Pharmacology of GABA\textsubscript{A} Receptors of Retinal Dopaminergic Neurons

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Feigenspan, Andreas, Stefano Gustincich, and Elio Raviola. Pharmacology of GABA\textsubscript{A} receptors of retinal dopaminergic neurons. J Neurophysiol 84: 1697–1707, 2000. When the vertebrate retina is stimulated by light, a class of amacrine or interplexiform cells release dopamine, a modulator responsible for neural adaptation to light. In the intact retina, dopamine release can be pharmacologically manipulated with agonists and antagonists at GABA\textsubscript{A} receptors, and dopaminergic (DA) cells receive input from GABAergic amacines. Because there are only 450 DA cells in each mouse retina and they cannot be distinguished in the living state from other cells on the basis of their morphology, we used transgenic technology to label DA cells with human placental alkaline phosphatase, an enzyme that resides on the outer surface of the cell membrane. We could therefore identify DA cells in vitro after dissociation of the retina and investigate their activity with whole cell voltage clamp. We describe here the pharmacological properties of the GABA\textsubscript{A} receptors of solitary DA cells. GABA application induces a large inward current carried by chloride ions. The receptors are of the GABA\textsubscript{A} type because the GABA-evoked current is blocked by bicuculline. Their affinity for GABA is very high with an EC\textsubscript{50} value of 7.4 µM. Co-application of benzodiazepine receptor ligands causes a strong increase in the peak current induced by GABA (maximal enhancement: CL-218872 220%: flunitrazepam 214%: zolpidem 348%) proving that DA cells express a type 1 benzodiazepine-receptor (BZ1). GABA-evoked currents are inhibited by Zn\textsuperscript{2+} with an IC\textsubscript{50} of 58.9 ± 8.9 µM. Furthermore, these receptors are strongly potentiated by the modulator alphaxalone with an EC\textsubscript{50} of 340 ± 4 nM. The allosteric modulator loreclezole increases GABA receptor currents by 43% (1 µM) and by 107% (10 µM). Using outside-out patches, we measured in single-channel recordings a main conductance (29 pS) and two subconductance (20 and 9 pS) states. We have previously shown by single-cell RT-PCR and immunocytochemistry that DA cells express seven different GABA\textsubscript{A} receptor subunits (α1, α3, α4, β1, β3, γ1, γ2\textsubscript{δ}, and γ2\textsubscript{L}) and by immunocytochemistry that all subunits are expressed in the intact retina. We show here that at least α1, β3 and γ2 subunits are assembled into functional receptors.

INTRODUCTION

In the vertebrate retina, dopamine is synthesized by a class of neurons that can be either amacrine or interplexiform cells. When the vertebrate retina is stimulated by light, dopamine synthesis and release increase, modulating many of the events that lead to neural adaptation to light. These effects are mediated by metabotropic receptors that are expressed by a large number of cell types throughout the entire retina (see Djamgoz and Wagner 1992; Witkovsky and Deary 1991). In contrast to the wealth of information available on the pharmacological effects of dopamine, little is known about the mechanisms that control its release in the dark and light. Several experiments were carried out measuring the amount of dopamine synthesized or released by the intact retina in different physiological conditions or after addition of various pharmacological agents (see Djamgoz and Wagner 1992). These studies have shown that the release of dopamine can be manipulated with agonists and antagonists at the GABA\textsubscript{A} receptors. GABA and its agonist muscimol can in fact block the synthesis and release of dopamine evoked by light (Kirsch and Wagner 1989; Morgan and Kamp 1980, 1983). Furthermore, antagonists like bicuculline and picrotoxinin induce dopamine synthesis and release in dark-adapted retinas (Critz and Marc 1992; Ishita et al. 1988; Kamp and Morgan 1981; Kirsch and Wagner 1989; Kolbinger and Weiler 1993; Morgan and Kamp 1983; O’Connor et al. 1987; Piccolino et al. 1987).

These results suggest that GABAergic inhibition plays an important role in the regulation of dopamine release in both light and darkness. However, the identification of the target cell is left unsolved because dopaminergic amacrine cells (DA cells) receive multiple inputs and the pathway responsible for the control of dopamine release consists of multiple neurons. DA cells are postsynaptic to various types of amacrine cells (Kolb et al. 1990), and some of them are GABAergic (Kolb et al. 1991; Yazulla and Zucker 1988) suggesting that GABA receptors reside on the DA cells themselves. However, direct evidence can only be obtained by recording from these cells. Because there are only 450 DA cells in each mouse retina and they cannot be distinguished from neighboring cells on the basis of their morphology, we used transgenic technology to label the surface of DA cells with human placental alkaline phosphatase (PLAP) (Gustincich et al. 1997). We were then able to identify DA cells in vitro after dissociation of the retina.

