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Benzodiazepine modulation of homomeric $GABA_A\rho1$ receptors: Differential effects of diazepam and 4′-chlorodiazepam

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ABSTRACT

$GABA_A$ receptors ($GABA_ARs$) are ligand-gated ion channels that mediate inhibitory neurotransmission in the central nervous system (CNS). They are members of the Cys-loop receptor family and display marked structural and functional heterogeneity. Many $GABA_ARs$ receptor subtypes are allosterically modulated by benzodiazepines (BDZs), which are drugs extensively used as anxiolytics, sedative-hypnotics and anticonvulsants. One high-affinity site and at least three additional low-affinity sites for BDZ recognition have been identified in several heteromeric and homomeric variants of the $GABA_ARs$ (e.g.: $\alpha1\beta2\gamma2$, $\alpha1\beta2/3$, $\beta3$, etc.). However, the modulation of homomeric $GABA_A\rho Rs$ by BDZs was not previously revealed, and these receptors, for a long a time, were assumed to be fully insensitive to the actions of these drugs. In the present study, human homomeric $GABA_A\rho1$ receptors were expressed in *Xenopus* oocytes and GABA-evoked responses electrophysiologically recorded in the presence or absence of BDZs. $GABA_A\rho1$ receptor-mediated responses were modulated by diazepam and 4′-chlorodiazepam in the micromolar range, in a concentration-dependent, voltage-independent and reversible manner. Diazepam produced potentiating effects on GABA-evoked Cl^- currents and 4′-Cl diazepam induced biphasic effects depending on the GABA concentration, whereas Ro15-4513 and alprazolam were negative modulators. BDZ actions were insensitive to flumazenil. Other BDZs showed negligible activity at equivalent experimental conditions. Our results suggest that $GABA_A\rho1$ receptor function can be selectively and differentially modulated by BDZs.

1. Introduction

Benzodiazepines (BDZs) are minor tranquilizers widely used in clinics to produce anxiolytic, sedative-hypnotic, anticonvulsant and muscle relaxant effects (Johnston, 2005; Möhler, 2011; Rudolph and Möhler, 2006). The major action of BDZs in the central nervous system (CNS) is to enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at $GABA_A$ receptors ($GABA_ARs$) (Rudolph et al., 1999). $GABA_ARs$ are GABA-gated chloride (Cl^-) channels, members of the Cys-loop receptor superfamily, which mediate most of the inhibitory neurotransmission (Farrant and Nusser, 2005; Nys et al., 2013). A large variety of functionally and pharmacologically distinct $GABA_ARs$ subtypes originate by combination of diverse subunit classes (e.g.: $\alpha1$–6, $\beta1$–3, $\gamma1$–3, δ, ε, π, θ, $\rho1$–3), usually forming a pentameric array of hetero-oligomeric complexes (Moss and Smart, 2001). The more abundant $GABA_AR$ subtypes in brain synapses are composed by α, β and γ subunits. Responses mediated by these heteromeric receptors, for example the widespread $GABA_A\alpha1\beta2\gamma2Rs$, are typically antagonized by bicuculline or picrotoxin, and allosterically modulated by BDZs and other clinically relevant drugs (Miller and Smart, 2010; Sigel and Steinmann, 2012). In contrast with these $GABA_ARs$, $GABA_A\rho$ receptors ($GABA_A\rho Rs$; which are also commonly called $GABA_C$ receptors), appear to be exclusively composed of ρ subunits ($\rho1$, $\rho2$, $\rho3$), which were found in several regions of the CNS, but are strongly expressed in the retina (Boue-Grabot et al., 1998; Enz and Brandstätter, 1995). $GABA_A\rho Rs$ display both high affinity for GABA and poor desensitization, properties that allow them to mediate several modes of inhibitory signaling (Hull et al., 2006). Ionic currents mediated by homomeric $GABA_A\rho Rs$ receptors can also be antagonized by picrotoxin, but

Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$ receptors, $GABA_ARs$; BDZs, benzodiazepines; TPMPA, (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid; TACA, *trans*-aminocrotonic acid; CACA, *cis*-aminocrotonic acid; CNS, central nervous system; TEVC, two electrode voltage clamp.

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distinguishingly they are insensitive to the classic competitive $GABA_A$ antagonist bicuculline (Feigenspan et al., 1993; Goutman and Calvo, 2004; Polenzani et al., 1991).

Previous work has indicated the existence of $GABA_A$ receptors which are insensitive to the classical BDZ, typically those subtypes containing the α4 and α6 subunits (e.g.: α4β2γ2 and α6β2γ2) (Knoflach et al., 1996). A number of studies have also reported that $GABA_A$ρRs are fully insensitive to the actions of BDZs (Feigenspan et al., 1993; Qian and Ripps, 1999; Shimada et al., 1992; Wang et al., 1994). However, neither a set of different BDZs were systematically tested before, nor a wide range of concentrations had been used for evaluation of their effects on native and recombinant $GABA_A$ρRs. In the present work, we analyzed the effects of a group of BDZs, whose actions on $GABA_A$αβγRs have been well characterized, on the activity of human homomeric $GABA_A$ρ1Rs expressed in *Xenopus* oocytes. GABA-evoked responses were recorded in the absence or presence of the BDZs, using two-electrode voltage-clamp (TEVC). Contrary to the widely held belief that these receptors are insensitive to BDZs, we found that $GABA_A$ρ1R function can be selectively and differentially modulated by diazepam, 4′-Cl diazepam, Ro15-4513 and alprazolam.

