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Pharmacological modulation of adrenal medullary $GABA_A$ receptor: consistent with its subunit composition

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1 Muscimol, the specific $GABA_A$ receptor agonist, increased the secretion of catecholamines by chromaffin cells with an EC_{50} of 2.9 ± 0.4 μM.

2 $GABA_A$ receptors of these cells were modulated by the same drugs which modulate $GABA_A$ receptors in brain tissue.

3 Benzodiazepines enhanced muscimol-evoked catecholamine secretion by between 20 and 80%. This effect seems to be mediated by binding to a central type of benzodiazepine receptor because it was completely blocked by the specific antagonist, Ro 15 1788. This antagonist was able to displace [^{3}H]-flunitrazepam binding with an EC_{50} of 0.26 ± 0.05 nM.

4 β-Carbolines weakly inhibited muscimol-induced catecholamine secretion and were able to displace [^{3}H]-flunitrazepam binding with an EC_{50} between 0.2 and 0.9 nM, depending on the β-carboline used.

5 Pregnanolone and related neuroactive steroids enhanced muscimol-evoked catecholamine secretion by up to 87%, in a dose-dependent fashion. In contrast pregnenolone weakly inhibited muscimol-evoked catecholamine secretion.

6 Zn^{2+} did not affect $GABA_A$ receptor-induced catecholamine secretion.

7 These pharmacological results are absolutely concordant with the theoretical properties given by the $GABA_A$ receptor subunit composition of bovine adrenal medulla -α_1, α_4, β_{1-3}, γ_2- previously characterized by Western blot analysis.

Keywords: $GABA_A$ receptors; catecholamine secretion; chromaffin cells; benzodiazepines; β-carbolines; steroids; Zn^{2+}

Introduction

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS) is also shown to have important functional roles in the peripheral nervous system (PNS), such as the regulation of hormone secretion. This is the case with the adrenal medulla, where GABA regulates catecholamine (CA) secretion (Castro *et al.*, 1989). This function is mainly mediated by binding to $GABA_A$ receptors (Kataoka *et al.*, 1984; Castro *et al.*, 1988), through a mechanism dependent on the membrane potential (González *et al.*, 1992).

The $GABA_A$/benzodizepine ($GABA_A$/BZ) receptor complex is a hetero-olimeric protein composed of several distinct polypeptides. Five well characterized homologous subunits (α, β, γ, δ and ρ) form a family of subtypes (α_1-α_6, β_1-β_4, γ_1-γ_3, ρ_1-ρ_2), with similar but distinct gene sequences (Levitan *et al.*, 1988; Lüdens & Wisden, 1991), whose combinations result in heterogeneous populations of $GABA_A$ receptors with different pharmacological properties (see Sigel *et al.*, 1990; Verdoon *et al.*, 1990; Smart *et al.*, 1991; Wafford *et al.*, 1992).

In the adrenal medulla, the $GABA_A$ receptor was shown to be formed by α_1, α_4, β_{1-3} and γ_2 subunits by using Western blot analysis (Parramón *et al.*, 1994). Although the relevant pharmacology of $GABA_A$ receptors has been extensively studied in central structures, only partial pharmacological studies in adrenal medulla have appeared in the literature (Bormann & Clapham, 1985; Cottrel *et al.*, 1987; Peters *et al.*, 1988; 1989; Kitayama *et al.*, 1989). Moreover, these studies did not take into account the molecular structure of the receptor.

In this work we have performed a complete pharmacological characterization of adrenal chromaffin cell $GABA_A$ receptors with a wide range of substances known to modulate the action of $GABA_A$ receptors (Sieghart, 1992) and, for the first time, we have compared these pharmacological properties with theoretical characteristics conferred by the previously demonstrated $GABA_A$ receptor structure. Results show that the pharmacological characteristics found experimentally are absolutely concordant with the molecular composition.

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Methods

Cell isolation and culture

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Bader *et al.* (1981) (with minor modifications). Cell viability was checked with trypan blue and chromaffin cell purity was assayed by the specific incorporation of neutral red to these cells. Both parameters were routinely higher than 90%. Cells were suspended in DMEM containing 10% FCS, antibiotics (199 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 40 μg ml^{-1} gentamicin), and cytostatics (10 μM fluoro-deoxiuridine and 10 μM cytosine arabinoside), and plated in 24 well Costar cluster dishes, at a density of 5×10^5 cells/well, and used 3-7 days after plating for CA measurements.

