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Pharmacology of $GABA_A$ receptors exhibiting different levels of spontaneous activity

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Abstract

The present study examines the spontaneous channel activity of $GABA_A$ receptors and the pharmacology of various full agonists (γ-aminobutyric acid (GABA), isoguvacine), partial agonists (4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol (THIP), piperidine-4-sulphonic acid (P4S), imidazole-4-acetic acid), competitive antagonists (bicuculline, 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide (SR95531)) and non-competitive antagonists (picrotoxinin, zinc). Experiments were performed on oocytes separately expressing human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors using two-electrode voltage clamp electrophysiology. Quantifying spontaneous channel activity showed this varied significantly between the $\alpha_1\beta_2\gamma_{2S}$ ($0.2 \pm 0.07\%$), $\alpha_1\beta_3\varepsilon$ ($20 \pm 3\%$) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ ($83 \pm 4\%$) receptors. A direct correlation was found between the relative agonist potencies and the level of spontaneous activity of the $GABA_A$ receptors. Furthermore, the maximum responses for partial agonists were increased as a function of increased levels of spontaneous activity. There was no relationship between the potency/efficacy of competitive antagonists and the degree of spontaneous activity. However, the non-competitive allosteric inhibitor picrotoxinin showed an opposite dependence on spontaneous activity compared to that seen for agonists, whereas zinc showed a more complex dependence on the receptor subunit composition. These novel findings indicate that the potency and efficacy of ligands acting on $GABA_A$ receptors are highly dependent on the level of spontaneous activity of the receptor.

Keywords: $GABA_A$ receptor; Spontaneous activity; Potency; Partial agonist; Oocyte; Electrophysiology

1. Introduction

Previous reports of spontaneous channel activity in recombinant $GABA_A$ receptors have shown that dimeric $\alpha_4\beta_1$, as well as homomeric β_1 or β_3 subunit-containing receptors are generally insensitive to activation by γ-aminobutyric acid (GABA) (Khrestchatisky et al., 1989; Sigel et al., 1989; Krishek et al., 1996b; Wooltorton et al., 1997a), and therefore possess a pharmacological profile significantly different from $GABA_A$ receptors composed of subunits from three (or more) different families.

Few reports have demonstrated the phenomenon of spontaneous activity in heteromeric $GABA_A$ receptors, in the absence of GABA, using recombinant expression systems. These include channel mutants and ε-containing receptors. Two groups independently mutated a conserved pore-lining leucine residue (L259S) in the human $GABA_A$ β_2 subunit, which resulted in picrotoxin sensitive spontaneous channel activity and increased agonist potencies on functional $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ $GABA_A$ receptors expressed in oocytes (Thompson et al., 1998, 1999; Chang and Weiss, 1999a).

Only one wild type $GABA_A$ receptor, with more than two different subunits ($\alpha_1\beta_3\varepsilon$), has been reported as having spontaneous picrotoxinin sensitive activity, and this receptor is also gated by the application of extracellular GABA (Neelands et al., 1999).

Based on these previous studies, it was decided to attempt to quantify the levels of spontaneous activity of selected $GABA_A$ receptors in comparison to the pharmacological profiles of a number of $GABA_A$ receptor ligands.

In order to study a broad spectrum of receptors exhibiting spontaneous activity, we expressed the following subunit

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combinations in *Xenopus* oocytes: $\alpha_1\beta_2\gamma_{2S}$, (no spontaneous activity), $\alpha_1\beta_3\varepsilon$ (low level of spontaneous activity) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ (high level of spontaneous activity). Compounds were chosen, so that a number of full agonists (GABA and isoguvacine), partial agonists (4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol, THIP; piperidine-4-sulphonic acid, P4S; and imidazole-4-acetic acid, IAA), competitive antagonists (2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide, SR95531; and bicuculline) and non-competitive antagonists (picrotoxinin and zinc) were included in the study.

2. Materials and methods

2.1. Human $GABA_A$ receptor cDNAs

Isolation and sequencing of cDNAs encoding human α_1, β_2, β_3, γ_{2S} and ε have been described elsewhere (Hadingham et al., 1993a,b; Whiting et al., 1997). The $\beta_{2(L259S)}$ mutation has previously been described by Thompson et al. (1998, 1999).