We have previously reported that, in absence of synaptic inputs, solitary DA cells fire action potential in a rhythmic fashion. Furthermore, extracellular application of GABA reversibly inhibits the spontaneous discharge through bicuculline-sensitive receptors (Gustincich et al. 1997), thus proving that DA cells express functional GABA\textsubscript{A} receptors. GABA\textsubscript{A} receptors are pentameric structures that consist of...
various combinations of at least 16 subunits (Barnard et al. 1998; Bormann 2000; Sieghart 1995). In situ hybridization and immunocytoc hemical studies demonstrated that each subunit has a unique distribution in the CNS (Fritschy et al. 1992; Laurie et al. 1992; Wisden et al. 1992); furthermore, transfection experiments proved that the various combinations of sub units found in specific regions of the brain have different pharmacological properties (Rabow et al. 1995).

We have recently shown by single-cell RT-PCR experiments that solitary DA cells contain the messages for seven GABA\(A\) subunits (a1, a3, a4, b1, b3, y1, y2, and y2\(_C\)), and immunocytocytochemistry with subunit-specific antibodies proved that all subunits are expressed by DA cells in the intact retina, where they are associated to form at least two types of synaptic and multiple extrasynaptic receptors.

In this paper, we characterize the pharmacological properties of GABA\(A\) receptors of solitary DA cells, seeking evidence for the functional expression of their repertoire of subunit transcripts.

**METHODS**

**Dissociation of the retina and identification of DA cells**

Anesthetized, 1- to 3-mo-old mice homozygous for the PLAP transgene (Gustincich et al. 1997) were used. PLAP-expressing cells can be identified in the living state by labeling of their membrane with a monoclonal antibody to PLAP (E6) (De Waele et al. 1982) conjugated to the fluorochrome Cy3 (E6-Cy3) (Gustincich et al. 1997). Dissociation of the retina by enzymatic digestion and mechanical trituration was performed as previously described (Gustincich et al. 1997), with a few small changes. After removal of cornea, lens, and vitreous body, the eyecups including the retinas were transferred to 5 ml of digestion buffer (20 U/ml papain, 200 U/ml DNase I; both from Worthington Biochemical, Freehold, NJ) or directly on the screen of a Gateway 4DX2–66 computer. The sample frequency was 20 Hz and 10 kHz in whole cell and single-channel experiments, respectively. Patch pipettes were prepared as 10-mM stock solutions in DMSO and stored at –20°C. The maximal final concentration of DMSO was 0.03%. Bicuculline methiodide (RBI) and picrotoxinin (Sigma) were freshly prepared and added to the GABA-containing solution. A 10-mM stock solution of ZnCl\(_2\) (Fluka, Buchs, Switzerland) and pentobarbital (RBI) were prepared in extracellular solution and kept frozen at –20°C. CL-218872 was a gift from Dr. J. Bormann, and loreclezole was generously provided by Janssen Pharmaceutica (Beerse, Belgium).

**Data analysis**

Effects of modulatory drugs were expressed as \(I_{\text{IC}}\), the ratio of the GABA-induced peak current in the presence of the drug (\(I_d\)) relative to the control GABA response (\(I_c\)), or as percentage drug-induced change of \(I_c\). Dose-response curves were fitted with a logistic sigmoidal equation. GABA-activated currents in outside-out patches were analyzed after low-pass filtering at 1 kHz (–3 dB, 4-pole Bessel filter). The analysis of single-channel amplitudes was carried out with a semi-automatic procedure, where each current step was measured on the computer screen using two cursors. Current-voltage (I-V) relations were obtained by measuring 30 current amplitudes at different membrane potentials (range –70 to 70 mV). The single-channel conductance of the main state and subconductance levels were derived from the slope of the I-V curves by linear regression. Data are expressed as means ± SE; \(n\) is number of DA cells.

