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Antagonistic action of pitrazepin on human and rat $GABA_A$ receptors

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1 Pitrazepin, 3-(piperazinyl-1)-9H-dibenz(c,f) triazolo(4,5-a)azepin is a piperazine antagonist of GABA in a variety of electrophysiological and *in vitro* binding studies involving GABA and glycine receptors. In the present study we have investigated the effects of pitrazepin, and the $GABA_A$ antagonist bicuculline, on membrane currents elicited by GABA in *Xenopus* oocytes injected with rat cerebral cortex mRNA or cDNAs encoding $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ human $GABA_A$ receptor subunits.

2 The three types of $GABA_A$ receptors expressed were reversibly antagonized by bicuculline and pitrazepin in a concentration-dependent manner. GABA dose-current response curves for the three types of receptors were shifted to the right, in a parallel manner, by increasing concentrations of pitrazepin.

3 Schild analyses gave pA_2 values of 6.42 ± 0.62, $n=4$, 6.41 ± 1.2, $n=5$ and 6.21 ± 1.24, $n=6$, in oocytes expressing rat cerebral cortex, $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ human $GABA_A$ receptors respectively (values are given as means $\pm$ s.e.mean), and the Hill coefficients were all close to unity. All this is consistent with the notion that pitrazepin acts as a competitive antagonist of these $GABA_A$ receptors; and that their antagonism by pitrazepin is not strongly dependent on the subunit composition of the receptors here studied.

4 Since pitrazepin has been reported to act also at the benzodiazepine binding site, we studied the effect of the benzodiazepine antagonist Ro 15-1788 (flumazenil) on the inhibition of $\alpha_1\beta_2\gamma_{2S}$ receptors by pitrazepin. Co-application of Ro 15-1788 did not alter the inhibiting effect of pitrazepin. Moreover, pitrazepin did not antagonize the potentiation of GABA-currents by flunitrazepam. All this suggests that pitrazepin does not affect the GABA receptor-chloride channel by interacting with the benzodiazepine receptor site.

Keywords: Benzodiazepine; bicuculline; central nervous system; $GABA_A$ receptors; pitrazepin; *Xenopus* oocytes

Abbreviations: TBPS, t-butylbicyclophosphorothionate; Pitrazepin, 3-(piperazinyl-1)-9H-dibenz(c,f) triazolo(4,5-a)azepin

Introduction

The N-aryl piperazine derivatives exhibit both antidepressant (amoxapine, mianserine) and antipsychotic (clozapine, clothiapine and metiapine) clinical activities (Squires & Saederup, 1988; 1991); effects which may be partially due to $GABA_A$ receptor blockade. On the other hand $GABA_A$ receptors are heteromeric complexes made up of four or five subunits that form ligand-gated Cl^- channels (Whiting *et al*., 1995). Receptors made up of different combinations of these subunits have different affinities for GABA, as well as for various allosteric modulators (Levitan *et al*., 1988; Pritchett *et al*., 1989a,b; Luddens *et al*., 1990; Puia *et al*., 1991).

The N-aryl piperazine derivatives fully, or partially, reverse the inhibitory action of GABA on the specific binding of t-butylbicyclophosphorothionate (TBPS) to rat brain membranes *in vitro* (Squires & Saederup, 1993). Moreover, binding experiments indicate that pitrazepin, the most potent N-aryl piperazine, interacts competitively with muscimol and bicuculline binding sites (Squires & Saederup, 1987). However, unlike bicuculline, pitrazepin inhibits [^{3}H]-flunitrazepam and [^{3}H]-diazepam binding, and interacts with glycine receptors as a potent inhibitor of [^{3}H]-strychnine binding (Gähwiler *et al*., 1984; Braestrup & Nilsen, 1985). Electrophysiologically, pitrazepin reduces the inhibitory postsynaptic potentials and induces bursting activity in hippocampal and hypothalamic neurons, with a potency greater than that of bicuculline (Gähwiler *et al*., 1984). Moreover, the bursting activity induced by pitrazepin persists longer than that induced by bicuculline, and it is not affected by the benzodiazepine antagonist flumazenil, questioning the notion derived from binding assays, where pitrazepin antagonized [^{3}H]-flunitrazepam binding.

In view of this, the present study was undertaken to investigate further the effects of pitrazepin on GABA/benzodiazepine receptor complexes heterologously expressed in *Xenopus laevis* oocytes. For that purpose we have compared the modulation of responses to GABA in oocytes expressing receptors encoded by poly(A)$^+$mRNA isolated from the rat cerebral cortex (containing therefore a variety of $GABA_A$ receptor subtypes), or by human $GABA_A$ receptor subunit cDNAs: $\alpha_1\beta_2\gamma_{2S}$ representing the most abundant GABA/benzodiazepine sensitive $GABA_A$ receptor in the brain (Fritschy *et al*., 1992; Gao & Fritschy, 1994); and $\alpha_1\beta_2$, for insensitivity to benzodiazepine modulation (Smart *et al*., 1991; Poulter, *et al*., 1996).

