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γ-Aminobutyric Acid A or C Receptor? γ-Aminobutyric Acid ρ_1 Receptor RNA Induces Bicuculline-, Barbiturate-, and Benzodiazepine-Insensitive γ-Aminobutyric Acid Responses in *Xenopus* Oocytes

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SUMMARY

Xenopus oocyte expression of the recently cloned γ-aminobutyric acid (GABA) ρ_1 receptor subunit cDNA yields a pharmacologic profile characteristic of the $GABA_C$ responses described by Johnston [*Benzodiazepine/GABA Receptors and Chloride Channels. Receptor Biochemistry and Methodology* (R. W. Olsen and J. C. Venter, eds.), Vol. 5. Alan R. Liss, New York, 57–71 (1986)] and the responses to retinal mRNA recently reported in the *Xenopus* expression system [*Proc. Natl. Acad. Sci. USA* **88**:4318–4322 (1991)]. A rationale for defining GABA ρ_1 as forming a $GABA_C$ receptor is discussed.

Cloning of the cDNAs and genes encoding neurotransmitter receptors is adding to the array of receptor subtypes previously elucidated in classical pharmacologic studies (1–3). Elucidation by cloning of previously undefined α, β, γ, and δ subunits of the receptors for the major inhibitory brain neurotransmitter GABA underscores this point (1–31). The exact receptor subunit profiles expressed on specific neuronal or glial cells may confer substantially different cellular responsiveness to GABA or related drugs. This is most well documented for the benzodiazepine recognition sites on the GABA receptor complex. GABA receptors with α_5 subunits display specific type II benzodiazepine responses (5). Expression of the γ receptor subunit is necessary for full benzodiazepine physiology (16). Expression of an α_6 subunit creates a distinctive pharmacology for two drugs that can modify alcohol withdrawal symptoms, RO15–4513 and flumazanil (RO15–1788) (6).

With the increasing diversity of the possible GABA receptors that could be formed *in vivo*, the exact criteria for defining "$GABA_A$" receptors have become debatable. Classical pharmacologic studies suggest that responsiveness to GABA and inhibition by the competitive GABA antagonist bicuculline define $GABA_A$ receptors, whereas $GABA_B$ receptors are baclofen sensitive (23, 30). Alternatively, molecular cloning studies suggest that the $GABA_A$ receptors are a family of multimeric ligand-gated chloride channels, whereas $GABA_B$ responses may be mediated by GTP-binding protein links to second messenger systems (2, 24).

In central visual pathways, Sivilotti and Nistri (25–28) have defined a GABA responsiveness that is mimicked by the GABA agonist muscimol, is inhibited by the competitive GABA antagonist picrotoxin, but is insensitive to both the classical competitive $GABA_A$ antagonist bicuculline and $GABA_B$ antagonists. These receptors are also insensitive to barbiturates and benzodiazepines. GABA responses of *Xenopus* oocytes injected with mRNA extracted from retina have also recently been shown to display the same bicuculline and baclofen resistance (29). Based on the pharmacologic criteria noted above, such responses would thus be neither $GABA_A$ nor $GABA_B$. GABA responses in other brain areas that are insensitive to both bicuculline and baclofen have been called "$GABA_C$" by Johnston (30). However, other features of the observed responses, such as their rapid activation, are consistent with their mediation by the ligand-gated ion channels that are characteristic of $GABA_A$ receptors (23). Conceivably, bicuculline-insensitive responses could be conferred by drugs acting on a receptor homologous to other known $GABA_A$ receptors. The previously elucidated ability of different GABA subunits to form receptors with different pharmacologies makes such a possibility even more plausible (1–22).

Recently, we have cloned a cDNA for a receptor subunit, GABA ρ_1, whose mRNA is highly expressed in retina (31).

