Mechanism of action of γ-aminobutyric acid on frog melanotrophs

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ABSTRACT

We have previously demonstrated that γ-aminobutyric acid (GABA) is a potent regulator of secretory and electrical activity in melanotrophs of the frog pituitary. The aim of the present study was to investigate the intracellular events which mediate the response of melanotrophs to GABA.

We first observed that GABA (1–100 μM) inhibited both basal and forskolin-stimulated cyclic AMP (cAMP) formation. The inhibitory effect of GABA on cAMP levels was mimicked by the GABA_B receptor agonist baclofen (100 μM) and totally abolished by a 4-h pretreatment with pertussis toxin (0.1 μg/ml). In contrast, the specific GABA_A agonist 3-aminopropane sulphonic acid (3APS) did not affect cAMP production. Both GABA and 3APS (100 μM each) induced a biphasic effect on α-MSH release from perifused frog neurointermediate lobes, i.e. a transient stimulation followed by an inhibition of α-MSH secretion. Administration of forskolin (10 μM) prolonged the stimulatory phase and attenuated the inhibitory phase evoked by GABA and 3APS, indicating that cAMP modulates the response of melanotrophs to GABA_A agonists. Ejection of 3APS (1 μM) in the vicinity of cultured melanotrophs caused a massive increase in intracellular calcium concentration ([Ca^{2+}]). The stimulatory effect of 3APS on [Ca^{2+}] was abolished when the cells were incubated in a chloride-free medium. The formation of inositol trisphosphate was not affected by 3APS, suggesting that the increase in [Ca^{2+}] cannot be ascribed to mobilization of intracellular calcium stores. α-Conotoxin did not alter the secretory response of frog neurointermediate lobes to 3APS, while nifedipine blocked the stimulation of α-MSH secretion induced by 3APS.

In conclusion, the present data indicate that, in frog pituitary melanotrophs, (i) the stimulatory phase evoked by GABA_A agonists can be accounted for by an influx of calcium through L-type calcium channels, (ii) the inhibitory effect evoked by GABA_B agonists can be ascribed to inhibition of adenylate cyclase activity and (iii) cAMP attenuates the inhibitory phase evoked by GABA_A agonists. Taken together, these data suggest that activation of GABA_B receptors may modulate GABA_A receptor function.

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INTRODUCTION

The neurotransmitter γ-aminobutyric acid (GABA) is present, along with dopamine and neuropeptide Y, within axon terminals that make synaptic contacts with melanotrophs of the frog pars intermedia (Tonon et al. 1992). All these factors are involved in the inhibitory control of α-melanocyte-stimulating hormone (α-MSH) release (Adjeroud et al. 1986a,b, Tonon et al. 1989, Danger et al. 1990, Chartrel et al. 1991).

GABA is known to bind to two subtypes of receptors which differ in their molecular structure, transduction pathways and pharmacological profiles. The GABA_A receptor is a supramolecular complex associated with a chloride channel (Herb et al. 1992, Knoflach et al. 1992) which is activated by muscimol, isoguvacine or 3-aminopropane sulphonic acid (3APS), and competitively antagonized by bicuculline (Enna 1988). The action of GABA on the GABA_A receptor can be allosterically modulated by various compounds including benzodiazepines, endozepines, barbiturates and neurosteroids (Bormann 1988, Knapp et al. 1990, Burt & Kamatchi 1991, Lüddens & Wisden 1991, Majewska 1992). The molecular structure of the GABA_B receptor has not yet been characterized. The GABA_B receptor is negatively coupled to
adenylate cyclase (Hill 1985) and regulates potassium and/or calcium channels through G proteins (Bormann 1988). The GABA_B receptor is selectively activated by baclofen (Hill & Bowery 1981).