2.2. Expression in Xenopus oocytes

Stage V and VI oocytes were removed from anaesthetised adult female *Xenopus laevis* frogs. Following manual isolation with fine forceps, the oocytes were treated with a mild collagenase solution (type A1 [0.5 mg/ml] for 6 min) to remove follicle cells. The oocyte nuclei were injected with 18–25 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7.0, sterile filtered) containing different combinations of human $GABA_A$ subunit cDNAs engineered into the expression vector pCDM8 or pcDNA1/Amp. The injection ratio of cDNAs encoding the various subunits were 1:1:1 for $\alpha_1\beta_2\gamma_{2S}$ (~ 0.07 ng of each subunit per oocyte) and for $\alpha_1\beta_3\varepsilon$ (~ 0.14 ng of each subunit per oocyte), whereas $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ were injected in a ratio of 1:10:1 (~ 0.26 ng of α_1 and γ_{2S}, and ~ 2.6 ng of $\beta_{2(L259S)}$ per oocyte). The raised concentration of cDNA encoding the $\beta_{2(L259S)}$ subunit was necessary due to poor expression.

2.3. Electrophysiology and data analysis

Following incubation (24 h for $\alpha_1\beta_2\gamma_{2S}$ versus 48 h for $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$), oocytes were placed in a 300-μl bath and superfused continuously (6.5 ml/min) with modified Barth's medium (MBS) consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, at pH 7.5. Cells were impaled with two 0.5–1.5 MΩ electrodes containing 2 M KCl and voltage clamped at −60 mV.

In all experiments, drugs were applied in the perfusate, allowing up to 10 min between each drug application to prevent desensitisation.

Using the maximum inhibition by saturating concentrations of picrotoxinin as a probe for spontaneous channel activity and GABA for estimating the maximum agonist response, we quantified the full *response capacities* of the individual $GABA_A$ receptor populations by summing up

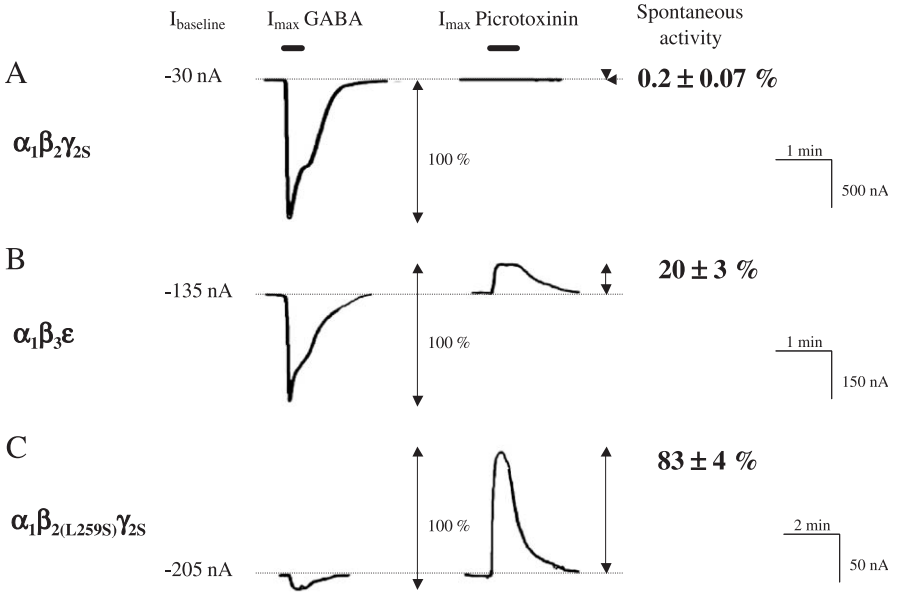

Fig. 1. Sample traces obtained from three different Xenopus oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ (A), $\alpha_1\beta_3\varepsilon$ (B) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ (C) receptors, demonstrating the effect of GABA (3000 μM on $\alpha_1\beta_2\gamma_{2S}$, and 100 μM at $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$) and picrotoxinin (1000 μM on all subunit combinations) on the baseline current. The apparent outward current seen with picrotoxinin on $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ indicates that these receptors are spontaneously active. The spontaneous activities are quantified as fractions (*righthand side arrows*) of the total response capacity (100% *arrows*). Baseline current values for each example are represented by the dotted lines.

these two oppositely directed responses on each oocyte tested. The spontaneous channel activity was subsequently quantified as a fraction of the total *response capacity*.

