GABA_B receptors modulate catecholamine secretion in chromaffin cells by a mechanism involving cyclic AMP formation

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1 The function of γ -aminobutyric acid_B (GABA_B) receptors in modulation of catecholamine secretion by chromaffin cells and the possible mechanism involved in this action have been examined.

2 The GABA_B agonists (–)-baclofen and 3-aminopropylphosphinic acid (3-APPA) were found to induce a dose-dependent increase of basal catecholamine secretion. The EC₅₀s were $151 \pm 35 \mu\text{M}$ and $225 \pm 58 \mu\text{M}$ for baclofen and 3-APPA, respectively. This stimulatory effect was specific since it could be blocked by 0.5 mM of the specific GABA_B antagonist CGP-35348.

3 In contrast, preincubation of chromaffin cells with the GABA_B agonists was found to inhibit, in a dose-dependent manner, the catecholamine secretion evoked by $10 \mu\text{M}$ nicotine and $200 \mu\text{M}$ muscimol.

4 The effects of GABA_B agonists on both basal and evoked catecholamine secretion were found to be accompanied by parallel changes in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). GABA_B agonists produced a dose-dependent increase in $[\text{Ca}^{2+}]_i$ which was partially blocked by CGP 35348, but they produced a strong inhibition of the $[\text{Ca}^{2+}]_i$ increase induced by nicotine and muscimol.

5 The GABA_B agonists also produced a dose-dependent increase in intracellular cyclic AMP levels, there being a direct correlation between both increase in catecholamine secretion and in intracellular cyclic AMP levels.

6 The pretreatment of chromaffin cells with pertussis toxin doubled the catecholamine secretion and increased by four times the intracellular cyclic AMP levels evoked by GABA_B agonists.

7 The possible involvement of adenylate cyclase in the mechanism of GABA_B receptor modulation of catecholamine secretion is discussed.

Keywords: GABA_B receptors; catecholamine secretion; chromaffin cells; cyclic AMP; adrenal medulla

Introduction

Different biochemical and physiological evidence concerning the regulation of catecholamine secretion by chromaffin cells show that, in addition to nicotinic cholinergic receptors (nAChR) whose role in adrenal medulla function is well established, there are other receptors implicated in the modulation of this response. These include muscarinic cholinergic receptors (Cheek & Burgoyne, 1985), opioid peptide (Lemaire *et al.*, 1981), substance P (Livett *et al.*, 1979), bradykinin (Owen *et al.*, 1989), histamine H₁ (Chaliss *et al.*, 1991), dopamine D₁ and D₂ (Stauderman & Pruss, 1990; Huettl *et al.*, 1991), prostaglandin E₂ (Negishi *et al.*, 1989; Ito *et al.*, 1991), adenosine (Chern *et al.*, 1988), angiotensin II (Marley *et al.*, 1989; O'Sullivan & Burgoyne, 1989) and GABA_A receptors (Castro *et al.*, 1989).

In relation to γ -aminobutyric acid (GABA) regulation of chromaffin cell secretion, previous work by our group has demonstrated the presence of all the enzymes implicated in GABA metabolism in the bovine adrenal medulla (Fernández-Ramil *et al.*, 1982; 1983) as well as the existence of an active uptake (Oset-Gasque & Aunis, 1989), and an exocytotic release of this neurotransmitter (Oset-Gasque *et al.*, 1990). Moreover, the presence of the two subtypes of GABA receptors, one sensitive to muscimol (GABA_A) and the other insensitive to muscimol but sensitive to baclofen (GABA_B) has also been described (Castro *et al.*, 1988).

The function and molecular mechanism of GABA_A receptors in the adrenal medulla is now well established. Our recent studies demonstrated that when GABA binds to GABA_A receptors in the adrenal medulla it modulates catecholamine secretion by a mechanism dependent on the membrane potential (González *et al.*, 1992). Nevertheless the

function and mechanism of action of GABA_B receptors in adrenal medulla function has not yet been investigated.

