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Electrophysiological actions of γ-aminobutyric acid and clomethiazole on recombinant $GABA_A$ receptors

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Abstract

Clomethiazole is a γ-aminobutyric acid (GABA)-mimetic agent with anticonvulsant, sedative and neuroprotective properties. The pharmacological actions of clomethiazole that underlie its functional profile have not been fully explored, but are known to result from an interaction with the $GABA_A$ receptor. Here, we present a quantitative electrophysiological study of clomethiazole action at human recombinant $GABA_A$ receptors. Whole-cell currents were recorded from murine L(tk-) cells stably transfected with either α1, β1 and γ2 or α1, β2 and γ2 $GABA_A$ receptor subunits. Clomethiazole directly activated $GABA_A$ currents in α1/β1/γ2- and α1/β2/γ2-containing cells, with EC_{50} values of 0.3 and 1.5 mM, respectively. A low concentration of clomethiazole (30 μM) also potentiated the action of GABA in both cell types, equivalent to a 3-fold increase in potency and up to 1.8-fold increase in maximal current. Both direct activation and gamma-aminobutyric acid potentiation are likely to contribute to the in vivo profile of clomethiazole.

Keywords: $GABA_A$ receptor; GABA (γ-aminobutyric acid)-mimetic; Clomethiazole; Neuroprotective agent; Bicuculline

1. Introduction

Clomethiazole is a well-established γ-aminobutyric acid ($GABA_A$)-mimetic drug that has been used clinically as an anxiolytic, sedative and anticonvulsant drug (see Green, 1998). Clomethiazole is also an effective neuroprotective agent in animal models of cerebral ischaemia (Green, 1998; Green et al., 2000a) and against 3,4-methylenedioxymethamphetamine-induced neurotoxicity (Colado et al., 1998). Biochemical evidence suggests that clomethiazole interacts with the $GABA_A$ receptor complex. It inhibits the binding of [^{35}S]butyl-bicyclophosphorothionate (TBPS), an effect indicative of $GABA_A$ receptor-channel activation (Cross et al., 1989; Green et al., 1996; Moody and Skolnick, 1989), by increasing the rate of [^{35}S]TBPS dissociation (Cross et al., 1989) and decreasing the binding affinity (Cross et al., 1989; Moody and Skolnick, 1989). Clomethiazole, like the barbiturates, also enhances [^{3}H]muscimol binding, though this is only a modest effect, with a 20% increase at 1 mM clomethiazole (Cross et al., 1989; Green et al., 1996; Leeb-Lundberg et al., 1981). However, in contrast to the barbiturates, it does not potentiate [^{3}H]flunitrazepam binding (Cross et al., 1989; Green et al., 1996; Zhong and Simmonds, 1997). These studies suggest that clomethiazole interacts with the $GABA_A$ receptor in a manner that is similar, but not identical, to that of the barbiturates (Cross et al., 1989; Green et al., 1996; Zhong and Simmonds, 1997). This suggestion is supported by the fact that clomethiazole is an effective neuroprotective agent against cerebral damage caused by either focal or global ischaemia (see Green, 1998) or MDMA administration (Colado et al., 1998, 1999), while pentobarbitone is not (Cross et al., 1991). The present study has been undertaken to clarify the unique interaction between clomethiazole and the $GABA_A$ receptor.

Gamma-aminobutyric acid (GABA), acting at $GABA_A$ receptors, is the main fast inhibitory neurotransmitter in mammalian central nervous system (Costa, 1998; Mehta and Ticku, 1999). At least 19 different $GABA_A$ receptor subunits have been identified, including α1–6, β1–3 and γ1–3 subunits, in addition to δ, ε and others (see Cherubini and Conti, 2001; Costa, 1998; McKernan and Whiting, 1996). The $GABA_A$ receptor is known to be a pentameric complex

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with an integral chloride-selective ion channel, and fluorescence energy transfer suggests that the most prevalent subunit composition is 2α:2β:1γ, with δ or ε being able to substitute for the γ subunit (Farrar et al., 1999; McKernan and Whiting, 1996). Antibody co-labelling methods suggest that the most abundant co-localization of protein subunits in adult rat brain is α1/β2/γ2, this combination being present in most brain regions, with overall abundance of 43% (Fritschy et al., 1992; Fritschy and Mohler, 1995; McKernan and Whiting, 1996).

