British Journal of Pharmacology (2001) 133, 539–549

www.nature.com/bjp

Effects of $GABA_A$ receptor partial agonists in primary cultures of cerebellar granule neurons and cerebral cortical neurons reflect different receptor subunit compositions

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1 Based on an unexpected high maximum response to piperidine-4-sulphonic acid (P4S) at human $\alpha1\alpha6\beta2\gamma2$ $GABA_A$ receptors expressed in *Xenopus* oocytes attempts to correlate this finding with the pharmacological profile of P4S and other $GABA_A$ receptor ligands in neuronal cultures from rat cerebellar granule cells and rat cerebral cortex were carried out.

2 GABA and isoguvacine acted as full and piperidine-4-sulphonic acid (P4S) as partial agonists, respectively, at $\alpha1\beta2\gamma2$, $\alpha6\beta2\gamma2$ and $\alpha1\alpha6\beta2\gamma2$ GABA receptors expressed in *Xenopus* oocytes with differences in potency.

3 Whole-cell patch-clamp recordings were used to investigate the pharmacological profile of the partial $GABA_A$ receptor agonists 4,5,6,7-tetrahydroisoxazolo-(5,4-c)pyridin-3-ol (THIP), P4S, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL), and 3-(4-piperidyl)isoxazol-5-ol (iso-4-PIOL), and the competitive $GABA_A$ receptor antagonists Bicuculline Methbromide (BMB) and 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyridazinium bromide (SR95531) on cerebral cortical and cerebellar granule neurons. In agreement with findings in oocytes, GABA, isoguvacine and P4S showed similar pharmacological profiles in cultured cortical and cerebellar neurones, which are known to express mainly $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ containing receptors and $\alpha1$, $\alpha6$ and $\alpha1\alpha6$ containing receptors, respectively.

4 4-PIOL and iso-4-PIOL, which at $GABA_A$ receptors expressed in oocytes were weak antagonists, showed cell type dependent potency as inhibitors of GABA mediated responses. Thus, 4-PIOL was slightly more potent at cortical neurones than at granule neurones and iso-4-PIOL was more potent in inhibiting isoguvacine-evoked currents at cortical than at granule neurons. Furthermore the maximum response to 4-PIOL corresponded to that of a partial agonist, whereas that of iso-4-PIOL gave a maximum response close to zero.

5 It is concluded that the pharmacological profile of partial agonists is highly dependent on the receptor composition, and that small structural changes of a ligand can alter the selectivity towards different subunit compositions. Moreover, this study shows that pharmacological actions determined in oocytes are generally in agreement with data obtained from cultured neurons.
British Journal of Pharmacology (2001) **133,** 539–549

Keywords: $GABA_A$ receptor; subunits; cerebellar granule neurons; cerebral cortical neurons; *Xenopus* oocytes; whole-cell patch-clamp; two-electrode voltage-clamp; partial agonists; antagonists; efficacy; potency

Abbreviations: BMB, Bicuculline Methbromide; IGU, isoguvacine; iso-4-PIOL, 3-(4-piperidyl)isoxazol-5-ol; 4-PIOL, 5-(4-piperidyl)isoxazol-3-ol; P4S, piperidine-4-sulphonic acid; SR95531, 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyridazinium bromide; Thio-muscimol, 5-aminomethyl-3-isothiazol; THIP, 4,5,6,7-tetrahydroisoxazolo-(5,4-c)pyridin-3-ol

Introduction

The $GABA_A$ receptor complex is believed to be a heteropentameric assembly of transmembrane protein subunits, forming a chloride-permeable ion channel. The subunits exist in several forms and/or splice variants. Molecular cloning has identified several distinct $GABA_A$ receptor proteins, which can be grouped into subunit families, i.e., α, β, γ, δ, ε, θ, π and ρ (Sieghart, 1995; Whiting *et al*., 1995; 1997; Davies *et al*., 1997; Hedblom & Kirkness, 1997; Bonnert *et al*., 1999; Neelands & Macdonald, 1999). The number of naturally occurring subtypes are probably far less than the number of theoretically possible subtypes and the presence of 2 α, 1–2 β, and either 1–2 γ, or 1 δ, or 1 ε, or 1 θ, or, π subunit seems to be a prerequisite to form functional receptors (Chang *et al*., 1996; Davies *et al*., 1997; Tretter *et al*., 1997; Bonnert *et al*., 1999; Neelands & Macdonald, 1999), with the $\alpha1\beta2\gamma2$ subtype making a major contribution (McKernan & Whiting, 1996).

Numerous studies have demonstrated that the pharmacological profile of GABAergic modulators and gating properties of the channel vary with the receptor composition (Sigel *et al*., 1990; Verdoorn *et al*., 1990; Angelotti & Macdonald, 1993; Macdonald & Olsen, 1994; Whiting *et al*., 1995; Saxena & Macdonald, 1996). The benzodiazepine binding site is by far the best characterized pharmacologically and it is well

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known that the α- and γ-subunits exert major influence on the pharmacology. The type of α subunit forms the basis for the commonly used division of $GABA_A$ receptors into categories of benzodiazepine receptor ligand selectivity (Pritchett *et al*., 1989; Sigel *et al*., 1990; Lüddens *et al*., 1990; Wisden *et al*., 1991; Angelotti & Macdonald, 1993; Yang *et al*., 1995; Hadingham *et al*., 1993; 1996). However, only very few modulators have been found to be subtype specific, such as the loop diuretic, furosemide, being a selective antagonist for α6 containing receptors (Korpi *et al*., 1995) and L-655,708 being a selective benzodiazepine ligand for α5 containing receptors (Quirk *et al*., 1996). Binding assays and/or various expression systems are commonly used to investigate the relationship between receptor composition and pharmacological profile because of the ease and capacity of such assays. However, this strategy does have limitations, in that affinity does not necessarily correlate with potency and the subtype studied may not exist in cell membranes *in vivo*. Furthermore, the phosphorylation state of the receptor can influence both gating and pharmacological properties (Moss & Smart, 1996). Since the brain has extraordinarily active systems for protein phosphorylation, the phosphorylation state of receptor expressed in an artificial system most likely differs from those found *in vivo*. Therefore, it is important to correlate findings in artificial expression systems with measurements in more natural systems, such as neurons in primary culture.

Preliminary studies in this laboratory using human $GABA_A$ receptors expressed in oocytes showed that whereas the efficacy of the GABA analogue isoguvacine remained relatively insensitive to variations in the subunit composition, this was clearly not the case for P4S. Thus, the relative efficacy of P4S varied from 30 and 15% at $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ containing receptors, respectively, to 75% at $\alpha1\alpha6\beta2\gamma2$ containing receptors. This prompted us to investigate if this difference in efficacy for P4S might be reflected in differences observed at two different neuronal cultures. Cultures of cerebellar granule neurons were chosen because they comprise a relatively simple model system with a nearly homogeneous population of neurons probably expressing only a few receptor subtypes. Furthermore, cerebellar granule neurons have been shown to alter subunit composition during development whereby the unique α6 subunit occur (Laurie *et al*., 1992; Mathews *et al*., 1994). In contrast, cultures of cerebral cortical neurons consist of a heterogeneous population of cells potentially expressing an extensive repertoire of $GABA_A$ receptor subunits (Wisden *et al*., 1992), allowing for several subtypes of the $GABA_A$ receptor to be expressed.