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *tert*-butylbicyclophosphorothionate; β-CCE, *n*-butyl β-carboline-3-carboxylate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Initial studies of the cDNA, expressed as a single subunit in *Xenopus* oocytes, reveal that it confers a picrotoxin-sensitive GABA response whose reversal potential indicates changed chloride conductance (31). In this report, we further describe the pharmacologic profile of the receptor whose expression is induced in *Xenopus* oocytes after injection of GABA ρ_1 mRNA. The profile obtained displays baclofen- and bicuculline-insensitive responses characteristic of the responses of the "unusual GABA receptor in central visual pathways," the bicuculline-insensitive responses recently reported in *Xenopus* oocytes injected with retinal mRNA, and the responses of the $GABA_C$ receptor described by Johnston (26–30). In light of data reported here, we discuss the merits of describing this receptor as a $GABA_A$ subtype and those of labeling it as a $GABA_C$ receptor subtype.

Materials and Methods

The cDNA templates for mRNA synthesis were full length subclones of the GABA ρ_1 cDNA, designated pCDM-rho1 (31), rat α_1 cDNA (pAlpha A2), and rat β_1 cDNA (clone p15.9) (p15.9 and pAlpha A2 were generous gifts of Dr. Allan Tobin, UCLA). Bovine γ_2 cDNA was a generous gift of Dr. Paul Whiting, Merck Laboratories. After digestion with *Not*I (α, β, and ρ) or *Pst*I (γ), capped RNA transcripts were synthesized from these clones using T7 RNA polymerase, as described (32). Rat brain mRNA was isolated as described (33). Adult female *Xenopus* were obtained from Nasco and Xenopus 1. The animals were anesthetized with ice, and oocytes were removed and placed in Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) with 2 mg/ml collagenase for 2 hr, with gentle agitation. Stage 5 and 6 oocytes were manually defolliculated and injected with 30 ng of mRNA dissolved in 50 nl of 100 mM RNase-free KCl, using a positive-displacement pipette (Drummond). For most experiments, synthetic ρ_1 mRNA was injected alone. For coexpression studies, 30 ng of ρ_1 mRNA and equimolar amounts of $GABA_A$ α_1, β_1, or γ_2 mRNAs were injected in 50 nl; equivalent amounts of α_1 and β_1 mRNAs were also injected together to serve as a positive control. Control oocytes were injected with KCl or with 50 ng of rat brain mRNA. Oocytes were incubated for 2–7 days at 19° in ND96 medium containing 1.8 mM $CaCl_2$, 2.5 mM pyruvate, 50 units/ml penicillin, and 50 mg/ml streptomycin, which was changed daily (32).

Oocyte responses to bath application of various drugs were recorded under two-electrode voltage-clamp conditions at −70 mV, using a WPI amplifier and micropipettes filled with 3 M KCl, as described (33). GABA, muscimol, baclofen, faclofen, bicuculline, picrotoxin, TBPS, pentobarbital, diazepam, and β-CCE were obtained from Research Biochemicals Inc. and Sigma.

Results

Oocytes injected with GABA ρ_1 mRNA displayed inward currents upon bath application of GABA or muscimol but not upon application of glycine or glutamate (Fig. 1). GABA displayed an EC_{50} of approximately 0.9 μM, whereas muscimol displayed half-maximal activity at 1.3 μM (data not shown).

Responses to repeated applications of 1 μM GABA showed modest desensitization. When a second 3-min application of GABA followed the first application after 5 min of washing, depolarizations were ~93% of the original response amplitudes (Fig. 1A). After 1 hr of washing, however, response amplitudes returned to virtually the initial size (data not shown).

Picrotoxin and TBPS displayed potency in this system. When coapplied with 1 μM GABA, the IC_{50} of picrotoxin was 4×10^{-7} M, and TBPS displayed an IC_{50} of 1.9×10^{-6} M (Fig. 1, B and C; Table 1).