$GABA_A$-mimetic agents, which activate $GABA_A$ receptors either directly, or by potentiating the agonist action of GABA, have found widespread clinical use as anxiolytics, general anaesthetics, antiepileptic drugs and sedative/hypnotics (Chebib and Johnston, 2000; Lees, 1998; Mehta and Ticku, 1999; Sieghart, 1995). In addition, some have been shown to act as neuroprotective agents in animal models of acute ischaemic stroke (see (Green et al., 2000a; Schwartz-Bloom and Sah, 2001). $GABA_A$ receptor activation has also been suggested to be a possible target for relief of neuropathic pain (Stubley et al., 2001).

Previous studies of clomethiazole have suggested an allosteric GABA-potentiating action of the compound, via a non-benzodiazepine, non-barbiturate binding site (Cross et al., 1989; Green et al., 1996; Harrison and Simmonds, 1983; Zhong and Simmonds, 1997) and, in addition, a direct $GABA_A$ receptor activating action (Hales and Lambert, 1992; Moody and Skolnick, 1989; Nelson et al., 2000) possibly via the GABA binding site (Hales and Lambert, 1992). Here we present the first quantitative electrophysiological study of clomethiazole, using whole-cell recording from mammalian cells transfected with recombinant α1/β2/γ2 $GABA_A$ receptor subunits, and fast extracellular perfusion. To test the influence of β subunit, we compared cells transfected with α1/β1/γ2 subunits. Some data have been previously reported in abstract form (Nelson et al., 2001a).

2. Materials and methods

2.1. Cell culture conditions

Mouse L(tk-) cells stably transfected with human α1, β1 and γ2L or α1, β2 and γ2S $GABA_A$ receptor subunits (Hadingham et al., 1992), were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C, 5% CO_2. Transfected subunits were under the control of a dexamethasone-sensitive promoter and selection pressure (1 mg/ml geneticin) applied every 4 weeks. Cells that were to be used for measurement of whole-cell currents were grown on glass cover slips coated with poly-L-lysine (2 μg/ml) using DMEM supplemented with 10% fetal bovine serum for 24 h at 37 °C, 5% CO_2. After this period, the medium was replaced and supplemented with dexamethasone (100 nM). Cells were then incubated for an additional 48 h to induce $GABA_A$ receptor expression.

2.2. Electrophysiological procedures

Cover slips were placed in a 35 mm Petri dish (Falcon, Beckton-Dickinson, CA, USA) on a Nikon TMS inverted microscope and continuously perfused with HEPES buffered saline (HBS) consisting of (in mM): NaCl (135), KCl (2.5), $CaCl_2$ (2.0), $MgSO_4$ (0.5), NaH_2PO_4 (1.0), HEPES acid (10), glucose (10), pH 7.4 with NaOH.

Patch-pipettes were pulled from 1.5 mm borosilicate capillary glass (Clarke Electromedical, Pangbourne, UK), coated with beeswax and fire-polished immediately before use. Pipette resistances were typically 5 MΩ when filled with a CsCl-based intracellular solution containing (in mM): CsCl (140), EGTA (3.0), $MgSO_4$ (8.0), HEPES (10), Na_2-ATP (4.0), pH 7.4 with CsOH. Standard whole-cell recordings were made at room temperature using an Axopatch-200 patch clamp amplifier (Axon Instruments, Foster City, CA, USA) and a CED1401 interface (Cambridge Electronic Design, Cambridge, UK), driven by Strathclyde software (freeware kindly supplied by Dr. J. Dempster; see http://innovol.sibs.strath.ac.uk/physpharm/ses). The holding voltage for cells was -60 mV unless stated otherwise. Records were filtered at 500 Hz (− 3 dB, 8-pole Bessel response, Fylde Electronics, Preston, UK) and sampled at 1 kHz.