Preparation of membranes from bovine adrenal medulla

Membranes from bovine adrenal medulla were prepared as previously described (Stephenson *et al.*, 1982). Basically, 40 g of adrenal tissue were chopped and homogenized in a Waring blender in 10 mM HEPES (pH 7.5), 1 mM EDTA, 300 mM sucrose, 0.5 mM dithiothreitol, 1 mM benzamidine/HCl, 0.3 mM phenylmethylsulphonyl fluoride (PMSF), (400 ml final volume). All operations were performed at 4°C. The homogenate was centrifuged at 1,000 *g* for 35 min. The pellet was resuspended, using glass-Teflon homogenizer, in 200-300 ml of the same medium except that sucrose and PMSF were omitted. After re-centrifugation the pellet was resuspended in the latter medium (final volume 40 ml; 12-16 mg protein ml^{-1}).

Measurement of catecholamine secretion

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, $KHPO_4$ 1.2, glucose 11, ascorbic acid 0.5 and HEPES 15, pH 7.5, at 37°C. This medium was removed from the well and cells were stimulated, for a 10 min period at 37°C, with 0.25 ml of fresh Locke medium containing the different secretagogues used, as indicated for each type of experiment. The pharmacological study with β-carbolines and benzodiazepines was performed by pre-incubating the cells for 15 min with different concentrations of modulators and further incubations with modulator plus muscimol. This treatment ensures the complete saturation of the $GABA_A$ receptor by the modulator. At the end of each incubation the medium was removed and the cells lysed with 0.4 mM perchloric acid and scraped off the plates. Both, incubation medium and cell lysates were used for the determination of CA secretion and total CA content, respectively. CA determination in both sample types was performed with an electrochemical detector (Metrohm 641 VA-detector) adjusted to +580 mV and registered in an LKB recorder model 2210, using a standard curve of commercial noradrenaline for their calibration. Results were expressed as a percentage of CA release in the incubation medium with respect to the total CA content (incubation medium + pellet).

Binding assays in solution

Radioligand binding assays were performed on adrenal medulla membranes as previously described (Duggan & Stephenson, 1988). Membranes were diluted into 20 mM K phosphate (pH 7.4), 0.1 mM EDTA, 0.1% (w/v) Triton X-100. Diluted membranes (160 μl; 160 μg protein) were incubated on ice with 10 nM [^{3}H]-flunitrazepam and either unlabelled flunitrazepam (10 μM final concentration), giving values for non-specific binding, or β-carbolines and Ro 15-1788 at different concentrations so as to displace the binding of the labelled flunitrazepam. Incubations were carried out at 0°C for 1 h. Duplicates of both incubations were filtered on GF/C glass fibre filters under suction. The filters were washed 3 times with 4 ml of 20 mM K phosphate, pH 7.4, 0.1 mM EDTA, dried and counted in a scintillation counter.

Materials

The following were used: Dulbecco's Modified Eagle's Medium (DMEM) from ICN, Biomedicals Ltd. (Irvine, Scotland); foetal calf serum (FCS) from Sera-Lab. (Sussex, England); collagenase (E.C.3.4.4.19) from Boehringer Mannheim (Barcelona, Spain); N-methyl-[^{3}H]-flunitrazepam (85 Ci mmol^{-1}) was purchased from Amersham International (Aylesbury, Bucks); steroids from Sigma Chemical Co. (St. Louis, MO, U.S.A.); β-carbolines, Ro 15 1788 (see legend to Figure 4) and flunitrazepam were kindly donated by Hoffman la Roche (Basel, Switzerland). All other chemicals were reactive grade products from Merck (Darmstadt, Germany). Glass microfibre filters (GF/C) from Whatman were used in the binding assays.

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. Fitting of concentration-response curves for estimation of EC_{50} values were made by weighted non-linear regression of minimum squares, using logistic curves.