GABA_B receptors have recently assumed additional importance because of evidence that they could mediate both pre- and postsynaptic actions in the brain (see Bowery, 1989 for a review). Their presence has also been demonstrated in peripheral tissues and enteric nerve terminals, where they appear to have a function in the regulation of the evoked release of excitatory neurotransmitters and hormones (Bowery *et al.*, 1980; Huston *et al.*, 1990), in the regulation of muscular contraction and tone (see Bowery, 1989, for a review) and in the facilitation of the induction of long-term potentiation (Mott & Lewis, 1991).

These actions seem to be mediated by molecular mechanisms involving the participation of second messenger systems such as adenylate cyclase, inositolphosphate turnover or regulation of Ca²⁺ or K⁺ conductance. However it is not clear whether or not these reactions occur sequentially or independently and which component is directly coupled with GABA_B receptors.

In this paper we examine (1) the role of GABA_B receptors in the regulation of chromaffin catecholamine secretion, (2) the possible interactions between this GABA_B and other receptor systems known to regulate this process and (3) the possible molecular mechanism linked to these actions.

Methods

Cell isolation and culture

Bovine chromaffin cells were isolated from adrenal glands and cultured as described by Bader *et al.* (1981) with minor

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modifications. Cell viability was checked with trypan blue and chromaffin cell purity was assessed by the specific incorporation of 3 mg ml⁻¹ neutral red, which dyes red specifically only the cells which contain catecholamines, to these cells. Viability and purity were always higher than 90%. Cells were suspended in DMEM containing 10% FCS, antibiotics (100 u ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 40 µg ml⁻¹ gentamicin), and cytostatics (10 µM fluoro-deoxyuridine and 10 µM cytosine arabinoside), plated in 24 well Costar cluster dishes, at a density of 0.5×10^6 cells per well, and used 3–7 days after plating.

Measurement of catecholamine secretion

Chromaffin cells were washed twice, at 10 min intervals with 1 ml of a Krebs HEPES solution (Locke medium) containing (mM) NaCl 140, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, glucose 11, ascorbic acid 0.5 and HEPES 15, pH = 7.5, at 37°C. This medium was removed and cells were stimulated, during 10 min periods at 37°C, with 0.5 ml of a fresh Locke medium containing the different secretagogues used as indicated in each type of experiment. At the end of incubation the medium was removed and cells lysed with 0.4 M perchloric acid, and then scraped off the plates. Incubation medium and cell lysates were both used for the determination of catecholamine secretion and total catecholamine content, respectively. The catecholamine determination, in both sample types, adequately diluted and neutralized (see below), was performed with an electrochemical detector (Metrohm 641 VA-detector) adjusted to +580 mV and registered in an LKB recorder model 2210, using for this calibration a standard curve with noradrenaline. Results are presented as a percentage of catecholamine released in the incubation medium in respect of the total catecholamine content (incubation medium + pellet).

Measurement of intracellular levels of cyclic AMP

Chromaffin cells, prepared as indicated above, were stimulated for 10 min with appropriate secretagogues as indicated. Cells were incubated in the presence of 0.5 mM isobutyl methyl xanthine (IBMX) in order to prevent adenosine 3':5'-cyclic monophosphate (cyclic AMP) degradation. The medium was removed, cells lysed with 200 µl of 0.4 M HClO₄, and scraped off the plates. These cell lysates were neutralized with 1 M KOH, centrifuged in an Eppendorf centrifuge and supernatants were used for assay of cyclic AMP. Intracellular cyclic AMP measurements were performed by using the cyclic AMP Radioimmunoassay Kit of Amersham Int., as described in the manufacturer's instructions.

