Effect of the combined administration of 10 μ*M* GABA with 20 μ*M* forskolin on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$; (**B**) effect of the combined administration of 10 μ*M* GABA with 1 m*M* 8-Br-cAMP on $[Ca^{2+}]_i$ transients evoked by 35 m*M* $[K^+]_o$. The traces represent typical responses obtained in 10 independent experiments performed for each condition.

Discussion

In the present study, with the use of fura-2 fluorometric recordings in monolayer networks of immortalized GnRH neurons of the GT1-7 lineage, we demonstrated that GABA inhibited neuronal excitability and that this effect was secondary to the inhibition of the adenylyl cyclase/cAMP pathway. Our results also show that both of these effects of GABA are triggered by the activation of $GABA_A$ receptors.

GABA has the ability to stimulate a rapid and transient episode of GnRH secretion in GT1 neurons *(20)*. This finding suggested that GT1 cells maintained elevated levels of intracellular chloride, which could be the result of a chloride intrusion mechanism, such as the one mediated by the sodium-potassium-(2)-chloride cotransporter *(24,25)*, or the lack of an active chloride extrusion mechanism, such as the electroneutral potassium-chloride cotransporter *(26, 27)*, or the inwardly rectifying chloride channel *(28,29)*, all of which would result in a chloride reversal potential above the membrane potential. Consistent with this interpretation is the finding that GABA triggers the efflux of chloride ions, the depolarization of the membrane, and a transient increase in $[Ca^{2+}]_i$ *(19,21,22)*. The level of expression of these chloride-intruding and -extruding molecules in GT1 cells remains to be determined.

The evanescent (lasting for only 10–15 min) increase in $[Ca^{2+}]_i$, observed here in the recorded population of immortalized GnRH neurons in response to GABA and muscimol, is consistent with previous reports in single GT1-7 cell recordings *(19)* and GT1-7 cell populations *(30)*. It is possible that this initial $[Ca^{2+}]_i$ transient triggers the inhibition of adenylyl cyclase, leading to the decreased cell excitability observed 15 min after the onset of GABA infusion. The regulation of adenylyl cyclase by Ca^{2+} provides a confluence of two major signaling pathways with profound physiological significance. For instance, Ca^{2+} inhibition of adenylyl cyclase has been proposed to contribute to oscillations and pacemaking in cardiac tissue *(31)* and the maintenance of endothelial cell permeability *(32)*. Calcium has been recognized as playing a critical regulatory role in modulating adenylyl cyclase activity, since two of the nine distinct mammalian adenylyl cyclases that have been cloned to date *(31,33)*, namely types V and VI, are directly inhibited by submicromolar concentrations of calcium *(31,34)*. Adenylyl cyclase type VI is widely expressed in the central nervous system *(35)*. Moreover, the expression of adenylyl cyclase type V was recently reported in GT1-1 and GT1-7 cells *(36)*, providing the basis for a putative $GABA_A$-triggered ionotropic regulation of cAMP formation. In spite of this attractive possibility, the actual mechanism by which $GABA_A$ receptors trigger the inhibition of adenylyl cyclase remains to be determined.

We previously reported that baclofen inhibited the amplitude of the pulsatile release of GnRH in GT1-1 cells, without affecting the basal levels of secretion *(20)*. Expression of low levels of $GABA_B$ receptors was previously shown by the binding of ^{3}H-baclofen to synaptosomal membranes from GT1-7 cells *(37)*. Because $GABA_B$ receptors usually are negatively coupled to adenylyl cyclase, in this study we tested the effect of GABA on cAMP formation. Our results show a robust inhibitory effect of GABA on the accumulation of cAMP in GT1-7 cells that reached a minimum value 10 min after the onset of GABA and lasted for at least 30 min, without any sign of desensitization. These experiments were performed in the presence of an inhibitor of phosphodiesterases (IBMX), suggesting that GABA inhibited the synthesis of cAMP rather than increased the degradation of the cyclic nucleotide. The inhibitory effect of GABA on cAMP accumulation was blocked in a dose-dependent fashion by the antagonist bicuculline, suggesting that this effect was mediated by the activation of $GABA_A$ receptors. This is consistent with the observation that the $GABA_A$-receptor agonist muscimol dose and time dependently decrease the intracellular content of cAMP in GT1-7 cells. GABA and muscimol also decrease the formation of cAMP in GT1-7 and GT1-1 cells, stimulated by dopamine, norepinephrine, and forskolin, suggesting a robust inhibitory effect of $GABA_A$ receptors on the activity of adenylyl cyclase (unpublished observations).

However, baclofen did not affect the formation of cAMP, nor did it affect basal or K^+-triggered $[Ca^{2+}]_i$ transients. Thus, in accordance with Hales et al. *(19)* and Spergel et al. *(30)*, our present results show that $GABA_B$ receptors are not coupled to calcium metabolism in GT1 cells. Altogether,

these observations suggest that the inhibitory effects of baclofen on pulsatile GnRH secretion reported in GT1-1 cells *(20)*, on K^+-triggered GnRH secretion in GT1-7 cells *(37)*, on GnRH secretion from hypothalamic fragments *(38)*, and on GnRH gene expression *(39)*, do not involve the inhibition of the cAMP pathway nor the modulation of calcium signaling. Even though our study was not aimed at resolving this issue, the present results appear to be inconsistent also with an increased potassium conductance or a decreased calcium influx, the other actions attributed to $GABA_B$ receptor stimulation *(40)*. Thus, the nature of the signaling pathway coupling the effects of baclofen on GnRH neurons remains to be determined.

