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Response kinetics and pharmacological properties of heteromeric receptors formed by coassembly of GABA ρ- and γ_2-subunits

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Two of the γ-aminobutyric acid (GABA) receptors, $GABA_A$ and $GABA_C$, are ligand-gated chloride channels expressed by neurons in the retina and throughout the central nervous system. The different subunit composition of these two classes of GABA receptor result in very different physiological and pharmacological properties. Although little is known at the molecular level as to the subunit composition of any native GABA receptor, it is thought that $GABA_C$ receptors are homomeric assemblies of ρ-subunits. However, we found that the kinetic and pharmacological properties of homomeric receptors formed by each of the ρ-subunits cloned from perch retina did not resemble those of the $GABA_C$ receptors on perch bipolar cells. Because both $GABA_A$ and $GABA_C$ receptors are present on retinal bipolar cells, we attempted to determine whether subunits of these two receptor classes are capable of interacting with each other. We report here that, when coexpressed in *Xenopus* oocytes, heteromeric ($\rho_{1B}\gamma_2$) receptors formed by coassembly of the ρ_{1B}-subunit with the γ_2-subunit of the $GABA_A$ receptor displayed response properties very similar to those obtained with current recordings from bipolar cells. In addition to being unresponsive to bicuculline and diazepam, the time-constant of deactivation, and the sensitivities to GABA, picrotoxin and zinc closely approximated the values obtained from the native $GABA_C$ receptors on bipolar cells. These results provide the first direct evidence of interaction between GABA ρ and $GABA_A$ receptor subunits. It seems highly likely that coassembly of $GABA_A$ and ρ-subunits contributes to the molecular organization of $GABA_C$ receptors in the retina and perhaps throughout the nervous system.

Keywords: $GABA_C$ receptors; ρ-subunits; $GABA_A$ γ_2-subunit; *Xenopus* oocytes; bipolar cells; retina

1. INTRODUCTION

Three classes of γ-aminobutyric acid (GABA) receptor ($GABA_AR$, $GABA_BR$ and $GABA_CR$) are known to mediate the actions of GABA, the main inhibitory neurotransmitter in the central nervous system (CNS). Whereas $GABA_B$Rs act via G proteins to regulate potassium and calcium channels, the ionotropic $GABA_A$Rs and $GABA_C$Rs directly gate a chloride channel. However, the GABA-evoked currents of $GABA_A$Rs and $GABA_C$Rs reveal several important differences. Unlike the transient, rapidly desensitizing response of $GABA_A$Rs, activation of $GABA_C$Rs results in a response that is sustained during GABA application. Moreover, $GABA_C$Rs are more sensitive to GABA, and are insensitive to bicuculline as well as to drugs that modulate $GABA_A$Rs (cf. Johnston 1996).

$GABA_C$Rs are thought to be formed by the GABA ρ-subunits that were first cloned from a human retinal cDNA library (Cutting *et al*. 1991). When expressed in *Xenopus* oocytes, these subunits form homo-oligomeric receptors that are highly sensitive to GABA and insensitive to bicuculline, features similar to those of neuronal $GABA_C$Rs (Cutting *et al*. 1992; Shimada *et al*. 1992; Wang *et al*. 1994). However, neither a quantitative analysis of their response kinetics nor a detailed comparison of their pharmacology has yet been performed.

In the vertebrate retina, both $GABA_A$Rs and $GABA_C$Rs are expressed on the bipolar cells of every species studied thus far. Although there is a high degree of similarity between the amino-acid sequences of GABA ρ- and $GABA_A$R-subunits (Barnard *et al*. 1998), earlier studies had shown that subunits comprising these two classes of GABA receptor do not coassemble with each other. The responses elicited from oocytes that coexpress GABA ρ- with either α- or β-subunits are virtually identical to those from homomeric ρ-receptors (Shimada *et al*. 1992), and no interaction between GABA ρ-subunits and the α- and β-subunits of the $GABA_A$R has been detected by immunoprecipitation (Hackam *et al*. 1998), or by immunocytochemistry (Koulen 1998). Nevertheless, the exact composition of native $GABA_C$Rs has yet to be determined, and a possible interaction between GABA ρ-subunits and the $GABA_A$R γ-subunit has not been fully explored.