The present study has focused on $GABA_A$ receptor partial agonists since subtype selective partial agonists displaying a range of efficacies may have a therapeutic potential in certain neurological disorders (Krogsgaard-Larsen *et al*., 1997). Moreover, the potency and efficacy of some agonists has previously been shown to be highly dependent on different subunit compositions expressed in oocytes, whereas the affinity, as measured in binding assays, remains relatively insensitive to differences in subunit composition (Ebert *et al*., 1994; 1997). GABA and isoguvacine were chosen as full agonists; GABA is the endogenous ligand of the receptors and isoguvacine is a specific $GABA_A$ receptor agonist due to its low affinity for $GABA_B$ receptors and GABA uptake systems (Krogsgaard-Larsen *et al*., 1977). P4S has previously been shown to be a partial agonist highly sensitive to variation of the receptor composition as demonstrated with recombinant receptors expressed in *Xenopus* oocytes (Ebert *et al*., 1994; 1997). 4-PIOL is a low efficacy partial $GABA_A$ agonist (Kristiansen *et al*., 1991; 1995). The related structure iso-4-PIOL is putatively a very low efficacy partial agonist with unknown subtype selectivity (Frølund *et al*., 1995). In addition, efficacy estimates were obtained for two structural analogues of the $GABA_A$ agonist muscimol, Thio-muscimol and THIP (Lykkeberg & Krogsgaard-Larsen, 1976; Krogsgaard-Larsen *et al*., 1977; 1978; Ebert *et al*., 1994).

Methods

Electrophysiological recordings using the whole-cell patch-clamp technique were performed on cultured mouse cerebellar granule neurons and mouse cerebral cortical neurons and two-electrode voltage-clamp was performed on *Xenopus* oocytes.

Cell cultures

Primary cultures of cerebellar granule neurons and cortical neurons were prepared from 7 day old NMRI mice and 15 day old embryos, respectively, essentially as previously described (Herts *et al*., 1989; Schousboe *et al*., 1989). For granule neurons 50 μM kainic acid was added to the above medium to selectively eliminate stellate, basket and Golgi cells. Cells were plated on poly-L-lysine coated 35 mm culture dishes and cultured at 37°C in a humidified atmosphere of 5% CO_2. After 36–48 h *in vitro*, cytosine arabinoside (20 μM final concentration) was added to prevent replication of non-neuronal cells. This procedure yields neuronal cultures with minimal contamination of astrocytes and for the granule cultures also other types of neurons.

Oocyte expression.

Xenopus oocytes were removed from anaesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment to remove follicle cells (Type IA (0.5 mg ml^{-1}) for 6 min) the oocyte nuclei were then directly injected with 20–30 nl of injection buffer ((in mM): NaCl 88, KCl 1, HEPES 15, at pH 7.0 (nitrocellulose filtered)) containing different combinations of human $GABA_A$ subunit cDNAs (6 ng ml^{-1}) engineered into the expression vector pCDM8 or pcDNAAmp and incubated for 1–4 days. cDNAs were gifts from Dr Paul Whitting, MSD, Harlow, U.K.

Electrophysiological recordings

For whole-cell patch-clamp recordings cortical neurons were used at the age of 7–9 days and granule neurons were used at the age of 10–12 days. The culture dish was placed on the stage of a Zeiss Axiovert 10 inverted phase microscope (Zeiss, Germany), where the individual neurons were viewed at 200× magnification. The culture medium was replaced with about 4 ml of artificial balanced salt solution (ABSS), which was continuously renewed by constant perfusion at 0.5 ml/min at room temperature. The composition of ABSS

was (in mM): NaCl 140, KCl 3.5, Na_2HPO_4 1.25, $MgSO_4$, 2, $CaCl_2$ 2, glucose 10, and HEPES 10; pH was 7.3. Standard patch-clamp techniques (Hamill *et al*., 1981) were used for recordings in the whole-cell patch-clamp configuration using an EPC-9 patch-clamp amplifier (HEKA, Germany). The patch pipettes were pulled from 1.5 mm O.D. glass (World Precision Instruments, U.S.A.) on a BB-CH-PC electrode puller (Mecanex, Switzerland) and had resistances of 3–5 MΩ. Tight seal recordings were achieved with series resistances ranging from 5–25 MΩ. The composition of the pipette solution was (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, MgATP 2, and HEPES 10; pH was 7.35. A holding potential of −60 mV was used except for 1–5 experiments. For experiments with high efficacy agonists 60% series resistance compensation was used. Signals were recorded at 2.3 kHz on a computer and on video tape using a digital data recorder (VR-10B, Instrutech, U.S.A.), and were subsequently analysed without further filtering. Stock solutions of the drugs were prepared in distilled water at a concentration of at least one hundred times the final concentration used for perfusion and premixed by diluting solutions in ABSS. Drug solutions were applied from a gravity feed multi-barrel application pipette (List, Germany) placed ~100 μm of the recorded neuron. To prevent hydrodynamic perfusion artifacts and to hasten recovery the neuron was superfused with normal ABSS from one of the barrels between drug applications. Drugs were applied for approximately 5 s at 1 min intervals. During drug application the responses always peaked or reached a plateau and between applications a stable baseline was reached.

For two-electrode voltage-clamp experiments, oocytes were placed in a 50 μl bath and perfused with modified Barth's medium (MBS) consisting of (in mM): NaCl 88, KCl 1, HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.91, $NaHCO_3$ 2.4, pH 7.5. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage clamped between −40 and −70 mV using a GeneClamp 500 amplifier (Axon, U.S.A.). The cell was continuously perfused with saline at 4–6 ml/min, and drugs were applied in the perfusate. GABA or $GABA_A$ agonists were applied until the peak of the response was observed, usually 30 s or less. At least 3 min wash time was allowed between each agonist application to prevent desensitization.

4-PIOL, iso-4-PIOL, P4S, thio-muscimol and THIP were gifts from Dr Erik Falch, Department of Medical Chemistry, The Royal Danish School of Pharmacy. All other drugs were purchased from Sigma (St. Louis, MO, U.S.A.).

Data analysis

In patch-clamp experiments responses were quantified by measuring the peak current (mean-value at maximum response) during the agonist application and the end current (mean of data points just before termination of agonist application after 5 s). Peak and end currents were subsequently normalized to a control peak response unless otherwise stated. Reversal potentials for the modulators were established by interpolation, using the currents elicited at holding potentials of −10, 0, and +10 mV.

For oocyte experiments the maximum of each response was measured and subsequently normalized to either the plateau level for a full concentration-response curve for GABA or the response to 3 mM GABA (no difference) in the same oocyte.