Concentration response curves were fit using a nonlinear least square fitting routine to the equation $I/I_{max} = 1/[1+(EC_{50}/x)^n]$ where I and I_{max} represent the peak currents activated by a ligand concentration, x, and a saturating concentration of the agonist, GABA, or antagonist, picrotoxinin, EC_{50} is the concentration of drug eliciting a half-maximal response and n is the slope factor. Individual EC_{50} values were transformed into logarithmic pEC_{50} values for statistic evaluations using the following equation, -Log $EC_{50} = pEC_{50}$. Mean pEC_{50} values were reconverted into mean EC_{50} values. To estimate the inhibition equilibrium constant (K_i values) for competitive antagonists at the $\alpha_1\beta_2\gamma_{2S}$ receptor, GABA concentration–response curves were displaced by fixed concentrations of antagonist (10 μM bicuculline; 1 μM SR95531). The K_i values were calculated by Schild analysis using the following equation, K_i=[*Antagonist*]/(*Shift*-1), where *Shift* is the ratio between EC_{50} values with or without antagonist. To determine the potency of the non-competitive antagonists picrotoxinin and Zn^{2+} at $\alpha_1\beta_2\gamma_{2S}$ receptors, the response of a fixed concentration of GABA (30 μM) was inhibited by increasing concentrations of the non-competitive antagonists, and the data were fitted to the equation $I/I_{max} = 1/[1+(x/IC_{50})^n]$ to obtain values of antagonist concentrations eliciting half maximal inhibition (IC_{50}). Values presented are mean values ± S.E. of at least four individual experiments. P4S and THIP were synthesised in-house. All other compounds were obtained from Sigma (St. Louis, MO), RBI (Natick, MA) or Tocris (Langford, Bristol, UK). The computer program, GraFit 4.0.12 (Erithacus Software, Staines, UK), was used to analyse and plot data, and SigmaStat 2.03 was used for statistical analyses.

3. Results

In oocyte experiments, a baseline (leak) current is often detected at a holding potential of − 60 mV. As illustrated in Fig. 1, this leak was significantly increased for $\alpha_1\beta_3\varepsilon$

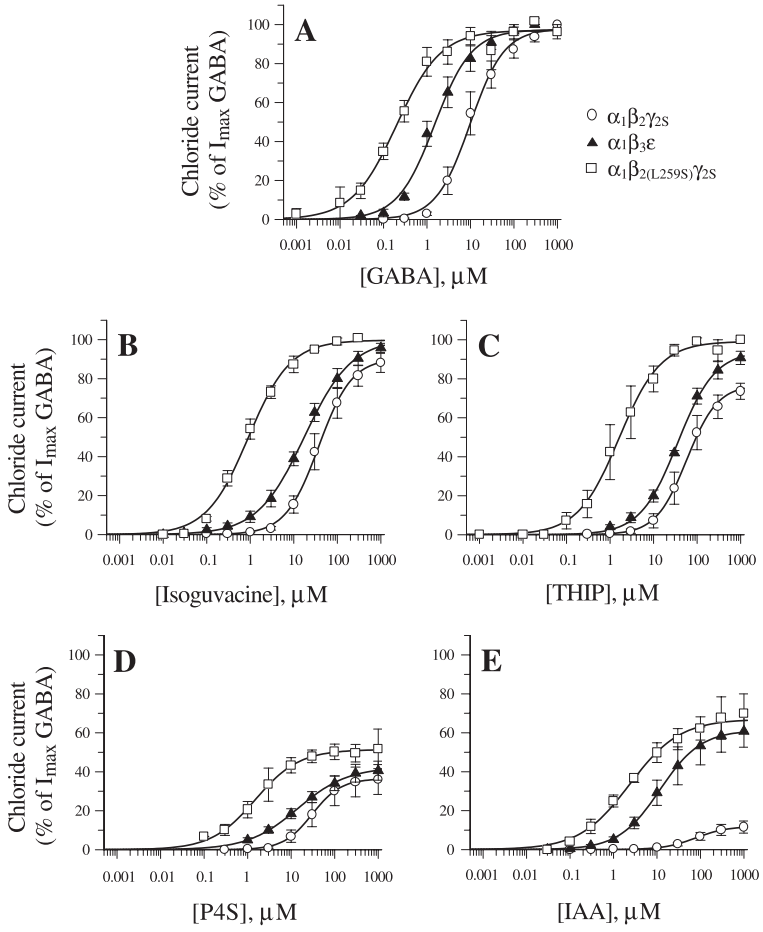

Fig. 2. Concentration response curves of GABA (A), isoguvacine (B), THIP (C), P4S (D) and IAA (E) obtained from $\alpha_1\beta_2\gamma_{2S}$ (*open circles*), $\alpha_1\beta_3\varepsilon$ (*filled triangles*) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ (*open squares*) receptors. Nonlinear regression analysis of the curves was performed as described in the Methods section, and the maximum responses (efficacy) and half maximum concentrations (potency) are summarised in Table 1. The data are expressed relative to a maximum GABA evoked current and represents the mean ± S.E. from 4 to 6 oocytes. In some cases, the error bars are smaller than the points.