Measurement of cytosolic [Ca²⁺]

Changes in intracellular calcium concentration ([Ca²⁺]_i) were monitored by measuring Fura 2AM fluorescence. Cells were washed twice with Locke medium and incubated with 5 µM Fura 2AM for 45 min at 37°C. Excess dye was removed by washing the cells twice with fresh Locke medium and suspended in this medium at 10^6 cells ml⁻¹. Fluorescence (excitation wavelength = 380 nm, emission = 510 nm, slits, 5 nm exc. 10 nm em.) was monitored at 37°C in a well stirred cuvette containing 1 ml of this suspension with a Perkin-Elmer LS-50 spectrofluorimeter. At the end of each experiment 0.5% triton X-100 was added to permeabilize the cells, allowing the dye to gain access to the extracellular [Ca²⁺] ([Ca²⁺]_e). This [Ca²⁺]_e saturated the dye and provided a measure of the maximum fluorescence signal (F_{max}). To determine the intensity of the minimum fluorescence signals (F_{min}), 20 mM Tris base was added to raise the pH above 8.2

followed by 10 mM EGTA which reduced the [Ca²⁺]_i to less than 1 nM. The [Ca²⁺]_i was calculated with the equation:

$$[Ca^{2+}]_i = K_D (F - F_{min}) / (F_{max} - F)$$

the equilibrium dissociation constant (K_D) for [Ca²⁺]-Fura being 224 nM.

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and foetal calf serum (FCS) were from GIBCO. Collagenase (E.C.3.4.4.19) and 8-(4-chlorophenylthio)-adenosine 3':5' cyclic monophosphate (pClp cyclic AMP), were obtained from Boehringer Mannheim S.A. (Barcelona, Spain). Muscimol, nicotine, forskolin, and 3-isobutyl-1-methyl xanthine (IBMX) were from Sigma Chemical Co (St. Louis, MO, U.S.A.). (–)-Baclofen, CGP 35348 (3-aminopropyl-diethoxymethyl-phosphinic acid) and 3-aminopropylphosphinic acid (3-APPA) were a gift from Ciba-Geigy (Basel, Switzerland). Faclofen was purchased from Tocris Neuramin (Essex, U.K.). The kit for cyclic AMP assay was from Amersham Int. (Bucks., U.K.) and Fura-2 AM was obtained from Molecular Probes (Cambridge, U.K.). All other chemicals were reactive grade products from Merck (Darmstadt, Germany).

Statistics

The data shown are means ± s.e.mean of at least three experiments each one performed in duplicate. Statistical significance was estimated with Student's *t* test for unpaired observations. A *P* value of less than 0.05 was considered significant. Linear regressions were made by the method of minimum squares and fittings of concentration-response curves for estimation of EC₅₀ or IC₅₀ values by a weighed non linear regression of minimum squares, using logistic curves.

Results

Effect of GABA_B agonists on basal catecholamine secretion

Stimulation of GABA_B receptors with their specific agonists baclofen and 3-APPA increase basal catecholamine secretion from chromaffin cells in culture. This effect was dose-dependent in both cases with EC₅₀s of 151 ± 35 µM and 225 ± 58 µM for baclofen and 3-APPA, respectively (Figure 1). Maximal responses were achieved at concentrations between 0.5 and 1 mM of agonists and were 2.73 ± 0.52 and of 2.32 ± 0.44 times the basal catecholamine secretion for stimulation with 3-APPA and baclofen, respectively.

The stimulatory effect of GABA_B agonists on basal catecholamine secretion was Ca²⁺-dependent and specific since it was inhibited between 75 and 95% in an extracellular medium containing 200 nM CaCl₂ and it could be blocked by the specific GABA_B antagonist CGP 35348. At a dose of 0.5 mM this compound was able to inhibit the agonist response between 67 and 100%. In contrast, the antagonist faclofen was ineffective at the same dose (Figure 2). None of the antagonists had any significant effect on basal catecholamine secretion at doses between 10 µM and 1 mM (Figure 2).

To test the possible cell damage produced by GABA_B agonists on chromaffin cells, experiments of trypan blue permeation and LDH release were carried out. These experiments showed no significant differences from controls (cellular viability between 90–95%, and LDH release of $3.98 \pm 1.18\%$ over total cellular content).