Curve fitting and statistical analysis was carried out with Sigma Plot 2.0 and Sigma Stat 2.0, respectively, (Jandel Statistical Software, Germany). Concentration-response curves were fitted to the equation:

$$I = \frac{E_{max} \times X^n}{EC_{50}^n + X^n}$$

where I is the response as per cent of control, EC_{50} is the concentration of drug eliciting 50% of maximum response, E_{max}, X is the agonist concentration, and n is the Hill coefficient.

Antagonist experiments were carried out with three concentrations of GABA followed by the same three concentrations of agonist in the presence of 100 μM BMB or 10 μM SR95531, respectively. The shift of the concentration-response curve to GABA was determined in the response range where the two obtained curves were parallel. The dose ratio, calculated as the ratio of the concentration of GABA in the presence and absence of antagonist, respectively, was transformed to a K_i value by the equation:

$$K_i = [\text{Antagonist}]/(\text{dose ratio} - 1)$$

Data are presented as mean ± s.e.mean or median values with 25–75% percentile intervals. Unless otherwise stated, comparisons between two groups are performed as Mann–Whitney tests. For statistical analysis of fitting parameters, curve fitting are performed on single-cell concentration-response curves, from which mean and s.e.mean are calculated for each parameter on both cell types, and subsequently Student's *t*-test are performed. In the efficacy experiment, multiple comparisons are performed as Kruskall-Wallis One-Way Analysis of Variance.

Results

Agonist effects of P4S

Concentration-response curves to P4S at human $GABA_A$ receptors expressed in oocytes showed that the maximum response to P4S relative to GABA was highly dependent on the subunit composition. Thus, whereas P4S was a low efficacy partial agonist at $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ containing receptors, the compound acted as a high efficacy partial agonist at $\alpha1\alpha6\beta2\gamma2$ containing receptors (Figure 1, Table 1).

Individual concentration-response curves to P4S at cortical and granule cells were normalized relative to the response to 3 mM P4S and fitted as described in Methods section. As illustrated in Figure 2A,B the shapes of the two mean curves for peak currents were significantly different. Thus, whereas a relatively low variation in the potency at granule cells was observed, a large variation was seen at cortical neurones. This is further substantiated by the two insets in Figure 2, showing concentration-response curves for individual neurons of the two types normalized to the peak current induced by 2 mM GABA. The mean curves for end currents suggest EC_{50} values for this response of well below 20 μM.

Current-voltage relationships were constructed for P4S on cortical and granule neurons (data not shown). The reversal

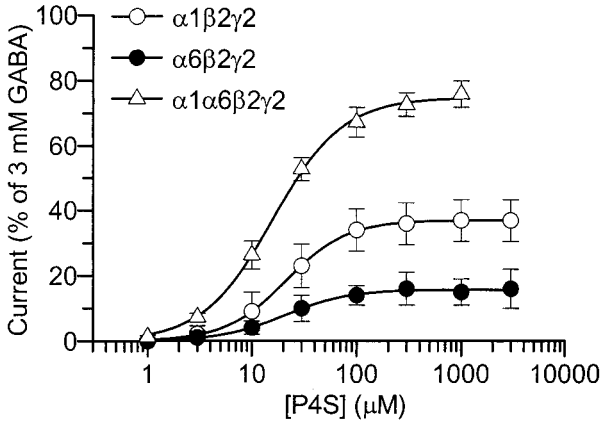

Figure 1 Concentration-response curves for P4S in oocytes injected with $\alpha1\alpha6\beta2\gamma2$, $\alpha1\beta2\gamma2$ or $\alpha6\beta2\gamma2$. Data are presented as mean ± s.e.mean from four individual oocytes. The parameters from the fits are presented in Table 1.

Table 1 EC_{50} and E_{max} for GABA, isoguvacine (IGU), and P4S in oocytes injected with α1, or α6, or α1α6 in combination with $\beta2\gamma2$

	$\alpha1\beta2\gamma2$		$\alpha6\beta2\gamma2$		$\alpha1\alpha6\beta2\gamma2$	
	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)
GABA	20±3	100	1.6±1	100	4.8±1	100
IGU	162±12	88±3	21±5	98±4	16±3	96±3
P4S	25±4	38±5	20±4	15±8	14±4	76±4

Data are presented as mean ± s.e.mean from four individual oocytes for agonist at all combinations.

potential was similar in cortical, 5.2±2.1 mV ($n=7$), and granule neurons, 6.6±2.3 mV ($n=8$), i.e. close to zero, as expected for a chloride channel connecting symmetrical chloride concentrations. In cortical neurons, 100 μM BMB and 10 μM SR95531 almost completely blocked 300 μM P4S-evoked peak currents (97±0.5%, $n=6$, $P<0.001$ and 95±1.6%, $n=7$, $P<0.001$ for BMB and SR95531, respectively). Also in cerebellar granule neurons the 300 μM P4S-evoked peak currents were significantly blocked by BMB (87±1.3%, $n=6$, $P<0.001$) and 85±5.5% by SR95531 ($n=7$, $P<0.001$). Together these results confirm that the effect of P4S is $GABA_A$-mediated.

Agonist effects of GABA and isoguvacine

GABA and isoguvacine both acted as full agonists at α1, α6 and α1α6 containing receptors expressed in oocytes. Both GABA and isoguvacine were significantly more potent at α6 and α1α6 containing receptors than at α1 containing receptors (Table 1). In contrast neither of the two compounds acted differently at cortical and granule neurons (Figures 3 and 4). In Figure 3 the end current of the GABA response relative to the control peak response is shown. Analysis of the end current indicated that the EC_{50} values in the cortical and granule neurones are close to 20 μM, a value 4–6 times weaker than the peak response.