Drugs were dissolved at a known concentration in HBS and applied via a custom-built fast-perfusion system for 500 ms, separated by rest periods of 60 s. The drug-induced current amplitude was measured from the computer screen using cursors manually placed at peak current amplitude.

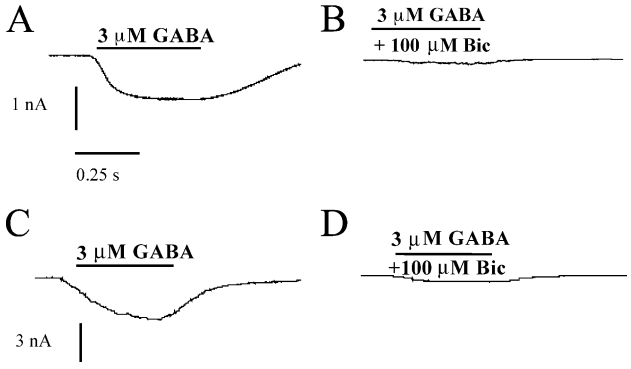

Fig. 1. GABA-activated whole-cell currents in L(tk-) cells transfected with $GABA_A$ receptor subunits α1/β1/γ2 (A–B) or α1/β2/γ2 (C–D). (A) Current evoked in response to application of GABA (3 μM, 500 ms). (B) Response of the same cell to application of GABA in the presence of bicuculline (Bic; 100 μM). (C, D) Currents evoked in response to application of GABA (3 μM, 500 ms) in the absence (C) or presence (D) of bicuculline. The holding voltage was -30 mV in each panel. Vertical scale bars show 1 nA (A–B) or 3 nA (C–D); horizontal scale bar shows 250 ms.

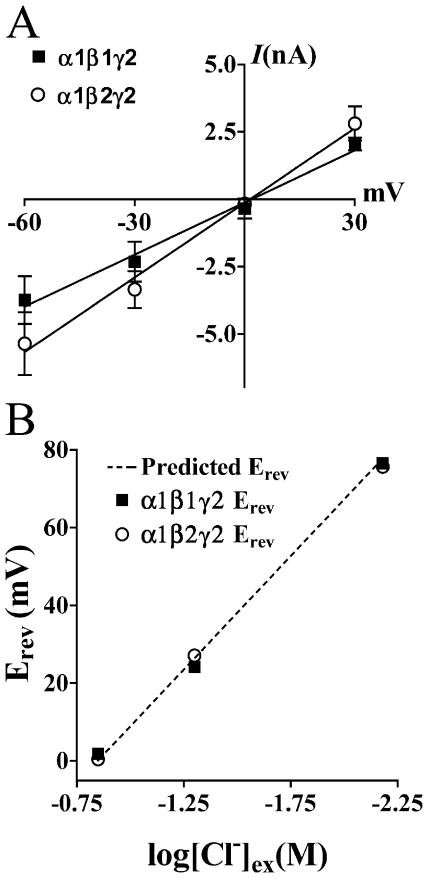

Fig. 2. Electrophysiological behaviour of GABA-activated currents. (A) Average current–voltage curves for α1/β1/γ2- and α1/β2/γ2-transfected cells, with GABA application (3 μM) as in Fig. 1, and standard recording conditions (142 mM external [Cl^-]). Points show mean ± S.E.M. from four to eight cells. Regression lines of best fit are shown for the two cell types. (B) Variation of zero current–voltage with logarithm of external [Cl^-]. Data points show values determined from current–voltage curves such as that in A, pooled from experimental recordings with external [Cl^-] of 6.5, 50 or 142 mM (n = at least 3 for each point). The dashed straight line shows the Nernst voltage, E_{Cl}, predicted for a perfectly chloride-selective conductance with internal [Cl^-] of 140 mM.