GABA_B agonists were also able to evoke secretion of the intravesicular component dopamine-β-hydroxylase (DBH) in a similar percentage to that for catecholamines (0.5 mM 3-

Figure 1 Dose-response curves for the effect of GABA_B agonists on basal catecholamine (CA) release by chromaffin cells. Cells were incubated with the indicated doses of 3-aminopropylphosphinic acid (3-APPA, ●) or baclofen (○) at 37°C. Catecholamine release over a 10 min period is expressed as percentage of maximal responses ($6.84 \pm 0.59\%$ and $5.8 \pm 0.45\%$ of total cellular catecholamine content for 3-APPA and baclofen, respectively) in which basal catecholamine secretion ($2.58 \pm 0.55\%$ of total cellular catecholamine content) was subtracted. Release data are means $\pm$ s.e. mean of three independent determinations each performed in duplicate. EC_{50} for 3-APPA = $225 \pm 58 \mu M$ and for baclofen = $151 \pm 35 \mu M$.

Figure 2 Effect of antagonists of GABA_B receptors on catecholamine (CA) secretion evoked by GABA_B agonists. Cells were incubated with Locke solution (■) or the antagonists baclofen 0.5 mM (▨) or CGP 35348 0.5 mM (▩) at 37°C for 10 min. The medium was removed and cells restimulated with a mixture of the indicated GABA_B agonists and antagonists. Results are expressed as ratios to their respective basal values, which were taken as the unit. Columns represent means and bars s.e. mean of three duplicate determinations. Significantly different from the corresponding controls without antagonist at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Basal catecholamine secretions were: Locke solution = 2.5 ± 0.1 , CGP 35348 0.5 mM = 2.47 ± 0.09 and baclofen 0.5 mM = $2.61 \pm 0.16\%$ over total cellular catecholamine content.

APPA and 0.5 mM baclofen produced an increase in DBH release of 2.03 ± 0.11 and 1.69 ± 0.09 times, respectively, the control values).

Effect of GABA_B agonists on evoked catecholamine secretion

In contrast to the effect of GABA_B agonists on basal catecholamine secretion, preincubation of chromaffin cells

Figure 3 Concentration-dependent inhibition of (a) nicotine and (b) muscimol-evoked catecholamine (CA) release by GABA_B agonists. Chromaffin cells were incubated with the indicated concentrations of the GABA_B agonists (○, baclofen; ●, 3-aminopropylphosphinic acid) at 37°C for 10 min before stimulation with (a) 10 μM nicotine or (b) 200 μM muscimol. Results are expressed as percentages of catecholamine release over total cellular content (c.c.). Values represent means $\pm$ s.e. mean of three experiments each one performed in duplicate. (a) IC_{50} for baclofen = $354 \pm 98 \mu M$ and for 3-APPA = $240 \pm 59 \mu M$. (b) IC_{50} for baclofen = $45 \pm 9 \mu M$ and for 3-APPA = $21 \pm 7 \mu M$.

with baclofen or 3-APPA inhibited catecholamine secretion evoked by 10 μM nicotine. This effect was dose-dependent (Figure 3a) with an IC_{50} estimated by log-logit analysis of $240 \pm 59 \mu M$ for 3-APPA and $354 \pm 98 \mu M$ for baclofen.

An inhibitory effect was also observed in the case of the catecholamine secretion evoked by 200 μM muscimol. The effect was dose-dependent the IC_{50} being $21 \pm 7 \mu M$ for 3-APPA and $45 \pm 9 \mu M$ for baclofen (Figure 3b), doses lower than those required for inhibition of 50% of nicotinic responses.

Effect of GABA_B agonists on intracellular levels of calcium

The effect of GABA_B agonists on basal catecholamine secretion was accompanied by a dose-dependent increase in the intracellular calcium concentration. The EC_{50} s were 25 ± 6 and $60 \pm 10 \mu M$ for 3-APPA and baclofen, respectively (Figure 4). Maximal effects were obtained with 0.5 mM of the agonists and were 250 ± 40 nM for 3-APPA and 225 ± 30 nM for baclofen. This effect was partially blocked by the specific antagonist CGP 35348 which, at 0.5 mM, inhibited 50% of the effect evoked by 0.5 mM 3-APPA (increase of about 200 nM in $[Ca^{2+}]_i$). This increase in $[Ca^{2+}]_i$ was also depen-