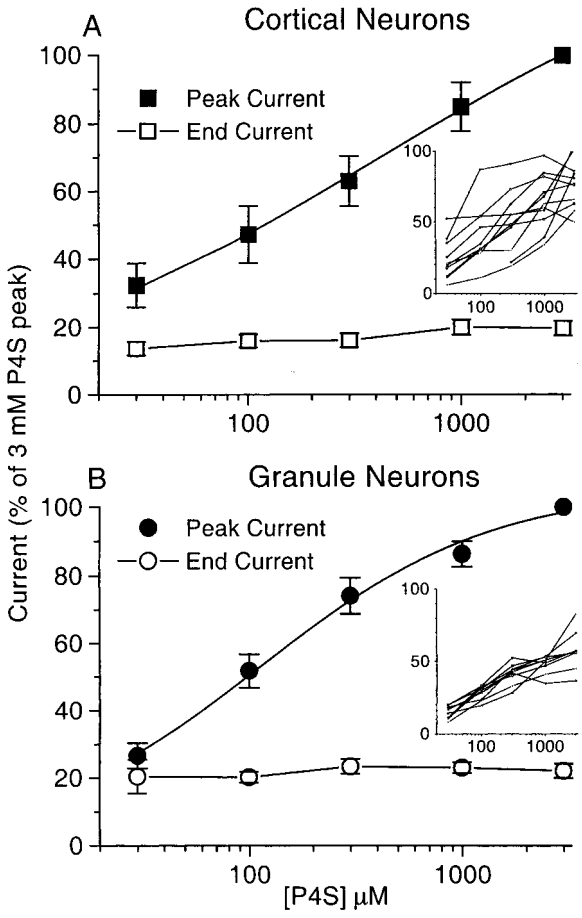

Figure 2 Concentration dependence of P4S induced peak currents in cortical (A) and granule (B) neurons normalized to 3 mM P4S. Data are presented as mean ± s.e.mean. Cortical neurons ($n=10$): EC_{50} 406 μM (95% confidence interval: 314–533 μM), Hill coefficient 0.5 (95% confidence interval: 0.4–0.6) and E_{max} of 139% (95% confidence interval: 132–147%). Granule neurons ($n=9$): EC_{50} 111 μM (95% confidence interval: 62–194 μM), Hill coefficient 0.8 (confidence interval: 0.5–1.3) and E_{max} 105% (95% confidence interval: 93–118%). Also shown is the end current for cortical and granule neurons, respectively. The inset graphs depict the single-cell concentration-response curves for P4S normalized to 2 mM GABA in order to illustrate the large intercellular variation for the cortical neurons in contrast to the granule neurons.

Effects of 4-PIOL and iso-4-PIOL

The concentration-response relationship for 4-PIOL is shown in Figure 5A. Parameters for the best fit to the data are presented in Table 2. The EC_{50} values in cerebral cortical neurons and cerebellar granule neurons were significantly different (Student's t-test, $P=0.025$).

For iso-4-PIOL the concentration-response relationship is shallow in most of the concentration range on both cortical and granule neurons (Figure 5B). Even at high concentrations, iso-4-PIOL only elicits very small currents as illustrated in the inset graph, where a 1 mM iso-4-PIOL evoked current is compared to a 2 mM isoguvacine evoked current in the same cortical neuron. The increased response at higher concentrations of iso-4-PIOL may only be partly $GABA_A$-mediated since the current evoked by 1 mM iso-4-PIOL was not completely blocked by the $GABA_A$ antagonists as

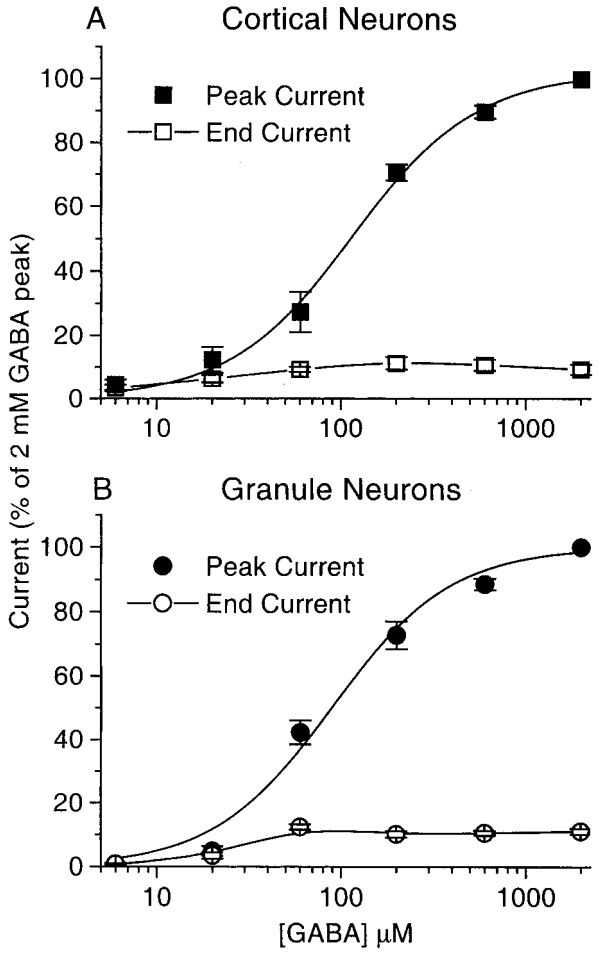

Figure 3 Concentration dependence of GABA induced peak currents in cortical ($n=7$) and granule neurons ($n=10$). The currents were normalized to 2 mM GABA. Data are presented as mean ± s.e.mean. Cortical neurons: EC_{50} 116 μM (95% confidence interval: 43–307 μM), Hill coefficient 1.3 (95% confidence interval: 0.5–2.3) and E_{max} 102% (95% confidence interval: 74–130%). Granule neurons : EC_{50} 89 μM (95% confidence interval: 34–244 μM), Hill coefficient 1.4 (95% confidence interval: 0.5–3.7) and E_{max} 100% (95% confidence interval: 72–126 μM). Also shown are the end currents for cortical and granule neurons, respectively.

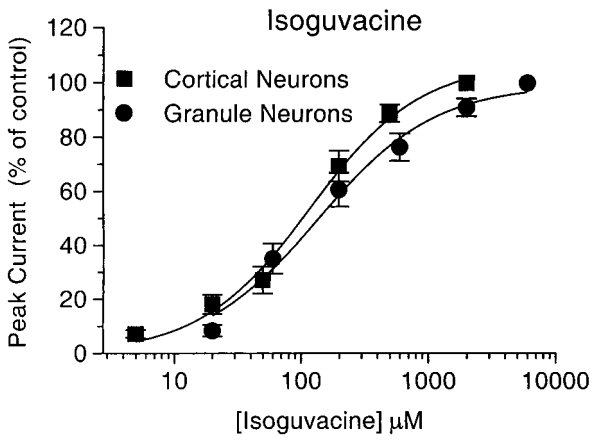

Figure 4 Concentration dependence of isoguvacine induced peak currents in cortical ($n=9$) and granule neurons ($n=9$). The currents were normalized to the maximum concentration used, i.e., 2 mM for cortical neurons and 6 mM for granule neurons, respectively. Data are presented as mean ± s.e.mean. Cortical neurons: EC_{50} 118 μM (95% confidence interval: 68–200 μM), Hill coefficient 1.0 (95% confidence interval: 0.6–1.8) and E_{max} 108% (95% confidence interval: 92–123%). Granule neurons: EC_{50} 137 μM (95% confidence interval: 69–261 μM), Hill coefficient 0.9 (95% confidence interval: 0.6–1.8) and E_{max} 99% (95% confidence interval: 86–114 μM).

discussed below. No attempts to fit these concentration-response curves were made.

Inhibition studies using 100 μM BMB or 10 μM SR95531 showed that both competitive $GABA_A$ antagonists were able to block the response to 1 mM 4-PIOL by more than 90%, whereas the response to 1 mM iso-4-PIOL was reduced far less.