**RESULTS**

**GABA concentration-response relationships**

After enzymatic digestion and mechanical trituration of the retina, DA cells were identified in the living state by labeling of their membrane with E6-Cy3 (Gustincich et al. 1997). Cells were voltage clamped at –70 mV with equal concentrations of chloride on either side of the membrane. Under these experimental conditions, we have previously shown that extracellular application of GABA induced a large inward current carried by chloride ions and blocked by bicuculline (Gustincich et al. 1997).

In this paper we present whole cell patch-clamp recordings carried out from 153 DA cells. A stable gigaseal was established in 82% of the cells. GABA was applied at concentrations ranging from 0.3 to 1,000 \(\mu\)M. Desensitization of GABA receptor (GABAR) currents was dose-dependent and became apparent at a GABA concentration of 10 \(\mu\)M (Fig. 1A). The maximal current measured at a saturating concentration of
GABA (1,000 μM) was variable and ranged in amplitude from 24.7 to 28.4 nA with a mean value of 26.3 ± 0.7 nA (mean ± SE, n = 6). Dose-response curves for GABA were obtained from six individual DA cells. Their responses to increasing concentrations of GABA were uniform with an increase in relative current amplitude (Fig. 1B). EC₅₀ values for individual cells ranged from 5.9 to 10.1 μM with a median value of 7.4 μM. For each concentration, the data from the cells were pooled and fitted to a sigmoidal logistic function (Fig. 1C). The mean EC₅₀ value was 7.4 ± 0.7 μM with a Hill coefficient of 1.6 ± 0.3 corresponding to two GABA binding sites on each GABAR. The maximum value of the GABAR current derived by the best fit to the logistic equation was 26,115 pA compared with a mean peak current value of 26,283 pA measured in six cells. This indicated that peak current amplitudes were not significantly affected by desensitization or by redistribution of chloride ions even at saturating GABA concentrations.

Inhibition by bicuculline and picrotoxin

The competitive GABAₐ receptor antagonist bicuculline (concentration range 0.1–100 μM) was co-applied with 10 μM GABA. The block of GABAR currents by increasing concentrations of bicuculline is shown in Fig. 2A. Complete dose-response curves were obtained for six DA cells. The sensitivity of the GABAR current for various concentrations of bicuculline was homogeneous for all neurons examined. Therefore the responses of individual cells were pooled, and the data were fitted to a sigmoidal logistic function (Fig. 2B). Bicuculline (0.1 μM) inhibited GABAR currents by 5%, whereas the current was completely blocked by 100 μM (Fig. 2A). Low concentrations of bicuculline (0.1–1 μM) selectively reduced the peak amplitude of the GABA response, but did not have a significant effect on the steady-state value. The IC₅₀ values for individual cells ranged from 0.8 to 2.2 μM with a median value of 1.4 μM. The mean IC₅₀ value obtained from the fit was 1.4 ± 0.1 μM with a Hill coefficient of 1.0 ± 0.1. These results show that the GABA responses of solitary DA cells are exquisitely sensitive to the inhibitory action of bicuculline and are thus mediated by GABAₐ receptors.

The open chloride channel blocker picrotoxin also inhibited the GABA response of DA cells. At a concentration of 10 μM, picrotoxin reduced the peak inward current evoked by GABA to 36% of control values (Table 1).

Benzodiazepine binding site shows BZ₁ receptor specificity

To study the effects of benzodiazepines on DA cells’ GABAₐ receptors, we co-applied flunitrazepam (1 μM) and 3 μM GABA. Flunitrazepam potentiated the peak current induced by GABA in all cells tested (Table 1). In addition, GABAR currents were uniformly inhibited by the inverse benzodiazepine agonist DMCM (Table 1).

Next we determined the pharmacology of the benzodiazepine binding site by applying agonists with BZ₁-receptor specificity.
When co-applied with 3 μM GABA, the imidazopyridine zolpidem (1 μM), a BZ1-selective drug, increased more than twofold the peak current evoked by GABA (Fig. 3A). Dose-response curves were obtained for seven DA cells; data were pooled and fitted with a sigmoidal logistic function (Fig. 3B). Zolpidem enhanced GABAR function of DA cells with an EC50 value of 126 ± 2 nM, a maximal enhancement of 348 ± 9% and a Hill coefficient of 0.9 ± 0.1.

CL-218872, another BZ1-selective drug with high affinity for the α1 subunit, had a very similar effect on the GABA response of DA cells (Fig. 3C). When co-applied with 3 μM GABA, CL-218872 (1 μM) increased more than twofold the peak current induced by GABA (Table 1), and its effects were completely reversible after wash out (2 min).