Results

$GABA_A$ receptor is a functional receptor in bovine adrenal chromaffin cells

The specific agonist of the $GABA_A$ receptor, muscimol, was able to increase CA secretion in a dose-dependent manner. Dose-response curves (Figure 1) yielded an EC_{50} value of 2.9 ± 0.4 μM.

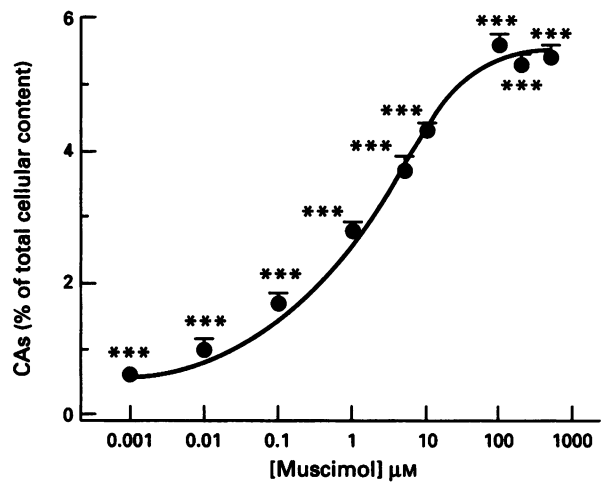

Figure 1 Dose-response curve of muscimol-evoked catecholamine (CA) secretion. Cells were incubated with the indicated doses of agonist and CA secretion was measured as described in Methods. Each point represents the percentage of secretion with respect to total cellular CA content in which basal value (4.11 ± 0.23%) was subtracted. Data points are the mean ± s.e.mean of 6 experiments performed in duplicate ($n = 12$). $EC_{50} = 2.9 \pm 0.4$ μM. Statistical analyses refer to basal value at (***)$P < 0.001$ (Student's *t* test).

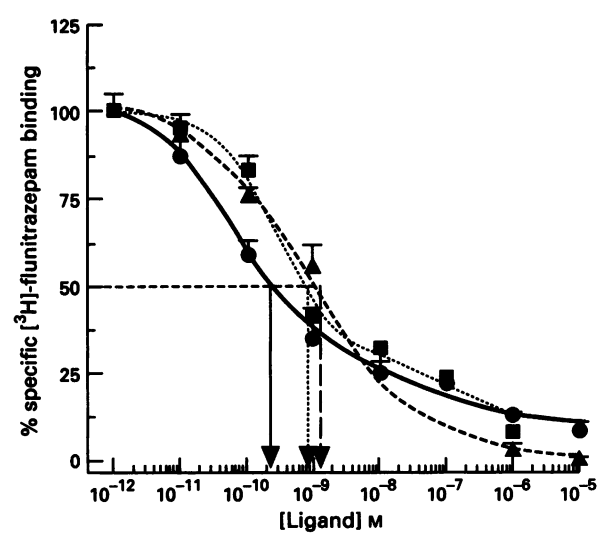

Figure 2 Specific [^{3}H]-flunitrazepam binding displacement by β-carbolines. Binding assay with adrenal medulla membranes was performed as described in Methods in the presence of indicated concentrations of the β-carbolines: (●) β-CCE, (■) β-CCM and (▲) β-CCP. Data points represent the percentages of specific binding, taking the specific binding of 10 nM [^{3}H]-flunitrazepam alone as 100%. Specific binding values were calculated from the difference between total and non-specific binding with cold 10 μM flunitrazepam. EC_{50} (nM) = 0.2 ± 0.07, 0.8 ± 0.2 and 0.9 ± 0.3 for β-CCE, β-CCM and β-CCP, respectively.