Table 1
Efficacy (% of maximum response to GABA) and potency (pEC_{50}) of $GABA_A$ receptor agonists on $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors

	$\alpha_1\beta_2\gamma_{2S}$			$\alpha_1\beta_3\varepsilon$			$\alpha_1\beta_{2(L259S)}\gamma_{2S}$		
	% of max_{GABA}	pEC_{50} (EC_{50}, μM)	Slope factor	% of max_{GABA}	pEC_{50} (EC_{50}, μM)	Slope factor	% of max_{GABA}	pEC_{50} (EC_{50}, μM)	Slope factor
GABA	100 ± 1	5.009 ± 0.178 (9.8)	1.31 ± 0.15	100 ± 2	5.761 ± 0.155 (1.7)	1.20 ± 0.27	100 ± 2	6.636 ± 0.114 (0.23)	0.88 ± 0.17
Isoguvacine	88 ± 5	4.458 ± 0.134 (35)	1.32 ± 0.04	100 ± 2	4.770 ± 0.099 (17)	0.85 ± 0.11	100 ± 1	6.044 ± 0.085 (0.90)	0.96 ± 0.06
THIP	77 ± 3	4.231 ± 0.179 (59)	1.41 ± 0.08	95 ± 3	4.438 ± 0.031 (36)	1.05 ± 0.15	97 ± 3	5.822 ± 0.210 (1.5)	1.64 ± 0.24
P4S	36 ± 8	4.463 ± 0.100 (34)	1.42 ± 0.07	43 ± 5	4.838 ± 0.049 (14)	0.76 ± 0.06	52 ± 3	5.792 ± 0.159 (1.6)	0.97 ± 0.12
IAA	11 ± 3	4.059 ± 0.027 (87)	1.24 ± 0.08	62 ± 8	4.843 ± 0.135 (14)	0.93 ± 0.13	67 ± 9	5.675 ± 0.219 (2.1)	0.91 ± 0.16

Values represent mean ± S.E. of at least four experiments. Numbers in paragraphs are mean EC_{50} values in μM concentrations.

(−180 ± 47 nA) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ (−341 ± 25 nA) receptor expressing oocytes, when compared to wildtype $\alpha_1\beta_2\gamma_{2S}$ receptors (−34 ± 18 nA). In order to establish if this leak current was a consequence of spontaneous channel opening, picrotoxinin was applied. The extent of the inhibition of the baseline current by 1000 μM picrotoxinin, manifested as a picrotoxinin-induced outward current, was directly correlated to the level of the leak current. Thus, a high degree of inhibition of baseline current was observed for $\alpha_1\beta_{2(L259S)}\gamma_{2S}$-injected oocytes, an intermediate inhibition of baseline current for $\alpha_1\beta_3\varepsilon$ receptors, and basically, no reduction in the baseline current for $\alpha_1\beta_2\gamma_{2S}$-containing receptors.

Current voltage plots (data not shown) revealed that the baseline leak current was mediated via a current with reversal potentials ($\alpha_1\beta_2\gamma_{2S}$: −27 ± 1.9 mV; $\alpha_1\beta_3\varepsilon$: −25 ± 1.4 mV; $\alpha_1\beta_{2(L259S)}\gamma_{2S}$: −22 ± 3.9 mV) close to the equilibrium potential for Cl^- (−25 mV; Dascal, 1987). Similarly, the reversal potentials obtained in the presence of GABA ($\alpha_1\beta_2\gamma_{2S}$: −23 ± 2.6 mV; $\alpha_1\beta_3\varepsilon$: −20 ± 4.1 mV; $\alpha_1\beta_{2(L259S)}\gamma_{2S}$: −22 ± 4.3 mV) were not significantly different for the leak currents of the three receptor combinations, also indicating that chloride channel activation occurs in the presence of the full agonist.