Methods

Xenopus laevis *oocytes*

Xenopus laevis frogs were anaesthetized (by immersion in ice-cold water) and stages IV–VI oocytes were manually isolated

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from the ovary by removing the external layers (epithelium and theca). The surrounding follicular cell envelope was enzymatically removed by collagenase treatment (SIGMA, type IA) 1 mg ml^{-1} for 45 min at room temperature in Ringer's solution (in mM: NaCl, 115; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH adjusted to 7 with NaOH; see Miledi *et al.*, 1982). Defolliculated oocytes were then stored for 24 h at 16–18°C in Barth's medium (in mM: NaCl, 88; KCl, 1; $CaCl_2$, 0.41; $Ca(NO_3)_2$, 0.33; $MgSO_4$, 0.82; $NaHCO_3$, 2.4; HEPES, 5; pH adjusted to 7.4 with NaOH; usually with 0.1 mg ml^{-1} gentamycin) before injection.

Recombinant plasmids

cDNAs encoding for different $GABA_A$ receptor subunits were introduced into the polylinker of plasmids pcDNA3 or pDMD8, so that the human cytomegalovirus (hCMV) promoter-enhancer would drive the constitutive transcription of the cDNAs, once they were injected into the oocyte nucleus. Plasmids were purified by the alkaline lysis method and dissolved at 1 mg ml^{-1} in Tris-EDTA buffer. Usually 5–15 ng of cDNAs mixture was injected to express $GABA_A$ receptors of the desired subunit composition ($\alpha_1\beta_2$ 1 : 1; $\alpha_1\beta_2\gamma_{2S}$ 2 : 2 : 1).

Preparation of mRNA

Total RNA was extracted from adult rat cerebral cortex using the method of Chomczynski & Sacchi (1987), and poly(A)$^+$mRNA was isolated by chromatography in oligo(dT)-cellulose. Oocytes were injected with 50–100 ng of poly(A)$^+$mRNA dissolved in water and were kept in Barth's medium until used for electrophysiological experiments.

Electrophysiology

Membrane currents were recorded 2–6 days after injection, using a conventional two-microelectrodes voltage-clamp technique (Miledi, 1982), after placing the oocyte in normal frog Ringer's solution. The membrane potential was held at −60 mV, and all drugs were diluted in Ringer's and applied to the oocytes by bath superfusion at 7–15 ml min^{-1} (bath volume 100 μl). When various drugs were applied to the same oocyte, the control response was allowed to recover to 80–100% before the next drug application. Current-voltage relationships were obtained by applying voltage pulses (3 s in 20 mV steps from −140 to +20 mV) before and during drug applications, and plotting their difference. Dose-response curve data were fitted to the equation:

$$y = 1/[1 + (EC_{50}/[x]^{n_H}],$$

where y is the agonist response as a percentage of the maximum response, n_H is the apparent Hill slope and EC_{50} is the concentration producing half maximal response. Results are plotted as the means ± s.e.mean.

Drugs

All bulk chemicals were purchased from Fisher. (+)-bicuculline, GABA, HEPES, flunitrazepam and flumazenil, from Sigma; and pitrazepin from Sandoz Ltd.