We recently cloned four orthologues of the human ρ_1- and ρ_2-subunits from a white perch retinal cDNA library (Qian *et al*. 1998). Like their human counterparts, each proved capable of forming functional GABA-gated Cl^- channels when expressed in *Xenopus* oocytes. However, none of the ρ-receptors exhibited the rapid time-course of deactivation seen in recordings of the $GABA_C$-mediated

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responses from perch retinal bipolar cells. This observation led us to consider the possibility that GABA ρ-subunits might interact with one or another of the $GABA_A$R-subunits in the same neuron to constitute a hetero-oligomeric receptor. Specifically, we attempted to determine whether the $GABA_C$Rs of perch bipolar cells are formed by coassembly of GABA ρ-subunits with the $GABA_A$R γ_2-subunit, the most abundant of the γ-subunits. The human γ_2-subunit was used for these studies for two reasons: (i) earlier work had shown that when expressed in oocytes, the γ-subunit does not independently form a functional receptor (Sigel *et al*. 1990; Sanna *et al*. 1995), and (ii) perch γ-subunits have not yet been cloned.

We report here that one of the perch ρ-subunits (ρ_{1B}) coassembled with the $GABA_A$R γ_2-subunit to form a heteromeric receptor with temporal and pharmacological properties that resembled in many respects the response characteristics of the $GABA_C$Rs on perch bipolar cells. This finding is the first to demonstrate interaction between subunits from these different classes of GABA receptor, and raises the possibility that some neuronal $GABA_C$Rs may be formed by coassembly of GABA ρ- and $GABA_A$-subunits.

2. METHODS

(a) *Subunit expression and current recording from* Xenopus *oocytes*

The procedures for expressing GABA receptor subunits in *Xenopus* oocytes have been described in detail previously (Qian *et al*. 1998). Briefly, stage V–VI oocytes were removed from gravid *Xenopus laevis* females, and held at 16 °C in a Ringer solution containing (in mM): NaCl (100), KCl (2), $CaCl_2$ (2), $MgCl_2$ (1), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) (5), glucose (10), at pH 7.4. The oocytes were defolliculated by immersing them for 30 min in a Ca^{2+}-free Ringer solution containing 2 mg ml^{-1} collagenase. White perch GABA ρ-subunits (Qian *et al*. 1998) and a human $GABA_A$R γ_2-subunit (a gift from Dr Paul Whiting, Merck Sharp & Dohme Research Laboratories, Essex, UK) were processed for injection by cloning each into the expression vector pcDNA1/Amp (Invitrogen, Carlsbad, CA, USA). The oocytes were then injected with 20 nl (20 ng) of one or another of the GABA ρ-subunit DNAs, either alone, or in combination with the DNA of the human $GABA_A$ γ_2-subunit. Recordings of GABA-activated currents were obtained after two to seven days of expression using a two-microelectrode voltage clamp amplifier (GeneClamp 500, Axon Instruments, Inc, Foster City, CA, USA). Current and voltage electrodes were pulled to a resistance of 1–2 MΩ, and filled with a solution containing 3 M KCl. A gravity flow system, controlled by a solenoid manifold and operated under computer command, was used to deliver drugs to the preparation. The rate of superfusion was 2 ml min^{-1}, and the time-constant of solution exchange was approximately 0.5 s. Protocols for voltage-clamp and data analysis were controlled by a computer running Patchit software (Grant & Werblin 1994) through a TL-1 interface (Axon), and have been described in detail in prior publications (cf. Qian & Dowling 1995; Qian *et al*. 1997*a*).

(b) *Patch-clamp recording from isolated retinal bipolar cells*

Solitary bipolar cells from white perch (*Morone amacricana*) and hybrid bass (*M. chrysops* crossed with *M. saxitilis*) retina were used in this study. The properties of the GABA-induced responses from bipolar cells of the two species were indistinguishable, and the results were pooled for data analysis. The cell isolation and recording procedures were the same as previously published (Qian & Dowling 1995). Briefly, the retina was isolated and incubated for 40 min in an enzyme solution made up of Leibovitz's L-15 culture medium (GIBCO, Grand Island, NY, USA) containing 25 units ml^{-1} papain (Worthington, Freehold, NJ, USA) and 1 mg of L-cysteine. Enzyme activity was arrested by washing the tissue five times in fresh L-15 medium. After trituration through a sterilized pipette, aliquots of the supernatant containing dissociated cells were placed in 35-mm Petri dishes containing 3 ml L-15 medium, and stored in an incubator at 15 °C. Bipolar cells were readily identified by their characteristic morphology (Qian & Dowling 1995), and those with prominent cell bodies were selected for study.