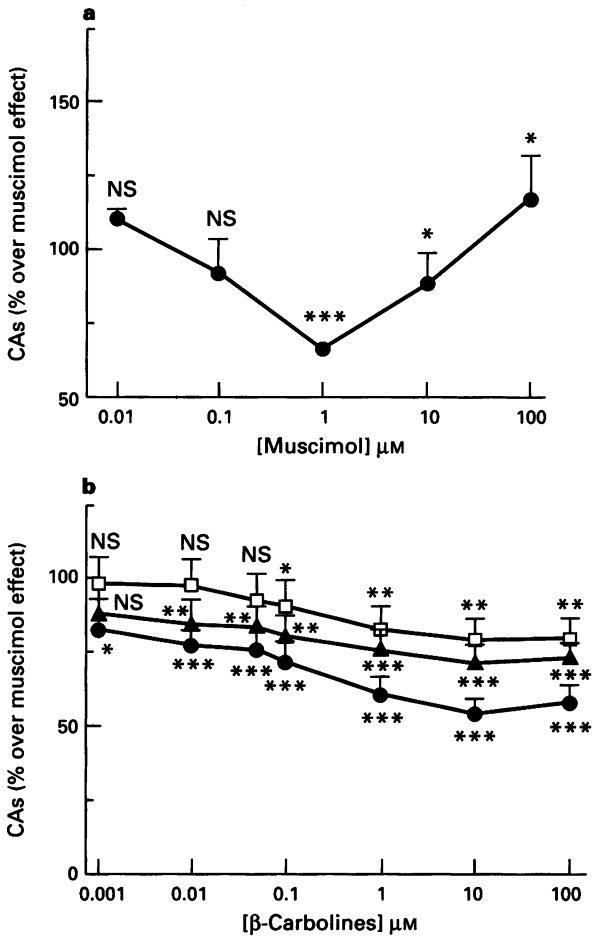

Figure 3 Modulation of the secretory effect of $GABA_A$ agonists by β-carbolines in adrenal chromaffin cells. Cells were treated with 10 μM β-CCE and different concentrations of muscimol (a) or 1 μM muscimol and different concentrations of β-carbolines (b): (□) β-CCM; (▲) β-CCP and (●) β-CCE. Secretion was measured as described in Methods. Data (mean ± s.e.mean, $n=8$) are represented as percentages over muscimol effect at each point. Statistical analyses are referred to muscimol effect at given concentrations. NS, non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$ (Student's t test).

$GABA_A$ receptor modulation by β-carbolines

β-Carbolines are convulsant compounds considered to be 'inverse agonists' of $GABA_A$ receptors. Binding experiments were performed with three available β-carbolines: ethyl-β carboline-3-carboxylate (β-CCE) and its methyl and propyl esters (β-CCM and β-CCP, respectively). All of them showed high affinity binding to $GABA_A$/BZ receptors. EC_{50} values for [^{3}H]-flunitrazepam displacement were 0.2 ± 0.07, 0.8 ± 0.2 and 0.9 ± 0.3 for β-CCE, β-CCM and β-CCP, respectively (Figure 2).

In addition, studies on CA secretion showed that β-carbolines themselves did not induce significant CA secretion at the concentrations indicated in Figure 3 (data not shown). Nevertheless, β-carbolines were able to modulate muscimol-evoked CA secretion showing a dose-response curve suggesting that they behave as inverse agonists of the BZ receptor (Figure 3a): that is to say an inhibitory effect at low doses of muscimol (between 1 and 10 μM) and an excitatory effect at high doses (100 μM), the inverse of the effect shown by BZs (see below). β-CCE (10 μM) had a maximum inhibitory effect upon muscimol-evoked CA secretion at 1 μM muscimol (44% inhibition). The dose-dependent nature of the inhibitory action of β-carbolines upon the effect of 1 μM muscimol was shown by the use of a range of concentrations (Figure 3b).