As illustrated in Fig. 1, the levels of constitutive activity were determined as 0.2 ± 0.07% for $\alpha_1\beta_2\gamma_{2S}$; 20 ± 3% for $\alpha_1\beta_3\varepsilon$; and 83 ± 4% for $\alpha_1\beta_{2(L259S)}\gamma_{2S}$. These levels of constitutive activity were significantly different for the three receptors ($P<0.001$). Despite differing levels of receptor expression, observed as different GABA maximum currents (1163 ± 111 nA for $\alpha_1\beta_2\gamma_{2S}$, 284 ± 74 nA for $\alpha_1\beta_3\varepsilon$ and 12.4 ± 1.5 nA for $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors), the levels of spontaneous activities were constant for both $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ and did not vary with expression levels between different oocytes (data not shown).

Interestingly, the level of desensitisation of GABA responses decreased as the level of spontaneous activity increased. The opposite was observed for the picrotoxinin induced outward currents, where desensitisation was observed for $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors but hardly at the $\alpha_1\beta_3\varepsilon$ receptors (Fig. 1).

Whether spontaneous activity affect agonist potency and efficacy was investigated using full concentration–response curves for GABA, isoguvacine, THIP and IAA on oocytes expressing each receptor combination.

All agonists were significantly more potent at $\alpha_1\beta_3\varepsilon$ compared to $\alpha_1\beta_2\gamma_{2S}$-containing receptors. The potency was further increased when the agonists were tested at $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors. The assessment of agonist efficacies was made by comparing the maximum responses, relative to that elicited by 3 mM GABA, obtained with each agonist on the three individual receptor combinations. Isoguvacine showed maximum responses similar to that of GABA at all three receptor combinations confirming previous reports of full agonism of isoguvacine on many $\alpha_x\beta_{2/3}\gamma_{2S}$-containing receptors (Ebert et al., 2001; Hansen et

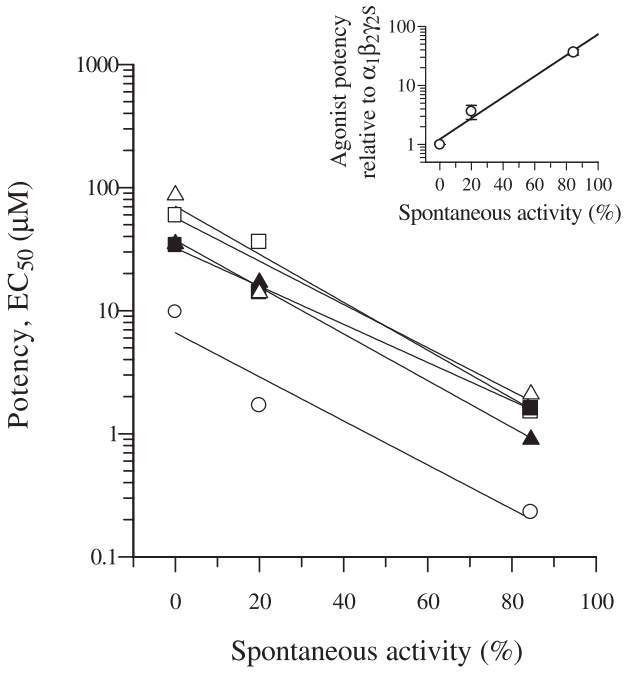

Fig. 3. Linear regression of potency as a function of spontaneous activity for GABA (*open circles*, $R^2=0.813$), isoguvacine (*filled triangles*, $R^2=0.929$), THIP (*open squares*, $R^2=0.834$), P4S (*filled squares*, $R^2=0.890$) and IAA (*open triangles*, $R^2=0.823$). All linear regressions were highly significant ($P<0.001$). The data points at 0.2%, 20% and 83% spontaneous activity represents EC_{50} values obtained from $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$, respectively. *Insert*: Linear regression plot of agonist potencies normalized relative to $\alpha_1\beta_2\gamma_{2S}$ as a function of spontaneous activity ($R^2=0.932$, $P<0.001$). Since all $\alpha_1\beta_2\gamma_{2S}$ values are normalised to unity, there are no error bars at this data point. Data points at 20 and 83% constitutive activity are represented as mean ± S.E. from normalised values from the 5 agonists, though at 83% the error bars are smaller than the point.