In all vertebrates studied so far, GABA exerts an inhibitory effect on α-MSH secretion. However, the mode of action of GABA on melanotrophs appears to differ considerably in the various species investigated. In rat and porcine pars intermedia cells, GABA induces a biphasic effect on α-MSH secretion; activation of GABA_A receptors causes stimulation of α-MSH release while activation of GABA_B receptors causes an inhibitory effect (Tomiko et al. 1983, Demeneix et al. 1984, 1986). In Xenopus laevis, GABA induces only inhibition of α-MSH release and the inhibitory action of GABA is mediated through both GABA_A and GABA_B receptors (Verburg-van Kemenade et al. 1987, Jenks et al. 1993). In the frog Rana ridibunda, GABA induces a transient stimulation followed by a prolonged inhibition of α-MSH release (Adjeroud et al. 1986b). Both effects are mimicked by GABA_A agonists and blocked by bicuculline, indicating that the stimulatory and inhibitory actions of GABA in frog melanotrophs can be ascribed to activation of GABA_A receptors. In this species however, baclofen is also capable of inhibiting α-MSH release (Tonon et al. 1989), suggesting the existence of a GABA_B receptor in the frog pars intermedia.

There is now increasing evidence for the involvement of multiple transduction mechanisms associated with activation of one type of receptor (Birnbaumer et al. 1990). For instance, in frog pituitary melanotrophs, activation of dopamine D_1 receptors causes a reduction in adenylate cyclase activity, a decrease in intracellular calcium concentration ([Ca^{2+}]_i), an inhibition of inositol phosphate production (Desruès et al. 1993), a reduction of calcium and sodium currents and an increase in potassium conductance (Valentijn et al. 1991a,b). In view of the complexity of the action of GABA on α-MSH secretion from pituitary cells, we have investigated the intracellular events associated with activation of both GABA_A and GABA_B receptors in frog melanotrophs.

MATERIALS AND METHODS

Animals

Adult male frogs (Rana ridibunda), of about 30 g body weight, were purchased from a commercial source (Couétard, St-Hilaire de Riez, France). The animals were maintained in a temperature-controlled room (8 ± 0.5 °C (s.e.m.)) under running water on a regimen of 12 h darkness:12 h light (lights on 0600–1800 h). Animal manipulations were performed according to the recommendations of the French Ethics Committee and under the supervision of authorized investigators.

Agents

GABA, 3APS, baclofen, forskolin, pertussis toxin (PTX) and α-conotoxin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Phaclofen was obtained from Research Biochemicals Inc. (Natick, MA, USA). Synthetic α-MSH was kindly provided by Drs R H Andreatta and V Rasetti (Ciba-Geigy, Basel, Switzerland). CGP35348 was a gift from Drs L Maitre and H Kaufmann (Ciba-Geigy).

Measurement of cyclic AMP (cAMP) production

Frogs were decapitated and the neurointermediate lobes (NILs) were dissected under the microscope. The NILs were preincubated for 30 min in 250 μl Krebs Ringer solution (112 mM NaCl, 2 mM KCl, 2 mM CaCl_2, 15 mM Hepes) containing 0.8 mg glucose/ml and 0.3 mg BSA/ml (pH 7.4) supplemented with 0.1 mM isobutylmethylxanthine (IBMX; Sigma Chemical Co.) to inhibit phosphodiesterase. The lobes were then incubated in the same solution, with test substances, at 21 °C for 2–20 min. The reaction was stopped by the addition of ice-cold 20% (w/v) trichloroacetic acid (TCA). After homogenization, the proteins were removed by centrifugation (13 000g, 4 min) and the supernatants were washed three times with 1 ml water-saturated diethylether, dried and reconstituted in RIA buffer (0.05 M sodium acetate, pH 5.8). The cAMP concentration was determined in each sample using a cAMP RIA kit (RPA 509; Amersham International, Les Ulis, France).

Measurement of inositol phosphate production

The effect of GABA on polyphosphoinositide metabolism was investigated as previously described (Desruès et al. 1990). Briefly, the NILs were incubated in Leibovitz culture medium with myo-[³H]inositol (200 μCi/ml, specific activity 100 Ci/mmol; Amersham International) at 21 °C for 18 h and then washed six times with Krebs Ringer solution supplemented with 1 mM inositol. After a 10-min preincubation with LiCl (10 mM)
to inhibit inositol monophosphate phosphatase and inositol polyphosphate-1-phosphatase, test substances were added for various periods (2–20 min). The reactions were stopped with ice-cold 20% TCA and the NILs were homogenized. After centrifugation (13 000 g, 4 min), the supernatants containing the phosphoinositols were washed three times with water-saturated diethyl ether, neutralized with NaHCO₃ (1 M) and stored at −20 °C until analysis.