Figure 4 Dose response curves of the effect of GABA_B agonists on intracellular calcium concentrations. Cytosolic calcium was measured, as indicated in Methods, after stimulation of cells with indicated doses of 3-aminopropylphosphinic acid (3-APPA, ○) or baclofen (Δ). $[Ca^{2+}]_i$ is expressed as percentage of maximal effects (250 ± 40 nM and 225 ± 30 nM for 3-APPA and baclofen, respectively) in which, basal $[Ca^{2+}]_i$ (175 ± 25 nM) was subtracted. Points are means $\pm$ s.e. mean of 4–5 independent determinations with three different cell batches. EC_{50} for 3-APPA = 25 ± 6 μ M and for baclofen = 60 ± 10 μ M.

dent on Ca^{2+} entry from extracellular medium since in a 200 nM Ca^{2+} -containing medium the increase in $[Ca^{2+}]_i$ was inhibited between 80 and 100%.

In addition, GABA_B agonists decreased the rise in $[Ca^{2+}]_i$ induced by nicotine and muscimol. Figure 5a shows that 0.5 mM 3-APPA or baclofen was able to reduce by 80–90% the increase in $[Ca^{2+}]_i$ evoked by 10 μ M nicotine. Similar results were obtained in the case of stimulation of cells with 200 μ M muscimol (Figure 5b).

GABA_B agonists increase intracellular levels of cyclic AMP

Stimulation of cells with GABA_B agonists produced a time-dependent increase in the intracellular concentration of cyclic AMP, reaching the maximum effect at 10 min of incubation (data not shown). This effect was dose-dependent (Figure 6a) with EC_{50} s for baclofen of 210 ± 47 μ M and 146 ± 20 μ M determined in the absence or presence, respectively, of 0.5 mM IBMX, an inhibitor of phosphodiesterase used to prevent cyclic AMP degradation.

Correlation between catecholamine secretion and intracellular cyclic AMP levels evoked by GABA_B agonists

Stimulation of GABA_B receptors produced an increase in basal catecholamine secretion as well as in intracellular levels of cyclic AMP. As shown in Figure 6b both parameters are directly related either in the absence or presence of 0.5 mM IBMX. This compound did not have any effect on catecholamine secretion and did not interfere with cyclic AMP determination.

Effect of forskolin and cyclic AMP analogues on catecholamine secretion and intracellular cyclic AMP levels evoked by GABA_B agonists

When cells were stimulated with 3-APPA or baclofen added together with forskolin, the intracellular levels of cyclic AMP increased significantly, as a result of an additive effect (Table 1). However, stimulation of cells with forskolin or pClp

Figure 5 Effect of GABA_B agonists on intracellular calcium concentrations evoked by nicotine and muscimol. Cytosolic Ca^{2+} transients elicited by stimulation of chromaffin cells with (a) 10 μ M nicotine and (b) 200 μ M muscimol in the absence or presence of 0.5 mM 3-APPA or baclofen. Superimposed records from different cell samples are traced. Drugs were added at times indicated by the arrows. Nic, 10 μ M nicotine; Mus, 200 μ M muscimol; 3-APPA, 0.5 mM 3-aminopropylphosphinic acid; Bac, 0.5 mM baclofen. Similar records were obtained from different samples in three different cell batches.

cyclic AMP together with GABA_B agonists, baclofen or 3-APPA, did not produce any further increase in catecholamine secretion but instead resulted in a reduction of secretion evoked by each agent independently (Table 1).

Effects of preincubation with bacterial toxins on GABA_B agonist-mediated responses in chromaffin cells

To study the participation of G proteins in the mechanism of action of GABA_B receptors, cells were preincubated with

