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Both $GABA_A$ and $GABA_B$ receptors participate in suppression of $[Ca^{2+}]_i$ pulsing in toad melanotrophs

Izumi Shibuya [1], Sathapana Kongsamut [*], William W. Douglas

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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Abstract

The receptor mechanisms involved in the inhibitory effect of γ-aminobutyric acid (GABA) in suppressing spontaneous $[Ca^{2+}]_i$ pulsing in melanotrophs of *Xenopus laevis* were investigated. The selective $GABA_B$ receptor agonist, baclofen reversibly arrested $[Ca^{2+}]_i$ pulsing. This inhibition was unaffected by the selective $GABA_A$ receptor antagonist, bicuculline methiodide, but was blocked by the selective $GABA_B$ receptor antagonist, CGP 35348 (3-aminopropyl diethyoxymethyl phosphinic acid). The selective $GABA_A$ receptor agonist, muscimol, also arrested $[Ca^{2+}]_i$ pulsing after causing a transient rise in $[Ca^{2+}]_i$. This biphasic response to muscimol was unaffected by CGP 35348, but was blocked by bicuculline. The inhibitory effect of GABA was unaffected by either CGP 35348 or bicuculline when given alone, but was blocked by both antagonists given together. In cells pretreated with pertussis toxin, the response to baclofen was completely lost, whereas responses to GABA and muscimol persisted; the response to GABA was blocked by bicuculline alone. Thus, both $GABA_A$ and $GABA_B$ receptors are involved in the inhibitory effect of GABA in suppressing spontaneous $[Ca^{2+}]_i$ pulsing in *Xenopus* melanotrophs.

Keywords: Melanotroph; $GABA_A$ receptor; Muscimol; $GABA_A$ receptor antagonist; Bicuculline; $GABA_B$ receptor; Baclofen; $GABA_B$ receptor antagonist; CGP 35348; Cytosolic free Ca^{2+}; Fura-2

1. Introduction

γ-Aminobutyric acid (GABA) is one of the most important physiological regulators in neuronal and endocrine cells. There are two distinct types of GABA receptors, namely $GABA_A$ and $GABA_B$ receptors. The former is a ligand-gated Cl^- channel and the latter is a G-protein-coupled seven helical transmembrane segment receptor that inhibits adenylate cyclases and modulates Ca^{2+} and/or K^+ channels (see, for example, reviews by Sieghart, 1992; Wojcik and Holopainen, 1992; Pearson et al., 1994).

Melanotrophs of the pituitary gland are directly innervated by hypothalamic neurons containing GABA (Schimchowitsch et al., 1991; Oertel et al., 1982; Vincent et al., 1982; Verburg van Kemenade et al., 1986a,b) and secretion of the products, various biologically active peptides derived from pro-opiomelanocortin, is modulated by GABA and various agonists and antagonists of GABA receptors (Tomiko et al., 1983; Verburg van Kemenade et al., 1987a; Shibuya et al., 1991). Both $GABA_A$ and $GABA_B$ receptors are reported to exist in melanotrophs of mammals and amphibians (Tomiko et al., 1983; Demeneix et al., 1984, 1986; Verburg van Kemenade et al., 1987a; Shibuya et al., 1991); however, little is known about the physiological significance of the dual GABA receptor mechanisms. It has been suggested that inhibitory effects of GABA on melanotroph secretion in the toad, *Xenopus laevis*, is predominantly mediated by $GABA_B$ receptors because (1) the secretory response to GABA was unaffected by a $GABA_A$ receptor antagonist and (2) it was mimicked by the $GABA_A$ receptor agonist, baclofen (Verburg van Kemenade et al., 1987a).

Although antagonists for $GABA_A$ receptors (such as bicuculline) have long been available, a useful antagonist to study $GABA_B$ receptor-mediated actions has not been available until recently. With the synthesis of CGP 35348 (3-aminopropyl diethyoxymethyl phosphinic acid), we have

[*] Corresponding author. Present address: Neuroscience Research, Hoechst Marion Roussel, Inc., P.O. Box 6800, Bridgewater, NJ 08807-0800, USA. Tel.: (1-908) 231-3072; fax: (1-908) 231-4335; e-mail: KONGSAM1@brwhcc3.hcc.com

[1] Present address: Department of Physiology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807, Japan.