One of the interesting findings in our experiments is that activation of adenylyl cyclase can block the inhibitory action of GABA on K^+-triggered $[Ca^{2+}]_i$ transients, suggesting that the inhibitory effect of GABA on GT1-7 cell excitability could be the consequence of decreased intracellular cAMP concentrations. Accordingly, blockade was also observed in cells treated with exogenous cAMP analogs. The elevation of cAMP levels in GT1 cells is clearly associated with cell depolarization and GnRH secretion. Increasing cAMP levels by administration of cAMP analogs, by activation of adenylyl–cyclase coupled receptors, and by direct activation of adenylyl cyclase, all result in facilitation of GnRH secretion *(41–43)*. Although it is generally accepted that cAMP functions via activation of cAMP-dependent protein kinase (protein kinase A [PKA]), it is also known that cAMP has a direct effect on cellular function independent of PKA activation *(44)*. GT1 cells were recently shown to express functional cyclic nucleotide-gated cation channels, suggesting that cAMP may have a direct effect on cellular excitability *(36)*.

Taken together, our results suggest that the activation of $GABA_A$ receptors on GT1-7-immortalized GnRH neurons, known to rapidly result in cell membrane depolarization and increased $[Ca^{2+}]_i$, leads to a sustained inhibition of adenylyl cyclase. This action, in turn, provides a sustained inhibition of cellular excitability and, hence, could help explain the long-term, desensitization-resistant inhibition of pulsatile GnRH secretion by GABA. The role that these mechanisms identified in immortalized GnRH neurons may play in the regulation of reproduction by GABA through the direct modulation of GnRH neuronal physiology remains to be determined.

Materials and Methods

Reagents

Fura-2 AM, γ-amino-*n*-butyric acid, muscimol hydrobromide, (±)-baclofen, (−)-bicuculline methiodide, picrotoxin, forskolin, 8-Br-cAMP sodium, and IBMX were purchased from Research Biochemicals (Natick, MA). Adenosine 3', 5'-cyclic phosphoric acid 2'-*O*-succinyl-3 [^{125}I] iodotyrosine methyl ester (2000 Ci/mmol) was purchased from Amersham (Little Chalfont, UK).

Cell Culture

The GT1-7 cell line (kindly provided by R. I. Weiner, University of California, San Francisco), was cultured in Dulbecco's modified eagle's medium supplemented with 10% of fetal bovine serum and penicillin/streptomycin. The cells were plated at a density of 2×10^5 cells/mL, directly on 24-well plates, or on 35-mm plastic Petri dishes containing 9×22 mm glass cover slips (Termanox, Miles, Naperville, IL) coated with poly-L-lysine. The cultures were maintained at 37°C in a water-saturated atmosphere of 95% O_2 and 5% CO_2. Cells were cultured until they reached 50–70% confluency, at which time medium was replaced by a defined medium (Opti-MEM; Gibco, Grand Island, NY) without serum for 2 d.

cAMP Studies

The GT1-7 cells were cultured with Opti-MEM supplemented with 1 m*M* IBMX for 90 min. Cells were then challenged with GABAergic drugs for the last 1–30 min. At the end of the incubation period, the cells were lysed in 1 mL of ice-cold 0.1*N* HCl and frozen on dry ice overnight. Then, lysates were sonicated and incubated at 4°C for 48 h. After centrifugation (10,000*g* for 30 min), the supernatants were stored at −20°C until radioimmunoassayed for cAMP.

cAMP Radioimmunoassay

cAMP from cell extracts was determined by radioimmunoassay, using rabbit anti-cAMP polyclonal antiserum AB505 (Chemicon, Temecula, CA), after acetylation of the samples with triethylamine-acetic anhydride (2:1). All samples from an experiment were analyzed in the same assay. The limit of detection was 10 fmol/mL and the intraassay coefficient of variation was 2.8%.

Measurement of $[Ca^{2+}]_i$ in Cell Populations

The cover slip–attached cultures were washed twice with Ringer's solution (140 m*M* NaCl; 2.8 m*M* KCl; 2.0 m*M* $MgCl_2$; 1.0 m*M* CaCl; 10 m*M* HEPES; and 10 m*M* glucose, pH 7.3), and the cells were then loaded with 2 μ*M* acetoxymethyl ester of the Ca^{2+}-sensitive dye fura-2 ([fura-2 AM], freshly dissolved in 10 μL of dimethyl sulfoxide and then diluted in 30 mL of Ringer's solution) for 60 min. Then, fura-2 AM solution was removed by rinsing twice with Ringer's solution for 20 min at 37°C for completion of ester hydrolysis. The cover slips were suspended in a quartz cuvet so that the plane of the cover slip was at a 45° angle to the incident light and turned away from the emission monochromator so that reflected light would be minimal. Cells were continously perfused with Ringer's solution and eventually replaced by Ringer's solution containing high K^+ (35 m*M*; NaCl was isosmotically replaced by KCl), GABA, GABA agonists, GABA antagonists, forskolin, or 8-Br-cAMP. Determinations of $[Ca^{2+}]_i$ were made in a spectrophotometer (SLM-Aminco, Rochester, NY) equipped with a 150-W xenon lamp. The cells were alternately exposed to 340- and 380-nm light, and fluorescence

emission at 540 nm was collected, digitalized, and analyzed by the software provided with the spectrophotometer. Constants were determined for each cover slip using 5 μ*M* ionomycin for R_{max} at 1 m*M* $[Ca^{2+}]_{ext}$ and 5 m*M* EGTA for R_{min}. The ratio of the fluorescence excited at the two wavelengths ($R = F_{340}/F_{380}$) was calibrated to express $[Ca^{2+}]_i$ using the formula proposed by Grynkiewicz et al. *(45)*.

Data Analysis

The statistical significance of the results was tested by using a one-way analysis of variance followed by Fisher's multiple comparison test at 0.05 level of significance.

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