Tritiated phosphoinositols were separated by anion-exchange chromatography (AG1-X8 resin, 100–200 mesh, formate form; Bio-Rad Labs, Richmond, CA, USA). Free [³H]inositol and [³H]inositol mono-, bis- and trisphosphates were sequentially eluted with 10 × 2 ml distilled water and with 20 × 2 ml each of solutions of 0-2, 0-45 and 0-8 M ammonium formate in formic acid (0-1 M). Scintillation fluid (6 ml; Quicksafe A; Zinsser Analytic, Frankfurt, Germany) was added to each fraction and the radioactivity was determined by counting in a 1217 Rackbeta spectrometer (63% efficiency).

Measurement of α-MSH release

The perifusion technique was used to determine the effect of test substances on α-MSH release. For each experiment, eight NILs were suspended in preswollen Bio-Gel P2 (Bio-Rad Labs) and perifused with Krebs Ringer solution at a constant flow rate (0-6 ml/min) and temperature (21 °C) as previously described (Tonon et al. 1980). The effluent perifusate, collected as 7-5-min fractions during the stabilization period and 1-min fractions during the infusion of test substances, was immediately chilled on ice until RIA. The α-MSH concentration was measured in each fraction on the same day as the perifusion experiment using a double-antibody RIA procedure (Vaudry et al. 1978). The perifusion profiles were calculated and expressed as percentages of the basal secretion rate. Each figure represents the mean profile of α-MSH release (± S.E.M.) from at least three independent experiments.

Measurement of [Ca²⁺]ᵢ

The effect of the GABAₐ agonist 3APS on [Ca²⁺]ᵢ was studied by using a double wavelength microfluorometric technique as previously described (Gracia-Navarro et al. 1992). Melanotrophs were cultured on coverslips in Leibovitz medium containing 2 mM CaCl₂ for 3–5 days, as described by Louiset et al. (1988). Before each experiment, the cells were incubated in the dark at room temperature for 30 min with 5 µM Indo-1 pentacetoxy-methylester and 0-2% Pluronic F127 (both from Molecular Probes, Eugene, OR, USA), in a Krebs Ringer solution containing 2 mM CaCl₂. The chloride-free Krebs Ringer solution was prepared with acetate salts of sodium, potassium and calcium. At the end of the incubation, the cells were washed twice with 1 ml fresh medium. [Ca²⁺]ᵢ was monitored by a dual-emission microfluorimeter system constructed from a Nikon Diaphot inverted microscope, equipped for epifluorescence with an oil-immersion objective (×100 CF Fluor series, numerical aperture 1-3). The fluorescence emission of Indo-1, induced by excitation at 355 nm, was recorded at two wavelengths (405 and 480 nm) by separate photometers (P1; Nikon). The 405:480 nm ratio was determined using an analogical divider (constructed by Dr B Dufy, CNRS URA 1200, Bordeaux, France) after conversion of single photon currents to voltage signals. All three values (405, 480 and 405:480 nm) were continuously recorded with a three-channel voltage recorder (BD 100/101; Kipp and Zonen, Delft, The Netherlands). [Ca²⁺]ᵢ was calculated from the formula established by Grynkiewicz et al. (1985):

\[ [Ca^{2+}]_i = K_d \times \beta \times \left[ \frac{(R - R_{min})}{(R_{max} - R)} \right] \]

where Rₘᵢₙ represents the fluorescence ratio obtained after incubation of cells with 10 mM EGTA and 10 µM ionomycin for 3 h, Rₘₐₓ the maximum fluorescence ratio obtained after incubation of cells with 10 mM CaCl₂ and 10 µM ionomycin for 3 h, and β the ratio of fluorescence readings yielded from the indicator at the minimum and maximum Ca²⁺ concentrations at 480 nm (Ca²⁺ₘᵢₙ:Ca²⁺ₘₐₓ). The averaged values of Rₘᵢₙ, Rₘₐₓ and β were 0-164±0-03 (S.E.M.) (n=55), 1-82±0-06 (n=50) and 1-62 (n=50) respectively. Kₜ is the dissociation constant for Indo-1 (250 nM), as previously determined (Grynkiewicz et al. 1985). 3APS was administered in the vicinity of the cells by a pressure ejection system.