These data indicate that benzodiazepine binding sites of DA cell GABA_A receptors conform to the traditional definition of the BZ1 type.

In addition, control GABA-induced currents of all DA cells were increased more than twofold by 50 μM pentobarbital (Table 1). At this concentration, application of pentobarbital alone did not induce an inward current.

**TABLE 1. Modulation of dopaminergic amacrine cell GABA_A receptors**

<table>
<thead>
<tr>
<th>Drug</th>
<th>(I/I_c)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.01 ± 0.01</td>
<td>13</td>
</tr>
<tr>
<td>Picrotoxinin (10 μM)</td>
<td>0.36 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>Flunitrazepam (1 μM)</td>
<td>2.14 ± 0.147</td>
<td>6</td>
</tr>
<tr>
<td>DMCM (1 μM)</td>
<td>0.52 ± 0.06</td>
<td>5</td>
</tr>
<tr>
<td>CL-218872 (1 μM)</td>
<td>2.00 ± 0.07</td>
<td>8</td>
</tr>
<tr>
<td>Pentobarbital (50 μM)</td>
<td>2.36 ± 0.19</td>
<td>9</td>
</tr>
<tr>
<td>Loreclezole (1 μM)</td>
<td>1.43 ± 0.07</td>
<td>6</td>
</tr>
<tr>
<td>Loreclezole (10 μM)</td>
<td>2.07 ± 0.14</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; \(n\) is the number of cells. \(I/I_c\) is the ratio of GABA-induced peak current in the presence (\(I\)) and absence of the drug (\(I_c\)). Drugs were co-applied with 3 μM GABA.
The incomplete inhibition of GABAR currents could reflect the presence of more than one subpopulation of GABA<sub>A</sub> receptors, one Zn<sup>2+</sup>-sensitive and the other Zn<sup>2+</sup>-insensitive. Likewise, we can assume a single GABAR population displaying incomplete block by Zn<sup>2+</sup>. Using recombinant expression systems, it has been shown that the presence of a γ-subunit leads to lower sensitivity to zinc ions (Fisher and MacDonald 1998; Horenstein and Akabas 1998; Smart 1992; Smart et al. 1991), and therefore Zn<sup>2+</sup>-insensitive GABARs are likely to be modulated by benzodiazepines. We then tested on seven DA cells the possibility that the residual GABAR current after Zn<sup>2+</sup> block was mediated by a flunitrazepam-sensitive GABAR subpopulation. First, inward currents elicited by 3 μM GABA were potentiated by 1 μM flunitrazepam (Fig. 5A). After wash out of the drug and complete recovery of the control response, GABA-induced currents of the same neuron were inhibited by 100 μM Zn<sup>2+</sup> to 43 ± 4% of control value (Fig. 5B). When 1 μM flunitrazepam was applied in addition to 100 μM Zn<sup>2+</sup>, the residual current was 47 ± 6% of the control response augmented by flunitrazepam. While flunitrazepam alone potentiated the GABA response of 214 ± 14%, in the presence of 100 μM Zn<sup>2+</sup> currents were enhanced by flunitrazepam of 206 ± 10%. Thus Zn<sup>2+</sup>-insensitive residual current was no more sensitive to benzodiazepines than the Zn<sup>2+</sup>-sensitive GABAR currents. The results of this experiment are summarized in Fig. 5C.

**Steroid enhancement of GABAR currents**

Varying concentrations of the neuroactive steroid alphaxalone (10–3,000 nM) were co-applied with 3 μM GABA. The lowest concentration tested (10 nM) augmented the GABA response by about 23%, and the GABAR currents were more than doubled with 0.3 μM alphaxalone (Fig. 6A). Dose-response curves were obtained from seven DA cells. Because the cells responded uniformly to increasing concentrations of alphaxalone, individual data were pooled and fitted with a sigmoidal logistic function (Fig. 6B). The EC<sub>50</sub> value obtained from the best fit to the data points was 360 ± 4 nM with a Hill slope of 1.4 ± 0.2. The maximal potentiation obtained with 3 μM alphaxalone was 452 ± 37% which is close to the value obtained from the fit (473 ± 17%).