Membrane currents were recorded using either conventional whole-cell voltage-clamp (Hamill *et al*. 1981) or amphotericin perforated-patch recordings (Rae *et al*. 1991). Similar results were obtained with both recording configurations, and no distinction is made in the presentation of data. During the recording, cells were bathed in a Ringer solution containing (in mM): NaCl (145), KCl (2.5), $CaCl_2$ (2.4), $MgCl_2$ (1.5), glucose (10) and HEPES (5), pH 7.6. For whole-cell recording, the pipettes were filled with an intracellular solution containing (in mM): CsCl (124), $CaCl_2$ (1), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (11), $MgCl_2$ (2), HEPES (10), pH 7.4. Macroscopic currents were recorded with the cells clamped initially at −60 mV, close to their initial resting potential. For perforated patch recording, the electrode solution contained (in mM) Cs acetate (120), CsCl (4), $CaCl_2$ (1), EGTA (11), $MgCl_2$ (2), HEPES (10), pH 7.4 and amphotericin (240 μg ml^{-1}). In this configuration, the GABA responses were elicited with the membrane potential held at 0 mV. Because both $GABA_A$Rs and $GABA_C$Rs are present on white perch bipolar cells, investigation of $GABA_C$R-mediated responses required bicuculline to block activation of $GABA_A$Rs.

Data analysis was performed with pCLAMP software (Axon). The time-constants (τ) of the individual deactivation curves were calculated using the Clampfit program to fit the falling phase of the 'off' response to a first-order exponential function. The EC_{50} and IC_{50} of dose–response curves were derived from Hill equations fit to the data with the program Origin™ (Microcal, Northampton, MA, USA). For each experiment, data were collected from five to eight cells, and are presented as means ± s.d.

3. RESULTS

(a) *Coexpression of the $GABA_A$ γ_2-subunit with perch ρ-subunits*

Four GABA ρ-subunits cloned from the white perch retina were shown to be capable of forming functional homo-oligomeric receptors with unique response characteristics in the oocyte expression system (Qian *et al*. 1998). Figure 1*a* presents a comparison of a typical $GABA_C$-mediated response from a perch bipolar cell (left) with recordings obtained from oocytes expressing the various ρ-subunits. In each case, the GABA offset response could be described by a single exponential function. The bar graph shown in figure 1*b* gives the time-constants (on the logarithmic scale of ordinates) of the off-responses following application of GABA for the bipolar cell (solid

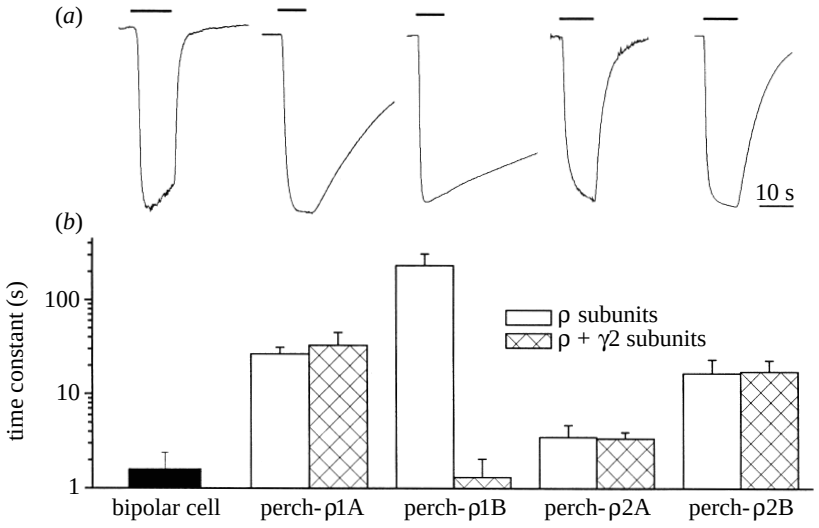

Figure 1. GABA-evoked responses from white perch bipolar cells, and from *Xenopus* oocytes expressing the various ρ-subunits. (*a*) The different deactivation kinetics seen in current recordings from a bipolar cell (to 100 μM GABA in the presence of 500 μM bicuculline), and from four homo-oligomeric perch ρ-receptors (to 10 μM GABA). To facilitate a comparison of response kinetics, the traces are plotted on the same time-scale, but with arbitrary vertical scales in order to give similar response amplitudes. Responses are single representative recordings. (*b*) Bar graphs depict the time-constants of the GABA offset responses from bipolar cells, from homo-oligomeric perch ρ-receptors, and from heteromeric receptors composed of ρ_{1B}- and γ_2-subunits.

bar), and for each of the GABA ρ-subunits (clear bars). It is evident that none of the GABA responses elicited from any of the ρ-receptors mimicked the $GABA_C$-mediated response of bipolar cells with respect to the time-course of deactivation following the GABA pulse.