Statistical analysis

All values presented in the figures and tables are means ± S.E.M. Student’s t-test for variation analysis was used to determine statistical differences between control and experimental values within the same set of experiments. When P<0.05 test values were considered significantly different from controls.
RESULTS

Effect of GABA on cAMP formation

The time-course of the effect of GABA on forskolin-stimulated cAMP production by whole NILs is shown in Fig. 1. GABA (100 μM) significantly decreased cAMP production within 5 min. Maximal inhibition occurred 10 min after the onset of the administration of GABA. The effect of GABA on both basal and forskolin-stimulated cAMP formation was dose-dependent (concentration range from 1 to 100 μM) (Table 1). The selective GABA_B receptor agonist baclofen (100 μM) reduced the forskolin-stimulated cAMP level, whereas the GABA_A receptor agonist 3APS (100 μM) was inactive (Fig. 2). After a 4-h incubation of the NILs with PTX (0.1 μg/ml), the GABA-induced inhibition of cAMP production was totally abolished (Fig. 3).

Effect of GABA_B receptor agonist and antagonists on α-MSH secretion

Administration of the GABA_B receptor agonist baclofen (100 μM, 20 min) to perfused frog NILs induced a significant inhibition (P<0.01) of α-MSH release (Fig. 4). Infusion of the GABA_B receptor antagonists phaclofen (100 μM) or CGP35348 (300 μM) did not modify the response of melanotrophs to baclofen.

Effect of forskolin on the secretory response of frog NILs to GABA agonists

In order to determine the possible action of cAMP on the response of frog NILs to GABA_A and GABA_B receptor agonists, the effects of baclofen, GABA and 3APS on α-MSH release were studied in the absence or presence of forskolin (Fig. 5). As previously shown (Adjeroud et al. 1986b), a 20-min pulse of GABA or 3APS (100 μM each) induced a transient stimulation (3 and 4 min) followed by a sustained inhibition of α-MSH secretion (−51.2 and −50.6% respectively). Infusion of forskolin prolonged the duration of the stimulatory effect of GABA and 3APS (by 2 and 3 min respectively) and significantly reduced the amplitude of the inhibitory phase to −23.8 and −17.7% respectively. In contrast, the kinetics of the inhibitory effect of baclofen on α-MSH release were not affected by forskolin.

Effect of a GABA_A agonist on [Ca^{2+}]_i

The effect of the GABA_A agonist 3APS on [Ca^{2+}]_i was studied by monitoring the fluorescence signal in a total of 41 melanotrophs. Ejection of 3APS (1 μM) in the vicinity of the cells (n=31) caused an increase in [Ca^{2+}]_i (Fig. 6a). The 3APS-evoked increase in [Ca^{2+}]_i was totally suppressed when the cells were incubated in a chloride-free Ringer solution (n=10; Fig. 6b).
**Effect of GABA on inositol phosphate production**

Frog NILs were labelled with [³H]inositol for 18 h and then preincubated with 10 mM LiCl for 10 min. Exposure of the tissues to GABA (100 µM) did not significantly affect the inositol trisphosphate (IP₃) level within the first 10 min (Table 2). Thereafter, GABA induced a significant inhibition of IP₃ formation (P<0.01). The inhibitory effect of GABA on the IP₃ concentration was mimicked by the GABAₐ agonist baclofen (100 µM) (Table 3). In contrast, the GABAₐ agonist 3APS did not modify the IP₃ level and the GABAₐ antagonist bicuculline did not reverse the GABA-induced inhibition of IP₃ production (Table 3).