Whole-cell currents were measured in response to GABA at concentrations ranging between 0.1 and 100 μM for α1/β1/γ2-transfected cells, and 0.1 and 300 μM for α1/β2/γ2-transfected cells. To determine if clomethiazole was able to activate $GABA_A$ receptors directly, varying concentrations of clomethiazole were applied (10 μM–30 mM) in the absence of any added GABA. The effect of bicuculline on GABA- or clomethiazole-induced whole-cell currents was also determined. GABA (3 μM) or CMZ (1 mM) was applied for 500 ms. After a 15 s wash, bicuculline (100 μM) was applied in conjunction with GABA or clomethiazole for a further 500 ms. After a further 30 s wash, GABA or clomethiazole alone was reapplied for 500 ms. To determine the potentiating effect of clomethiazole on GABA-induced whole-cell currents, clomethiazole (30 or 100 μM) was co-applied with varying GABA concentrations.

To test the ionic selectivity of the GABA- or clomethiazole-evoked currents, extracellular chloride concentrations were varied while the intracellular chloride concentration was maintained at 140 mM. Extracellular chloride concentrations of 6.5, 50 or 141.5 mM were obtained by varying the NaCl content (replacement with Na-acetate). Whole-cell currents were recorded in response to a single concentration of GABA (3 μM) or clomethiazole (1 mM), applied for 500 ms, with 10 mV voltage increments. The zero-current voltage in each case was then determined from current–voltage graphs.

2.3. Data analysis

Concentration–response data were fitted by least squares nonlinear regression (GraphPad Prism) and statistical analysis was performed using two-way analysis of variance with post hoc tests using Bonferroni's multiple comparison (GraphPad Prism).

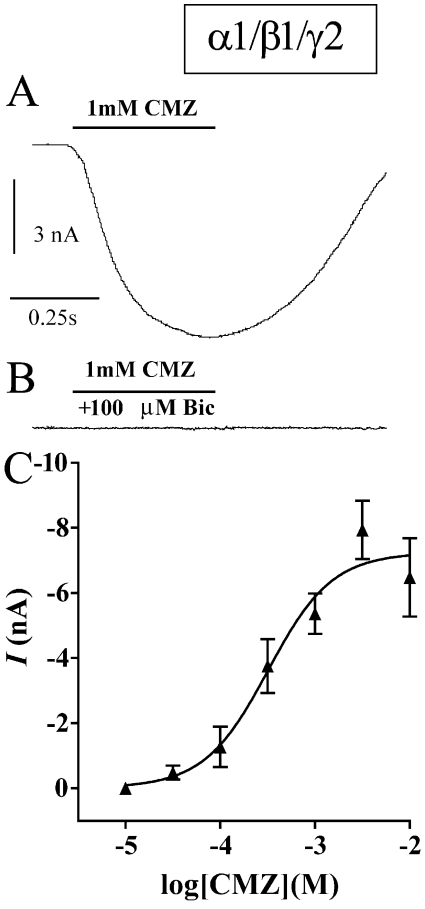

Fig. 3. Clomethiazole directly activated whole-cell currents in cells transfected with α1/β1/γ2 $GABA_A$ receptor subunits. (A, B) Current evoked in response to application of clomethiazole (CMZ, 1 mM, 500 ms) in the absence (A) or presence (B) of bicuculline (Bic; 100 μM); holding potential was −30 mV in each case. (C) Pooled concentration–response data. Points show clomethiazole-activated peak current (I), mean ± S.E.M. from six cells. The Langmuir–Hill curve of best fit is shown (solid line).

3. Results

3.1. Actions of GABA on α1/β1/γ2L or α1/β2/γ2S subunit-transfected cells

Application of GABA to L(tk-) cells, transfected with either α1/β1/γ2L or α1/β2/γ2S $GABA_A$ receptor subunits, evoked large whole-cell currents (Fig. 1). In both cell types, the currents evoked in response to GABA (3–10 μM) were almost completely inhibited by the $GABA_A$ receptor antagonist bicuculline (100 μM, $n=3–4$, see Fig. 1) and recovered on washing out the antagonist (not shown). The current–voltage relations for GABA-evoked currents in both cell types were approximately linear over the voltage range -60 to +30 mV (Fig. 2A). When the chloride ion concentration in the extracellular solution was varied (6.5–142 mM), the zero-current potential for GABA-evoked currents varied in a manner very similar to that predicted for a chloride-selective conductance (Fig. 2B).