**Effect of loreclezole**

Six DA cells were tested for enhancement of GABA (3 μM)-induced currents by 1 and 10 μM loreclezole, a drug selective for the β2 or β3 subunits. Loreclezole uniformly potentiated the GABA response in all of these cells in a concentration-dependent way, and the rate of apparent desensitization was enhanced with higher concentrations (Fig. 7A). Loreclezole (1 μM) increased GABAR currents by 43 ± 7%, whereas the GABA response was enhanced by 10 μM loreclezole to 207 ± 14%. The effects of different concentrations of loreclezole on GABAR currents are summarized in Fig. 7C.

**Single-channel recordings**

To determine the single-channel conductance, outside-out patches were pulled from the cell bodies of solitary DA cells. Channel openings induced by 2 s GABA (1 μM)
pulses were recorded at a holding potential of −70 mV. Because of the high-density of GABARs on the surface of DA cells, it was not possible to obtain patches that contained only a single GABAR channel. Therefore a detailed kinetic analysis of single-channel properties could not be carried out. Extracellular application of GABA induced bursts of channel openings to multiple conductance levels (Fig. 8A). The main conductance and a subconductance level were determined by recording GABA-induced single-channel openings at various holding potentials. The data points were fitted with a linear regression line, and the resulting conductances of the current-voltage relationships were 29 and 20 pS, respectively (Fig. 8B). Because of the small amplitude, the current-voltage relation of the second subconductance state was obtained by connecting the data points at −70 and 70 mV with a straight line. The slope of this line was 9 pS, representing the conductance of the second sublevel.

The amplitude distribution of the main and two subconductance states were determined in six patches at a holding potential of −70 mV with 1 μM extracellular GABA. The mean current values as determined by Gaussian distributions were −2.06 ± 0.14 pA for the main level and −1.55 ± 0.11 pA and −0.61 ± 0.08 pA, respectively, for the two sublevels (Fig. 8C).

**DISCUSSION**

In this study a transgenic mouse line was used in which DA cells were labeled with PLAP, an enzyme that resides on the outer surface of the cell membrane (Gustincich et al. 1997). After enzymatic digestion and mechanical trituration of the retina, DA cells were identified in the living state by labeling of their membrane with a monoclonal antibody to PLAP conjugated to the fluorochrome Cy3 (E6-Cy3). We have previously shown by single-cell RT-PCR that E6-Cy3–positive neurons are dopaminergic because they contain TH mRNA. Furthermore, we proved that the expression of PLAP on the cell surface did not affect the properties of GABA-gated chloride channels (Gustincich et al. 1997). Many studies of GABAR currents after enzymatic digestion of tissue have been reported, showing no alterations of the responses compared with those recorded from cultured neurons (Kapur and MacDonald 1996).

The pharmacological properties of the GABAR receptors of DA cells were analyzed studying the effects of the application of various drugs that are known to have distinct actions in the presence or absence of specific subunits. Drugs were applied only when stable recordings were obtained, and responses to consecutive applications of 3 μM GABA remained constant. We never observed “run-down” of GABA-induced currents.
during the time course of the recordings (15–20 min). After application and wash out of the drug, GABA was repeatedly applied to ensure complete recovery of the control GABA response.

**GABA sensitivity of DA cells**

The EC$_{50}$ value for acutely isolated DA cells was 7.4 µM, which is slightly lower than that in acutely dissociated pyramidal neurons (25.4 µM) (Celentano and Wong 1994), in adult cortical neurons (40.3 µM), and in thalamic neurons (23 µM) (Oh et al. 1995). It is one order of magnitude lower than the value measured in unidentified cultured amacrine cells of the rat retina (71 µM) (Feigenspan and Bormann 1994). In cultures of rat hippocampus, rapidly and slowly desensitizing responses to GABA have been observed with EC$_{50}$ values of 8.5 and 37.3 µM, respectively (Schonrock and Bormann 1993). High-affinity (EC$_{50}$ = 6–8 µM) GABA responses have been reported for rat hippocampal neurons, rat nucleus tracti solitarii neurons, and mouse retinal bipolar cells (Nakagawa et al. 1991; Shirasaki et al. 1991; Suzuki et al. 1990). The effect of subunit composition on the affinity of GABA$_A$ receptors for GABA has been studied by transient expression of $\alpha_1\beta_3\gamma_2\delta$, $\alpha_6\beta_3\gamma_2\delta$, and $\alpha_6\beta_3\gamma_2\delta$ isoforms in mouse fibroblast cells (Saxena and MacDonald 1996). The isoform containing the $\alpha_6$ subunit revealed an increased affinity for GABA (2.2 µM) when compared with the $\alpha_1$-containing receptor (16.4 µM). EC$_{50}$ values in the range of 6–17 µM have been reported with recombinant GABA receptors composed of $\alpha_1\beta_2\gamma_2\delta$, $\alpha_5\beta_1\gamma_2$, and $\alpha_5\beta_1\gamma_2$ (Ebert et al. 1994; Sigel et al. 1990, 1992). It has been recently shown that both $\alpha$ and $\beta$ subunits interact to influence EC$_{50}$ values of GABA$_A$ receptors (White et al. 1995).