2. Materials and methods

All experimental procedures were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the CONICET-University of Buenos Aires Animal Care and Use Committee.

2.1. RNA preparation, oocyte isolation and cell injection

Human cDNA encoding the ρ1 $GABA_A$ receptor subunit, cloned in the in vitro transcription-suitable vector pGEM, was used as a template to synthesize cRNAs in vitro. cRNA solutions (0.3–1 ng/nl) were prepared in Rnase-free H_2O and stored at −70 °C. *Xenopus laevis* (Nasco, Modesto, CA, USA) oocytes at stages V and VI were used for expression of exogenous cRNAs. Isolation and maintenance of cells were carried out as previously described (Miledi and Woodward, 1989). Briefly, frogs were anesthetized with 3-aminobenzoic-acid ethylester (~1 mg/ml) and ovaries surgically removed. Ovaries were incubated with 400 U/ml collagenase for 4 h at 23–24 °C and isolated oocytes maintained in an incubator at 18 °C in Barth's medium (in mM: 88 NaCl; 0.33 $Ca(NO_3)_2$; 0.41 $CaCl_2$; 1 KCl; 0.82 $MgSO_4$; 2.4 $NaHCO_3$; 10 HEPES and 0.1 mg/ml gentamicin; pH adjusted to 7.4 with NaOH). After 1 day, each oocyte was manually microinjected (microinjector Drummond Sci. Co., Broomall, PA, USA) with 50 nl of a solution containing 5–50 ng of cRNA.

2.2. Electrophysiological recordings

TEVC recordings were performed 3–7 days after oocyte injection, with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA). Standard glass recording electrodes were made in a Narishige PB-7 puller (Narishige Scientific Instrument Lab., Tokyo, Japan) and filled with 3 M KCl. Pipette resistance values were approximately 1 MΩ. The holding potential was set to −70 mV and current traces acquired by a PC through a Labmaster TL-1 DMA interface (Scientific solutions Inc., Solon, OH, USA) using AXOTAPE software (Axon Instruments). Cells were placed in a chamber (volume 100 μl) continuously superfused (12 ml/min) with frog Ringer's solution (in mM: 115 NaCl; 2 KCl; 1.8 $CaCl_2$; 5 HEPES; pH 7.0). GABA and other drugs were applied through the perfusion system (Goutman et al., 2005). Drugs were freshly prepared each day as concentrated stocks in DMSO and dissolved in frog Ringer's solution. Stocks were made up as 0.1 M for diazepam, 4′-Cl diazepam, flunitrazepam, flumazenil and clonazepam and 0.01 M for alprazolam, triazolam and RO15-4513. Control experiments, in the absence of BDZs, received equivalent amounts of DMSO. The maximal concentration of DMSO was 1% and had no effect on oocytes integrity and GABA responses. The pH of each test solution was always checked and adjusted if necessary to pH=7.0. All the experiments were carried out at room temperature (23–24 °C) and were replicated in at least 5 different oocytes isolated from at least two different frogs.

2.3. Materials

The transcription kit mMessage mMachine was purchased from Ambion (Austin, TX, USA), and type I or type II collagenase was from Worthington (Freehold, NJ, USA). The agonist, salts, HEPES and RNAse-free H_2O were purchased from Sigma-Aldrich (St Louis, MO, USA). BDZs were kindly donated by Hoffman-La Roche (Nutley, NJ).

2.4. Data analysis

Data were analyzed with Prism v. 5.0 (Graphpad Software, Inc. San Diego, CA, USA). Concentration–response curves for GABA and concentration–effect curve for BDZs were fitted with a logistic equation of the following form: $I_{GABA}/B=[A^n/(A^n+EC_{50}^n)]\times 100$ where A is the agonist concentration, B the maximal response, EC_{50} the concentration of agonist that elicits half-maximal responses, and n the Hill coefficient. Percentage of potentiation was calculated as $[(I_{GABA\rho 1BDZ}\times 100/I_{GABA\rho 1control})-100]$, where $I_{GABA\rho 1BDZ}$ indicates the current amplitude evoked at each particular GABA concentration in the presence of the BDZs and $I_{GABA\rho 1control}$ the corresponding responses in the absence of modulator. Student's *t*-tests (two tailed) were employed to evaluate significant differences between parameters. In all cases errors were expressed as S.E.M.

3. Results

3.1. Functional modulation of $GABA_A\rho 1$ receptors by benzodiazepines

GABA applications to oocytes expressing homomeric $GABA_A$ρ1Rs induce inward Cl^- currents displaying all features of the bicuculline resistant GABA receptor mediated responses found in the retina (Hull et al., 2006; Zhang et al., 2001). For example, they are non-desensitizing, can be antagonized by TPMPA and picrotoxin and display a distinctive pharmacological profile for agonists (TACA > GABA > CACA > muscimol) (data not shown).