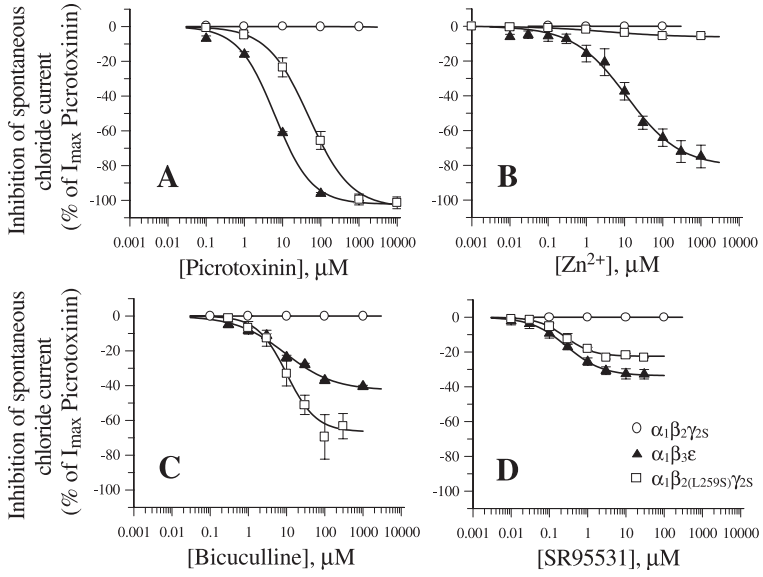

Fig. 4. Concentration response curves for the inhibitory effect of picrotoxinin (A), Zn^{2+}(B), bicuculline (C) and SR95531 (D) obtained from $\alpha_1\beta_2\gamma_{2S}$ (*open circles*), $\alpha_1\beta_3\varepsilon$ (*filled triangles*) and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ (*open squares*) receptors. Nonlinear regression analysis of the curves was performed as described in Materials and methods and the maximum responses (efficacy) and half maximum concentrations (potency) are summarised in Table 2. The data are expressed relative to a maximum suppression of baseline current by picrotoxinin and represents the mean ± S.E. from 4 to 6 oocytes. In some cases, the error bars are smaller than the points.

al., 2001). However, the partial agonists (THIP, P4S and IAA) all showed increased maximum responses at $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors probably as a function of the increased level of spontaneous activity (Fig. 2).

The slope factors for the agonist concentration–response curves were between 1.24 and 1.42 on $\alpha_1\beta_2\gamma_{2S}$ receptors, but generally low (<1) for most agonists on $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors, as summarised in Table 1.

By using linear regression analyses, significant correlations were observed between the levels of spontaneous activity and the potency for all of the agonists ($P<0.001$; Fig. 3). The consistent linear relationships between spontaneous activity and agonist potency was similar when normalised to $\alpha_1\beta_2\gamma_{2S}$. This normalised relationship is displayed in the insert of Fig. 3. It was observed that the agonist potencies increased 3.6 ± 0.98 fold by exchanging $\alpha_1\beta_2\gamma_{2S}$ receptors for $\alpha_1\beta_3\varepsilon$ receptors with a 20% spontaneous activity level. The increase in agonist potency from the non-spontaneously active $\alpha_1\beta_2\gamma_{2S}$ receptors to the $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors (83% spontaneous activity level) was 37 ± 3.9 fold.

Due to the insignificant level of spontaneous activity of $\alpha_1\beta_2\gamma_{2S}$ receptors, the potencies of selected antagonists were determined on the GABA induced responses. In the presence of GABA, picrotoxinin and Zn^{2+} behaved as non-competitive antagonists at $\alpha_1\beta_2\gamma_{2S}$ receptors, whereas bicuculline and SR95531 behaved as competitive antagonists as shown previously (Krishek et al., 1996a; Uchida et al., 1996; data not shown).

At the constitutively active $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors, the same antagonists all evoked outward currents in the absence of GABA suggesting that they blocked the

Table 2
$GABA_A$ receptor antagonist potency (pK_i/pIC_{50}) on $\alpha_1\beta_2\gamma_{2S}$ receptors, and efficacy (% of maximum response to picrotoxinin) and potency (pEC_{50}) on $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors

	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_1\beta_3\varepsilon$			$\alpha_1\beta_{2(L259S)}\gamma_{2S}$		
	pIC_{50} (IC_{50})[a] pK_i (K_i)[b]	% of max_{PTX}	pEC_{50} (EC_{50}, μM)	Slope factor	% of max_{PTX}	pEC_{50} (EC_{50}, μM)	Slope factor
Picrotoxinin[a]	5.472 ± 0.263 (3.4)	100 ± 1	5.203 ± 0.013 (6.3)	0.91 ± 0.05	100 ± 1	4.338 ± 0.168 (46)	0.87 ± 0.04
Zn^{2+} [a]	3.153 ± 0.055 (703)	80 ± 7	4.994 ± 0.066 (10)	0.71 ± 0.15	6 ± 1	‡	‡
Bicuculline[b]	5.590 ± 0.257 (2.6)	43 ± 1	5.039 ± 0.053 (9.1)	0.69 ± 0.07	67 ± 7	5.036 ± 0.098 (9.2)	1.22 ± 0.10
SR95531[b]	6.737 ± 0.025 (0.18)	34 ± 3	6.582 ± 0.099 (0.26)	1.00 ± 0.14	23 ± 1	6.570 ± 0.174 (0.27)	1.44 ± 0.19