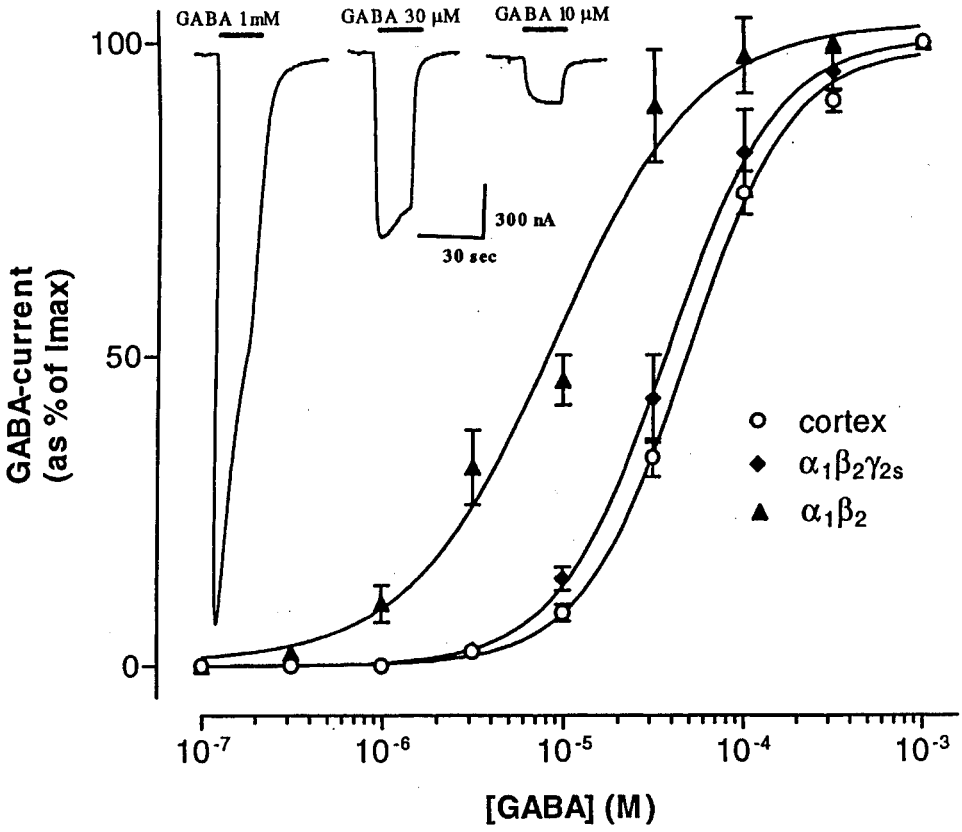

Figure 1 Dose-response relations for GABA-currents evoked by different types of $GABA_A$ receptors expressed by rat cerebral cortex mRNA or human cloned $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ subunits. Responses were normalized and fitted with the Hill relation. Each point shows the mean ± s.e.mean for 5–8 oocytes. Inset: Sample currents elicited in an oocyte injected with rat cortex mRNA. In this and subsequent traces the bars above the records indicate the times of drug applications to oocytes clamped at −60 mV; and inward currents are denoted by downward deflections of the traces.