In the present work we evaluated the sensitivity of $GABA_A$ρ1Rs to several BDZs whose effects on $GABA_A$αβγRs are well known (Bateson, 2004; Möhler, 2011). BDZs (100 μM) were applied on-top of GABA-evoked responses (0.4 μM GABA) recorded at −70 mV (equivalent results were obtained if oocytes were pre-incubated with the different BDZs, flanked by control responses to GABA, not shown), and modulation calculated as percentage of the control response. As shown in Fig. 1, $GABA_A$ρ1Rs sensitivity to BDZs is clearly distinct from that showed by $GABA_A$αβγRs (Ramerstorfer et al., 2010). Diazepam and 4′-Cl diazepam both produced substantial potentiating actions at this GABA concentration. In contrast, Ro15-4513 and alprazolam induced mild inhibiting actions, while triazolam, flunitrazepam and clonazepam did not produce significant effects. On the other hand, the BDZ antagonist, flumazenil also failed to modulate $GABA_A$ρ1Rs responses by itself. Results obtained for the different BDZs are as follows: diazepam: 42.7 ± 4.6%, $n=20$, $P<0.0001$; 4′-Cl diazepam: 58.2 ± 3.6%, $n=14$, $P<0.0001$; Ro15-4513: −14.5 ± 3.1%, $n=5$, $P<0.01$; alprazolam: −9.9 ± 2.9%, $n=4$, $P<0.05$; triazolam: −4.6 ± 1.1%, $n=3$, *n.s*; flunitrazepam: −4.0 ± 2.4%, $n=5$, *n.s*; clonazepam: −0.6 ± 2.7%, $n=5$, *n.s*; flumazenil: −2.5 ± 2.4%, $n=6$, *n.s*.

Based on these results we decided to further characterize the actions of the two more effective BDZ modulators, diazepam and 4′-Cl diazepam.

3.2. Functional modulation of $GABA_A\rho1$ receptors by diazepam

Current responses elicited by 0.4 μM (A) and 10 μM (B) GABA were significantly enhanced during short bath applications of diazepam (100 μM) performed at the response plateau (Fig. 2). These two representative experiments also show that diazepam potentiation had a fast onset, was stable, strongly depended on GABA concentration and was reversible. No appreciable effects on the oocyte properties such as membrane potential, membrane resistance or current baseline under voltage-clamp were observed during applications of diazepam (Fig. 2C) or any of the other BDZs examined, and no changes in the kinetics of the current responses were observed in the presence of the BDZs (data not shown).

In addition, we performed concentration–response curves (*C–R*) for GABA in the absence (control) or presence of diazepam (100 μM)

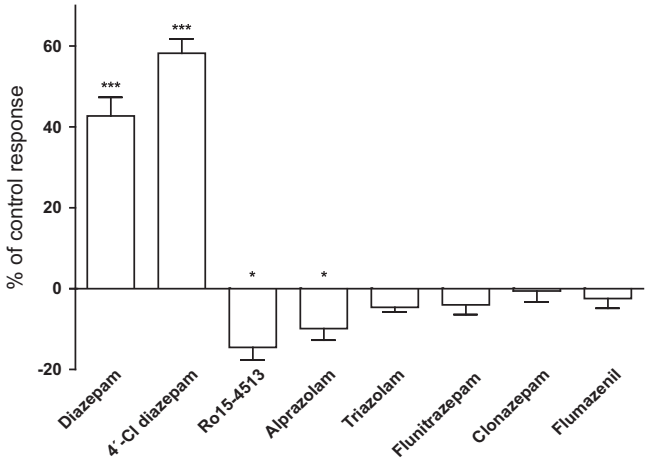

Fig. 1. Effects of different BDZs on responses mediated by $GABA_A\rho1$Rs expressed in *Xenopus laevis* oocytes. Bar graph summarizing the data obtained for different BDZs tested (as indicated). BDZs (100 μM) were applied on-top of GABA-evoked responses, flanked by control responses to GABA (0.4 μM GABA). Data were expressed as percentage of change relative to control values. For this and the subsequent figures, oocytes were voltage-clamped at −70 mV.

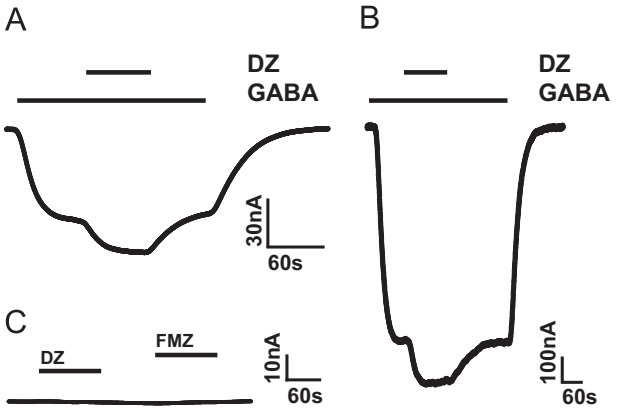

Fig. 2. Potentiating effects of diazepam on responses mediated by $GABA_A\rho1$Rs expressed in *Xenopus laevis* oocytes. Representative traces of $GABA_A\rho1$Rs mediated Cl^- currents elicited by 0.4 μM (A) or 10 μM (B) GABA applications (indicated as bars) in the absence (control) or presence of diazepam (100 μM). Diazepam was delivered on-top of the GABA-evoked responses, flanked by control responses to GABA. (C) Lack of effect of diazepam (100 μM) and flumazenil (100 μM) on a representative baseline current recorded from a transfected oocyte. Scale bars indicate current amplitude (*y*-axis) and time (*x*-axis).