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shown that this $GABA_B$ receptor antagonist antagonizes baclofen-induced inhibition of melanotroph secretion (Shibuya et al., 1991). This compound was also the most potent and selective $GABA_B$ receptor antagonist to inhibit baclofen-induced reductions in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in rat melanotrophs, and, moreover, this antagonism was competitive (Shibuya et al., 1992).

The purpose of the present experiments was to identify the GABA receptor mechanisms involved in the inhibitory action of GABA on $[Ca^{2+}]_i$ in *Xenopus* melanotrophs by using selective agonists and antagonists for the two GABA receptor subtypes as well as GABA itself. For this purpose, we have used isolated melanotrophs of *Xenopus laevis* in primary culture, where pulses in $[Ca^{2+}]_i$ occur spontaneously (Shibuya and Douglas, 1993a,b), and have assessed effects of various GABA-related compounds on this spontaneous $[Ca^{2+}]_i$ pulsing.

2. Materials and methods

2.1. Dissociation and culture of melanotrophs

Xenopus laevis (weighing 50–100 g, either sex) were kept in black containers for at least 3 weeks to disinhibit melanotroph secretion. After adaptation to a black background, they were decapitated under ether anesthesia and neurointermediate lobes of the pituitary gland were isolated and incubated in standard solution containing (in mM): NaCl, 112; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 15; glucose, 11 with 0.1% bovine serum albumin, and pH adjusted to 7.4 with NaOH. Melanotrophs were dissociated and cultured for 1–4 days according to procedures previously described (Shibuya and Douglas, 1993a).

2.2. Fluorescence measurement

Culture medium was removed and the cells were washed with standard solution and then loaded, in the same solution, with the acetoxymethyl ester of fura-2 (1 μM) at room temperature (about 23°C) for 1 h. The cells were then washed with dye-free standard solution and kept at room temperature until used. Methods for cell perifusion and fluorescence measurement have been described previously (Shibuya et al., 1992). In brief, a portion of the cell suspension was transferred to a perifusion chamber, the bottom of which consisted of a glass coverslip. This was placed on the stage of an inverted microscope (Nikon, Diaphot) and perifusion with standard solution was begun 5–10 min later when the cells had settled and attached to the coverslip. The fluorescence intensities at 510 nm with excitation at 340 and at 380 nm were recorded together and $[Ca^{2+}]_i$ was calculated from the ratio of the two fluorescence intensities using the equation of Grynkiewicz et al. (1985).

3. Results

3.1. $GABA_B$ receptor agonists and antagonists

As shown in Fig. 1a, the selective $GABA_B$ receptor agonist, baclofen (10 μM), produced arrest of spontaneous $[Ca^{2+}]_i$ pulsing with rapid onset and recovery. After removing baclofen, the amplitude of $[Ca^{2+}]_i$ pulsing often increased for several minutes. The arrest of $[Ca^{2+}]_i$ pulsing produced by baclofen was abolished by pretreatment with the selective $GABA_B$ receptor antagonist, CGP 35348 (300 μM), in a rapidly reversible manner. The baclofen-induced arrest of $[Ca^{2+}]_i$ pulsing was also reversed by addition of CGP 35348 in the continued presence of baclofen. However, the effect of baclofen was unaffected by addition of the selective $GABA_A$ receptor antagonist, bicuculline (50 μM; Fig. 1b).

3.2. $GABA_A$ receptor agonists and antagonists

The selective $GABA_A$ receptor agonist, muscimol (3 μM), also arrested spontaneous $[Ca^{2+}]_i$ pulsing in a reversible manner (Fig. 2). The pattern of the effect of muscimol was different from that of baclofen in that muscimol often caused a brief rise in $[Ca^{2+}]_i$ before arresting $[Ca^{2+}]_i$ pulsing. This is similar to what we have previously observed with measurement of secretion (Shibuya et al., 1991) and $[Ca^{2+}]_i$ (Shibuya and Douglas, 1993a) where an initial small spike is often observed following addition of muscimol or GABA. The arrest of $[Ca^{2+}]_i$ pulsing by muscimol was reversibly abolished by pretreatment with bicuculline (50 μM; Fig. 2a), or upon addition of bicuculline (50 μM) after muscimol (3 μM) had produced its inhibitory effect (Fig. 2b). The $GABA_B$ receptor antagonist CGP 35348 (300 μM) did not affect the initial rise in $[Ca^{2+}]_i$ (Fig. 2c) and did not arrest $[Ca^{2+}]_i$ pulsing induced by muscimol (3 μM; Fig. 2b and