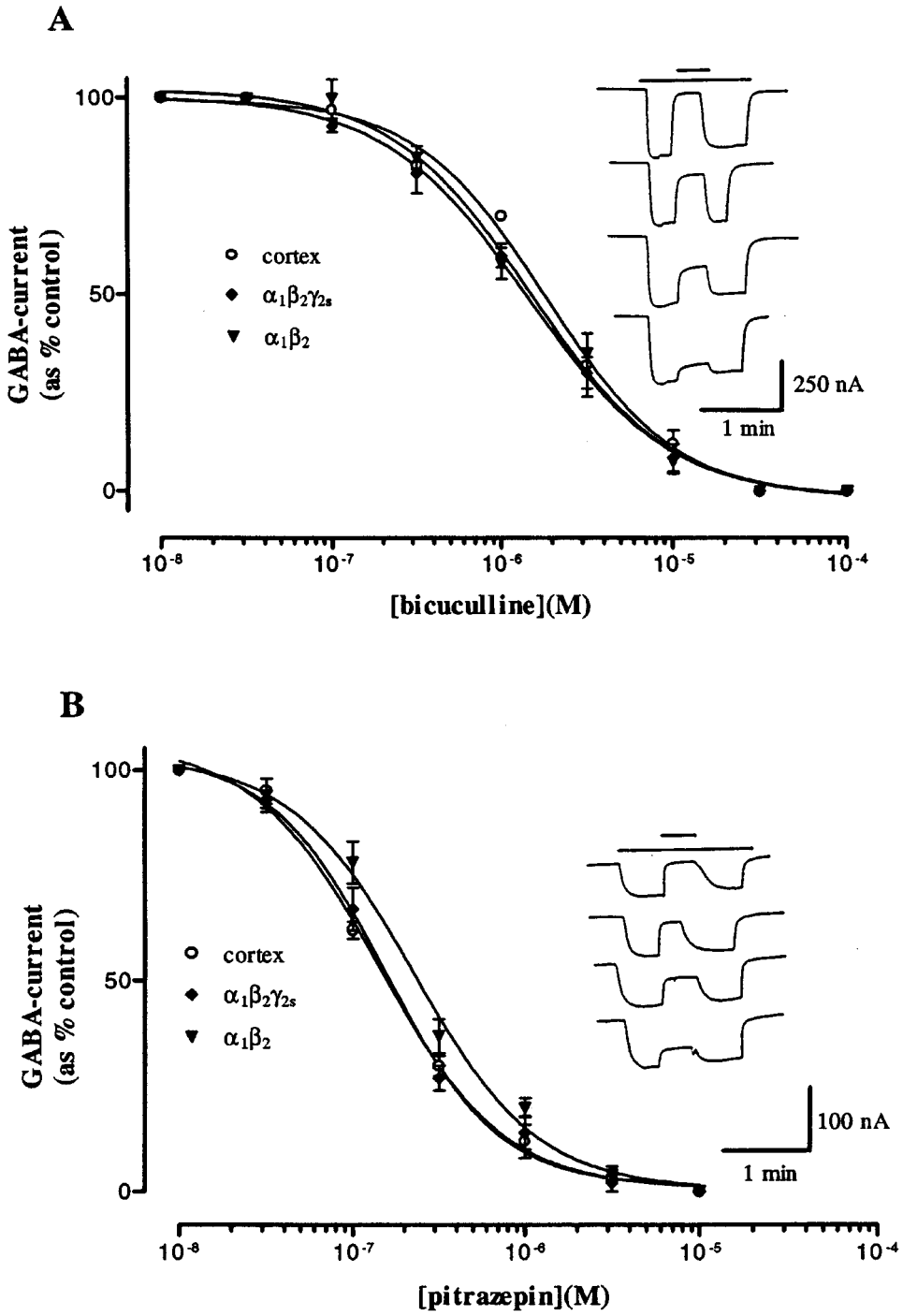

Figure 2 Antagonism of GABA-currents by bicuculline or pitrazepin in oocytes expressing rat cerebral cortex or human $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. The currents were normalized to the amplitudes of the currents obtained with GABA alone and fitted with the Hill relation. (A) Effect of bicuculline. Each point shows the mean ± s.e.mean for 5–7 oocytes. Inset: Sample traces of inhibition by bicuculline (upper bar, from top to bottom: 30, 10, 3 and 1 μM), on I_{GABA} (10 μM, lower bar), in an oocyte expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. (B) Effect of pitrazepin. The concentration of GABA was 10 μM for rat cortex and $\alpha_1\beta_2\gamma_{2s}$ and 2 μM for $\alpha_1\beta_2$ human $GABA_A$ receptors. Inset: Sample traces of inhibition by pitrazepin (upper bar, from top to bottom: 3, 1, 0.3 and 0.1 μM), on I_{GABA} (10 μM, lower bar) in an oocyte expressing $\alpha_1\beta_2\gamma_{2S}$ receptors.

Results

Effects of flunitrazepam, pentobarbitone, zinc and bicuculline

Oocytes expressing receptors encoded by either rat cerebral cortex mRNA, human $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ subunits, all responded to GABA with an inward membrane current, due mainly to a flux of chloride ions (*c.f.* Gundersen *et al.*, 1984). The inset in Figure 1 shows typical responses to GABA in an oocyte expressing rat brain cortex mRNA. At low concentrations, GABA induced a steady inward current, whilst the current evoked by higher concentrations declined with time, even though the drug was continuously applied. Therefore, low concentrations of GABA $\cong EC_{10}$ (10 μM for rat cortex and $\alpha_1\beta_2\gamma_{2S}$ receptors and 2 μM for $\alpha_1\beta_2$ receptors) were routinely

used to reduce time-dependent desensitization of the receptors. The sigmoidal GABA dose-current response curves (Figure 1) for the rat cortex, $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2$ human receptors, yielded EC_{50} values of 48 ± 1.3 μM, $n=5$, 37 ± 1.2 μM, $n=7$, and of 8.8 ± 1.2 μM $n=8$, respectively. The Hill coefficients were 1.5 for both rat cortex and $\alpha_1\beta_2\gamma_{2S}$ receptors, whereas $\alpha_1\beta_2$ receptors gave a Hill coefficient of 1.1. These values are similar to those previously reported for oocytes injected with mRNA from rat cerebral cortex (Parker *et al*., 1986; Polenzani *et al*., 1991) and oocytes expressing rat $GABA_A$ receptors containing $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ subunits (Sigel *et al*., 1990).

To characterize further the three types of $GABA_A$ receptors expressed, we studied the effects of different modulators. For example, flunitrazepam (0.3 μM) potentiated the amplitude of the GABA-current elicited by activation of rat cortex or $\alpha_1\beta_2\gamma_{2S}$ receptors; but had little or no effect on the GABA-current of oocytes expressing $\alpha_1\beta_2$ receptors. The positive allosteric modulator pentobarbitone (30 μM) potentiated the GABA-currents (*c.f.* Gundersen *et al*., 1984; Parker *et al*., 1986) elicited by all three types of receptors, but its efficacy depended on the receptor subunit composition. In respect to the control (GABA-alone) the potentiation was: rat cortex, $424\pm45\%$, $n=5$, $\alpha_1\beta_2\gamma_{2S}$, $345\pm14\%$, $n=6$, and $\alpha_1\beta_2$, $197\pm35\%$, $n=5$. At 1 μM, zinc selectively blocked the GABA-current elicited by $\alpha_1\beta_2$ receptors (Smart *et al*., 1991), whereas it had little or no effect in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ or cerebral cortex receptors. This suggests that when the three subunits are co-injected they preferentially form $\alpha_1\beta_2\gamma_{2S}$ receptors.

It is well known that bicuculline blocks cerebral cortex $GABA_A$ receptors, but fails to block $GABA_C$ receptors (Polenzani *et al*., 1991; Woodward *et al*., 1993). However, the effects of bicuculline on recombinant $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ receptors expressed in *Xenopus* oocytes had not been well characterized. Therefore, we examined the effects of bicuculline on the three types of receptors. In all cases bicuculline blocked the receptors in a dose-dependent way (Figure 2A). IC_{50} values for bicuculline acting on rat cortex, $\alpha_1\beta_2$, or $\alpha_1\beta_2\gamma_{2S}$ receptors were 1.8 ± 0.5 μM, $n=4$, 1.4 ± 0.7 μM, $n=4$, and 1.4 ± 0.1 μM, $n=4$, respectively, and the corresponding Hill coefficients were 1.2 ± 0.1, 1.0 ± 0.1 and 1.0 ± 0.1.