**Involvement of voltage-sensitive calcium channels in the mechanism of action of GABA**

The effect of calcium channel blockers on the response of NILs to 3APS was studied using the perifusion technique (Fig. 7). In the absence of calcium channel blockers, a 20-min pulse of 3APS induced a transient stimulation followed by a sustained inhibition of α-MSH secretion. The N-type channel blocker ω-conotoxin did not significantly modify the profile of the response of melanotrophs to 3APS. In contrast, the L-type calcium channel blocker nifedipine totally abolished the early stimulatory phase of α-MSH secretion induced by 3APS.
DISCUSSION

Previous studies on the effects of GABA on frog pituitary melanotrophs have shown that GABA induces a biphasic response consisting of a rapid and transient stimulation followed by a more prolonged inhibition of the secretory activity (Adjeroud et al. 1986b, Tonon et al. 1989). Both the stimulatory and inhibitory phases are mimicked by GABA_A agonists and abolished by GABA_A antagonists, while activation of GABA_B receptors only induces an inhibitory response (Adjeroud et al. 1986b, Tonon et al. 1989). Concurrently, patch-clamp experiments have shown that GABA_A agonists cause an immediate hyperpolarization (Louiset et al. 1992), which is dissonant with the transient stimulation of α-MSH secretion observed during the first phase of the response. These data indicated that the mechanism of action of GABA on frog melanotrophs probably involves multiple transduction pathways.

Coupling of GABA_B receptors to adenylate cyclase

We observed that GABA induced a significant reduction in both basal and forskolin-stimulated cAMP production. The inhibitory effect of GABA was mimicked by the GABA_B receptor agonist baclofen but not by the GABA_A agonist 3APS. In addition, the effect of GABA was suppressed when the lobes were preincubated with PTX. These data show that, in frog melanotrophs, GABA inhibits adenylate cyclase activity through activation of a GABA_B receptor coupled to a PTX-sensitive G protein. Concurrently, baclofen decreased the concentration of IP_3 in the frog pars intermedia. The GABA-induced reduction of phosphoinositide

![Diagram of GABA effect on adenylate cyclase](image)

**FIGURE 4.** Effect of the GABA_B antagonists phaclofen and CGP35348 on baclofen-induced inhibition of α-MSH release from perifused frog NILs. After a 90-min stabilization period, the lobes were perifused in the absence (●) or presence (■) of (a) phaclofen (100 μM) or (b) CGP35348 (300 μM). Thirty minutes after the onset of the administration of the GABA_B antagonists, baclofen (100 μM) was infused for 20 min. Each profile represents the mean secretion pattern (± S.E.M.) of three independent experiments. For each experiment, the basal level of α-MSH secretion was calculated as the mean of four consecutive fractions preceding the infusion of baclofen. The basal rates of α-MSH secretion (100% basal level) in these experiments were 136.5 ± 10.8 (control), 121.4 ± 6.6 (phaclofen) and 119.5 ± 2.4 (CGP35348) pg/min per lobe. α-MSH-LI, α-MSH-like immunoactivity.
turnover was a delayed event, suggesting that the decrease in IP₃ production can be ascribed to an indirect effect on phospholipase C. Consistent with this notion, it has previously been shown that activation of GABA₉ receptors reduces [Ca²⁺], (Bormann 1988, Taraskevich & Douglas 1990, Shibuya et al. 1992), and that blockage of calcium influx inhibits IP₃ production in frog pars intermedia cells (Desrues et al. 1993).

Four subtypes of GABA₉ receptor have been recently characterized in the rat cerebral cortex (Raiteri et al. 1992). All of them are baclofen-sensitive, but each receptor type exhibits variable sensitivity to the novel antagonists phaclofen and CGP35348 (Raiteri et al. 1992). In rat melanotrophs, both CGP35348 and phaclofen are potent antagonists of GABA₉ receptors (Shibuya et al. 1992). In the toad Xenopus laevis, CGP35348 totally blocks the inhibitory effect of baclofen on α-MSH release (Shibuya et al. 1991), whereas phaclofen is only a weak antagonist (de Koning et al. 1993). We have now shown that, in the frog Rana ridibunda, neither phaclofen nor CGP35348 affects the inhibitory action of baclofen on α-MSH release. Previous studies have also demonstrated that, in our model, the inhibition of α-MSH secretion induced by baclofen is not attenuated by the GABA₉ antagonist SR95531 (Tonon et al. 1989). Taken together, these observations reveal the existence, on frog melanotrophs, of GABA₉ receptors negatively coupled to adenylate cyclase. Our data also indicate that the pharmacological profile of this receptor is distinct from those previously characterized in other vertebrate species.