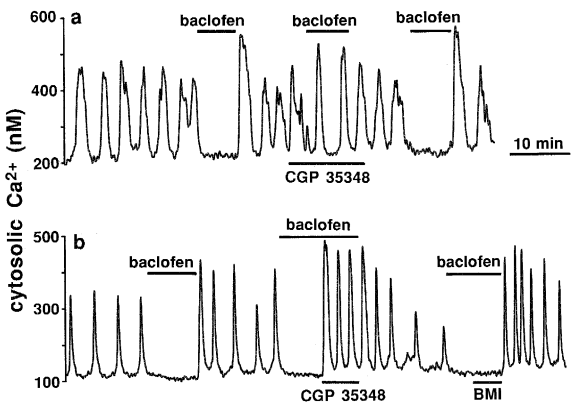

Fig. 1. Effect of baclofen (10 μM) on cytosolic Ca^{2+} pulsing occurring spontaneously in individual *Xenopus* melanotrophs and its block by CGP 35348 (300 μM; a). In b, CGP 35348 (300 μM), when added on top of baclofen (10 μM), intensified cytosolic Ca^{2+} pulsing. Bicuculline methiodide (BMI; 50 μM) had no effect on arrest of spiking by baclofen. These are examples of 4–6 similar experiments.

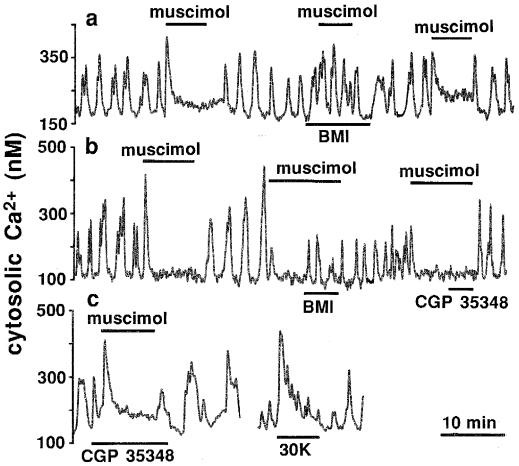

Fig. 2. (a) Muscimol (3 μM) caused a transient rise in $[Ca^{2+}]_i$ followed by arrest of pulsing, which was blocked by bicuculline methiodide (BMI, 50 μM). (b) BMI (50 μM), when added during the course of arrest by muscimol, initiated spiking, but CGP 35348 (300 μM) had no effect. (c) CGP 35348 (300 μM) had no effect on the response to muscimol (3 μM). In c, response to membrane depolarization by K^+ (30 mM) obtained in the last cell is shown for comparison. These are examples of 3–4 similar experiments.

2c). The size of the initial rise in $[Ca^{2+}]_i$ in response to muscimol (3 μM) was similar to that achieved by high K^+ (30 mM; Fig. 2c).

3.3. The physiological ligand, GABA

GABA (10 μM) arrested spontaneous $[Ca^{2+}]_i$ pulsing in a reversible manner (Fig. 3). Like muscimol, GABA often produced initial transient rises in $[Ca^{2+}]_i$ before arresting $[Ca^{2+}]_i$ pulsing; however, the frequency of appearance of this initial rise in $[Ca^{2+}]_i$ was smaller for GABA (65% (17 out of 26 experiments)) than for muscimol (86% (12 out of 14 experiments)). In cells where the initial rise in $[Ca^{2+}]_i$ in response to GABA (10 μM) was unclear, pretreatment with CGP 35348 (300 μM) unmasked this response (Fig. 3b and Fig. 4). When cells were pretreated with bicuculline (50 μM) the initial rise in $[Ca^{2+}]_i$ in response to GABA (10 μM) was abolished but the subsequent arrest persisted (Fig. 3a). Pretreatment with CGP 35348 (300 μM) did not affect the arrest of $[Ca^{2+}]_i$ pulsing in response to GABA (10 μM; Fig. 3b). Pretreatment with the combination of both bicuculline (50 μM) and CGP 35348 (300 μM) was needed to abolish the inhibitory action of GABA (10 μM; Fig. 3c).