Effects of pitrazepin on $GABA_A$ receptors

Pitrazepin (1 μM) inhibited almost completely the GABA-currents evoked by the three types of receptors. Like bicuculline, pitrazepin decreased the GABA-current in a concentration-dependent manner (Figure 2B). Using GABA concentrations that elicited 10–20% of the maximal currents, the dose-inhibition curves gave an IC_{50} of 138 ± 2.4 nM, $n=5$, 294 ± 11 nM, $n=5$, and 148 ± 10 nM, $n=5$, for rat cortex, $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ receptors respectively, and the corresponding Hill coefficients were 1.3 ± 0.1, 1.1 ± 0.1 and 1.4 ± 0.1. Pitrazepin alone, up to 100 μM, did not elicit detectable membrane currents.

For the three types of receptors the GABA current-voltage relationships (e.g. Figure 3) showed an outward rectification at potentials more negative than about -50 mV, and a reversal potential between -10 and -30 mV. Pitrazepin reduced significantly the GABA-currents at all membrane potentials tested; and the rectification and reversal potential of the GABA-currents remained practically unchanged. Although in the case of Figure 3 the inhibition by pitrazepin appears to show some voltage-dependence, the average of seven oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptor indicates that, in the range -140 to -20 mV, the inhibition is not voltage-dependent.

GABA dose-current response curves, obtained in the presence of different concentrations of pitrazepin, showed a parallel shift to the right for the three types of receptors, and the maximum currents obtained in the presence of pitrazepin were not significantly different from those elicited by GABA alone (Figure 4). The curves for $\alpha_1\beta_2$ receptors showed the smallest shift. Concentrations of GABA eliciting 50% of maximal response, in the absence or presence of pitrazepin, were used to obtain the dose ratio (DR) for each pitrazepin concentration. For each receptor type, IC_{50} values and Hill coefficients of dose-response relationships were significantly increased by pitrazepin (Table 1); and Schild plots obtained

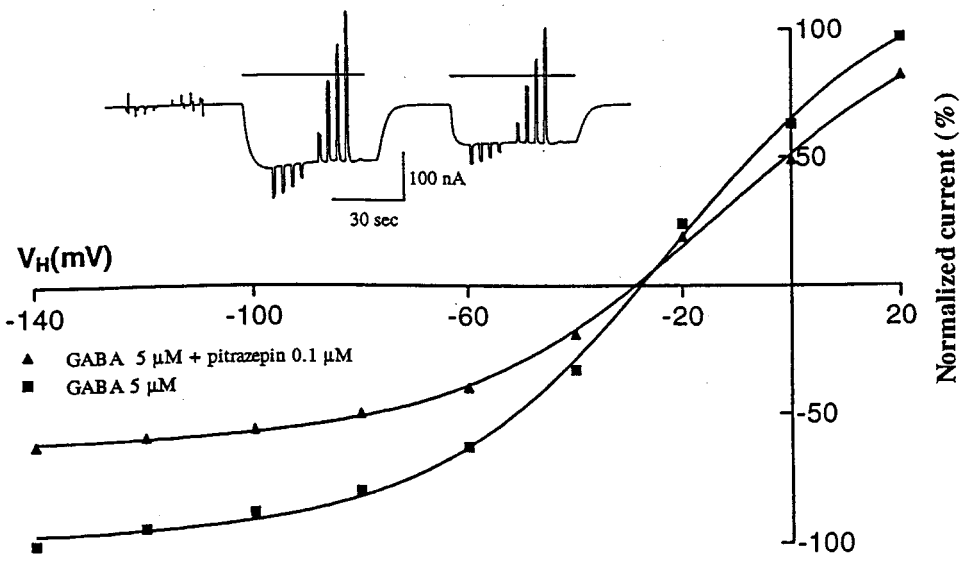

Figure 3 Current-voltage relations for currents evoked by GABA alone, or together with pitrazepin, in an oocyte expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. The responses were normalized to the current obtained with GABA alone at -140 mV. Each point represents the peak current elicited by GABA (5 μM) at the potentials indicated. Voltage steps (-140 mV to $+20$ mV, in 20 mV steps) were applied to the oocyte (held at -60 mV) during application of GABA alone or together with pitrazepin (0.1 μM) as illustrated in the inset.