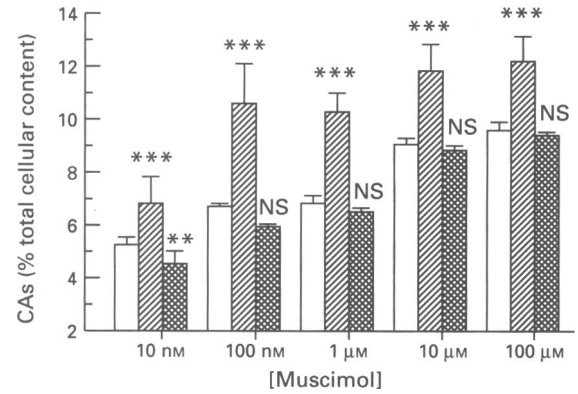

Figure 4 Modulation of the secretory effect of $GABA_A$ agonists by benzodiazepines in adrenal chromaffin cells. Specific inhibitory action of Ro 15 1788. Cells were treated and secretion was measured as described in Methods. Data represent CA secretion over total cellular CA content (basal value = 4.11 ± 0.23%). Open columns, muscimol alone; hatched columns, muscimol plus flunitrazepam (10 nM); cross-hatched columns, muscimol, flunitazepam (10 nM) and Ro 15 1788 (10 μM). Statistical analysis refers to the muscimol effect at each point. Data are means ± s.e.mean of 3 experiments performed in duplicate. NS, non-significant, $**P<0.01$, $***P<0.001$ (Student's t test). Ro 15 1788, ethyl-8-Fl-5,6,dihydro-5-methyl-6-oxo-4H-imidazol(1,5a)(1,4)benzodiazepine-3-carboxylate.

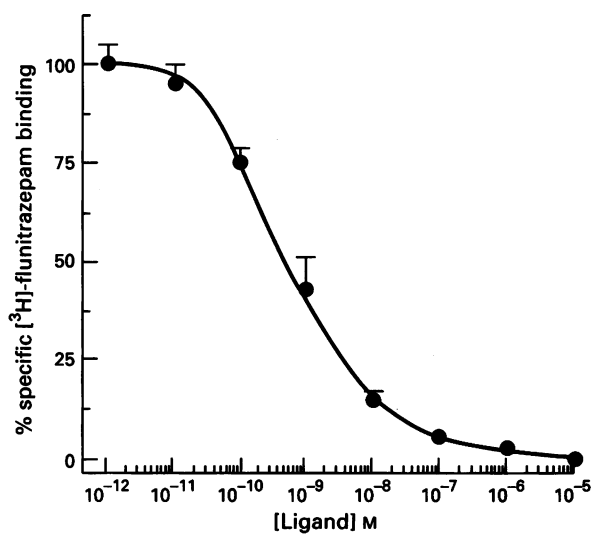

Figure 5 Specific [^{3}H]-flunitrazepam displacement by Ro 15 1788. Binding assay with adrenal medulla membranes was performed as described in Methods. Specific binding was obtained and data are represented as indicated in Figure 2. EC_{50} = 0.26 ± 0.005 nM.

$GABA_A$/BZ receptor can be characterized as a central type BZ receptor

The modulation of muscimol-evoked CA secretion by central BZ agonists (flunitrazepam and diazepam) and antagonists (Ro 15 1788), and Ro 5 4864 (which only acts at the central receptor in high concentrations) was analysed so as to study the central or peripheral nature of $GABA_A$/BZ receptor complex in chromaffin cells.

The effect of muscimol on CA secretion was modulated by BZs such as flunitrazepam and diazepam. Flunitrazepam (10 nM) enhanced muscimol-evoked CA secretion between 25–80% (Figure 4) and diazepam (10 μM) between 20–60% (data not shown). The enhancement produced by flunitrazepam was completely reversed by pretreatment with Ro 15

Table 1 Modulation of the secretory effect of $GABA_A$ agonist by steroids in adrenal chromaffin cells

A		*Steroids* (1 μM)	
	Pregnanolone	*Dexamethasone*	*Alphaxalone*
Muscimol 0.1 μM	1.13±0.05**	1.00±0.01NS	1.16±0.14**
Muscimol 1 μM	1.31±0.05***	1.12±0.14*	1.50±0.20***
Muscimol 10 μM	1.37±0.10***	1.47±0.10**	1.79±0.10***
B		*Steroids* (10 μM)	
	Pregnanolone	*Dexamethasone*	*Alphaxalone*
Muscimol 0.01 μM	1.00±0.03NS	1.0±0.01NS	1.14±0.13*
Muscimol 0.1 μM	1.46±0.50***	1.3±0.20***	1.24±0.03***
Muscimol 1 μM	1.73±0.08***	1.87±0.2***	1.67±0.20***
C	*Pregnenalone* (10 μM)		
Muscimol 0.01 μM	1.13±0.09*		
Muscimol 0.1 μM	1.00±0.09NS		
Muscimol 1 μM	0.85±0.08**		
Muscimol 10 μM	0.86±0.03***		

Values express the net effect on the muscimol effect at each concentration indicated on the left (for comparisons, see Figure 1). **A** and **B** are pregnanolone steroids at 1 μM (**A**) or 10 μM concentrations (**B**); (**C**) is pregnenolone. Data are means±s.e.mean of 4 experiments performed in duplicate. Pregnanolone: 5-β-pregnan-3-ol-20-one; dexamethasone: 9α-F1-16-methyl-prednisolone; alphaxalone: 5α-pregnan-3-α-ol-11,20-dione; pregnenolone: 5-pregnen-3β-ol-20-one. NS, non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$; Student's *t* test.