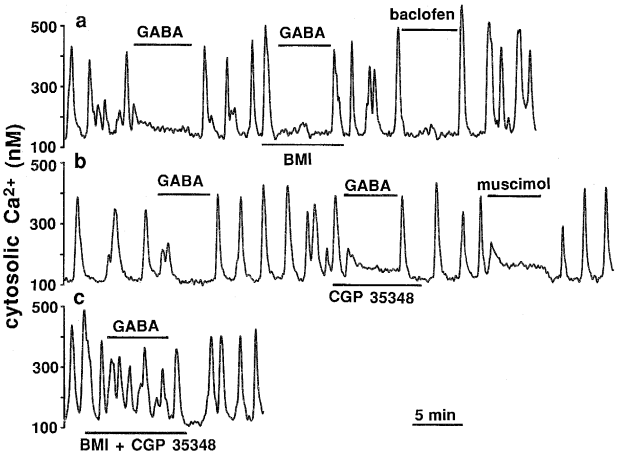

Fig. 3. (a–c) Effects of GABA as well as muscimol and baclofen, and effects of $GABA_A$ and $GABA_B$ receptor antagonists on the inhibitory effect of GABA obtained in a single melanotroph. (a) GABA (10 μM) arrested cytosolic Ca^{2+} pulsing after causing a small increase in $[Ca^{2+}]_i$. The arresting effect of GABA was unaffected by bicuculline methiodide (BMI, 50 μM) and the response to GABA in the presence of BMI resembles that to baclofen (10 μM). (b) The response to GABA (10 μM) was also unaffected by CGP 35348 (300 μM), and the response to GABA (10 μM) in the presence of CGP 35348 (300 μM) resembles that to muscimol (3 μM). (c) The response to GABA (10 μM) was blocked by prior exposure to a combination of BMI (50 μM) and CGP 35348 (300 μM). These are examples of 4–7 similar experiments.

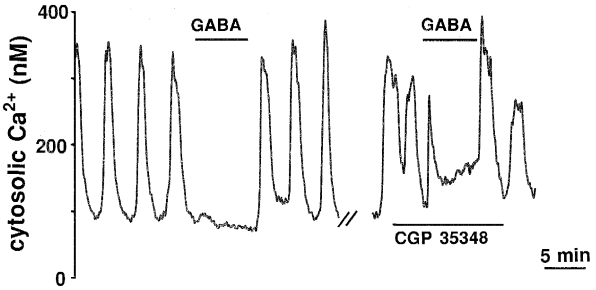

Fig. 4. In a cell where the response to GABA (10 μM) did not cause a clear initial rise in $[Ca^{2+}]_i$ (a), CGP 35348 (300 μM) unmasked the initial response (transient rise in $[Ca^{2+}]_i$) to GABA (b).

The involvement of dual GABA receptors was more clearly illustrated when the receptor antagonists were added one by one during the course of arrest of $[Ca^{2+}]_i$ pulsing produced by GABA (Fig. 5). Arrest of $[Ca^{2+}]_i$ pulsing produced by GABA (10 μM) was unaffected by addition