In order to determine whether the kinetics of the GABA response can be altered by assembly of $GABA_AR$ and $GABA_CR$ subunits, we coexpressed the $GABA_AR$ γ_2-subunit with each of the individual GABA ρ-subunits in *Xenopus* oocytes. The hatched bars in figure 1*b* show the time-constants of the off-response for each of the ρ-subunits when expressed in combination with the $GABA_AR$ γ_2-subunit. Except for the perch ρ_{1B}-subunit, the time-constants of the GABA offset responses elicited from oocytes coexpressing other perch ρ-subunits with the γ_2-subunit were virtually identical to the values obtained from oocytes expressing the perch ρ-subunits alone. On the other hand, membrane current recordings from oocytes coexpressing the perch ρ_{1B}-subunit with the γ_2-subunit showed that the time-constant of the GABA offset response was reduced more than 180-fold when compared with that obtained from homo-oligomeric ρ_{1B}-receptors. Moreover, the time-constant of deactivation for the heteromeric $\rho_{1B}\gamma_2$-receptor ($\tau = 1.3 \pm 0.7$ s) now closely approximated the value obtained from perch bipolar cells ($\tau = 1.6 \pm 0.8$ s).

(b) *Characteristics of the heteromeric $\rho_{1B}\gamma_2$-receptor*

Figure 2*a* provides a more detailed comparison of recordings from oocytes expressing the ρ_{1B}- and γ_2-subunits individually and in combination, together with the response from a perch bipolar cell. The sustained inward current evoked by 10 μM GABA in oocytes expressing the perch ρ_{1B}-subunit shows little evidence of desensitization, and decays toward baseline over a period of several minutes ($\tau = 234 \pm 76$ s) after GABA application has been terminated. The time-constant of deactivation is about two orders of magnitude greater than previously seen in recordings from retinal neurons that exhibit $GABA_C$-mediated responses (Feigenspan *et al.* 1993; Qian & Dowling 1993, 1995; Qian *et al.* 1997*b*; Bormann & Feigenspan 1995).

In contrast, when the $GABA_AR$ γ_2-subunit was injected into oocytes, no GABA-induced current could be detected. This is consistent with earlier findings indicating that the γ_2-subunit alone does not form a functional receptor in the oocyte expression system (Sigel *et al.* 1990; Sanna *et al.* 1995). However, coexpressing the perch ρ_{1B}-subunit together with the human $GABA_AR$ γ_2-subunit led to the formation of a heteromeric receptor with a response waveform very similar to that of the bipolar cell.

In addition, the dose–response curves shown in figure 2*b* reveal that the homomeric perch ρ_{1B}-receptor is far more sensitive to GABA ($EC_{50} = 0.17$ μM; Hill coefficient of 1.6) than is the $GABA_CR$ on bipolar cells ($EC_{50} = 12.5$ μM; Hill coefficient of 1.4). On the other hand, the heteromeric receptor formed by incorporating the γ_2-subunit decreased the GABA sensitivity by more than tenfold. This reduced sensitivity ($EC_{50} = 4.04$ μM; Hill coefficient of 1.3) more closely approximates the GABA sensitivity of the bipolar $GABA_CR$.

(c) *Pharmacology of the heteromeric $\rho_{1B}\gamma_2$-receptor*

Further evidence that the $GABA_AR$ γ_2-subunit co-assembles with the perch ρ_{1B}-subunit to form a heteromeric receptor with unique properties can be seen in the results obtained with the Cl^- channel-blocker picrotoxin, and the inhibitory divalent cation zinc. Homomeric perch ρ_{1B}-receptors are relatively insensitive to picrotoxin inhibition (Qian *et al.* 1998); at a GABA concentration of 2 μM, the IC_{50} for picrotoxin inhibition is about 110 μM (figure 3*a*). However, when coexpressed with the γ_2-subunit, there is a significant increase in sensitivity to picrotoxin inhibition; the IC_{50} value for picrotoxin inhibition is decreased by more than two orders of magnitude ($IC_{50} = 0.63$ μM), again in good agreement with the picrotoxin inhibition of $GABA_CR$-mediated responses on perch bipolar cells ($IC_{50} = 1.1$ μM).

Coassembly of GABA ρ- and γ-subunits also influences the zinc sensitivity of the receptor. Figure 3*b* illustrates the zinc inhibition curves of homo-oligomeric and heteromeric perch ρ_{1B}-receptors. While both receptors are inhibited by zinc in a dose-dependent manner, the hetero-oligomeric