Figure 6 Effect GABA_B agonists on intracellular levels of cyclic AMP in chromaffin cells. Cells were incubated with indicated concentrations of baclofen in the absence (○) or presence (●) of 0.5 mM isobutyl methylxanthine (IBMX) for 10 min and then cyclic AMP levels measured as indicated in Methods. (a) Dose-response curves at indicated doses of baclofen in the presence or absence of IBMX. Results are expressed as percentages of maximal effects (7.2 ± 0.5 and 6.16 ± 0.34 pmol cyclic AMP/ 10^6 cells for baclofen in the presence or absence, respectively of IBMX) in which basal [cyclic AMP] values (3.85 ± 0.15 pmol cyclic AMP/ 10^6 cells in the absence and 3.6 ± 0.2 pmol cyclic AMP/ 10^6 cells in the presence of 0.5 mM IBMX) were subtracted. Values are means $\pm$ s.e. mean of three independent determinations each performed in duplicate. $EC_{50} = 146 \pm 20$ μ M in the presence of IBMX and 210 ± 47 μ M in the absence of IBMX. (b) Correlation between catecholamine (CA) secretion and cyclic AMP level rises induced by GABA_B agonists. Data represent % of variation over the corresponding controls of catecholamine secretion ($3.2 \pm 0.1\%$ of total cellular content) and of cyclic AMP levels (4.16 ± 0.23 pmol cyclic AMP/ 10^6 cells). Rects were fitted by minimum squares and were of $y = 5.18 \times - 5.18$; $***P < 0.001$, $n = 13$ (without IBMX); and $y = 1.77 \times - 1.18$; $**P < 0.01$, $n = 16$ (with IBMX). In the absence of IBMX $r = 0.87$ and in its presence $r = 0.96$.

cholera toxin (CTX) or pertussis toxin (PTX) for 4 h and then the effect of GABA_B agonists on catecholamine secretion and intracellular cyclic AMP levels determined.

Figure 7a shows that the effect of baclofen on cyclic AMP levels was approximately doubled in cells pretreated with PTX, but was inhibited by pretreatment with CTX. Moreover, pretreatment of cells with PTX increase by 4.5 times the catecholamine secretion evoked by baclofen but the effect of baclofen was not inhibited after pretreatment with CTX (Figure 7b).

The same effects on cyclic AMP levels and catecholamine secretion were shown with 1 μ M forskolin when cells were preincubated with PTX but was different when cells were pretreated with CTX. In the latter case there was a rise in intracellular levels of cyclic AMP and, as a consequence, an increase in catecholamine secretion (Figure 7a and b).

Figure 7 Effect of bacterial toxins on (a) intracellular levels of cyclic AMP and (b) catecholamine (CA) secretion induced by baclofen and forskolin. Cells were incubated with Locke solution (control), 300 ng ml⁻¹ choleric toxin or 100 ng ml⁻¹ pertussis toxin for 4 h at 37°C. Then the medium was removed and cells incubated with normal Locke (□), 100 μ M baclofen (▨) or 1 μ M forskolin (▩) for 10 min and cyclic AMP levels (a) or catecholamine secretion (b) measured. Results are means $\pm$ s.e. mean of three determinations each one performed in duplicate. Significantly different from the corresponding controls without toxins at $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

Table 1 Effect of preincubation with forskolin on catecholamine secretion and cyclic AMP levels evoked by GABA_B agonists

Drug	Intracellular cyclic AMP (pmol/ 10^6 cells)	Catecholamine secretion (% of total c.c.)
<i>- Forskolin</i>		
Control	4.43 ± 14	2.79 ± 0.10
Baclofen 100 μ M	$6.20 \pm 0.19^{***}$	$4.30 \pm 0.27^{***}$
Baclofen 500 μ M	$8.95 \pm 0.61^{***}$	$6.28 \pm 0.80^{**}$
3-APPA 100 μ M	$8.42 \pm 0.42^{***}$	$4.60 \pm 0.52^{**}$
3-APPA 500 μ M	$13.00 \pm 0.83^{***}$	$7.40 \pm 1.04^{**}$
<i>+ Forskolin 1 μM</i>		
Control	25.36 ± 1.97	6.13 ± 0.47
Baclofen 100 μ M	$30.25 \pm 0.52^*$	$2.34 \pm 0.05^{***}$
Baclofen 500 μ M	$33.70 \pm 1.97^{**}$	$3.42 \pm 0.05^{***}$
3-APPA 100 μ M	$34.10 \pm 0.50^{**}$	$3.18 \pm 0.04^{***}$
3-APPA 500 μ M	$38.09 \pm 0.98^{***}$	$2.62 \pm 0.18^{***}$

Cells were incubated with Locke solution (control) or GABA_B agonists at the indicated concentrations in the absence or presence of 1 μ M forskolin and then catecholamine secretion and [cyclic AMP] measured. Results are means $\pm$ s.e. mean of three duplicate determinations. Significantly different from the corresponding controls (in the absence or presence of forskolin) at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. c.c. = cellular content.