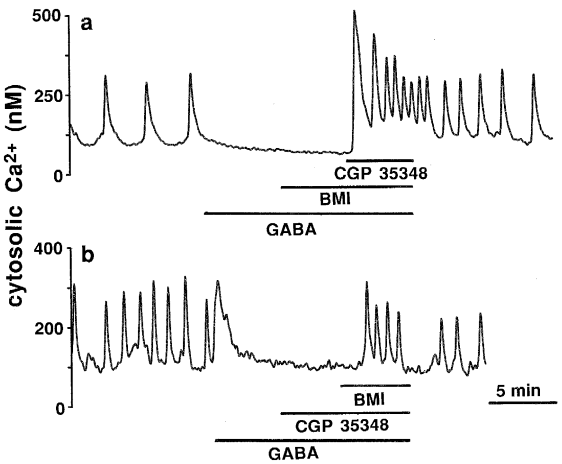

Fig. 5. (a) Addition of bicuculline methiodide (BMI, 30 μM) alone had no effect on the response to GABA (10 μM), but further addition of CGP 35348 (300 μM) initiated cytosolic Ca^{2+} pulsing with an intensified form. (b) Addition of CGP 35348 (300 μM) alone had no effect on the response to GABA (10 μM), but further addition of BMI (30 μM) initiated cytosolic Ca^{2+} pulsing. These are examples of 3 similar experiments.

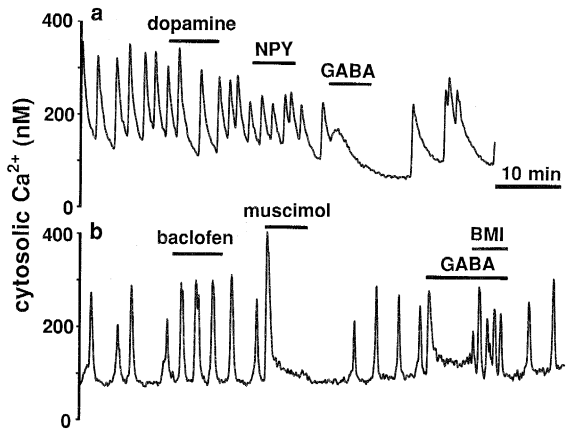

Fig. 6. In melanotrophs pretreated with pertussis toxin (100 ng/ml; 16 h), (a) dopamine (100 nM) and neuropeptide Y (10 nM) no longer arrested Ca^{2+} pulsing whereas GABA (10 μM) arrested it. (b) Baclofen (10 μM) did not arrest Ca^{2+} pulsing but muscimol (3 μM) did. The response to GABA (10 μM) was not antagonized by bicuculline methiodide (BMI, 50 μM) alone. These are examples of 3 similar experiments.

of bicuculline (50 μM) alone or CGP 35348 (300 μM) alone (Fig. 5a and 5b); however, it was reversed when both receptor antagonists were present.

3.4. Pertussis toxin pretreatment

Pretreatment with pertussis toxin (100 ng/ml, 16 h), which is known to block the actions of inhibitory G-proteins (G_i and G_o), abolished responses to the two inhibitory transmitters reported to exist in nerves innervating melanotrophs, dopamine (100 nM) and neuropeptide Y (10 nM) (Cote et al., 1984; Kongsamut et al., 1991); however, it did not abolish the response to GABA (10 μM; Fig. 6a). In pertussis toxin-treated cells, the response to baclofen (10 μM) was abolished but that to muscimol (10 μM) persisted; moreover, the response to GABA (10 μM) was reversed by bicuculline (50 μM) alone (Fig. 6b).

3.5. Concentration-response relations

The concentrations used in the illustrations presented above were chosen for their ability to obtain near-maximal effects; other concentrations of all the compounds were also studied (not shown). The concentration-response relation for inhibition of cytosolic $[Ca^{2+}]_i$ pulsing in single melanotrophs is essentially all or nothing (Shibuya and Douglas, 1993a); a full study of concentration-response relations would require averaging across a large population of cells.

4. Discussion

The present study unequivocally shows that both $GABA_A$ and $GABA_B$ receptors are involved in the inhibitory actions of GABA on pituitary melanotrophs of *Xenopus laevis*. We show that neither of the selective antagonists for $GABA_A$ and $GABA_B$ receptors, bicuculline and CGP 35348, could effectively antagonize the inhibitory response to GABA when the antagonists were given alone; however, the combination of the two antagonists completely abolished the response to GABA. These results indicate that GABA, released from the nerve terminal of the hypothalamic neurons innervating pituitary melanotrophs, acts on either or both $GABA_A$ and $GABA_B$ receptors to suppress cytosolic $[Ca^{2+}]_i$ pulsing. This study extends previously reported evidence for the involvement of $GABA_A$ and $GABA_B$ receptors in the inhibitory regulation by GABA obtained with secretion measurements (Kongsamut et al., 1991; Shibuya et al., 1991) and measurements of $[Ca^{2+}]_i$ pulsing (Shibuya and Douglas, 1993a; Scheenen et al., 1994). Shibuya and Douglas (1993a) and Scheenen et al. (1994) examined GABA receptor pharmacology using different pharmacological agents as part of a larger study of different secretagogues. The sensitivity and the time resolution of the measurements of $[Ca^{2+}]_i$ reported in the present study is superior to what is possible with secretion measurements and reveals details of the subcellular mechanisms of the two GABA receptor subtypes.