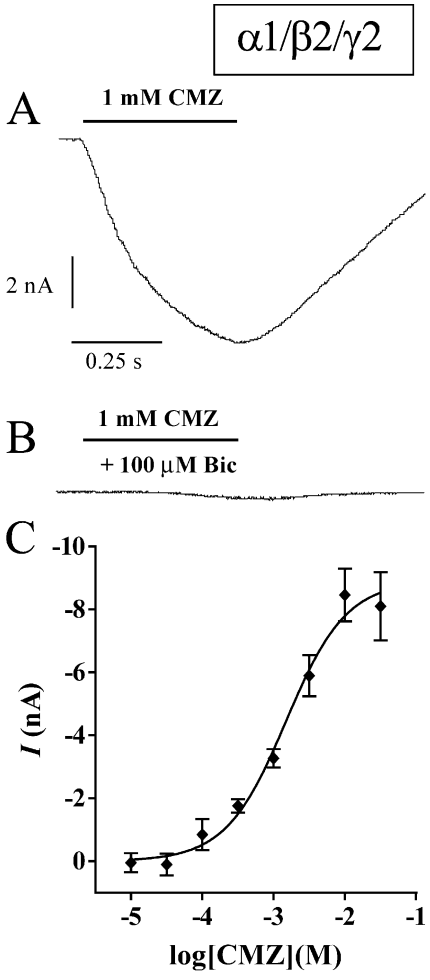

Fig. 4. Clomethiazole directly activated whole-cell currents in cells transfected with α1/β2/γ2 $GABA_A$ receptor subunits. (A, B) Current evoked in response to application of clomethiazole (CMZ, 1 mM, 500 ms) in the absence (A) or presence (B) of bicuculline (Bic; 100 μM); holding potential was −30 mV in each case. (C) Pooled concentration–response data. Points show clomethiazole-activated peak current (I), mean ± S.E.M. from five cells. The Langmuir–Hill curve of best fit is shown.

Table 1
Recombinant $GABA_A$ receptor activation

	α1/β1/γ2		α1/β2/γ2	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
GABA	2.4 ± 0.23	1.4	11 ± 0.32	1.2
GABA + clomethiazole[a]	0.8 ± 0.27	1.7	3.4 ± 0.24	2.3
Clomethiazole	300 ± 0.17	1.8	1500 ± 0.11	2.8

L(tk-) cells were transfected with either α1, β2 and γ2 or α1, β2 and γ2 human $GABA_A$ receptor subunits. Columns show half-maximal concentration (EC_{50}) values together with the S.E.M. and Hill coefficient (n_H) for Langmuir curves of best fit to pooled data in Figs. 3–6.

[a] Parameters for GABA in the presence of a fixed concentration of clomethiazole (30 μM).

3.2. Direct activation of $GABA_A$ currents by clomethiazole in α1/β1/γ2L or α1/β2/γ2S subunit-transfected cells

Application of clomethiazole (1 mM), in the absence of GABA, to α1/β1/γ2 or α1/β2/γ2 subunit-containing cells produced large whole-cell currents (Figs. 3 and 4). In each cell type, the currents evoked by clomethiazole (1 mM) were almost completely inhibited by bicuculline (100 μM; $n=3$, see Figs. 3 and 4), indicating a $GABA_A$ receptor-dependent mechanism. These currents that were directly evoked by clomethiazole, like those evoked by GABA, displayed a near-linear current–voltage relation and chloride-selective zero-current potential with external [Cl^-] in the range 6.5–142 mM ($n=4$, data not shown). Maximal current activation was obtained with concentrations of 3–10 mM clomethiazole (Figs. 3 and 4). EC_{50} values obtained from the fitted Langmuir–Hill curves are given in Table 1.