The individual EC$_{50}$ values measured in this study were evenly distributed around the mean, within a range of 5-µM GABA concentration. The best fit to both the individual and the mean data points was obtained with a first-order sigmoidal function, suggesting a uniform sensitivity of the GABA$_A$ receptors of DA cells. It must be noted, however, that with only six different GABA concentrations tested, small differences in affinities of two or more hypothetical GABA receptor subpopulations could not be resolved.

**Benzodiazepine pharmacology of DA cells**

Central benzodiazepine receptors have been traditionally classified into two different pharmacological subtypes. BZ1
sites show high affinity for the triazolopyridazine CL-218872, zolpidem, and some β-carbolines, whereas BZ2 sites display low affinity for these ligands and high affinity for flunitrazepam. It is now clear, however, that benzodiazepine-binding sites of GABA receptors are much more heterogeneous in their affinity for the ligands listed above. For instance a subtype in cerebellar granule cells designated BZ3 is associated with the α6 subunit and is insensitive to diazepam (see MacDonald and Olsen 1994).

We studied the effects of the BZ1-preferring agonists CL-218872 and zolpidem, as well as the benzodiazepine agonist flunitrazepam and the inverse agonist DMCM. The GABA response of all DA cells tested were modulated by these allosteric regulators.

The EC_{50} value for zolpidem was 126 nM and thus intermediate between BZ1 and BZ2 receptors. However, maximal enhancement of GABA_{A} receptors currents was 348% compared with 214% by flunitrazepam and 220% by CL-218872.

The benzodiazepine sensitivity of DA cell GABA_{A} receptors can be explained by various combinations of subunits. Expression in oocytes or mammalian cell lines suggested that benzodiazepine sensitivity is conferred by the presence of the γ2 subunit (Pritchett et al. 1989). Since all DA cells showed enhancement of the GABA response by flunitrazepam, a likely explanation would be that all DA neurons express the γ2 subunit. Furthermore, all GABA_{A} receptor currents were positively modulated by CL-218872 and zolpidem, compounds indicative of a BZ1 benzodiazepine binding site; these findings are therefore consistent with the presence of the α1 subunit.

Recombinant GABA_{A} receptors containing the α4 subunit (Wisden et al. 1991) in combination with one β and the γ2 subunits are insensitive to benzodiazepines such as diazepam. Because DA cells, however, express multiple types of GABA_{A} receptors, the observed benzodiazepine sensitivity does not rule out the presence of a functional receptor containing α4.

**Zn^{2+} sensitivity of the GABA_{A} receptors**

GABA receptor currents from all DA cells displayed low sensitivity to Zn^{2+}. Currents were maximally inhibited to 6% of control values with an IC_{50} of 59 μM. The effects of Zn^{2+} on GABA responses depend on the isoforms of GABA_{A} receptors that are expressed (Celentano et al. 1991; Draguhn et al. 1990; Smart and Constanti 1990; Smart et al. 1991; Xie and Smart 1991). Recombinant GABA receptor isoforms expressed in human embryonic kidney cells composed of α1 or β1 subunits (Smart et al. 1991) or both (Draguhn et al. 1990) were highly sensitive to Zn^{2+} (IC_{50} < 2 μM). On addition of the γ2
subunit to these GABA receptor subtypes, Zn$^{2+}$ (100 μM) had no inhibitory effect on GABA-induced currents. In addition, Zn$^{2+}$ sensitivity was also modified by the α subunit, with GABA$_A$ receptors that contained α6 being more sensitive to Zn$^{2+}$ than those that contained α1 (Saxena and MacDonald 1996). Finally, expression of the γ subunit together with α6 and β3 confers a 10-fold higher sensitivity to Zn$^{2+}$ when compared with the α6β3γ2 GABA receptor isoform (Saxena and MacDonald 1996). Therefore it has been proposed that when the α1 or γ2 subunit is present in recombinant GABA receptor isoforms, the sensitivity to Zn$^{2+}$ inhibition is lost (Draguhn et al. 1990; Smart et al. 1991).