In secretion experiments previously published (Shibuya et al., 1991; Kongsamut et al., 1991), we observed an initial small peak of secretion in response to $GABA_A$ receptor stimulation. This initial peak has also been observed in measurements of $[Ca^{2+}]_i$ (Shibuya and Douglas, 1993a) and was seen even in cells that did not exhibit spontaneous $[Ca^{2+}]_i$ pulsing (Shibuya and Douglas, 1993a). The initial rise in $[Ca^{2+}]_i$ in response to GABA or muscimol has also been observed in the present study. These observations are consistent with membrane depolarization caused by Cl^- channel activation, and the subsequent arrest of $[Ca^{2+}]_i$ pulsing could also be the result of such action (clamping the membrane potential at the Cl^- equilibrium potential). In rat melanotrophs, electrophysiological studies have revealed that GABA produces a transient burst of action potential discharge which then leads to arrest of spontaneous action potentials (Taraskevich and Douglas, 1985). Moreover, in another type of endocrine cell, the adrenal chromaffin cell of the rat, where only $GABA_A$ receptors function to inhibit $[Ca^{2+}]_i$, GABA and muscimol also produced similar biphasic effects on both action potentials and spontaneous oscillations in $[Ca^{2+}]_i$ (Busik et al., 1996). Since spontaneous $[Ca^{2+}]_i$ pulsing in *Xenopus* melanotrophs is primarily due to Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Shibuya and Douglas, 1993b), the suppression of $[Ca^{2+}]_i$ pulsing by GABA or muscimol, by $GABA_A$ receptor activation, is likely to be the result of clamping the membrane potential at the Cl^- equilibrium potential (which is less negative than the resting membrane potential).

Changes in the size of the initial peak and changes in

the basal level of intracellular Ca^{2+} were also observed, on occasion, with continuous or repeated administration of muscimol (Fig. 2b). This may be due to either desensitization of the $GABA_A$ receptor or changes in the Cl^- equilibrium potential following prolonged opening of the $GABA_A$-Cl^- channel complex.

Mechanisms involving $GABA_B$ receptor activation are different from those involving $GABA_A$ receptors. Increases in cyclic AMP concentrations have been shown to stimulate $[Ca^{2+}]_i$ pulsing (Shibuya and Douglas, 1993c; Scheenen et al., 1994), and GABA and baclofen have also been shown to decrease cyclic AMP in *Xenopus* melanotrophs (Verburg van Kemenade et al., 1987b; Jenks et al., 1991; De Koning et al., 1992; Leenders et al., 1995). Thus, it would seem that the effects on $[Ca^{2+}]_i$ pulsing mediated by $GABA_B$ receptors use a mechanism of action involving cyclic AMP. However, dopamine and baclofen are able to arrest $[Ca^{2+}]_i$ pulsing in the presence of a saturating concentration of membrane-permeable cyclic AMP analogs (Shibuya and Douglas, 1993c; and unpublished observations). Additionally, it would be more difficult to explain the rapid rebound phenomenon on this basis.