Discussion

Exocytotic release of catecholamines is a very complex event in the regulation of which numerous proteins are involved. A few years ago, the only receptor clearly linked to the stimulation of the exocytosis of catecholamine in bovine chromaffin cells was nAChR, but at present, a large number of other receptors are known to be able to stimulate catecholamine secretion by themselves or modulate nicotine-induced secretion (for a review see Burgoyne, 1991). At present, one of the most important neurotransmitters implicated in this regulation is GABA.

GABA has two binding sites in the adrenal medulla, characterized as GABA_A and GABA_B receptors because of their capacity to be blocked with saturating concentrations of muscimol or baclofen, respectively and their sensitivity to Ca²⁺ ions (Castro *et al.*, 1988). The function of GABA_A receptors has been clearly established (Kataoka *et al.*, 1986; Kitayama *et al.*, 1986; Castro *et al.*, 1989). It is known that stimulation of cells by GABA_A agonists depolarizes the chromaffin cell membrane producing an increase in Cl⁻ efflux through the Cl⁻ channel associated with the GABA_A receptor and as a consequence, a membrane depolarization which culminates in an increase in basal catecholamine secretion (González *et al.*, 1992).

The function of GABA_B receptors in adrenal chromaffin cells has not been studied before. Results in this paper show for the first time that the GABA_B agonists baclofen and, the most potent agonist available, 3-APPA increase basal catecholamine secretion in a dose-dependent manner. The EC₅₀ of this effect was estimated as 225 ± 58 μM for 3-APPA and 151 ± 35 μM for baclofen, respectively. These doses are high compared with the concentrations of other agonists needed to stimulate 50% of catecholamine secretion (i.e. nicotine, muscimol and histamine have an EC₅₀ of 5–10 μM), and even higher than the doses required to stimulate other GABA_B responses in the mammalian brain (i.e. between 10–100 μM in GABA_B mediated inhibitory post-synaptic potentials or hyperpolarizations in the pyramidal cells of the hippocampus). However they are of the same order of magnitude as those required to stimulate GABA_B responses in other peripheral tissues such as the inhibition of ileum peristalsis or inhibition of glutamate, acetylcholine and noradrenaline release in the peripheral nervous system (100–300 μM), although in all these cases GABA_B responses were inhibitory. These findings together with the fact that GABA_B responses on chromaffin cell catecholamine secretion represent between 40–50% of those obtained with nicotine at the same doses, seem to suggest either a low affinity of GABA_B receptors or the existence of a low number of GABA_B binding sites in adrenal medullary cells (Castro *et al.*, 1988).

Nevertheless, in spite of the high doses required to stimulate these receptors, the GABA_B-mediated increase in catecholamine secretion from chromaffin cells seems to be a true exocytotic and specific effect. This is supported by the findings that (1) this effect was accompanied by a dose-dependent increase in [Ca²⁺]_i measured by Fura-2 methods (Figure 5a) and was almost completely blocked when catecholamine secretion by these agonists was measured in a low Ca²⁺-containing medium (200 nM), (2) GABA_B agonists were able to release other secretory granular components such as DBH to a similar extent as catecholamines and (3) their effects were able to be blocked by the specific GABA_B antagonist CGP-35348, which, at a dose of 0.5 mM inhibited by 70 to 100% the agonist-evoked responses (Figure 2). Faclofen, another specific GABA_B antagonist, was unable to inhibit GABA_B agonist responses. On the basis of the pharmacological classification of GABA_B receptors of Dutar & Nicholl (1988), this insensitivity to faclofen could mean that GABA_B receptors in the adrenal medulla belong to a presynaptic type, although the finding that the same data were obtained with well characterized postsynaptic GABA_B

receptors (Huston *et al.*, 1990) suggests that this rule may not be universally applicable. Faclofen may be a non-specific and potent GABA_B antagonist in some tissues. In fact this compound has been shown to have GABA_B agonist behaviour in some responses (Michler & Erdő, 1989). Therefore, the precise nature, pre- or post-synaptic, of adrenal medullary GABA_B receptors remains to be determined.