(Fig. 3A). Diazepam produced significant potentiation of the maximal GABA response (14.9 ± 4.8%, $n=5$; $P<0.05$), concomitantly with a leftward shift in $GABA_{EC50}$ ($EC_{50\ GABA}=0.90\pm0.03$ μM, $nH=2.5\pm0.2$, $n=5$; $EC_{50\ GABA+DZ}=0.71\pm0.04$ μM, $nH=3.2\pm0.5$, $n=5$; $P<0.001$). The degree of potentiation exerted by diazepam (100 μM) on $GABA_A\rho1$R-mediated responses decreased as GABA concentration increased (Fig. 3B). For example, the amplitude of currents evoked by 0.4 μM GABA was enhanced by 41.3 ± 4.3% ($n=25$), whereas potentiation of currents evoked by 10 μM GABA was 15.7 ± 4.9% ($n=5$). Potentiation induced by diazepam (100 μM) was significant for all the GABA concentrations tested ($P<0.05$). To determine the concentration range for effective diazepam modulation, we tested the effect of increasing concentrations of diazepam on 0.4 μM GABA-evoked responses (Fig. 3C). Diazepam effects were significant for concentrations above 50 μM ($P<0.05$), suggesting that this BDZ exclusively exerted a low affinity modulatory action on the $GABA_A\rho1$ receptor, in contrast to the modulation produced by diazepam on $GABA_A\alpha1\beta2\gamma2$ receptors that showed two components (Walters et al., 2000). At the highest diazepam concentration tested (500 μM) no saturation of the potentiating effects was observed. Current–voltage relationships (*I–V* curves) in the presence or absence of diazepam indicate that potentiating effects were independent of the membrane potential (Fig. 3D). A significant change in the slope without alteration in the linearity of the *I–V* relationship or reversal potential (between −120 and 40 mV for 0.4 μM GABA-elicited responses) was observed in the presence of diazepam (100 μM).

3.3. Functional modulation of $GABA_A\rho1$ receptors by 4′-Cl diazepam

Fig. 4 illustrates the effects of 4′-Cl diazepam on $GABA_A\rho1$R-mediated responses recorded at −70 mV and elicited by 0.4 μM (panel A) and 10 μM (panel B) GABA. 4′-Cl diazepam (100 μM) produced a biphasic action that depended on the GABA concentration tested. Current amplitudes elicited by 0.4 μM GABA were significantly enhanced, whereas responses elicited by 10 μM GABA were significantly inhibited by 4′-Cl diazepam. Effects on current by 4′-Cl diazepam were rapid, stable and completely reversible after washout. No appreciable effects on oocyte properties, such as membrane potential, membrane resistance or current baseline under voltage-clamp were observed during 4′-Cl diazepam applications (Fig. 4C). *C–R* curves for GABA either in the absence (control) or the presence of 4′-Cl diazepam (Fig. 5A) were performed. At concentrations below 1 μM GABA, 4′-Cl diazepam (100 μM) produced a leftward shift on the *C–R* curve ($EC_{50\ GABA}=1.04\pm0.03$ μM, $nH=2.8\pm0.3$, $n=5$; $EC_{50\ GABA+4'\text{-Cl diazepam}}=0.85\pm0.04$ μM, $nH=2.5\pm0.3$, $n=5$; $P<0.01$) and significant inhibition of the maximal GABA responses (18.8 ± 2.8%; $P<0.05$) were observed at higher concentrations of GABA. Fig. 5B illustrates the changes exerted by 4′-Cl diazepam (100 μM) on $GABA_A\rho1$ receptor responses amplitude for the different GABA concentrations. For example, currents evoked by 0.4 μM GABA were enhanced by 58.8 ± 4.2% ($n=17$), whereas currents evoked by 10 μM GABA were inhibited by 18.7 ± 3.7% ($n=4$). To determine the concentration range for effective modulation, we tested the effect of increasing concentrations of 4′-Cl diazepam on 0.4 μM GABA-evoked responses (Fig. 5C). Effects were significant for concentrations above 50 μM ($P<0.05$). No saturation of the potentiation was observed at the higher 4′-Cl diazepam concentration tested. Current–voltage relationships (*I–V* curves), performed in the presence or absence of 4′-Cl diazepam, indicate that its effects were independent of the membrane potential (Fig. 6). A significant change in the slope without alteration in the linearity of the *I–V* relationship or reversal potential (between −120 and 40 mV) was observed for 0.4 μM