As shown by Saxena and MacDonald (1996), the presence of the γ subunit causes a decrease in the susceptibility to Zn$^{2+}$ inhibition rather than a total loss. Thus the moderate to low effect of Zn$^{2+}$ on DA cell GABA responses is compatible with the presence of γ2 in all receptors expressed. However, the incomplete inhibition of GABA-induced currents by saturating concentrations of Zn$^{2+}$ may result from the presence of at least two subpopulations of GABA receptors, one containing γ2 (Zn$^{2+}$ insensitive), the other lacking γ2 (Zn$^{2+}$ sensitive). Based on the studies of recombinant GABA$_A$ receptors, the Zn$^{2+}$-insensitive isoform containing γ2 should display high affinity for benzodiazepines like flunitrazepam. The Zn$^{2+}$-insensitive residual current, however, was no more benzodiazepine sensitive than were the total GABA-induced currents, resulting from the hypothetical activation of more than one GABA$_A$ receptor population. These data suggested a single population of moderately Zn$^{2+}$-sensitive GABA receptors rather than two or more subpopulations.

**Modulation by neurosteroids and loreclezole**

Although there is no absolute subunit specificity for steroid modulation of GABA$_A$ receptor function, it has been shown that subunit composition affects their action (Gee and Lan 1991; Korpi and Luddens 1993; Lan et al. 1991; Puia et al. 1990, 1993; Shingai et al. 1991). The greater potentiation of neurosteroid activity than those containing α6, whereas the type of β subunit does not appear to play a significant role (Zhu et al. 1996).

We have observed a large potentiation of GABA-induced chloride currents (452%) when the neuromodulator alphaxalone was applied together with GABA. The variability of the effect at higher steroid concentrations might be due to a combination of allosteric modulation and direct opening of GABA-gated chloride channels. The strong enhancement of DA cell GABA responses by alphaxalone is in agreement with the expression of a GABA$_A$ receptor that lacks the δ subunit.

A new allosteric modulatory site on the GABA$_A$ receptor β subunit has been described (Wafford et al. 1994; Wingrove et al. 1994). The action of the broad-spectrum anticonvulsant loreclezole depends on the type of β subunit present in the receptor complex: receptors containing β2 or β3 have more than 300-fold higher affinity for loreclezole than receptors containing β1. A single amino acid residue present in both β2 and β3, but not β1 has been shown to confer sensitivity to the modulatory action of loreclezole (Wingrove et al. 1994). In addition, loreclezole potentiation does not require the presence of either an α or a β subunit (Wafford et al. 1994).

We have observed a dose-dependent potentiation of GABA responses by loreclezole, indicating the presence of GABA$_A$ receptors containing the β2 and/or β3 subunits. However, the presence of β1 cannot be ruled out from these experiments, since a loreclezole-insensitive receptor population would remain undetected. The modulatory effects of loreclezole on GABA responses of DA cells were less pronounced than previously reported for recombinant receptors. Because receptors that contained β1 did not contribute to the overall potentiation of GABA-evoked currents, these results could indicate that DA cells did not express a unique population of GABA receptors.

**Which GABA$_A$ receptor subunits are expressed by DA cells?**

We have previously shown with single-cell RT-PCR experiments, that DA cells contained the messages for seven GABA$_A$ subunits: α1, α3, α4, β1, β3, γ1, γ2, and γ3 (Gustincich et al. 1999). By immunocytochemistry with subunit-specific antibodies, we have confirmed that all subunits were translated and expressed by DA cells in the intact retina. Such a complexity in GABA$_A$ receptor composition is not new. Three other types of neurons, hippocampal pyramidal cells (Nusser et al. 1996) and granule cells of both cerebellum and dentate gyrus (Laurie et al. 1992; Nusser et al. 1995, 1998), exhibit a similar richness in subunits.

With the exception of α4, six of the subunits formed clusters on the surface of DA cells that were interpreted as postsynaptic active zones containing GABA$_A$ receptors. The postsynaptic clusters distributed throughout the dendritic tree contained the α3 subunit associated with β3 and, less frequently, β1, whereas clusters containing the α1 subunit were confined to large dendrites. We were surprised that γ subunits were not colocalized with the α3 subunit, but the antibodies to γ1 and γ2 stained fewer synapses.