1788 (Figure 4). By contrast, Ro 5 4684 did not significantly modify the effect of flunitrazepam upon muscimol-evoked CA secretion. In this latter case the maximal inhibition observed was 15±5% ($n=4$, NS). Furthermore Ro 15 1788 produced a highly specific displacement of [^{3}H]-flunitrazepam binding with an EC_{50} of 0.26±0.05 nM (Figure 5).

As well as the main stimulatory effect of BZs an additional inhibitory effect was shown but only at high concentrations of BZs (100 μM) (data not shown).

$GABA_A$ receptor modulation by steroids

Different steroids, both natural (pregnanolone) and synthetic (dexamethasone, alphaxalone), which are known to be positive modulators of $GABA_A$ receptors in the CNS, were tested on basal and muscimol-evoked CA secretion in chromaffin cells. Basal CA secretion was not modified by these substances at the concentrations given (data not shown). These steroids at 1 μM concentration produced a dose-dependent enhancement of 0.1–1 μM muscimol-evoked CA secretion (Table 1A). A 10 fold higher concentration of steroids (10 μM) required a 10 fold lower concentration of muscimol in order to observe a similar rate of enhancement of the muscimol-evoked CA secretion (Table 1B).

Pregnenolone, another natural steroid which in the CNS has no effect or even an inhibitory effect (Steiger *et al.*, 1993), was also tested: at 10 μM it had no effect on basal CA secretion but weakly inhibited muscimol-evoked CA secretion, the inhibition being dose-dependent (Table 1C).

Zn^{2+}-insensitivity of $GABA_A$ receptor

Muscimol (10 μM) evoked CA secretion was not modified by different concentrations of Zn^{2+} between 0.01 and 100 μM (data not shown). Zn^{2+} at the same concentrations did not affect basal CA secretion either (data not shown).

Discussion

Different authors have shown that the specific composition of the $GABA_A$ receptor subunits in a given brain area determines not only the channel properties of the receptor but also its pharmacological profile (Pritchett *et al.*, 1989; Tseng *et al.*, 1994). Adrenal medullary $GABA_A$ receptors have been shown to be formed by α_1, α_4, β_1, β_2, β_3 and γ_2 subunits by Western blot analysis (Parramón *et al.*, 1993; 1994). Most of these subunits have also been shown to be present by Northern blotting of $GABA_A$ receptor subunit mRNAs (Pritchett *et al.*, 1989; Ymer *et al.*, 1989). Homo-olimeric or hetero-olimeric receptors expressed in different systems such as *Xenopus* oocytes (i.e. Wafford *et al.*, 1992) or human embryonic kidney cells (Verdoon, 1994) have allowed some authors to study specific subunit-associated characteristics. From these data, some properties linked with adrenal medulla $GABA_A$ receptors can be deduced: (i) BZ_1 subtype of pharmacologically classified BZ receptors, thus high affinity BZ and β-carboline binding sites; (ii) susceptibility to modulation by BZ and steroids; (iii) Zn^{2+}-insensitivity; (iv) receptor inactivation by phosphorylation by different protein kinases, such as protein kinase A (PKA).

From our studies of the modulation of CA secretion by $GABA_A$ receptor agonists and/or pharmacological modulators, we draw the following conclusions: (1) the $GABA_A$ receptor is modulated by BZ and β-carbolines, and possesses high affinity binding sites for these substances; (2) the $GABA_A$ receptor is modulated by steroids; (3) the $GABA_A$ receptor is insensitive to Zn^{2+}. The relationships between properties (i)-(iv) and pharmacological properties found in this study (1)-(3) are discussed below.