In cortical neurons, 100 μM BMB blocked the 1 mM iso-4-PIOL induced current 34 ± 13% ($n=8$) and 10 μM SR95531 blocked the 1 mM iso-4-PIOL evoked current 47 ± 8% ($n=8$). The blockage was significant for both BMB ($P=0.01$) and SR95531 ($P<0.001$), indicating that the iso-4-PIOL current is at least partly $GABA_A$ mediated. For granule neurons, 100 μM BMB and 10 μM SR95531 blocked the 1 mM iso-4-PIOL evoked current by 21 ± 17 and 13 ± 31% ($n=8$). In neither case was the current evoked by iso-4-PIOL blocked significantly.

In Figure 6A, 4-PIOL is demonstrated to antagonize the response of the full agonist isoguvacine (20 μM) in a concentration-dependent manner in both cortical and granule neurons, the response in granule cells being most sensitive to inhibition by 4-PIOL. Figure 6B shows the antagonistic effect of iso-4-PIOL on isoguvacine induced currents, which at all concentrations was significantly more pronounced in cortical neurons than in cerebellar granule neurons (Student's t-test).

In order to obtain rough estimates of the efficacies, the peak currents of selected agonists at a concentration of 2 mM were compared to the maximum GABA-induced peak current (Figure 7). In addition to the compounds presented above, the full agonist Thio-muscimol (THIO) and the partial agonist THIP were included in this experiment. Figure 7 illustrates that in both cerebellar granule cells and cortical neurons isoguvacine and thio-muscimol act as full $GABA_A$ agonists. In contrast, currents evoked by P4S, THIP, and 4-PIOL were significantly smaller than the GABA evoked current on both cell types ($P<0.001$). The relative response to P4S and THIP at the two types of neurons was not significantly different, and with responses of 50 to 70% of the maximum response to GABA, these compounds probably act as high efficacy partial $GABA_A$ receptor agonists. On the other hand, 2 mM 4-PIOL only evoked a response which was 5% of that evoked by GABA, indicating that 4-PIOL acts as a low efficacy partial $GABA_A$ agonist at both cortical and granule neurons.

Antagonist effects of BMB and SR95531 on GABA evoked currents

In order to compare the potency of the two competitive $GABA_A$ antagonists, BMB and SR95531, the shifts of GABA dose-response curves were compared in the two cell types. Based on previously published K_i-values (Yu & Ho, 1990; Ito

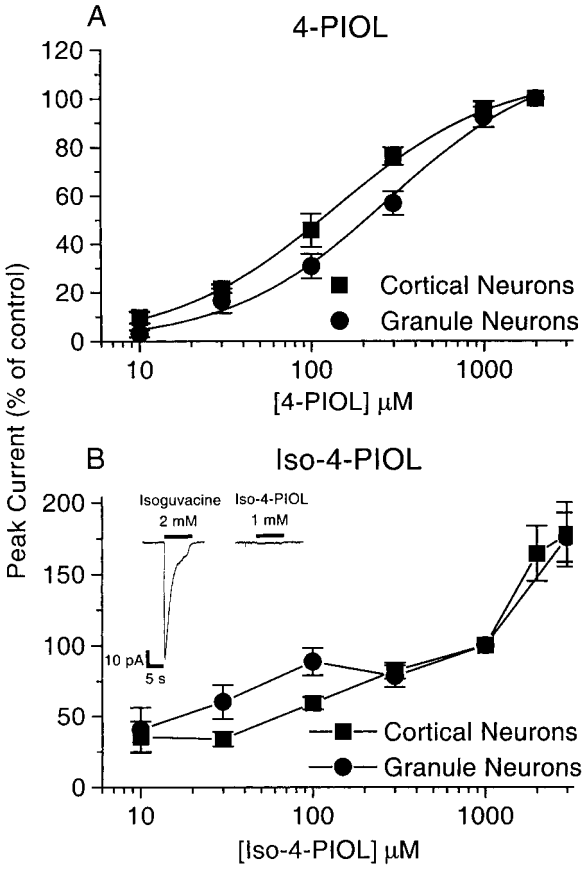

Figure 5 Concentration-reponse curves for 4-PIOL (A) and iso-4-PIOL (B) in cortical and granule neurons. The 4-PIOL currents were normalized to the current induced by 2 mM 4-PIOL and the iso-4-PIOL induced currents to the current induced by 1 mM iso-4-PIOL in each cell. Data are presented as mean ± s.e.mean. Fitting of the concentration-response curves for 4-PIOL data gave the following results: Cortical neurons EC_{50}: 139 μM (95% confidence interval: 105–177 μM), Hill coefficient 0.9 (95% confidence interval: 0.8–1.2) and the E_{max} was 110 (95% confidence interval: 103–117%), $n=10$. Granule neurons: EC_{50} 283 μM (95% confidence interval: 184–452 μM), Hill coefficient 0.9 (95% confidence interval: 0.6–1.4) and E_{max} 117 (95% confidence interval: 102–132%), $n=9$. No attempt to fit the concentration-response curves for iso-4-PIOL in cortical ($n=17$) and granule ($n=12$) neurons were made. The inset graph in (B) shows representative current responses induced in a cortical neuron by 2 mM isoguvacine and 1 mM 4-PIOL.

Table 2 EC_{50}, E_{max}, and Hill coefficients for GABA, isoguvacine (IGU), P4S, and 4-PIOL on cortical and granule neurons

	Cortical neurons		*Granule neurons*	
	EC_{50} (μM)	*Hill*	*EC_{50} (μM)*	*Hill*
GABA	116 [43–307]	1.3 [0.5–2.3]	89 [34–244]	1.4 [0.5–3.7]
IGU	118 [68–200]	1 [0.6–1.8]	137 [69–261]	0.9 [0.6–1.8]
P4S	406 [314–533]	0.5 [0.4–0.6]	111 [61–193]	0.8 [0.5–1.3]
4-PIOL	139 [104–177]	0.9 [0.8–1.2]	283 [184–452]	0.9 [0.6–1.4]

Data are presented as mean and 95% confidence intervals. Parameters were obtained from fittings of concentration-response curves. The number of experiments is mentioned in the individual Figure legends.