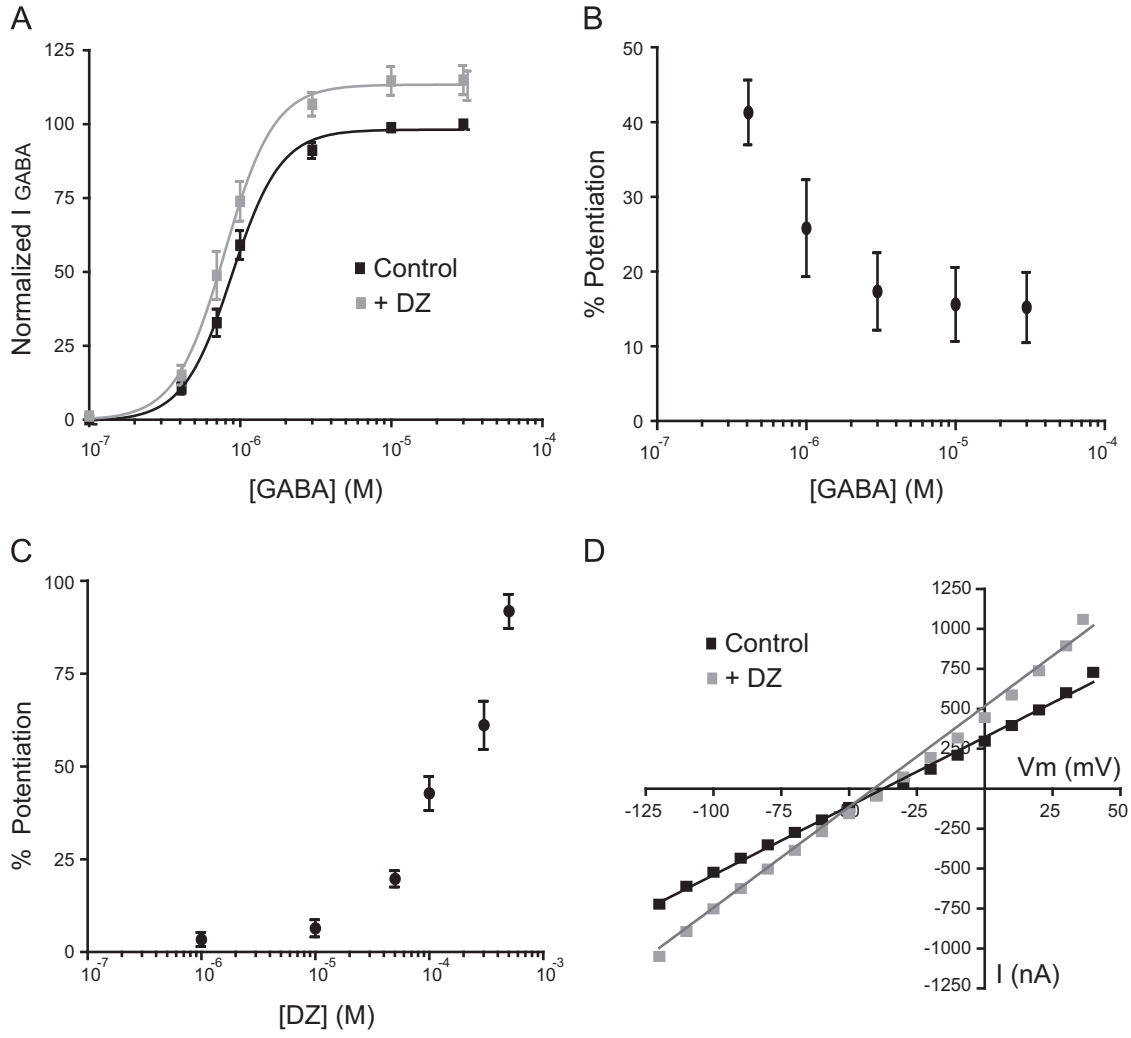

Fig. 3. Analysis of diazepam effects on $GABA_A\rho1$Rs. (A) *C–R* curves for GABA in the absence (■) or presence (■) of diazepam (100 μM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). (B) GABA concentration dependence of the induced diazepam (100 μM) potentiation of $GABA_A\rho1$R responses. (C) Potentiation of $GABA_A\rho1$R responses (0.4 μM GABA) by increasing concentrations of diazepam. (D) *I–V* relationship for $GABA_A\rho1$R responses evoked by 0.4 μM GABA in the absence (■) or presence (■) of diazepam (100 μM).

(A) or 10 μM (B) GABA-elicited responses in the presence of 4′-Cl diazepam (100 μM).

3.4. *Modulation of $GABA_A\rho1$ receptors by diazepam or 4′-Cl diazepam are both insensitive to flumazenil*

To further analyze the modulation of $GABA_A\rho1$Rs by diazepam and 4′-Cl diazepam we performed co-applications of flumazenil, a classic antagonist of the benzodiazepine site at diverse $GABA_A$R subtypes (Fig. 7). Flumazenil (100 μM) failed to antagonize the modulation elicited by 100 μM diazepam (A) or 100 μM 4′-Cl diazepam (B) on responses elicited by 0.4 μM or 10 μM GABA ($n=5–9$; *n.s*). These results indicate that BDZ modulation of responses mediated by $GABA_A\rho1$Rs is insensitive to flumazenil.

4. Discussion

The present findings demonstrate that the function of the human homomeric $GABA_A\rho$Rs can be effectively modulated by BDZs and that the sensitivity of $GABA_A\rho1$Rs to BDZs noticeably differs from that showed by other $GABA_A$Rs subtypes (Bonetti et al., 1988; Harris et al., 1995; Möhler, 2011; Ramerstorfer et al., 2010; Sigel and Steinmann, 2012). The activity of $GABA_A\rho1$Rs expressed in oocytes was substantially potentiated by diazepam in the micromolar concentration range, whereas 4′-Cl diazepam, at equivalent concentrations, produced biphasic effects on current responses mediated by these receptors. Ro15-4513 and alprazolam exerted mild inhibitory actions while triazolam, flunitrazepam and clonazepam produced negligible effects at the concentration tested. In addition, the classic BDZ antagonist flumazenil failed to prevent modulatory actions of BDZ on the $GABA_A\rho1$Rs.

A number of previous in vitro studies proved that testing BDZs in the high micromolar range could be useful to compare and characterize the general pharmacological profile of the different $GABA_A$ receptor subtypes which are so diverse and heterogeneous (Puia et al., 1989; Rudolph et al., 1999; Walters et al., 2000; Whittemore et al., 1996). As previously mentioned, the present results are in sharp contrast with those observed for the same BDZs acting on other $GABA_A$Rs subtypes. For example, concerning $GABA_A\alpha1\beta2\gamma2$Rs, diazepam potentiation of GABA has two components, one at the nanomolar and other at the micromolar range (Walters et al., 2000) (see more details below), 4′-Cl diazepam acts as a monophasic allosteric inhibitor (Puia et al., 1989) and alprazolam is a positive allosteric modulator with high intrinsic efficacy (Mihic et al., 1994). Meanwhile, Ro15-4513 acts as a negative modulator at both $GABA_A\rho1$Rs and

$GABA_A\alpha1\beta2\gamma2$Rs, whereas is a potentiating agent at BDZ-insensitive receptors containing the α4 subunit (Knoflach et al., 1996).