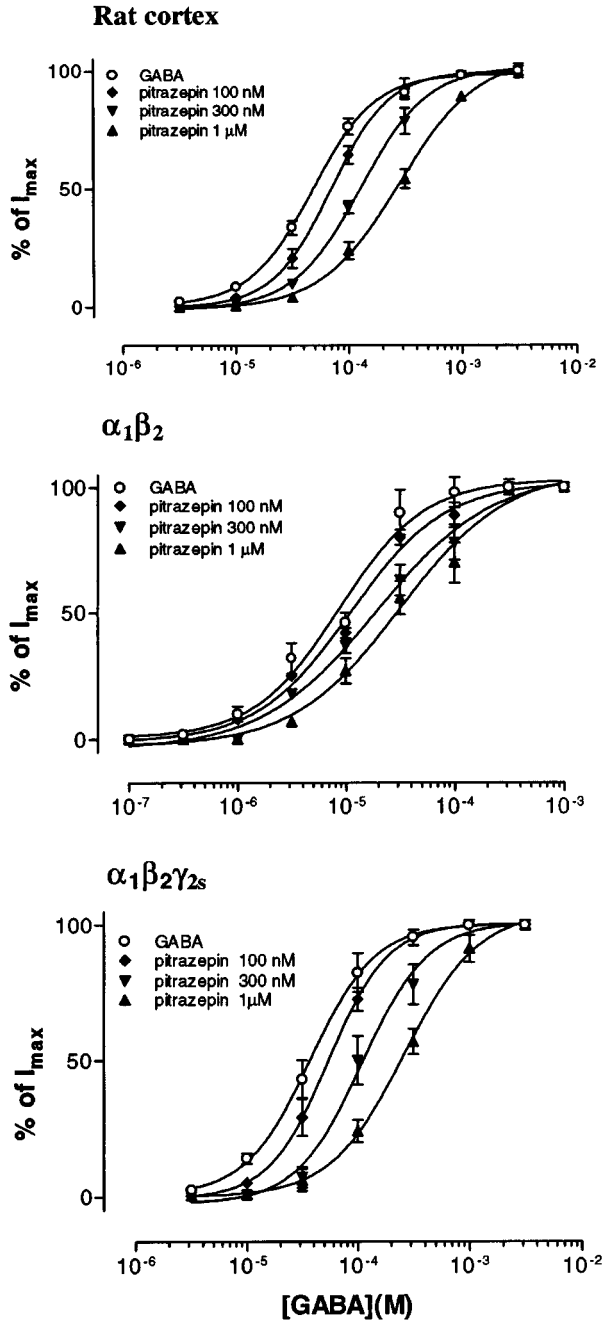

Figure 4 Antagonism of GABA-currents by pitrazepin. Normalized GABA dose-response curves obtained, for the three types of receptors, in the absence or in the presence of pitrazepin. Data fitted to the logistic equation used to determine IC_{50} and Hill coefficients ($n=5$).

without slope constraint for the three types of receptors gave slopes close to 1 and comparable pA_2 values (Table 2).

Pitrazepin, flunitrazepam and flumazenil interaction

In order to see if the binding sites for a benzodiazepine and for pitrazepin are different, we compared the effect of flumazenil, a potent benzodiazepine antagonist, on GABA currents elicited by GABA plus flunitrazepam, or by GABA plus pitrazepin. For that purpose we used oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors, which displayed the greatest pitrazepin effect. For example, in the oocyte used to illustrate Figure 5, pitrazepin (0.1 μM) decreased the GABA-current to 40% whilst flunitrazepam (0.3 μM) increased it 230%. Flumazenil (0.1 μM) alone did not alter the resting membrane current and did not alter the inhibitory effect of pitrazepin. However, flumazenil abolished the potentiating action of flunitrazepam.

To investigate the possibility that pitrazepin acts as a benzodiazepine antagonist we studied the effect of flunitrazepam on GABA currents elicited by 6 μM GABA alone or by 10 μM GABA plus 0.1 μM pitrazepin, at these concentrations the currents elicited in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors were similar in amplitude. As illustrated in Figure 6, flunitrazepam (0.3 μM) caused the same potentiation in both experimental conditions.

Discussion

As far as we know this is the first report on the effects of pitrazepin on the currents evoked by activation of rat cortex and human $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. These receptors display properties very similar to those of native receptors present in the vertebrate central nervous system. Such properties include the dose-response relationship (Tyndale *et al*., 1995), the time-dependent inactivation (Poulter *et al*., 1996) and a reversal potential similar to the chloride equilibrium potential (Kusano *et al*., 1982; Miledi *et al*., 1982). The EC_{50} values obtained here for rat cerebral cortex and for $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ human $GABA_A$ receptors are consistent with those found in previous studies, demonstrating that functional $GABA_A$-receptors are formed also in the absence of the γ_2-subunit (McKernan and Whiting, 1996). $GABA_A$ receptors, studied electrophysiologically in neurons or after injection of poly(A)$^+$ mRNA from rat or chick brain into *Xenopus* oocytes, exhibited Hill coefficients of 1.4–2.0, indicating a positive cooperativity (Choi & Fischbach, 1981; Miledi *et al*., 1982; Hattori *et al*., 1984; Bormann & Clapham, 1985; Smart *et al*., 1987; Bormann, 1989). The observation that the Hill coefficient of $\alpha_1\beta_2$ receptors was 1.1 might be due to increased desensitization of these GABA receptors (Poulter *et al*., 1996). However, this interpretation is not likely since a relatively fast perfusion was used, and also because, using the same experimental conditions, other types of $GABA_A$ receptors (native or $\alpha_1\beta_2\gamma_{2S}$) showed Hill coefficients of 1.5. Therefore, our findings confirm that recombinant $GABA_A$ receptors can fail to show effects attributed to cooperativity of GABA binding (Burt & Kamatchi, 1991). Similar results have been reported for transfected L929 cells, where $\alpha_1\beta_2$ receptors displayed a low Hill coefficient ($n_H=1.1$) compared with that of $\alpha_1\beta_2\gamma_{2S}$ receptors ($n_H=1.7$) (Angelotti *et al*., 1993).