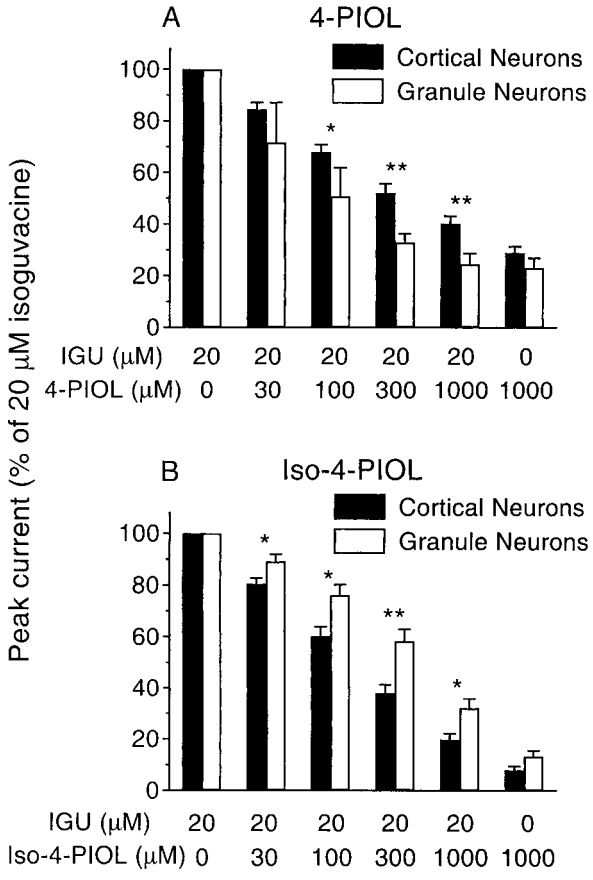

Figure 6 Concentration-inhibition plot for 4-PIOL (A) and iso-4-PIOL (B) in cortical and granule neurons. For iso-4-PIOL $n=10$ and $n=12$ and for 4-PIOL $n=14$ and $n=9$ for cortical and granule neurons, respectively. The responses were normalized to 20 μM isoguvacine (first column on the left). Data are presented as mean ± s.e.mean. The 20 μM isoguvacine induced current was progressively reduced by increasing concentrations of iso-4-PIOL and 4-PIOL, respectively. Significant differences between cortical and granule neurons at the same concentration are indicated by asterisks (*, $P<0.05$ and **, $P<0.01$, Student's *t*-test).

et al., 1992; Ebert *et al*., 1997), concentrations of 100 μM BMB and 10 μM SR95531 were used. Preliminary investigations of the effect of different pre-treatment times showed that no further blockage of a 3 mM GABA evoked current was achieved going from 30 s to 60 s pre-treatment, so pre-treatment for 30 s was used in both sets of experiments. Representative traces from these experiments are presented in Figure 8 and data are presented in Table 3. The pK_i-values show that BMB and SR95531 inhibits a GABA evoked current at the two types of neuronal cultures equally effectively.

Discussion

The purpose of this study was to investigate if pharmacological differences observed in oocytes injected with the $GABA_A$ receptor subunit cDNA combinations $\alpha1\beta2\gamma2$, $\alpha6\beta2\gamma2$ or $\alpha1\alpha6\beta2\gamma$ were reflected in $GABA_A$ receptors of neurons in culture. In order to address this question, a few selected compounds were characterized in oocytes and

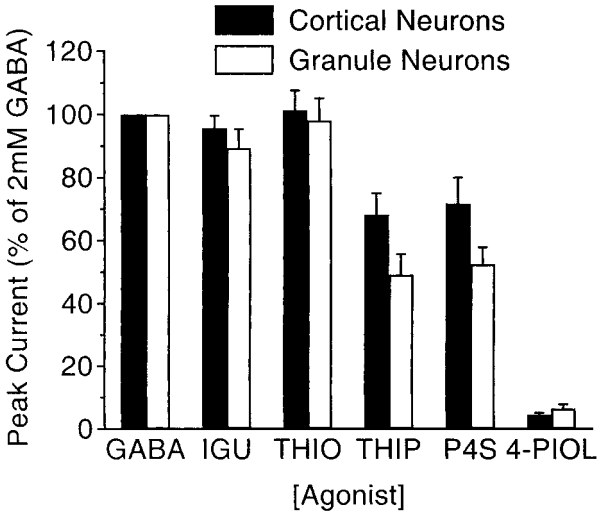

Figure 7 Efficacy estimates for agonists and partial agonists normalized to the full agonist GABA. All substances were applied at a concentration of 2 mM. Data are presented as mean ± s.e.mean. Cortical neurons: $n=17$. Granule neurons: $n=13$. Abbreviations: isoguvacine (IGU), and thio-muscimol (THIO), others as in text.

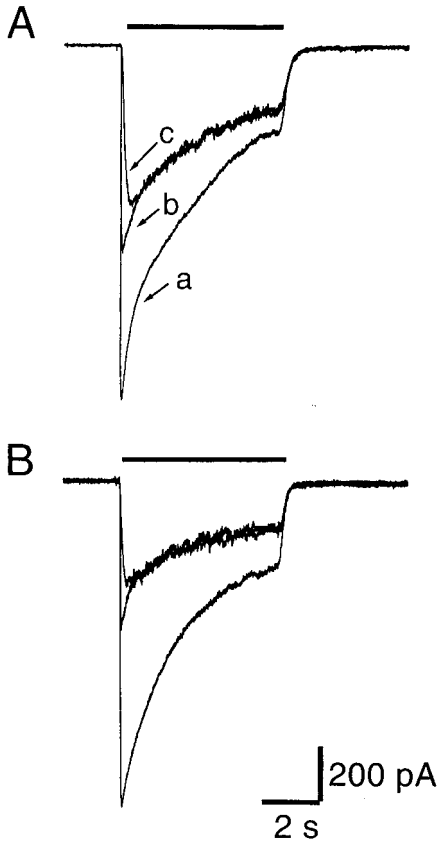

Figure 8 Representative traces of the effect of bicuculline (BMB) on GABA evoked currents in cortical neurons (upper panel) and granule neurons (lower panel). a: agonist, b: agonist + BMB, and c: agonist + BMB pretreated for 30 s. The bar on top indicates application of drugs, i.e., approximately 6 s.

Table 3 pK_i for bicuculline (BMB) and SR95531 on GABA-induced currents in cortical and granule neurons

Agonist	*Antagonist*	*Cortical neurons* pK_i	*Granule neurons* pK_i
GABA	BMB	4.6 ± 0.06	4.9 ± 0.08
	SR	5.2 ± 0.06	5.4 ± 0.07

Data are presented as mean ± s.e.mean, $n=8$.

neuronal cultures originating from either cerebral cortex or cerebellum.

Different molecular biological techniques have demonstrated differences in the subunits being expressed in the two neuronal types making it plausible that also the receptor compositions differ. Almost all known $GABA_A$ subunit mRNAs, with the notable exception of α6, have been detected in rat cortical neurons (Wisden *et al.*, 1992), from which a vast number of possible functional subunit compositions may be formed. Presently, however, the number and compositions of receptor complexes formed *in vivo* are not known (McKernan & Whitting, 1996). In contrast, the cerebellar granule neurons comprise a much simpler system. A detailed study of the developmental profile of α1–6, β1–3, γ1–3, and δ subunits of the $GABA_A$ receptors in the rat cerebellum has revealed some interesting transitions of subunit expression (Laurie *et al.*, 1992). Around postnatal day 6, there is a significant upregulation in the mRNA expression of the α1, α6, β2, β3, γ2, and δ subunits while the mRNA expression of the α2, α3, and β1 subunits is decreased. This profile is also expressed in cultured systems (Mathews *et al.*, 1994). Most studies relating the expression of subunit compositions to specific cell types have been performed in rats, including the above mentioned. One study showed that cerebellar granule neurons taken from mice at P5 and grown in 25 mM $[K^+]$ did not express the α6 subunit gene whereas cells grown in 5 mM $[K^+]$ for the same period of time did (Mellor *et al.*, 1998). The time seems to be an important determinant for expression of the α6 gene, since if cells initially are grown in 5 mM $[K^+]$ for 3 days or more and then moved to 25 mM $[K^+]$, the α6 gene is still expressed. The cerebellar granule cells used in this study are from P7 mice and single-cell RT–PCR analysis has shown that after 10–12 DIV (in 25 mM K^+) 75% of the cells express detectable amounts of α_6 mRNA (Alsbo *et al.*, 2001).