Values represent mean±S.E. of at least four experiments. Numbers in paragraphs are respective mean K_i, IC_{50} and EC_{50} values in μM concentrations.
‡ Not determined due to low efficacy.
[a] Non-competitive antagonists.
[b] Competitive antagonists.

spontaneous current. The maximum current evoked by picrotoxinin was consistently the largest and was used as a comparator for the other antagonists/inverse agonists. At these spontaneously active receptors, concentration–response curves were obtained to determine the efficacy (% of maximum response relative to that seen with picrotoxinin) and potency (pEC_{50}) of the $GABA_A$ receptor antagonists/inverse agonists Fig. 4).

Minor differences in both potencies and maximum responses of the competitive antagonists bicuculline and SR95531 were observed (see Table 2), however, these were not correlated with the extent of spontaneous activity.

The potency of picrotoxinin was highest at $\alpha_1\beta_2\gamma_{2S}$ receptors (3.4 μM) and lowest on $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors (46 μM) therefore showing an inverse correlation to the levels of spontaneous activity of the $GABA_A$ receptors as that seen for the agonists.

Zinc was a weak antagonist on both γ_{2S}-subunit containing $GABA_A$ receptors revealing a low potency on wild-type receptors (703 μM), and such a low maximum response on $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors ($6 \pm 1\%$) that the EC_{50} and Hill coefficients could not be estimated. However, zinc was an effective and relatively potent antagonist on $\alpha_1\beta_3\varepsilon$ receptors ($80 \pm 7\%$, 10 μM; Table 2).

4. Discussion

The aim of the present study has been to investigate the effects of full and partial agonists as well as competitive and non-competitive antagonists on heteromeric $GABA_A$ receptors exhibiting different levels of spontaneous activity. By quantifying the levels of spontaneous activity with picrotoxinin for $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\varepsilon$ and $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors, we were able to investigate possible relationships between spontaneous activity and the pharmacology of different agonists and antagonists.

We observed increased maximum responses and potencies of the partial $GABA_A$ receptor agonists, THIP, P4S and IAA, on spontaneously active $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors when compared to wildtype $\alpha_1\beta_2\gamma_{2S}$ receptors. For the full agonists, isoguvacine and GABA, higher potencies were similarly observed for $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors when compared with wildtype $\alpha_1\beta_2\gamma_{2S}$ receptors. Previous reports of the effect of the β_2 mutation in $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors on the potencies and efficacies of agonists (Thompson et al., 1998, 1999; Chang et al., 1996) are in agreement with our results. The importance of the 9′ leucine residue in the transmembrane 2 domain of ligand-gated ion channels has been similarly observed on nicotinic acetylcholine receptors where mutations of this conserved residue also resulted in leftward shifts of agonist concentration–response curves towards higher potencies (Revah et al., 1991; Labarca et al., 1995; Filatov and White, 1995).

Similar trends were also observed by exchanging $\alpha_1\beta_2\gamma_{2S}$ to $\alpha_1\beta_3\varepsilon$ receptors where the potency and, in the case of partial agonists, efficacy, was increased on the spontaneously active receptor. In accordance with our results, Whiting et al. (1997) have reported a lower EC_{50} for GABA at $\alpha_1\beta_1\varepsilon$ receptors compared to $\alpha_1\beta_2\gamma_{2S}$ receptors. We observed a strong positive correlation between the potency of the agonists, their maximum responses, and the level of spontaneous activity, suggesting that the increased spontaneous opening and the increased potency/efficacy of the agonists may reflect a common mechanism. This is exactly what we might have predicted following consideration of a kinetic scheme describing a simple receptor model. We suggest that the gating efficacy is altered for spontaneously active $GABA_A$ receptors favouring channel opening (higher values of E).

$$\begin{array}{ccccc} A+R & \leftrightarrow & A+AR & \leftrightarrow & A_2R \\ \updownarrow E & & \updownarrow E[A] & & \updownarrow E[A] \\ R^* & & AR^* & & A_2R^* \end{array}$$

Up to two agonist molecules (A) can bind to the closed receptor conformation (R) causing the receptor to change conformation into an open channel state (R^*). At the microscopic level, this conformational change from a closed to an open receptor channel is controlled by the gating constant (E).