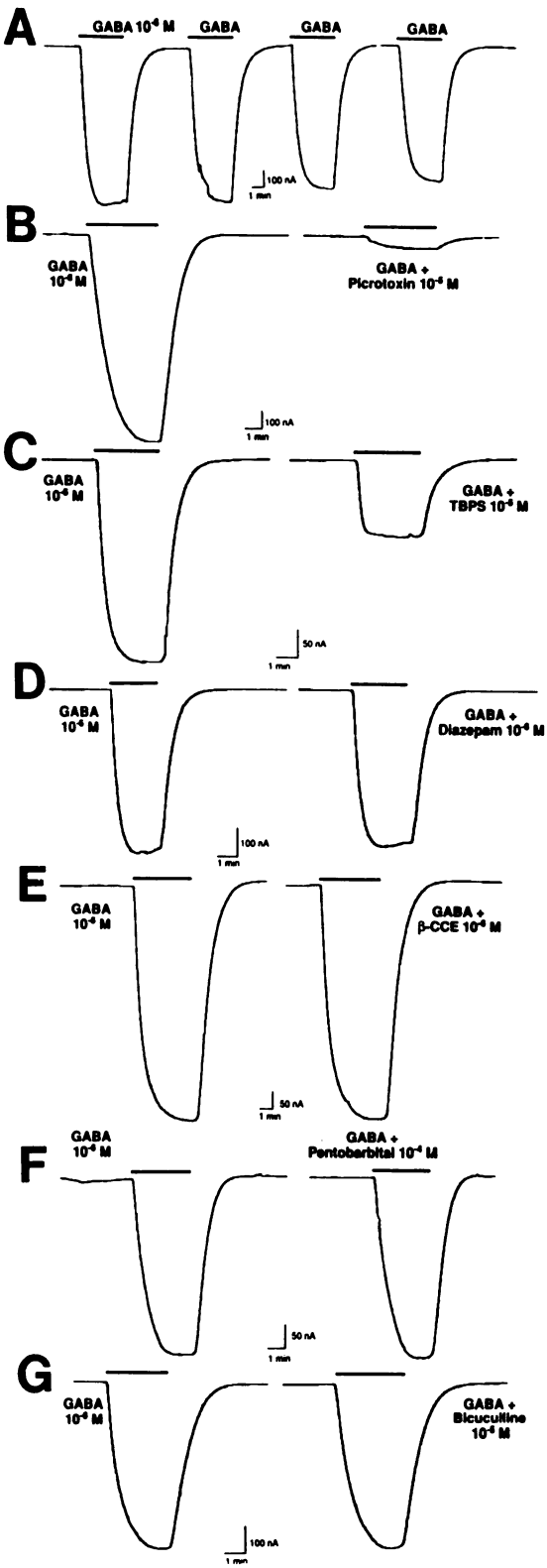

Fig. 1. Tracings of current fluxes induced in GABA ρ_1 mRNA-injected *Xenopus* oocytes superfused with GABA (1 μM) (*bars*) and other agents. *Left tracing*, control depolarization for each oocyte; *right tracing*, subsequent testing with repeated GABA superfusions separated by 5-min washes (A) or reapplication of GABA with other agents, as shown, after a 20-min wash (B–G). Scales as shown.

TABLE 1

Influence of various drugs on GABA responses in *Xenopus* oocytes expressing GABA ρ_1 mRNA or whole brain mRNA

	GABA response[a]							
	10^{-8} M	10^{-7} M	$10^{-6.5}$ M	10^{-6} M	$10^{-5.5}$ M	10^{-5} M	10^{-4} M	10^{-3} M
					%			
A. Oocytes expressing ρ_1 mRNA								
Picrotoxin	95 ± 1.1	79 ± 0.4	59 ± 1.4	35 ± 0.1		6.0 ± 0.48		
TBPS		95 ± 1.3	86 ± 1.9	63 ± 1.3	40 ± 1.8	27 ± 0.5		
Pentobarbital						100 ± 2.7	98 ± 2.1	82 ± 3.4
Diazepam		99 ± 1		99 ± 2.1		100 ± 1.6		
β-CCE		100 ± 2.3		98 ± 1.7		98 ± 1.9		
Bicuculline		103 ± 4.35		100 ± 1.1		99 ± 0.5	115 ± 5.3	
B. Oocytes expressing rat brain mRNA								
Pentobarbital							7-fold increase	
Diazepam				2-fold increase				
β-CCE		30% Inhibition						
Bicuculline						80% Inhibition		

[a] 100% = response to 1 μM GABA (ρ_1-injected oocytes) or 10 μM GABA (brain RNA-injected oocytes).