The lack of effect of flunitrazepam (1 μM) on GABA-currents in oocytes expressing the $\alpha_1\beta_2$ receptor is in accord with the observation that the γ_2-subunit is required for the formation of benzodiazepine sites on the receptors (e.g. Gunther *et al*., 1995). Also consistent with previous data are the facts that GABA-currents were inhibited by bicuculline in a concentration-dependent manner (Simmonds, 1982), and that bicuculline acts as a competitive inhibitor with a Hill coefficient of approximately 1 (Krishek *et al*., 1996; Ueno *et al*., 1997). The similar IC_{50} values obtained here for the three types of receptors suggest that their affinity for the antagonist is not strongly dependent on the subunit composition of the $GABA_A$ receptor. Moreover, although 30 μM pentobarbitone enhanced GABA-currents in native as well as in $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_1\beta_2$ $GABA_A$ receptors, the potentiation was significantly greater for native and $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. This suggests a role for the γ subunit in the modulatory effects of GABA-current by barbiturates.

Table 1 Analysis of the concentration-response curves for GABA in the absence or presence of pitrazepin in oocytes injected with rat cortex or human $GABA_A$ receptor cDNAs

		IC_{50} (μM)			Hill Coefficient	
Receptor subtype	*Control*	+	*Pitrazepin*	*Control*	+	*Pitrazepin*
Rat cortex	48±1.2	(1)	284±5.4	1.6±0.1	(1)	1.2±0.1
		(0.3)	128±3.5		(0.3)	1.5±0.1
		(0.1)	70±2.0		(0.1)	1.6±0.1
$\alpha_1\beta_2$	8.8±1.1	(1)	32±2.7	1.1±0.1	(1)	0.9±0.1
		(0.3)	19±2.2		(0.3)	0.8±0.1
		(0.1)	11±2.0		(0.1)	1.1±0.1
$\alpha_1\beta_2\gamma_{2S}$	38±0.7	(1)	264±3.0	1.5±0.1	(1)	1.3±0.1
		(0.3)	110±2.5		(0.3)	1.5±0.1
		(0.1)	54±1.0		(0.1)	1.6±0.8

Concentration-response data for GABA in the absence and in the presence of pitrazepin ($n=4-5$). Data obtained by fitting the logistic equation to the normalized curves. Numbers in parenthesis indicated pitrazepin concentration in μM. All values are the means±s.e.mean.

Table 2 Schild analysis of pitrazepin antagonism of GABA-induced current

Subunit composition	*Slope*	pA_2
Rat cortex	0.96±0.1	6.4±0.6
$\alpha_1\beta_2$	0.99±0.2	6.4±1.2
$\alpha_1\beta_2\gamma_{2S}$	0.93±0.2	6.2±1.2

Values are derived from the Schild plots of data shown in Figure 4. Data were subjected to linear regression analysis (without slope constraint) and calculated with 95% confidence limits.

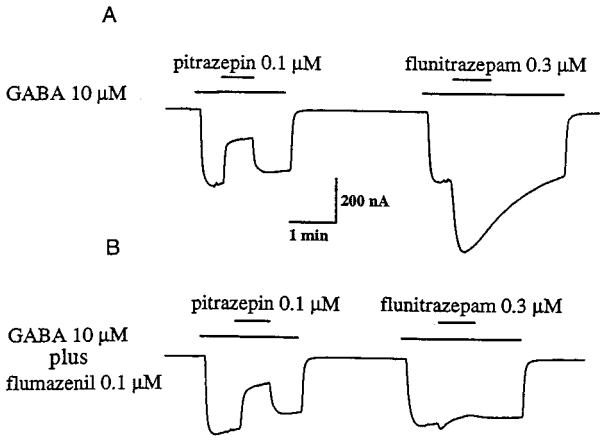

Figure 5 Pitrazepin does not interact with the benzodiazepine site of $\alpha_1\beta_2\gamma_{2S}$ receptors. (A) Pitrazepin inhibits the current induced by GABA whereas flunitrazepam potentiates it. (B) Co-application of GABA plus flumazenil did not affect the inhibition by pitrazepin, but strongly antagonized the flunitrazepam potentiating effect.