The present study shows that administration of forskolin to perifused frog NILs prolonged the stimulatory phase and attenuated the inhibitory phase induced by GABA₉ agonists, indicating that

FIGURE 5. Effect of forskolin on GABA agonist-induced modulation of α-MSH release from perifused frog NILs. After a 90-min stabilization period, the lobes were perifused in the absence (●) or presence (■) of forskolin (10 μM). Forty-five minutes after the onset of forskolin administration, (a) baclofen, (b) GABA or (c) 3APS (100 μM each) was infused for 20 min. Each profile represents the mean secretion pattern (±S.E.M.) of three independent experiments. For each experiment, the basal level of α-MSH secretion was calculated as the mean of four consecutive fractions preceding the infusion of GABA or GABA agonist. The basal rates of α-MSH secretion (100% basal level) in these experiments were 161 ± 7 ± 12.6 (control) and 202.5 ± 15.0 (forskolin) pg/min per lobe. *P<0.05 compared with forskolin treatment (Student’s t-test). α-MSH-LI, α-MSH-like immunoreactivity.

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cAMP modulates the activity of the GABA<sub>A</sub> receptor in pituitary melanotrophs. Since activation of GABA<sub>B</sub> receptors causes a reduction in both cAMP and IP<sub>3</sub> formation, these data suggest that GABA<sub>B</sub> receptors may indirectly affect the functioning of GABA<sub>A</sub> receptors through inhibition of cAMP-dependent protein kinases (PKA) and calcium-dependent protein kinases (PKC). In support of this hypothesis, biochemical studies have demonstrated that the GABA<sub>A</sub> receptor can be directly phosphorylated by both PKA (Kirkness et al. 1989, Browning et al. 1990, Moss et al. 1992) and PKC (Browning et al. 1990, Moss et al. 1992).

TABLE 3. Effect of GABA agonists and an antagonist on [Ca<sup>2+</sup>]<sub>i</sub> mobilization by frog NILs. After a 10-min incubation with LiCl (10 mM), the lobes were incubated for 20 min in the absence or presence of test substances (100 μM each). The results are expressed as percentages of the [Ca<sup>2+</sup>]<sub>i</sub> level in control lobes (270 ± 20 c.p.m. per lobe). Values are the means ± s.e.m. of four determinations.

<table>
<thead>
<tr>
<th>GABA agonist</th>
<th>% of control ± s.e.m.</th>
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<tr>
<td>Control</td>
<td>100.0 ± 7.4</td>
</tr>
<tr>
<td>GABA</td>
<td>71.8 ± 4.4*</td>
</tr>
<tr>
<td>3APS</td>
<td>108.1 ± 7.8</td>
</tr>
<tr>
<td>Baclofen</td>
<td>61.1 ± 5.9**</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>93.3 ± 5.6</td>
</tr>
<tr>
<td>Bicuculline + GABA</td>
<td>69.8 ± 5.9*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 compared with control (Student’s t-test).

It has been reported that phosphorylation of the GABA<sub>A</sub> receptor complex causes either inhibition (Harrison & Lambert 1989, Heuschenieder & Schwartz 1989, Tehrani et al. 1989, Porter et al. 1990) or stimulation of the GABA-gated chloride current (Sessler et al. 1989, Farfitt et al. 1990, Kano & Konnerth 1992). The results presented herein thus suggest that, in frog melanotrophs, the inhibition of adenylate cyclase and phospholipase C mediated by GABA<sub>B</sub> receptors can modulate the activity of GABA<sub>A</sub> receptors.

**Coupling of GABA<sub>A</sub> receptors to calcium mobilization**

The present report demonstrates the existence of a functional link between GABA<sub>A</sub>-induced stimulation of α-MSH secretion and elevation of cytosolic calcium concentration. We first observed that 3APS caused a massive stimulation of [Ca<sup>2+</sup>]<sub>i</sub> in cultured melanotrophs. The fact that GABA<sub>A</sub> receptor agonists did not affect IP<sub>3</sub> formation suggested that the increase in [Ca<sup>2+</sup>]<sub>i</sub> could be accounted for by calcium influx through plasma membrane channels, rather than mobilization of intracellular calcium pools. In support of this hypothesis, our data show that the transient stimulation of α-MSH secretion induced by 3APS is totally suppressed by nifedipine (but not affected by ω-conotoxin), indicating that, in frog melanotrophs, GABA<sub>A</sub> receptors are functionally coupled to L-type calcium channels. Consistent with this finding, we have recently observed that the stimulation of [Ca<sup>2+</sup>]<sub>i</sub> induced by 3APS is also blocked by nifedipine (data not shown).