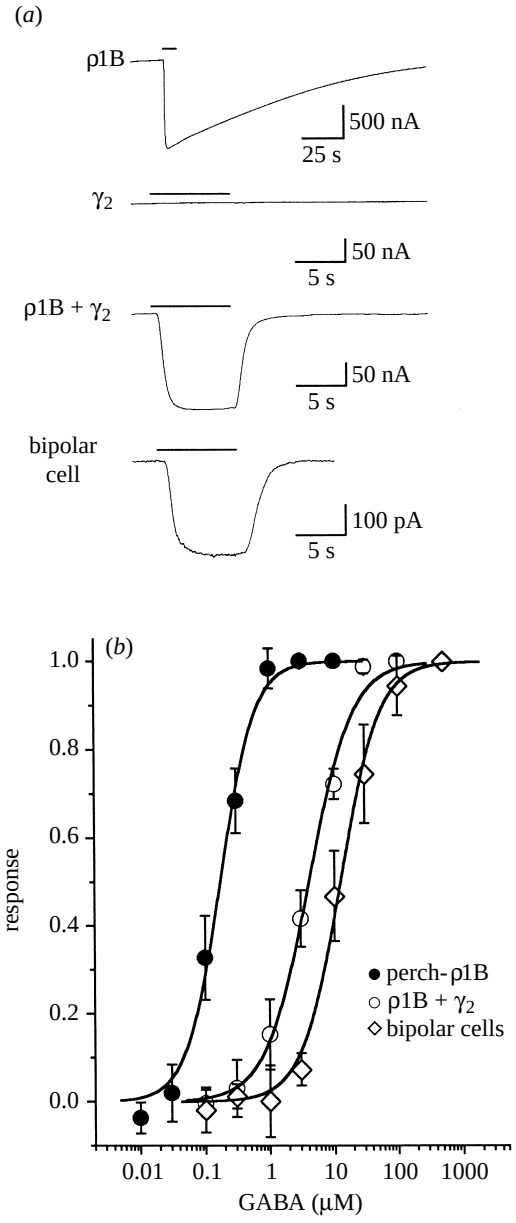

Figure 2. Coassembly of perch ρ_{1B}-subunit with the $GABA_A$ γ_2-subunit alters the kinetics and sensitivity of the response to GABA. (*a*) GABA-elicited responses from oocytes expressing perch ρ_{1B}-subunit, human γ_2-subunit, and both the ρ_{1B}- and γ_2-subunits, compared with the $GABA_C$-mediated response of a bipolar cell. GABA at 10 μM was used to activate the responses on oocytes. For the bipolar cell, the response was elicited by 30 μM GABA in the presence of 100 μM bicuculline. Note that the GABA response from the homomeric perch ρ_{1B}-receptor is shown on a time-scale five times longer than for the other traces. (*b*) GABA dose–response curves obtained from homomeric perch ρ_{1B}-receptors and heteromeric perch ρ_{1B}- and γ_2-receptors expressed in *Xenopus* oocytes, and from $GABA_C$Rs on perch bipolar cells. The data for bipolar cells were obtained in the presence of 500 μM bicuculline to block the activity of the $GABA_A$R. The curves are Hill equations with EC_{50} values of 0.17, 4.04 and 12.5 μM and Hill coefficients of 1.6, 1.3 and 1.4 for homomeric (ρ_{1B}), heteromeric ($\rho_{1B}\gamma_2$), and bipolar cell ($GABA_C$) receptors, respectively.

$\rho_{1B}\gamma_2$-receptor exhibits a significantly greater sensitivity to zinc inhibition ($IC_{50} = 6.5$ μM; Hill coefficient of 0.9) than does the homo-oligomeric perch ρ_{1B}-receptor ($IC_{50} = 125$ μM; Hill coefficient of 1.9). Here too the