Despite the efficacy of these GABA-related agents in this system, this receptor was strikingly insensitive to several other GABA-related agents. There were no responses to baclofen or faclofen, at concentrations of up to 100 μM (data not shown). When coapplied with 1 μM GABA, neither diazepam nor the β-carboline β-CCE altered responses at concentrations of up to 10^{-5} M (Fig. 1, D and E; Table 1). Pentobarbital was similarly ineffective at this concentration, although 10^{-3} M pentobarbital did occasionally reduce the response to 1 μM coapplied GABA by approximately 11% (Fig. 1; Table 1). When applied singly, neither pentobarbital, diazepam, nor β-CCE showed any response (data not shown).

The GABA antagonist bicuculline was remarkable ineffective in this system. Concentrations of up to 10^{-5} M were devoid of activity in inhibiting responses to coapplied GABA (Fig. 1G; Table 1), although there was a variable and modest enhancement of GABA responses when bicuculline concentrations were raised to 10^{-4} M.

Studies using oocytes injected with mRNA prepared from whole rat brain supported the efficacy, in altering GABA responses at other $GABA_A$ receptors, of the drug solutions used (Table 1B). Responses to 10^{-5} M GABA were inhibited approximately 80% by 10^{-5} M bicuculline and 30% by 10^{-7} M β-CCE and were potentiated 2-fold by 10^{-6} M diazepam and 7-fold by 10^{-4} M pentobarbital.

Coexpression of the α_1 and β_1 mRNAs also yielded substantial current flux when GABA (10 μM) was applied. However, coexpression of either the α or the β subunit with the ρ_1 mRNA, as described in Materials and Methods, did not modify either the bicuculline insensitivity or the barbiturate insensitivity of GABA responses (data not shown). Thus, in oocytes injected with 30 ng of ρ_1 and 30 ng of either α_1 or β_1 mRNAs, responses to 1 μM GABA were unaltered by simultaneous administration of bicuculline (10^{-5} M) or pentobarbital (10^{-4} M) (data not shown). Similar lack of efficacy was found in oocytes coinjected with 30 ng of ρ_1 and 30 ng of γ_2 mRNA, in two experiments (data not shown).

Discussion

Classical pharmacologic studies have defined several binding sites on postulated GABA/benzodiazepine receptor complexes (23). This work suggested that the GABA receptor site itself was the place where muscimol displayed agonist activity and where bicuculline displayed competitive antagonist properties. Noncompetitive sites on the GABA/benzodiazepine receptor complex were thought to recognize benzodiazepines, allowing benzodiazepines to potentiate GABA responses. Action of inverse agonists at the benzodiazepine sites was thought to result in inhibition of responses of coadministered GABA. The GABA receptor ionophore was thought to be a ligand-gated chloride channel, whose function was blocked by cage convulsant drugs, such as picrotoxin and TBPS. Finally, barbiturates were assigned a site somewhat different from each of the above, with binding properties suggesting noncompetitive interactions with the GABA site, chloride channel, and benzodiazepine site (23).

Cloning the GABA ρ_1 receptor subunit cDNA and finding its RNA expressed at high levels in vision-associated areas led to the current examination of the pharmacologic profile of the receptor expressed as a single subunit type (31). Several other $GABA_A$ receptor subunits can form functional ligand-gated channels when expressed in various combinations in oocytes or in cultured mammalian cells (1–22). Such responses typically reveal modest affinity for GABA and sensitivity to pentobarbital, picrotoxin, and (where tested) bicuculline. Although responses recorded upon expression of single subunits can show more variability, they have been reported for γ_2, δ, α_1, α_2, α_3, α_4, β_1, and β_2 subunits (8, 10, 18, 20, 21).