The mechanism by which GABA<sub>A</sub> receptor activation induces opening of voltage-operated calcium channels has not been fully elucidated. Stimulation of GABA<sub>A</sub> receptors has been shown to...
increase [Ca^{2+}]_i in various cell types including
oligodendrocytes (Kirchhoff & Kettenmann 1992),
neonatal neurones (Cherubini et al. 1991), newborn
rat pituitary cells (Horvath et al. 1993), rat
melanotrophs (Shibuya et al. 1992) and chromaffin
cells (Doroshenko 1989). In these studies, it was
suggested that the calcium transient resulted from
chloride efflux through GABA_A receptors, leading
to membrane depolarization and activation of
voltage-sensitive calcium channels. In particular, in
chromaffin cells, a diminution of the extracellular
chloride concentration increases the amplitude of
calcium transients, suggesting that an outward
chloride current was responsible for [Ca^{2+}]_i eleva-
tion (Doroshenko 1989). Conversely, our data
show that, in frog melanotrophs, the increase in
[Ca^{2+}]_i, associated with the activation of GABA_A
receptors was abolished when the cells were
incubated in a chloride-free solution, indicating that
the elevation of [Ca^{2+}]_i resulted from chloride efflux
rather than chloride influx. This observation is also
consistent with electrophysiological studies which
revealed that, in the presence of physiological
concentrations of chloride, GABA_A agonists induce
an inward chloride current in frog melanotrophs
(Louiset et al. 1992).

The molecular mechanism associating chloride
influx (and thus hyperpolarization) with activation
of voltage-operated calcium channels is currently a
matter of speculation. In a recent report, Rupnik &
Zorec (1992) have shown that high cytosolic
chloride concentrations increase membrane capaci-
tance in rat pituitary melanotrophs. On the other
hand, Higashijima et al. (1987) have shown that
chloride ions increase the affinity of the α_6 subunit
for GTP, suggesting that cytosolic chloride concentra-
tions may directly activate G proteins. Since

**FIGURE 7.** Effect of voltage-dependent calcium channel
blockers on 3APS-induced α-MSH release from
perifused frog NILs. After a 90-min stabilization period,
the lobes were perifused in the absence (a) or the
presence of (b) ω-conotoxin (1 μM) or (c) nifedipine
(10 μM). Twenty minutes after the onset of the
administration of the calcium channel blockers, 3APS
(0.1 mM) was infused for 20 min. Each profile represents
the mean secretion pattern (± S.E.M.) of three
independent experiments. For each experiment, the
basal level of α-MSH secretion was calculated as the
mean of four consecutive fractions preceding the
infusion of 3APS. The basal rates of α-MSH secretion
(100% basal level) in these experiments were (a)
134.2±5.2, (b) 150.8±6.5 and (c) 132.1±6.3 pg/min per
lobe. *P<0.05 compared with corresponding control (a)
(Student’s t-test). α-MSH-L1, α-MSH-like
immunoreactivity.
electrophysiological studies have shown that, in frog melanotrophs, G proteins directly couple to voltage-operated calcium channels (Valentijn et al. 1991a,b), these observations suggest that an influx in chloride ions may activate L-type calcium channels through a G protein-mediated mechanism.

**Towards a comprehensive model**

A recapitulation of our data is schematically presented in Fig. 8. In frog melanotrophs, activation of GABA_A receptors causes a chloride influx which stimulates L-type calcium channels, yielding a transient increase in hormone secretion. Concurrently, activation of GABA_B receptors causes inhibition of both adenylate cyclase and phospholipase C. The modification of PKA and PKC activities mediated by GABA_B receptors may modulate the functional properties of GABA_A receptors.

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