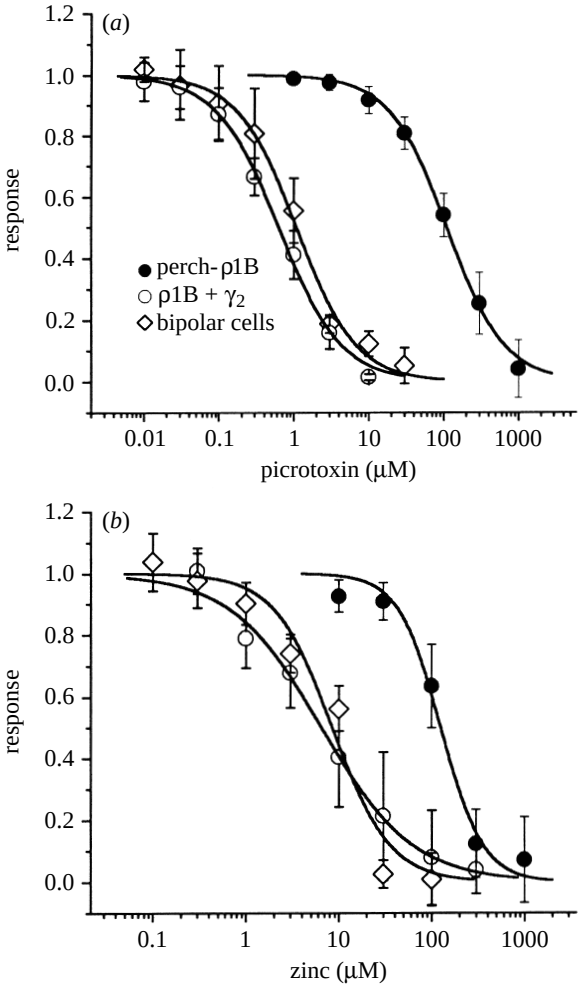

Figure 3. Dose–response data for the inhibitory actions of picrotoxin and zinc. At each drug concentration, the response represents the fractional change in the current evoked by GABA when applied at concentrations of 2 μM (for homomeric perch ρ_{1B}-receptors), 10 μM (for heteromeric $\rho_{1B}\gamma_2$-receptors), and 30 μM (for bipolar cells). (*a*) Inhibition by picrotoxin. Data were fitted by Hill equations with IC_{50} values of 110, 0.63 and 1.1 μM, and Hill coefficients of 1.1, 1.0 and 1.1 for ρ_{1B}-receptors, $\rho_{1B}\gamma_2$-receptors,and the $GABA_C$Rs of bipolar cells, respectively. (*b*) Inhibition by zinc. Curves are Hill equations with IC_{50} values of 125, 6.5 and 8.5 μM, and Hill coefficients of 1.9, 0.9 and 1.4 for homo- and heteromeric GABA ρ-receptors and bipolar cells, respectively.

zinc sensitivity of heteromeric receptors is similar to the value observed for $GABA_C$Rs on white perch bipolar cells ($IC_{50} = 8.5$ μM) although the Hill coefficient (1.4) was somewhat greater.

(d) *The effects of imidazole-4-acetic acid, bicuculline, barbiturates and diazepines*

Imidazole-4-acetic acid (I4AA) serves both as an agonist and competitive antagonist on the receptors formed by various GABA ρ-subunits (Qian *et al*. 1998). On homomeric perch ρ_{1B}-receptors, I4AA acts primarily as an agonist. As shown in figure 4*a*, the amplitude of the response elicited by 100 μM I4AA was about 85% of the saturating current evoked by 10 μM GABA. However, on heteromeric $\rho_{1B}\gamma_2$-receptors, the I4AA-activated currents were greatly reduced. The response to 100 μM I4AA was

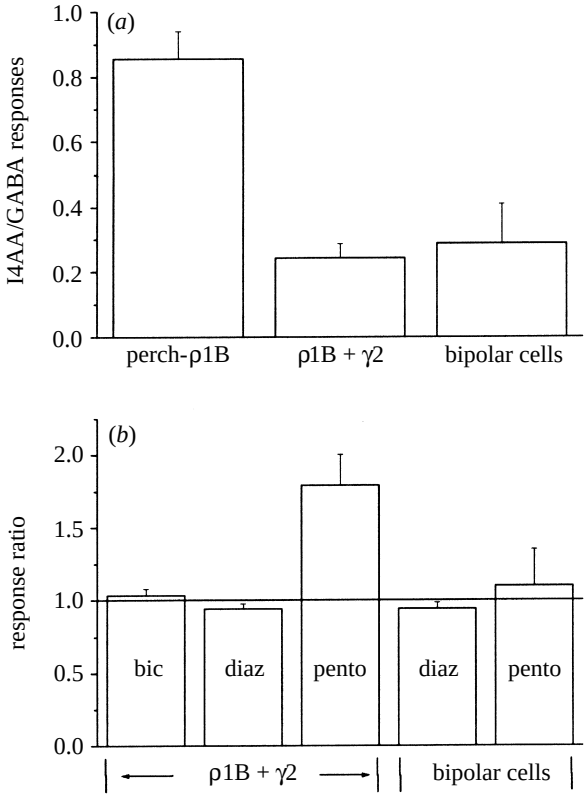

Figure 4. Effects of I4AA, bicuculline, barbiturates and diazepines on $GABA_C$Rs. (*a*) Normalized amplitude of I4AA-activated response from oocytes expressing perch ρ_{1B}-subunit alone, the ρ_{1B}- and γ_2-subunits, and from bipolar cells. For oocytes, responses to 100 μM I4AA were normalized to the responses activated by 10 μM GABA; for bipolar cells, response to 200 μM I4AA were normalized to responses activated by 100 μM GABA. The different GABA concentrations were required to elicit maximal responses under the two conditions. (*b*) Effects of bicuculline (Bic) (100 μM), diazepam (Diaz) (10 μM) and pentobarbital (Pento) (100 μM) on heteromeric ρ_{1B}- and γ_2-receptors and on bipolar cells. The data are presented as ratios of the responses elicited by coapplication of GABA plus test agents versus control (horizontal line); control responses were elicited by 3 μM GABA on heteromeric receptors and 10 μM GABA on bipolar cells.

only a small fraction (approximately 22%) of the saturating GABA response; this level of activity is similar to that seen with the $GABA_C$R of bipolar cells (approximately 25%).