When comparing results from oocytes injected with cDNA with results from cultured neurons some difficulties are immediately apparent: (A) Due to slow drug application, results from oocytes are based on desensitized responses, whereas the relatively fast drug application used for the cultured neurons allows estimation of peak currents. A possible consequence of this is revealed in Figure 1, where potency estimates in cultured neurons based on the desensitized end currents are significantly higher than those based on peak currents. This is not a problem as long as different agonists are affected equally, but when comparing potency of agonists with high and low efficacy a bias may occur. (B) The subunit composition of $GABA_A$ receptors in the cultured neurons are not known in detail. The picture obtained from mRNA analysis can be misleading, because translation efficiency and stability of mRNA as well as of

subunit protein may vary between subunits. Furthermore the presence of some subunits can influence the expression of others (Jones *et al*., 1997). (C) Differences in post-translational modifications (e.g. phosphorylation) may result in different properties of receptors composed of the same subunits.

The agonists and partial agonists investigated in this study were demonstrated to cover a wide range of efficacies in both cerebellar granule neurons and in cortical neurons (Figure 7): Isoguvacine, and thio-muscimol reached the same level as GABA and were full agonists, 4-PIOL had a low (approximately 5%) efficacy, and iso-4-PIOL had close to zero efficacy (1 mM iso-4-PIOL evoked currents that were 1–3% of a 2 mM isoguvacine evoked current). Intermediate and similar response levels at 2 mM relative to GABA was seen for P4S and THIP (around 50 and 70% at cortical and granule neurons respectively). However, since the full concentration-response relationships for THIP in these neurons have not been established this level may not reflect the efficacy, and since the maximum response levels of P4S has not been reached at the concentrations used in Figure 7, the efficacy of P4S will be somewhat higher than this level.

In agreement with earlier studies using recombinant receptors expressed in oocytes (Ebert *et al*., 1997) BMB and SR95531 were potent inhibitors at both neuronal types, and no difference in the potency between the cell types was detected. The pK_i values for SR95531 and BMB obtained in this study indicate SR95531 to be three times as potent as BMB (Table 3). This is in agreement with a study by Lüddens & Korpi (1995) who found SR95531 to be three times as potent as bicuculline in most receptor combinations composed of $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$ or $\alpha6$ with any of three β subunits and $\gamma2S$ or $\gamma3$. The two most pronounced exceptions were $\alpha2\beta1\gamma3$ and $\alpha6\beta1\gamma3$, where SR95531 was much more potent than bicuculline (Lüddens & Korpi, 1995).

A low-efficacy partial agonist will occupy the receptor, but will not be very effective in transforming the ionophore to the conducting state. At the same time, however, it will prevent a full agonist from gaining access to the receptor and will therefore exhibit the characteristics of an antagonist. In agreement with results from recombinant receptors expressed in oocytes (Ebert *et al*., 1997) and from cultured hippocampal neurons (Kristiansen *et al*., 1991), we found 4-PIOL to be a much weaker antagonist than BMB and SR-95531 (Figure 6A). The structural analogue (5-isoxazolol isomer) of 4-PIOL, iso-4-PIOL showed a similar antagonist profile (Figure 6B). However, whereas for 4-PIOL the isoguvacine induced currents in granule cells were more sensitive to inhibition than in cortical neurons, the reverse was true for iso-4-PIOL, suggesting a difference in receptor subtype selectivity for the two compounds. This implies that small structural changes can alter the sensitivity towards different subunit compositions.

The low-efficacy $GABA_A$ agonist activity of 4-PIOL confirms results from cultured hippocampal neurons (Kristiansen *et al*., 1991) and acutely dissociated olfactory bulb neurons (Kristiansen *et al*., 1995). We found 4-PIOL to be approximately equipotent with GABA and isoguvacine in cortical neurons, whereas in granule neurons 4-PIOL was approximately one-third as potent as the full agonist GABA (Table 1). In previous studies the EC_{50} for 4-PIOL was estimated to 91 μM in hippocampal neurons and 31 μM in olfactory bulb neurons (Kristiansen *et al*., 1991; 1995). Together with the significant difference between the potency of 4-PIOL at cortical and granule neurons found in the present study, this suggests the agonist potency of 4-PIOL to be highly dependent upon the receptor composition. In cultured hippocampal neurons the EC_{50} and K_i of 4-PIOL was found to coincide, suggesting that the agonist and antagonist activities of 4-PIOL are indeed due to the same binding site (Kristiansen *et al*., 1991). The low efficacy of 4-PIOL in olfactory bulb neurons has been shown to be caused by a low incidence of long-lasting bursts in 4-PIOL evoked responses compared to GABA evoked responses (Kristiansen *et al*., 1995). In accordance with observations in the present study (results not shown) the response to 4-PIOL did not desensitize (Kristiansen *et al*., 1991). Recently 4-PIOL has been shown to be devoid of agonist activity on recombinant $GABA_A$ receptors of the $\alpha6\beta2\gamma2$ subtype expressed in HEK-293 cells, whereas the efficacy in the $\alpha1\beta2\gamma2$ subtype was 5% (Rabe *et al*., 2000). This study did not investigate receptors containing both $\alpha1$ and $\alpha6$ subunits in the same complex or receptors containing the δ subunit. Still, in light of the 5% efficacy of 4-PIOL found in our cerebellar granule cells, this result suggests that receptors containing $\alpha6$ as the only α subunit makes only a minor contribution to the total population of $GABA_A$ receptors in our cerebellar granule cells.

The agonist activity of iso-4-PIOL is more enigmatic. The concentration-response curve of iso-4-PIOL indicates that this substance is a low efficacy partial $GABA_A$ receptor agonist at both neuronal types (Figure 5). However, at high concentrations (above 1 mM), an increase in the response is observed, which could be caused by a nonspecific action of iso-4-PIOL. A similar unspecific action at high concentrations has been shown for 4-PIOL in cultured hippocampal neurons (Kristiansen *et al*., 1991). For cortical neurons both BMB and SR95531 blocked the iso-4-PIOL induced current significantly, whereas for the granule neurons the blockage was not significant. Nevertheless, for both cell types the amount of blockage was small considering the concentrations of antagonists, which at responses to e.g. GABA would produce a high degree of inhibition. In any case the small currents evoked by even high concentrations of iso-4-PIOL (shown as insert in Figure 5) indicates that iso-4-PIOL is a very low efficacy partial agonist in agreement with previous work (Frølund *et al*., 1995).