$GABA_A$ receptor modulation by BZ and β-carbolines

BZs flunitrazepam and diazepam enhanced muscimol-evoked CA secretion. These results agree with previous studies by Kitayama *et al.* (1989) using midazolam. The magnitude of the enhancement is not very great (maximal effect was 80%), but it is very similar to this effect in a recombinant receptor α_1, β_1, γ_{2L} expressed in *Xenopus* oocytes (Wafford *et al.*, 1992).

It is generally accepted that two BZ receptors, peripheral and central, coexist within the nervous system. Distinct pharmacological properties allowed both types to be identified because those of the central type are specifically blocked by Ro 15 1788 and those of the peripheral type are specifically stimulated by Ro 5 4864 and blocked by PK11195 (Ohara-Imaizumi, 1991). The adrenal medulla BZ receptor could be considered a central type for several reasons: (a) the coexistence in purified adrenal medullary $GABA_A$ receptors of β subunits (where muscimol binds; Casalotti *et al.*, 1986) together with α subunits (Parramón *et al.*, 1994) (where BZs bind; Casalotti *et al.*, 1986); (b) the antagonist of the central BZ receptor, Ro 15 1788, specifically inhibited the BZ effect on muscimol-evoked CA secretion (Figure 4); (c) the Ro 15 1788 produces a specific displacement of [^{3}H]-flunitrazepam binding (Figure 5); (d) the specific agonist of the peripheral BZ re-

ceptor, Ro 5 4864, did not induce any effect either on basal CA secretion or on flunitrazepam enhancement of muscimol-evoked CA secretion.

The adrenal medullary $GABA_A$ receptor was shown to have an α_1 immunoreactivity in both affinity and immunoaffinity purified receptor preparations (Parramón *et al.*, 1994). The existence of this subunit means that this receptor may be classified as BZ_1 subtype (Schofield, 1989; Sieghart, 1989). BZ_1 receptors are pharmacologically characterized by possession of a high affinity for BZ inverse agonists, β-carbolines (Sieghart, 1989). In fact, it has been suggested that coexpression of α_1, β_1, γ_2 subunits yields a channel with high affinity BZ binding sites which either potentiate (agonists) or reduce (inverse agonists) the GABA-induced Cl^- current (Schofield, 1989). In our hands, β-CCE, β-CCM and β-CCP are able to displace the binding of [^{3}H]-flunitrazepam, thus confirming property (i). The EC_{50} values are very similar for this effect on cerebellum or high affinity sites of the cortex and hippocampus (Duggan & Stephenson, 1988). Moreover, K_D values of flunitrazepam binding were also of the same order of magnitude as those for high affinity binding sites in brain membranes (10 ± 4 nM, Sigel *et al.*, 1983; 2.6 nM, Edgar & Schwartz 1992), purified $GABA_A$ receptors (3.0 ± 0.9 nM, Edgar *et al.*, 1992) and in adrenal medulla membranes (4 ± 0.6 nM, Parramón *et al.*, 1994; 9.8 nM, Kataoka *et al.*, 1984). These observations lead us to suggest that there is a strong homology between BZ binding site of adrenal medulla and brain structures.

β-Carbolines weakly modulate muscimol-evoked CA secretion with a dose-dependent curve corresponding to its role as inverse agonist of the BZ receptor (Figure 3a). In fact, at doses of muscimol in which BZ produced less enhancement of the muscimol effect, β-carbolines produced less inhibition (compare Figures 3 and 4). The inhibitory effect of β-carbolines has been demonstrated to be dose-dependent not only in the case of β-CCE but also with other β-carbolines such as β-CCM and β-CCP.

$GABA_A$ receptor modulation by steroids

The study of the effect of steroids on chromaffin cells is particularly interesting because some of the naturally-occurring steroids are generated by the adrenal cortex. In this way the effect could be considered an autoregulatory effect of the gland itself.

Although steroids by themselves did not have any effect on CA secretion they were able to enhance muscimol-evoked CA secretion (Table 1A and B). This effect was strongly dependent upon the concentration of both muscimol and steroids and compensating for a reduction in the concentration of steroids by an increase in the concentration of muscimol maintained the effect at the same order of magnitude. Dose-dependency for the steroid effect has also been observed in $GABA_A$ receptors expressed in *Xenopus* oocytes (Lin *et al.*, 1992). Note that although significant effects were observed, the enhancement in chromaffin cells was not very high. Moreover, steroids at low doses had a potency similar in magnitude to that proposed by McEwen (1991) in studies on brain tissue.