As we have shown, the responses elicited by GABA from the heteromeric $\rho_{1B}\gamma_2$-receptor are sustained and show little desensitization, typical of neuronal $GABA_C$Rs. However, it was important to determine whether the heteromeric receptor exhibits properties similar to those of the $GABA_C$R on bipolar cells with respect to its reactions to agents that typically distinguish $GABA_C$Rs from $GABA_A$Rs. This distinction can best be assessed in the responses to the $GABA_A$R-blocker bicuculline, and to diazepam and pentobarbital, substances that tend to enhance the responses of $GABA_A$Rs. Figure 4*b* shows that oocytes expressing heteromeric $\rho_{1B}\gamma_2$-receptors are not blocked by bicuculline, and are insensitive to benzodiazepines, features that are evident also in the $GABA_C$R-mediated responses of bipolar cells (Feigenspan *et al*. 1993; Qian & Dowling 1995). In the presence of 100 μM bicuculline, the GABA response was virtually identical to that evoked by GABA alone (1.03 ± 0.05), and with 10 μM diazepam, the GABA responses of the $\rho_{1B}\gamma_2$-receptors and the bipolar cells were not significantly different from control (0.94 ± 0.04 and 0.94 ± 0.05, respectively). It should be noted that although γ-subunits are essential for $GABA_A$Rs to display maximal enhancement by benzodiazepines (Pritchett *et al*. 1989; Krampfl *et al*. 1998), the GABA responses elicited from the heteromeric $\rho_{1B}\gamma_2$-receptors were not modulated by diazepam. In contrast, the response of the $\rho_{1B}\gamma_2$-receptor was significantly enhanced by pentobarbital, a feature usually associated with $GABA_A$Rs, and not evident in the $GABA_C$-mediated response of bipolar cells. These pharmacological data indicate that the heteromeric receptors formed by coassembly of the $GABA_A$R γ_2-subunit with the GABA ρ_{1B}-subunit possess many, but not all, of the characteristic features of $GABA_C$Rs.

4. DISCUSSION

The present results provide strong evidence that when coexpressed in *Xenopus* oocytes, the perch ρ_{1B}-subunit assembles with the human $GABA_A$ γ_2-subunit to form a novel heteromeric receptor. Although the γ_2-subunit itself did not form a functional homomeric receptor, it altered significantly the kinetic and pharmacological properties displayed by the homomeric ρ_{1B}-receptor. The most notable effect was on the kinetics of deactivation, but there were also marked changes in GABA sensitivity, the IC_{50} of picrotoxin inhibition, and in the responses to I4AA and zinc. In each instance, the change resulting from the formation of the heteromeric $\rho_{1B}\gamma_2$-receptor led to response properties that approximated those of the $GABA_C$R on bipolar cells. In addition, other characteristics of neuronal $GABA_C$Rs were retained; i.e. the GABA-induced response of the $\rho_{1B}\gamma_2$-receptor was insensitive to bicuculline, and diazepam had no effect on the magnitude of the response. However, a notable exception was seen in the enhanced response to pentobarbital, a feature typically associated with the response properties of $GABA_A$Rs. Thus, although there is overall a striking similarity between the response properties of the heteromeric $\rho_{1B}\gamma_2$-receptor and the $GABA_C$R on perch bipolar cells, it cannot be said that they are alike in all respects. Nevertheless, the profound changes induced by coexpression of the human γ_2-subunit with the perch ρ_{1B}-subunit provide strong evidence that the two subunits are capable of coassembly. Unfortunately, there are as yet no specific antibodies to perch GABA ρ- and γ-subunits with which to detect the heteromeric receptor biochemically (e.g. by immunoprecipitation).

It is interesting to note the vastly different effects of the γ_2-subunit when it coassembles with the GABA ρ_{1B}-subunit, compared with when it coassembles with $GABA_A$R αβ-subunits. The marked decrease in the time-constant of deactivation is perhaps the most striking, particularly in view of recent evidence that coexpression of the γ_2-subunit with the $\alpha_1\beta_3$-heteromer significantly prolongs the time-course of deactivation (Haas &

Macdonald 1999). Moreover, recombinant receptors formed by coexpression of α_1- and β_2-subunits require the γ_2-subunit for coactivation by benzodiazepines (Pritchett *et al*. 1989; Krampfl *et al*. 1998), whereas addition of the γ_2-subunit decreases significantly the receptor's sensitivity to zinc (Draguhn *et al*. 1990). In contrast, we find that incorporating the γ_2-subunit into the GABAρ_{1B}-receptor had no effect on the sensitivity to diazepam, and that the inhibitory effect of zinc was greatly increased.