In addition to the effect of GABA_B agonists on basal catecholamine secretion, stimulation of chromaffin cells by these compounds also has an effect on the catecholamine secretion evoked by nACh and GABA_A agonists. When cells were preincubated with GABA_B agonists before nicotine was added, these agonists strongly inhibited nicotine-evoked catecholamine secretion in a dose-dependent manner; the IC₅₀s obtained were of the same order of magnitude as those for activation of basal catecholamine secretion (Figure 3a). This inhibitory effect was accompanied by a reduction in the intracellular Ca²⁺ levels induced by nicotine, results which support the hypothesis of a true inhibitory modulation of nACh receptors by GABA_B ones (Figure 5a). Similar results were also obtained for catecholamine secretion evoked by GABA_A agonists (Figure 3b) and for increases in [Ca²⁺]_i (Figure 5b).

With respect to the molecular mechanism by which GABA_B agonists produce these effects in chromaffin cells it seems to be mediated by the activation of adenylate cyclase (AC). This conclusion is supported by the following experimental data: (1) GABA_B agonists increase intracellular levels of cyclic AMP in a dose-dependent fashion, effects which are well correlated with increases obtained in catecholamine secretion (Figure 6). (2) Several activators of AC as well as forskolin and cyclic AMP analogues increase basal catecholamine secretion in a dose-dependent manner, a direct correlation existing between both parameters, intracellular cyclic AMP levels and catecholamine secretion (Oset-Gasque *et al.*, 1991). (3) Pretreatment of cells with PTX, which by ADP-ribosylation of G_i proteins produces an inhibition of negative control of AC, potentiates cyclic AMP levels and catecholamine secretion induced by GABA_B agonists as well as by forskolin (Figure 7a and b). (4) Pretreatment of cells with CTX, which permanently activates AC, does not produce additional increases in either the cyclic AMP levels or in catecholamine secretion induced by baclofen.

The inhibitory actions of GABA_B agonists on catecholamine secretion evoked by nicotinic and GABA_A receptors could also be explained by this mechanism. This conclusion seems to be supported both by our results which show that forskolin and pClp cyclic AMP have a dose-dependent inhibitory effect on catecholamine secretion evoked by nicotine and muscimol (Oset-Gasque *et al.*, 1991) and by other work showing that cyclic AMP, or agonists which activate AC, may produce an inactivation of nAChR and GABA_A receptors by phosphorylation (Albuquerque *et al.*, 1986; Haganir *et al.*, 1986; Harrison & Lambert, 1989; Leidenheimer *et al.*, 1991). However, although GABA_B agonists seem to mediate their effects on catecholamine secretion by a mechanism implicating cyclic AMP formation, we do not exclude the possibility of the existence of an additional mechanism for the action of these receptors.

Specific interactions between GABA_A and GABA_B receptors have not been described until very recently (Hahner *et al.*, 1991). Our results seem to support a specific modulation of GABA_A receptors by the GABA_B ones, since the IC₅₀ required for inactivation of GABA_A receptors by GABA_B agonists was very much smaller than that required for inactivation of nAChR.

In conclusion, GABA through both its GABA_A and GABA_B receptors, seems to play an important role in the modulation of chromaffin cell function and therefore, GABA could be added to the long list of neurotransmitters and hormones implicated in the modulation of this complex function. The physiological significance of the results presented here is not yet known. It could be possible that ionotropic

GABA receptors, as well as nACh receptors, play a major role in the initiation of catecholamine release *in vivo* while metabotropic receptors, including those for GABA_B, have a more important role in the modulation of the secretion evoked by ionotropic receptors.

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