Remarkably, the sensitivity of diverse native or cloned $GABA_A\rho$ receptors to BDZs had been previously assessed in various functional studies with negative results. Those studies included the expression in frog oocytes of native mRNA coding for $GABA_A\rho$Rs isolated from bovine retina (Polenzani et al., 1991) and in vitro transcribed mRNA coding for wild-type and mutant ρ subunits of different species (Qian and Ripps, 1999; Shimada et al., 1992; Walters et al., 2000; Wang et al., 1994) followed by recording of the $GABA_A\rho$Rs-mediated responses. BZD effects on native bicuculline-insensitive GABA responses mediated by $GABA_A\rho$ receptors from retinal slices and dissociated retinal neurons were also evaluated (Feigenspan and Bormann, 1994; Feigenspan et al., 1993; Sivilotti and Nistri, 1991). However, all these previous pharmacological examinations were invariably performed at high, frequently saturating, GABA concentrations that did not fully prevent but significantly counteracted BDZ actions. Since BDZs were never systematically tried in those experiments at the high micromolar range, concentrations tested were also regularly insufficient to elicit any effect. Based on the before mentioned cumulative evidence, $GABA_A\rho$Rs were long time assumed to be fully insensitive to BDZs. Here we show that effective modulation of the $GABA_A\rho1$Rs function can be induced by BDZ and that effects turn out to be relevant at low GABA concentrations.

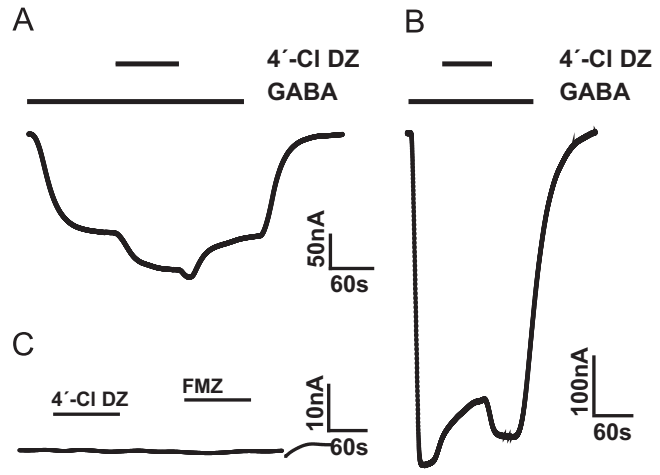

Fig. 4. Biphasic effects of 4′-Cl diazepam on GABA elicited responses mediated by $GABA_A\rho1$Rs expressed in *Xenopus laevis* oocytes. Representative traces of $GABA_A\rho1$Rs mediated Cl^- currents elicited by 0.4 μM (A) or 10 μM (B) GABA applications (indicated as bars) in the absence (control) or presence of 4′-Cl diazepam (100 μM). 4′-Cl diazepam was delivered on-top of the GABA-evoked responses, flanked by control responses to GABA. (C) Lack of effect of 4′-Cl diazepam (100 μM) and flumazenil (100 μM) on a representative baseline current recorded from a transfected oocyte. Scale bars indicate current amplitude (*y*-axis) and time (*x*-axis).

BDZ actions on $GABA_A$Rs are very complex; thus major efforts have focused to understand their mechanisms of action (Berezhnoy et al., 2004; Bergmann et al., 2013; Hanson and Czajkowski, 2008; Lüscher et al., 2012; Miller and Smart, 2010; Morlock and Czajkowski, 2011; Sharkey and Czajkowski, 2008; Sigel and Steinmann, 2012). Many BDZ binding sites were reported at the different $GABA_A$Rs, but given the great diversity of $GABA_A$Rs subtypes found, it is highly probable that other multiple BDZ recognition sites may exist within the different receptor variants (Ernst et al., 2005; Olsen and Sieghart, 2009; Ramerstorfer et al., 2011). The classic pharmacological effects of BDZ are mediated through a high-affinity (nM) site located at the α/γ interface of $GABA_A\alpha\beta\gamma$Rs. Thus, high affinity diazepam actions are restricted to only a few heteromeric $GABA_A$Rs subtypes, because they depend on the α subunit isoforms involved as well as on the presence of the γ subunit. Conversely, low affinity (μM) diazepam actions are believe to be less specific, since they can be found in many $GABA_A$Rs subtypes, either heteromeric or homomeric (e.g.: $\alpha1\beta2\gamma2$, $\alpha1\beta2/3$, β3, etc.) (Baur et al., 2008; Sieghart et al., 2012; Walters et al., 2000). Walters and colleagues, in a seminal work, demonstrated that during activation of $GABA_A\alpha1\beta2\gamma2$Rs by low GABA concentrations, diazepam can act via two distinct and separable mechanisms to produce potentiation. The low-affinity component of diazepam potentiation was independent of the presence of the γ2 subunit, insensitive to the selective BDZ antagonist flumazenil and was specifically disrupted by mutations at equivalent residues within the TM2 of α, β and γ subunits, without affecting the high-affinity component (Walters et al., 2000). Interestingly, these authors also observed that diazepam, which was unable to induce pharmacological effects on wild-type $GABA_A\rho1$Rs up to 30 μM, produced significant potentiation in $GABA_A\rho$Rs carrying converse mutations of the corresponding TM2 residue and a TM3 residue. Now we show that, in fact, wild-type $GABA_A\rho1$Rs are not fully insensitive to diazepam actions, but more likely diazepam acts as a low potency modulator at these receptors. One possible interpretation for the previous and present results is that those mentioned critical mutations might significantly increase the affinity of these receptors to diazepam, instead of conferring sensitivity to a reluctant receptor. The present results also raise the question whether BDZ effects on $GABA_A\rho1$ receptors are directly exerted through BDZ-sensitive sites