We therefore speculated that a receptor consisting of α1, β1, and γ2 subunits was localized at postsynaptic active zones on large dendrites, whereas a second type of receptor containing α3 and β3 subunits was postsynaptic at contacts distributed throughout the dendritic tree. Both types of synapses would be present on the vitreal aspect of the perikaryon. It is unclear which postsynaptic structures are present in solitary DA cells after enzymatic digestion and mechanical trituration of the retina. We do not know whether the recorded GABA-evoked currents derive exclusively from extrasynaptic receptors of the cell body or from postsynaptic receptors localized on the most vitreal aspect of the perikaryon and/or along the larger dendrites. Therefore the effects of drug application could reflect the participation of different receptor populations.

The physiological data show that α1, β3, and γ2 subunits are incorporated into functional receptors at the surface of solitary DA cells. In fact, the BZ1 pharmacology indicates that these cells express GABA$_A$ receptors containing α1 and γ2 subunits. The combination α1β2γ2 is the most abundant in the brain (McKernan and Whiting 1996) and is considered as the equivalent to the BZ1 subtype, but the nature of the β subunit does...
not appear to be essential for determining benzodiazepine pharmacology (Benke et al. 1994; Hadingham et al. 1993). We can then speculate that we are recording currents mediated by α1β1γ2 or α1β2γ2 receptors. These could be localized extrasynthetically and, for α1β1γ2, on large dendrites. Furthermore, in the rat cerebral cortex, 19% of the GABA_A receptors have both β1 and β3 subunits (Li and De Blas 1997), raising the possibility of a receptor consisting of α1β1γ3.

Small populations of native receptors containing both α1 and α3 have been detected in the CNS, and evidence for the combination α1α3β2γ2 has been obtained (Duggan et al. 1991; McKernan et al. 1991; Mertens et al. 1993). However, we failed to detect co-immunolocalization of α1 and α3, but we cannot exclude the presence of this combination in the cell body. Because α3 has been colocalized with β3 on DA cells, we can speculate that these two subunits form a α3β3γ2 receptor. α3β2γ2/γ3 constitutes approximately 17% of the total GABA_A receptor repertoire (McKernan and Whiting 1996). This combination confers B2 benzodiazepine pharmacology, but unfortunately no specific agonists or antagonists at this subtype are available. Interestingly, α3β2γ2 is found in cholinergic and monoaminergic neurons, and thus could be involved in the control of the release of norepinephrine and dopamine in other regions of the brain (Fritschy et al. 1992; Gao et al. 1993). Alternatively, α3 and β3 could form a functional receptor by themselves.

GABA_A receptors containing the α4 subunit were shown to be insensitive to benzodiazepines and to be colocalized with the δ subunit (Laurie et al. 1992; Wisden et al. 1992), although α4 is also present in regions of the brain where δ is not expressed. Therefore α4 could be expressed extrasynthetically in a distinct GABA receptor population of DA cells.

Small populations of receptors containing more than one type of γ subunit have been identified (Quirk et al. 1994), but most receptors appear to contain only one type (Mossier et al. 1994; Quirk et al. 1994). Immunoprecipitation studies have shown that γ2 and γ3 can exist in the same receptor complex, but γ1 does not co-precipitate with another γ subunit (Quirk et al. 1994). Since we have detected both γ1 and γ2 subunits, this could indicate the presence of at least two receptor populations with different γ subunits.

In conclusion, we have provided evidence that in DA cells at least the α1, β3, and γ2 subunits are assembled into functional GABA_A receptors. In absence of pharmacological tools specific for the other subunits, we could not obtain definitive evidence that also α3, α4, β1, and γ1 are part of functional receptors, although this is a very likely possibility. Thus the meaning of the subunit diversity of the GABA_A receptors of DA cells remains to be clarified. On the basis of the physiology alone, we could not predict the complexity of the GABA_A receptor subunit composition revealed in DA cells by RT-PCR applied to individual cells. On the other hand, it is well known that the results of immunocytochemistry are complicated by the vagaries of the interaction between antibody epitopes and the chemical fixatives required for microscopy. Our results therefore emphasize the crucial importance of combining all available technical approaches to the study of a complex receptor in a minuscule cell population.

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REFERENCES


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