Several features of the responses observed in the present study suggest that a functional GABA receptor is produced by expressing homooligomeric GABA ρ_1 in oocytes. The receptor formed after injection of GABA ρ_1 RNA displays high sensitivity to GABA itself, compared with reports of other expressed homooligomeric receptors (8, 10, 18, 20, 21). The consistency and size of oocyte responses, as well as the apparent EC_{50} of GABA in producing them, compare favorably with other reports of expressed homooligomeric receptors. The reversal potential of GABA responses noted previously (31), as well as the potency of the cage convulsants, suggest that mechanisms recognizing the amino acid inhibitory transmitter and transducing this recognition to changes in ion channel function are intact in this expressed receptor.

The striking absence of inhibition of the GABA responses by bicuculline suggests that the GABA binding site expressed

on this receptor may show substantial differences from the GABA binding sites on α and β homooligomeric receptors (8, 10, 18, 20, 21). This finding would fit with the properties of the unusual bicuculline-resistant GABA receptor in visual pathways, with GABA responses in *Xenopus* oocytes injected with retina mRNA, and with the responses of the $GABA_C$ receptor defined by Johnston (26, 30). Interestingly, the rank order of potencies of muscimol and GABA also supports a difference in the GABA ρ binding site. In receptors found in brain homogenates, the potency of muscimol is approximately 2 orders of magnitude higher than that of GABA itself (23). In the singly expressed GABA ρ_1 system, however, GABA is virtually as potent as muscimol (31).

Our physiologic results could suggest that barbiturate and benzodiazepine sites are lacking on this receptor molecule. Alternatively, the sites could exist but be unable to influence the effects of GABA on ion conductance. Inability to demonstrate full potentiation by benzodiazepines is a feature of $GABA_A$ α and β subunits when expressed singly, together, or together with δ subunits (4, 18). However, the lack of any change in GABA response with barbiturates found for the GABA ρ_1 receptor is novel.

Expression of the GABA ρ_1 cDNA as a homooligomer thus creates a unique GABA binding site, a robustly gated channel potently blocked by picrotoxin, and no sites conferring responsiveness to barbiturates or benzodiazepines. When the primary structure of this receptor cDNA is compared with other known ligand-gated channels, its closest homology is with $GABA_A$ receptor subunits (31). Nevertheless, its sequence is more divergent from the other classes of GABA receptor subunits than they are from each other. If a $GABA_A$ receptor is defined based on its membership in this family and based on its ability to form a ligand-gated channel, then the GABA ρ_1 receptor belongs in this family. Accepting this parsimonious classification would lead to reference to this receptor as "$GABA_A$ ρ_1." However, to the extent that $GABA_A$ receptors are defined based on a pharmacologic feature, bicuculline sensitivity, this receptor must fall into the class of "unusual GABA receptors" or $GABA_C$ receptors (25, 30).

It is conceivable that this receptor could be expressed as a single subunit within the retina or brain. Studies defining which subunits might actually be coexpressed in the nervous system will be necessary before the exact significance of these *in vitro* observations for *in vivo* receptor function are known. Detailed characterization of extracted proteins could help to define whether a molecule that was expressed endogenously by *Xenopus* oocytes was a necessary participant in the responses that the oocytes acquired after expression of the exogenous ρ_1 mRNA. Nevertheless, our observation that the unique properties of the receptor are maintained when it is coexpressed with either an α or a β subunit would be consistent with its function as a homooligomer *in vivo*. Further, recent findings that the major component of GABA responses obtained in *Xenopus* expression studies of mRNA isolated from the retina is bicuculline insensitive would also be consistent with the possibility that the ρ_1 subunits may self-associate *in vivo*.

The current work provides substantial evidence for a distinct and unique physiologic and pharmacologic role for this newest member of the GABA receptor gene family. These pharmacologic distinctions are at least as great as those used in the past to subtype, for example, different adrenergic receptor subtypes. Recent identification of a second member of this receptor gene subfamily, with a closely linked chromosomal localization, points to the different evolutionary path that this receptor family has taken (34). Especially if further evidence supports a self-associating role for this subunit, these genetic, pharmacologic, and physiologic distinctions may be sufficiently unique to merit naming the receptors formed from these subunits $GABA_C$.

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