In contrast to the effect of these pregnanolone steroids, the steroid pregnenolone had a dose-dependent inhibitory effect on muscimol-evoked CA secretion (Table 1c). This steroid, then, seems to behave as an allosteric $GABA_A$ receptor antagonist as suggested in studies on brain areas (Steiger *et al.*, 1993). Finally, the positive or negative actions upon the muscimol effect, of the pregnanolone steroids or pregnenolone respectively, has been observed in the CNS (McEwen, 1991).

The susceptibility to modulation by steroids, which confirms property (ii), is due to the presence of β subunits in adrenal medulla tissue, the presence of which has been shown by Western blot analysis (Parramón *et al.*, 1994). In fact, steroid-sensitivity is apparent when the β alone, or the α plus β or α plus β plus γ subunit DNA is expressed (McEwen, 1991). β-Subunits have also consensus sites for PKA, consisting of serine residues than can be phosphorylated (Harrison & Lambert, 1989).

PKA and other protein kinases such as protein kinase C and protein tyrosine kinase-dependent phosphorylation desensitize and subsequently inhibit the action of the receptor (Leidenheimer, 1991). In a previous study, our group demonstrated that some substances which increase cyclic AMP levels, such as forskolin, non-hydrolyzable analogues of cyclic AMP and $GABA_B$ agonists, inhibit the action of $GABA_A$ receptor due to the formation of cyclic AMP (Oset-Gasque *et al.*, 1993), thus confirming property (iv). The above mentioned results clearly confirm that β subunit of GABA receptors on chromaffin cells also have a dual role, namely both as GABA binding sites and 'modulatory' receptor subunits.

$GABA_A$ receptor insensitivity to Zn^{2+}

The homo-olimeric $GABA_A$ receptor Cl^- channel formed by α_1 or β_1 subunits alone or in combination was inhibited by Zn^{2+}. This compound nevertheless had no effect when the channel contains the γ_2-subunit alone or in combination with α or/and β-subunits (Smart *et al.*, 1991; Sieghart, 1992). At least one α subunit, one β and one γ are needed for a complete functional receptor (Pritchett *et al.*, 1989). Studies using subunit-specific antibodies suggest that subunit combination α_1, $\beta_{2/3}$ and γ_2 is common in the CNS (Mohler *et al.*, 1992). The type I $GABA_A$/BZ receptor composed by these subunits has been recently named type Ia, while associations of α_1 with subunits other than $\beta_{2/3}$ and γ_2 are called type Ib (Ruano *et al.*, 1994). Examination of rat cortex has shown receptors of type Ib to be less abundant than Ia (Ruano *et al.*, 1994).

It is very likely that a hetero-olimeric $\alpha_1\beta_x\gamma_2$ receptor is the most abundant subunit combination. The Zn^{2+}-insensitivity confirms this idea and property (iii).

Conclusions

In the present work we have clearly demonstrated the concordance between the properties conferred by $GABA_A$ receptor subunit composition (Parramón *et al.*, 1994) and their pharmacological properties. Adrenal medullary $GABA_A$ receptors are likely to be composed of a heterogeneous population of receptors. However, the repetitive nature of the results of the assays suggests that one receptor type predominates. It would be very interesting to examine if the expression of $GABA_A$ receptor subunits in the adrenal medulla is cell-type dependent or if $GABA_A$ receptors with different subunit compositions can be expected to be co-expressed within a single cell, as has been proposed for retinal neurones (Grikorenko & Yeh, 1994). $GABA_A$ receptors of the adrenal medulla would have some common properties with other forebrain $GABA_A$ receptors with the same subunits present in the tissue (Person *et al.*, 1992), for instance, the same binding values for BZ and β-carbolines were observed. Surprisingly, in some of these areas, GABA, through $GABA_A$ receptors, depolarizes neurones and increases intracellular Ca^{2+} concentrations (Fiszman *et al.*, 1990) as observed in chromaffin cells (González *et al.*, 1992).

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