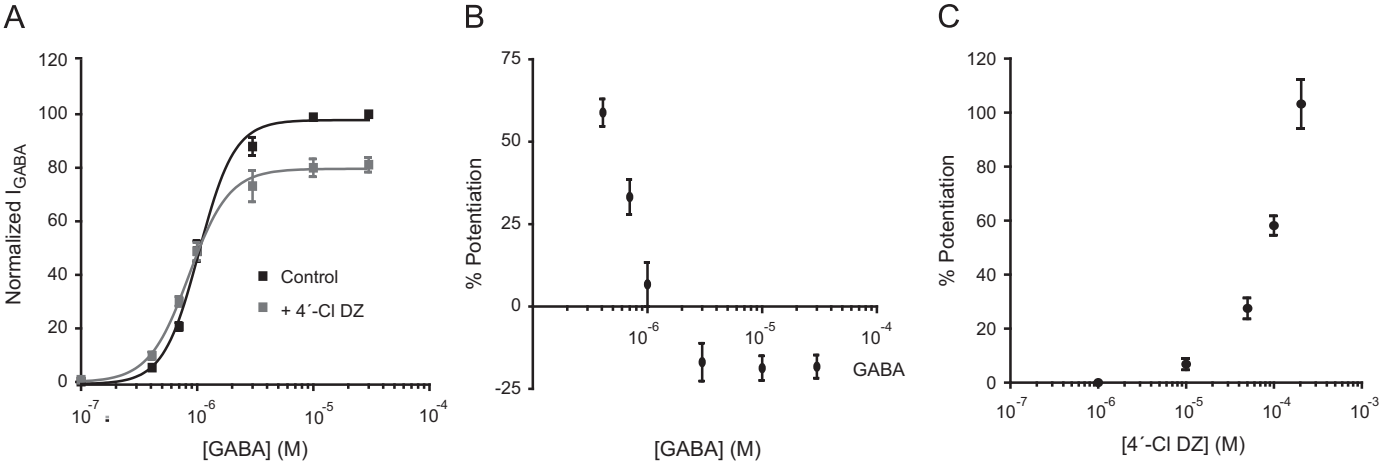

Fig. 5. Analysis of 4′-Cl diazepam effects on $GABA_A\rho1$Rs. (A) *C–R* curves for GABA in the absence (■) or presence (■) of 4′-Cl diazepam (100 μM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). (B) GABA concentration dependence of the induced 4′-Cl diazepam (100 μM) modulation of $GABA_A\rho1$ R responses. (C) Potentiation of $GABA_A\rho1$ R responses (0.4 μM GABA) by increasing concentrations of 4′-Cl diazepam.

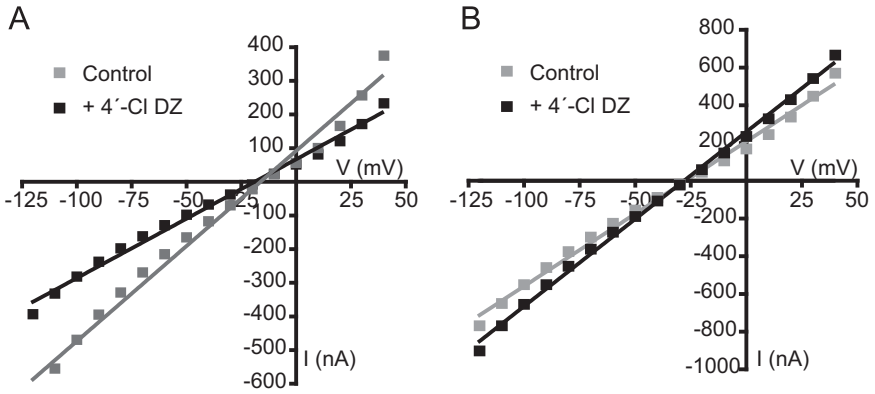

Fig. 6. *I–V* relationship for $GABA_A\rho1$ Rs responses evoked by 0.4 μM (A) or 10 μM (B) GABA in the absence (■) or presence (■) of 4′-Cl diazepam (100 μM).