$GABA_A$ receptors without any significant spontaneous channel activity must have very small intrinsic values of E and thus channel opening will only occur after the binding of at least one agonist molecule. The small value of E will reflect low agonist potencies and low efficacies for partial agonists. We propose that E increases significantly with the level of spontaneous activity of $GABA_A$ receptors. This increased efficacy will not only result in channel openings in the absence of bound agonist, but predicts that the agonist potency will increase (lower EC_{50} values) and the dose–response curve maximum will also increase to a saturating level. For a full agonist, this increase in E may manifest just as a parallel leftward shift in the dose–response curve but for the partial agonists with low efficacy, increasing E causes a leftward shift and an increase in the curve maximum. This is precisely what we observed and therefore seems a plausible explanation for the data.

An alternative reason for the increased apparent potency of agonists on spontaneously active $GABA_A$ receptors may be due to their constant activation in the absence of agonist causing the receptor to exist in one or more desensitised states. Binding studies using $GABA_C$ receptors (Chang and Weiss, 1999b) have confirmed the notion that prolonged presence of the agonist (as in the case of binding) results in a desensitised state of the receptor, which is reflected in the observed high affinity. Since two-electrode voltage-clamping of oocytes make use of a relatively slow application system, it is evident that the measured agonist activated

maximum peak currents reflects the activity of, at least partly, desensitised $GABA_A$ receptors.

Antagonists at $\alpha_1\beta_2\gamma_{2S}$ receptors were examined as inhibitors of GABA responses; whereas for constitutively active receptors, their action was characterised as a direct effect on the spontaneous current in the absence of the agonist. The potencies of the competitive antagonists were not significantly different in the presence of the agonist at $\alpha_1\beta_2\gamma_{2S}$ receptors and in the absence of agonists at constitutively active receptors. This corresponded well with the observations by Thompson et al. (1999) and may be accounted for by their competitive inhibitory mechanism. In line with these data on spontaneously active $GABA_A$ receptors, Burstein et al. (1997) reported that competitive antagonist potencies (reversing spontaneous activity or agonist induced activity) were similar at constitutive and non-constitutive G-protein coupled muscarinic receptors.

One would predict that the maximum responses of inverse agonist (competitive antagonists) would increase with constitutive activity. However, while the level of inverse agonism by bicuculline is increased at the $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ as compared to $\alpha_1\beta_3\varepsilon$ (67% and 43% of the maximum response evoked by picrotoxinin, respectively), the maximum response caused by SR95531 showed an inverse relationship (23% and 34% of the maximum picrotoxinin response, respectively). The reason for this ambiguity remains unclear.

When comparing the inhibitory actions of zinc at $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2\varepsilon$-containing receptors, it is evident that the ε subunit renders the receptors more sensitive to zinc. Similarly the δ and the γ_{2S} subunits have previously been shown to influence the potency of zinc (Krishek et al., 1998). Furthermore, since the effect of zinc is nearly completely abolished at the $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors, this clearly suggests that also the β_2 subunit is involved in zinc binding and functional effects. This observation is supported by the report that β_1 homomeric receptors are very sensitive to zinc (Krishek et al., 1996b), and that a mutation (His292Ala) in the transmembrane 2 region of β_3 homomeric receptors results in a 1000-fold reduction in zinc potency (Wooltorton et al., 1997b). More recently Hosie et al. (2003) have found specific amino acids in the β_3 subunit involved in binding of zinc to $\alpha_1\beta_3$ receptors.

That picrotoxinin responses induced more desensitisation on $\alpha_1\beta_{2(L259S)}\gamma_{2S}$ receptors than on $\alpha_1\beta_3\varepsilon$ receptors, and since picrotoxinin potency was also higher on less spontaneously active receptors, this indicated that picrotoxinin is acting through an allosteric inhibitory site rather than acting as a chloride channel blocker. This also supports our hypothesis that the conformational state of a highly spontaneously active receptor has shifted so that a putative higher energy barrier will have to be overcome for an allosteric inhibitor to close the receptor compared to when a receptor has little or no constitutive activity.

In conclusion, our data showed clear relationships between spontaneous channel opening activity and both efficacy and potency of agonists, and the potency of a non-competitive allosteric inhibitor, like picrotoxinin, indicating that these mechanisms are likely to be linked to the conformational state of the $GABA_A$ receptor channel complex.

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