Although there has been no report on the effect of $GABA_B$ receptor activation on K^+ channels or Ca^{2+} channels in *Xenopus* melanotrophs, both of these effects are known to occur during $GABA_B$ receptor activation in other cell types (see for example Gage, 1992; Pearson et al., 1994). Thus, the suppression of $[Ca^{2+}]_i$ pulsing by the $GABA_B$ receptor agonist baclofen may be due to membrane hyperpolarization caused by activation of K^+ channels and/or inhibition of Ca^{2+} channels. Such a mechanism would explain the lack of initial rise in $[Ca^{2+}]_i$ seen with baclofen. In addition, this mechanism can explain the rebound phenomenon seen upon removal of baclofen where $[Ca^{2+}]_i$ pulsing of larger amplitudes reappeared for several minutes. During hyperpolarization, inactivation of voltage-dependent Ca^{2+} channels would be removed and when membrane potential is again depolarized upon removal of baclofen, more Ca^{2+} channels are available to be recruited. Similar rebound phenomena have been observed in peptide release and $[Ca^{2+}]_i$ pulsing from these cells upon removal of other secreto-inhibitory transmitters, dopamine or neuropeptide Y (Shibuya et al., 1991; Kongsamut et al., 1991). Both of these transmitters are known to cause hyperpolarization and inhibition of Ca^{2+} currents. It is worthwhile noting that the rebound effect is particularly prominent when inhibition by dopamine is terminated by addition of the dopamine D_2 receptor antagonist, sulpiride at a supramaximal concentration (Shibuya et al., 1991). This observation is in good agreement with the present result that a clearer rebound effect was observed when CGP 35348 was added than with bicuculline during inhibition by GABA (Fig. 5a).

The present result that CGP 35348 unmasked the initial transient rise in response to GABA suggests that concomitant activation of $GABA_B$ receptors suppressed the initial response to $GABA_A$ receptor activation in some cells. The fact that 65% of cells examined in the present study showed the initial rise in response to GABA, as compared to 86% for the selective $GABA_A$ receptor agonist, muscimol, could be interpreted as activation of $GABA_A$ receptors exerts its effect more rapidly than activation of $GABA_B$ receptors. Since the former is a ligand-gated channel and the latter is a G-protein-coupled receptor, the delay in G-protein activation and the signal transduction to the effector(s) that would have to occur during $GABA_B$ receptor activation may explain the more rapid transduction through the $GABA_A$ receptor.

As has been seen in previous studies (Shibuya and Douglas, 1993a,b), the frequency of spontaneous $[Ca^{2+}]_i$ pulsing observed in this study varied somewhat from cell to cell. We found that the qualitative effects of the pharmacological agents was independent of the frequency of $[Ca^{2+}]_i$ pulsing.

Pretreatment with pertussis toxin has been a useful tool to distinguish G-protein (G_i or G_o)-coupled intracellular events from others. Dopamine D_2, $GABA_B$ and neuropeptide Y receptors in melanotrophs have been shown to be coupled to such G-proteins, and in previous studies, we have shown that inhibition of secretion by activation of these receptors was eliminated by pretreatment with pertussis toxin (Taraskevich and Douglas, 1990; Kongsamut et al., 1991). In contrast, inhibition of secretion in response to muscimol or to GABA was not blocked by pertussis toxin pretreatment (Kongsamut et al., 1991). The present results show that both the G-protein-coupled inhibitory mechanism and the ligand-gated receptor mechanism lead to suppression of spontaneous $[Ca^{2+}]_i$ pulsing in these cells and thereby inhibit melanotroph secretion.

Recently a third type of GABA receptor has been cloned and named $GABA_C$ (see Kusama et al., 1993; Bormann and Feigenspan, 1995). $GABA_C$ receptors resemble $GABA_A$ receptors in that they are both ligand-gated Cl^- channels and they are both sensitive to the Cl^- channel blocker, picrotoxin; however, $GABA_C$ receptors are not sensitive to the competitive $GABA_A$ receptor antagonist, bicuculline (Kusama et al., 1993). The observation that the response to muscimol (an agonist at both $GABA_A$ and $GABA_C$ receptors), and the response to GABA in pertussis toxin-treated cells was completely abolished by bicuculline suggests that the $GABA_C$ receptor is either not present in these cells or does not play an important role in the responses we measured.

The present results demonstrate, using a combination of selective receptor agonists and antagonists, that GABA-induced arrest of $[Ca^{2+}]_i$ pulsing is due to simultaneous activation of both $GABA_A$ and $GABA_B$ receptors. The physiological significance of the inhibitory mechanisms by the two receptors remains unclear. It could be that such redundant mechanisms assure inhibition of melanotrophs in conditions where G-protein-coupled inhibitory mechanisms do not function or ligand-gated channels desensitize.

Obviously further study will be required to answer this biologically important question.

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