Although the influence of specific $GABA_A$-subunits on the sensitivity to barbiturates has not been studied extensively, the results obtained with pentobarbital were clearly unexpected. There is evidence that barbiturates enhance $GABA_A$R-gated channel activity by increasing the mean open time, without affecting channel conductance (Study & Barker 1981; Macdonald *et al*. 1989; Porter *et al*. 1992), but the γ_2-subunit is not required to produce this effect (Porter *et al*. 1992). On the other hand, barbiturates do not potentiate the GABA responses either of perch ρ_{1B}-receptors or of neuronal $GABA_C$Rs. It was surprising, therefore, to find that with the addition of the γ_2-subunit, the GABA-evoked current of heteromeric $\rho_{1B}\gamma_2$-receptors was significantly enhanced. In all other respects, the pattern of responses obtained from oocytes expressing the $\rho_{1B}\gamma_2$-receptor mirrored that of $GABA_C$Rs on perch bipolar cells. When perch γ-subunits become available, we will be able to better judge whether coassembly with perch ρ-subunits yields a heteromeric receptor that more closely resembles the native $GABA_C$R of perch bipolar cells.

There is good evidence that both GABA ρ- and γ-subunits are expressed in retinal bipolar cells. The expression of GABA ρ-subunits has been confirmed both by *in situ* hybridization and immunocytochemical techniques (Qian *et al*. 1997*a*; Enz *et al*. 1995, 1996; Koulen *et al*. 1997), and $GABA_C$R-mediated responses have been recorded from every type of bipolar cell examined so far. The $GABA_A$R γ-subunit has also been detected in bipolar cells by immunocytochemistry (Wässle *et al*. 1998; Greferath *et al*. 1994, 1995), and the observation that the $GABA_A$R- mediated responses from these cells are resistant to zinc inhibition (Qian & Dowling 1995) is a further indication of the presence of γ-subunits (Draguhn *et al*. 1990; Saxena & Macdonald 1994). The presence of both GABA ρ- and γ-subunits in retinal bipolar cells, and the observation that they form heteromeric receptors with kinetic and pharmacological properties that resemble those of $GABA_C$Rs on bipolar cells, strongly suggest that the native $GABA_C$Rs on these cells are formed by coassembly of ρ- and γ-subunits.

There were, however, some obvious differences in the responses of oocytes expressing heteromeric $\rho_{1B}\gamma_2$-receptors as compared with those obtained from the $GABA_C$Rs of bipolar cells, specifically, the positive modulation by pentobarbital, and the greater sensitivity of the $\rho_{1B}\gamma_2$-receptors to GABA. Such differences could result from properties conferred by the oocyte expression system. On the other hand, both the modulation by barbiturates and increased GABA sensitivity may be linked to the number of γ-subunits incorporated into the heteromeric receptors. Differences in the stoichiometry of γ- and ρ-subunits in the recombinant receptors versus the native $GABA_C$Rs could account for the observed discrepancies. In addition, it is important to recognize that GABA ρ-subunits can themselves form heteromeric receptors (Enz & Cutting 1999; H. Qian and H. Ripps, unpublished observations), and that both GABA ρ_1- and ρ_2-subunits are expressed in retinal bipolar cells (Enz *et al*. 1995; Yeh *et al*. 1996; Zhang *et al*. 1995). In terms of their functional properties, however, receptors formed by the assembly of ρ-subunits do not compare favourably with the $GABA_C$Rs on perch bipolar cells. It seems likely, therefore, that the $GABA_C$Rs of bipolar cells have a more complex subunit composition, consisting perhaps of γ-subunits in combination with one or more of the GABA ρ-subunits.

Although GABA ρ-subunits are expressed predominantly in retina, recent studies indicate that they are distributed throughout the CNS. Using *in situ* hybridization and reverse transcription–polymerase chain reaction techniques, the expression of GABA ρ-subunits has been detected in superior colliculus, lateral geniculate nucleus, visual cortex, hippocampus, and cerebellum of rat and chick brain (Albrecht *et al*. 1997; Boue-Grabot *et al*. 1998; Wegelius *et al*. 1998). The $GABA_A$R γ-subunits have an even wider distribution in the brain, where more than 80% of the $GABA_A$Rs contain the γ_2-subunit (cf. McKernan & Whiting 1996; Seeburg *et al*. 1990). Thus, it is almost certainly the case that, in some regions of the brain, both ρ- and γ-subunits are coexpressed in the same neuron. It is possible that the heteromeric receptors formed by these subunits constitute the 'unusual' $GABA_C$Rs that have been observed in brain tissue (Sivilotti & Nistri 1991; Johnston 1986).

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