In cortical neurons, both THIP and P4S have an apparent efficacy of approximately 70% as compared to approximately 50% in granule neurons, suggesting the two compounds to be more efficacious in cortical than in granule neurons. P4S displayed a much larger variability in single-cell concentration-response curves in cortical neurons than in granule neurons, possibly due to a more heterogeneous pattern of receptor expression in cortical neurons derived from the wider spectrum of subunits available. Ebert *et al*. (1994) have shown that the α subunit is the major determinant of efficacy for P4S, and that α_1 conferred the lowest efficacy (20–40% depending on the actual combination with other subunits) while α_3 and α_5 conferred intermediate to high efficacies (50–100% in most combinations). The present experiments with oocytes injected with $\alpha1$ and/or $\alpha6$ in combination with $\beta2\gamma2$ (Table 2) showed that for P4S $\alpha6$-containing receptors conferred a lower efficacy ($15\pm8\%$) as compared to $\alpha1$-

containing receptors ($38 \pm 5\%$), and very surprisingly, the $\alpha1\alpha6$-containing receptors conferred a significantly higher efficacy ($76 \pm 4\%$). Taken together with data obtained in cell cultures, these results clearly indicate that colocalization of $\alpha1$ and $\alpha6$ in the same receptor complex takes place and point at P4S as a valuable tool for detection of this colocalization. The efficacy of P4S in cortical neurons of at least around 70% strongly suggests a major contribution of receptors with other α-subunits than α_1 (or α_6). For the granule cells, where the only α-subunits are believed to be $\alpha1$ and $\alpha6$, the apparent efficacy of at least around 50% points at a significant contribution from receptors with both of these subunits in the same complex. In this context the effect of receptors with the δ subunit instead of $\gamma2$ is not accounted for, but the efficacy of P4S on such receptors is presently not known. In line with this highly subunit dependent pharmacology of P4S, are data presented by Wafford *et al*. (1996) where P4S has been shown to act as an antagonist at $\alpha4\beta1\gamma2$ $GABA_A$ receptors expressed in oocytes. At present only the pharmacology of P4S has been shown to vary so dramatically with receptor type.

This study demonstrates GABA and isoguvacine to be full agonists with no differential action between cortical and granule neurons (Figures 3, 4 and 7). In contrast, in the oocytes both GABA and isoguvacine were significantly more potent at $\alpha6$- or $\alpha1\alpha6$-containing receptors compared with $\alpha1$-containing receptors (Table 2). Several reports have indicated that specific subunits confer differences in binding affinity for agonists. The $\alpha6$ subunit exhibits the highest affinity for agonists, i.e., GABA, muscimol and THIP, as compared to the other five α subunits (Ebert *et al*., 1997). Another study showed GABA to be 7 fold more potent in $\alpha6\beta3\gamma2$L than in $\alpha1\beta3\gamma2$L, and expression of the δ subunit confers greater sensitivity to GABA than the $\gamma2$L at least when expressed with $\alpha6\beta3$ (Saxena & Macdonald, 1996). The present findings with the $\alpha6\beta2\gamma2$ receptor subtype therefore are in agreement with previous reports. Results from receptors containing both the $\alpha1$ and $\alpha6$ subunits are more equivocal. A study by Mathews *et al*. (1994) reported GABA to have a lower potency ($EC_{50} = 34\ \mu M$) in HEK293 cells cotransfected with $\alpha1$ and $\alpha6$ in combination with $\beta2\gamma2$ than that for cells transfected with either $\alpha1$ ($EC_{50} = 14\ \mu M$) or $\alpha6$ ($EC_{50} = 2\ \mu M$). Another study using oocytes injected with mRNA for the subunit combinations $\alpha1\beta2\gamma2$, $\alpha6\beta2\gamma2$ and $\alpha1\alpha6\beta2\gamma2$ showed EC_{50} values of 41, 6.7 and 107 μM, respectively, for GABA (Sigel & Baur, 2000). These authors found the properties of the recombinant receptors to be very dependent on the ratio of $\alpha1$ to $\alpha6$ mRNA. By varying this ratio a spectrum of furosemide sensitivity ranging from $\alpha1\beta2\gamma2$ like to $\alpha6\beta2\gamma2$ like could be produced. In the present study using oocytes injected with cDNA we found $\alpha1\alpha6$-containing receptors to have an intermediate GABA sensitivity ($EC_{50} = 4.8\ \mu M$) compared to $\alpha6$-containing receptors ($EC_{50} = 1.6\ \mu M$) and $\alpha1$-containing receptors ($EC_{50} = 20\ \mu M$). This result cannot be caused by formation of a mixture of $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors in oocytes coinjected with the $\alpha1$ and $\alpha6$ subunits, since the high efficacy of P4S in these oocytes clearly demonstrates a significant contribution from $\alpha1\alpha6\beta2\gamma2$ receptors. Taken together these results suggest that expression of $\alpha1\alpha6\beta2\gamma2$ receptors is strongly dependent on the expression system and other experimental conditions. The fact that $GABA_A$ receptors of our cerebellar granule cells were not significantly more sensitive to GABA than those of the cerebral cortical neurons may then be due to preferential expression of $\alpha1$ and $\alpha1\alpha6$ containing receptors. This is in line with two other observations from cerebellar granule cells: The efficacy of 4-PIOL was around 5% compared to zero in recombinant $\alpha6\beta2\gamma2$ receptors (Rabe *et al*., 2000), and the efficacy of P4S in cerebellar granule cells was between that found in oocytes for $\alpha1\alpha6\beta2\gamma2$ and $\alpha1\beta2\gamma2$ receptors but considerably higher than that for $\alpha6\beta2\gamma2$ receptors. The presence of $\alpha6$ mRNA in the cerebellar granule cells does not guarantee a large amount of $\alpha6$ subunit protein in the receptors, and therefore receptors with $\alpha6$ as the only α subunit may be rare. While the effect of the δ subunit instead of $\gamma2$ in combination with a β subunit and $\alpha6$ is to increase the potency of GABA (Saxena & Macdonald, 1996), the effect in receptors with both $\alpha1$ and $\alpha6$ is not known.

Conclusion

In conclusion this study shows that the pharmacological profile of partial agonists and full agonists is highly dependent on the receptor composition as demonstrated on the two neuronal cell types, and that small structural changes of a ligand can alter the selectivity towards different subunit compositions. In contrast, the pharmacological profile of antagonists seems to be independent of neuronal type and thus of receptor type. Moreover, this study shows that, despite the problems encountered when using recombinant receptors expressed in oocytes to predict the properties of native receptors in cultured neurons, the pharmacological actions determined in oocytes generally are in agreement with data from cultured neurons.

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(Received November 28, 2000
Revised April 10, 2001
Accepted April 11, 2001)