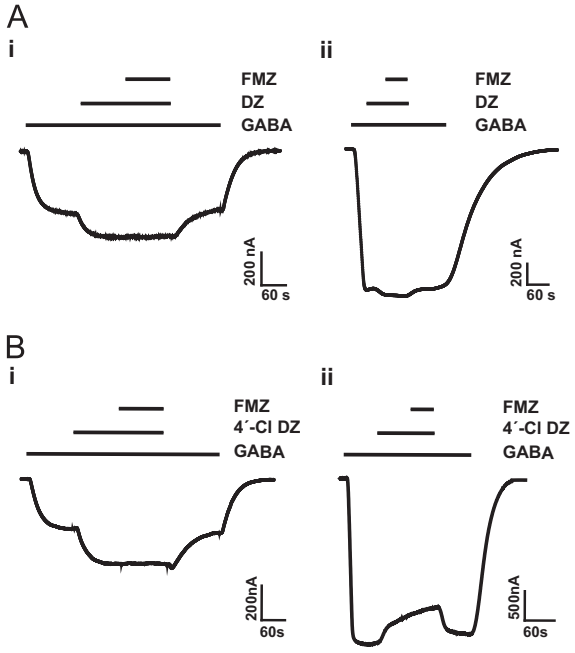

Fig. 7. Diazepam and 4′-Cl diazepam modulation of $GABA_A\rho1R$ responses are insensitive to flumazenil. Flumazenil (100 μM) failed to antagonize the modulation elicited by 0.4 μM (i) or 10 μM (ii) GABA in the presence of 100 μM diazepam (A) or 100 μM 4′-Cl diazepam (B). Flumazenil was co-applied with the benzodiazepines on GABA-evoked responses, flanked by control responses to GABA. Scale bars indicate current amplitude (*y*-axis) and time (*x*-axis).

or indirectly for example via interaction with membrane lipids in the annulae near to the Cl^- channel. Experimental evidence provided here indicates that $GABA_A\rho1Rs$ would be directly modulated by BDZs, because potentiation was easily reversible, concentration-dependent and strongly depended on the GABA concentration. In addition, BDZ treatment did not change the reversal potential of the *I–V* curves, thus, is unlikely that BDZ actions were due to a changes in the intracellular Cl^- levels. As BDZ effects washed out quickly and completely, the involvement of hydrophobic pocket/s facing the extracellular side is also unlikely. Crystal structures obtained using ELIC, a prokaryotic homolog that is also activated by GABA and modulated by BDZs with effects comparable to those at $GABA_A$ receptors (Thompson et al., 2012), revealed important features of both GABA recognition and BDZ interaction (Spurny and Ramerstorfer, 2012). Data indicated that BDZs, depending on their concentration, occupy two possible sites in ELIC, an intrasubunit site neighboring the GABA-recognition site and facing the channel vestibule and a second intersubunit site partially overlapping the GABA site. This later site would correspond to a low-affinity BDZ-binding site in $GABA_A$ receptors. Further studies will be necessary to know if $GABA_A\rho Rs$ actually carry an equivalent site.

BDZs bind to the $GABA_ARs$ and modulate the GABA-evoked Cl^- currents by triggering a global conformational rearrangement of the protein. It has been suggested that BDZs exert this allosteric effect by increasing the microscopic binding affinity for GABA agonist, altering the channel gating or shifting the equilibrium between the ligand-bound resting and pre-activated states that precedes channel opening (Baur and Sigel, 2005; Gielen et al., 2012). Something similar could occur with $GABA_A\rho Rs$ in the presence of BDZs. The leftward shift produced in *C–R* curves for GABA in the presence of BDZs is compatible with any of these scenarios. Simultaneous interaction of BDZs with more than one binding site at the ρ1 subunits, as in the case of other $GABA_AR$ subtypes (Ernst et al., 2005; Olsen and Sieghart, 2009; Ramerstorfer et al., 2011), cannot be discarded. Future experiments should be focused to properly establish the mechanisms of action, identify the residues involved and recognize how benzodiazepine ligands can be positioned in their binding pockets, these includes binding assays, site directed mutagenesis, concatenated receptor models, etc. It will be also interesting to know if other $GABA_A\rho R$ variants are also sensitive to BDZs.

On the other hand, bicuculline-insensitive $GABA_ARs$ were also described in insects and other invertebrates (Buckingham et al., 2005; Lummis and Sattelle, 1986). Particularly, the pharmacology of the homomeric *Rdl* (cyclodiene-resistant) receptors of *Drosophila* is similar to that of the $GABA_A\rho1Rs$. *Rdl* receptors were also shown capable to be modulated by BZDs (Buckingham et al., 2005; Hosie and Sattelle, 1996). However, they do not show significant structural similarity to $GABA_A\rho1Rs$ (Buckingham et al., 2005). Thus, our report appears to be the first indication of BDZ sensitive, bicuculline-insensitive ionotropic GABA receptor in vertebrates that is of $GABA_A\rho Rs$.

$GABA_A\rho Rs$ are involved in several modes of inhibitory actions in the retina (Lukasiewicz et al., 2004). Tonic inhibitory currents mediated by $GABA_A\rho Rs$ can be persistently activated in bipolar cells by low concentrations of ambient GABA, which is locally controlled by GABA transporters located on amacrine cells (Hull et al., 2006; Jones and Palmer, 2009). As $GABA_A\rho Rs$ show relatively high affinity for GABA, BDZ modulation at low GABA concentrations could be suited within the dynamic range of ion channel activation. Thus, extrasynaptic $GABA_A\rho Rs$, which would presumably be exposed to lower GABA concentrations, might be effectively modulated by BDZs. Several studies have reported both beneficial or adverse side effects on the human retina after consumption of high doses of BDZs (Hilton et al., 2004; Manners and Clarke, 1995). Thus, it will be important to analyze in future studies the pharmacological/physiological relevance of the BZDs actions on $GABA_A\rho Rs$ using animal models.

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