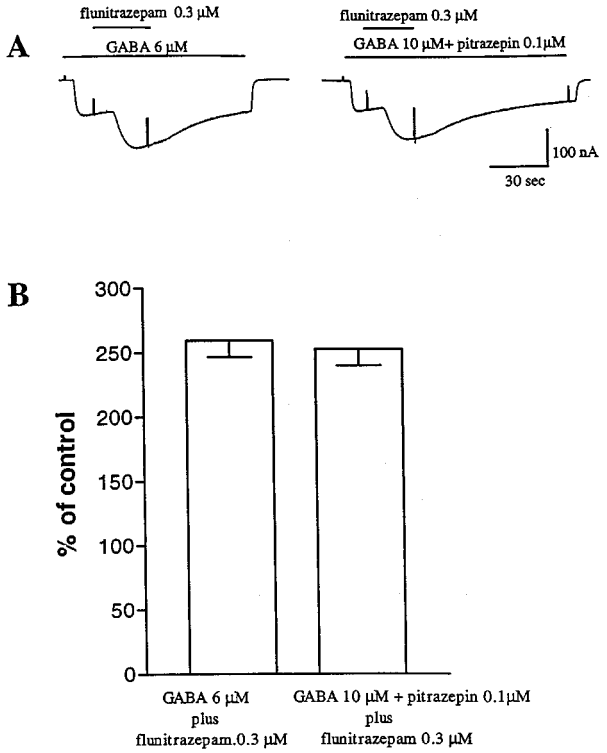

Figure 6 Failure of pitrazepin to antagonize the potentiating effect of flunitrazepam. (A) In an oocyte expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors GABA 6 μM (left) activated inward Cl^--currents of comparable amplitude elicited by GABA 10 μM in the presence of 0.1 μM pitrazepin (right). Co-application of flunitrazepam 0.3 μM in both experimental condition determined a similar potentiation of Cl^--currents. (B) Potentiation of GABA-currents by flunitrazepam. Each column shows the mean±s.e.mean of current amplitude from six oocytes.

The principal finding of this study is that pitrazepin blocks the currents elicited by GABA in *Xenopus* oocytes expressing rat cortex or cloned $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ human $GABA_A$ receptors with comparable efficacies. Like bicuculline, pitrazepin, appears to act as a competitive inhibitor, shifting the dose-response curve to the right without depressing the maximum response. Furthermore, Schild analysis of curves in the presence of pitrazepin gave a slope of approximately 1 for each type of receptor, suggesting a monomolecular action of pitrazepin on $GABA_A$ receptor sites.

The inhibition of GABA induced currents by pitrazepin was fully and rapidly reversible, again similar to the inhibition by bicuculline. Thus, our results agree with previous observations reporting that pitrazepin antagonizes GABA-responses in rat brain hippocampal slices (Gähwiler *et al*., 1984; Kemp *et al*., 1986). The IC_{50} values for bicuculline and pitrazepin show that pitrazepin was ten times more potent than bicuculline in inhibiting the GABA-current; and the current-voltage relations indicate that pitrazepin does not appreciably alter the voltage-dependence and selectivity of the $GABA_A$-gated

channel. Furthermore, the pA_2 values for inhibition of GABA-currents by pitrazepin determined in this study are similar to those derived using CA1 population spikes in a rat hippocampal slice preparation; which gave a slope of 1 and a pA_2 of 6.69 (Kemp *et al*., 1986). The similarity of pA_2 values for pitrazepin action on oocytes expressing rat cortex mRNA (therefore containing an heterogeneous population of $GABA_A$ receptors) and in oocytes expressing $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}$ human $GABA_A$ receptors, suggests strongly that the effect of pitrazepin on GABA-currents is relatively independent of the subunit composition of $GABA_A$ receptors; at least for those studied here.

Interestingly, even though in binding studies pitrazepin displaced [^{3}H]-flunitrazepam, in electrophysiological studies flumazenil did not alter the bursting activity induced by pitrazepin on rat hippocampal neurons (Gähwiler *et al*., 1984). Because of this, the authors discarded the possibility that the bursting induced by pitrazepin was due to its interaction with benzodiazepine sites. Our results on $\alpha_1\beta_2\gamma_{2S}$ receptors are in agreement with that notion, because flumazenil had practically no effect on the inhibition of GABA-currents by pitrazepin, whereas their potentiation by flunitrazepam was abolished. Furthermore, in our experimental conditions, where endogenous GABA or a GABA-reuptake system are absent, we found that pitrazepin did not alter the potentiation of GABA-currents by flunitrazepam. All of this suggest that pitrazepin does not have a positive or negative functional effect on the benzodiazepine site.

In short, our results provide further evidence that pitrazepin, similar to bicuculline, acts directly on the GABA binding site as a competitive antagonist, and that antagonistic action is not dependent upon the presence of the γ_{2S} subunit. Moreover, our results failed to disclose functional evidence of an interaction of pitrazepin with the GABA receptor benzodiazepine sites. The reason for the discrepancy between the binding and functional studies still remains to be determined.

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