3.3. Potentiation of GABA-evoked currents by clomethiazole in α1/β1/γ2L or α1/β2/γ2s subunit-transfected cells

In both α1/β1/γ2- and α1/β2/γ2-containing cells, the current evoked by a low concentration of GABA (3 μM) was greatly increased on co-application of clomethiazole (30–100 μM) (Figs. 5 and 6). The effect of clomethiazole (30 μM) on the concentration–response relation of GABA was determined. This concentration of clomethiazole produced negligible currents by direct activation (see Figs. 3 and 4). In cells containing α1/β1/γ2 subunits, the EC_{50} for current activation by GABA alone was 2.4 μM (Table 1) and a maximal effect achieved with 10 μM GABA (Fig. 5C). Clomethiazole (30 μM) potentiated current amplitude at all GABA concentrations in the range 0.1–30 μM (Fig. 5C). Potentiation of current at higher GABA concentrations was not significant. A Langmuir–Hill relation fitted to the data points in the concentration range 0.1–10 μM had an

EC_{50} of 0.8 μM (Fig. 5). The maximal current response, derived from the fitted curves, was augmented by a factor of about 1.8 (Fig. 5C).

GABA was less potent in cells containing α1/β2/γ2 subunits (Fig. 6). The fitted EC_{50} for GABA alone was 11 μM and a maximal effect was achieved at a concentration of 100 μM GABA (Fig. 6C). Clomethiazole (30 μM) potentiated GABA-evoked currents throughout the concentration range of 0.1–100 μM GABA (Fig. 6C). The potentiating effect of clomethiazole corresponded to a modified EC_{50} of 3.4 μM for GABA. The maximal response was augmented by a factor of 1.2 (Fig. 6C).

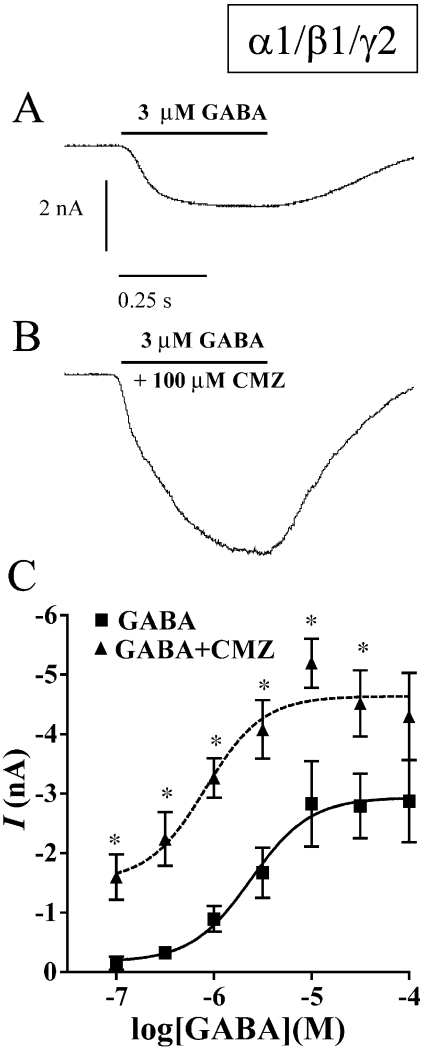

Fig. 5. Clomethiazole-potentiated GABA-activated currents in α1/β1/γ2-transfected cells. Currents evoked in response to application of GABA (3 μM, 500 ms) in the absence (A) or presence (B) of clomethiazole (CMZ; 100 μM); holding potential was −30 mV in each case. The current evoked by this low concentration of clomethiazole by direct activation is likely to be very low (see Figs. 3 and 4). (C) Pooled concentration–response data. Points show mean ± S.E.M. of GABA-activated peak current (*I*) in the absence (squares, data from eight cells) or presence (triangles, data from five cells) of a low concentration of clomethiazole (CMZ; 30 μM). Asterisks mark GABA concentrations where co-application of clomethiazole produced significant potentiation ($p < 0.05$). The Langmuir–Hill curves of best fit are shown for GABA alone (solid line) and GABA in the presence of clomethiazole (dashed line).

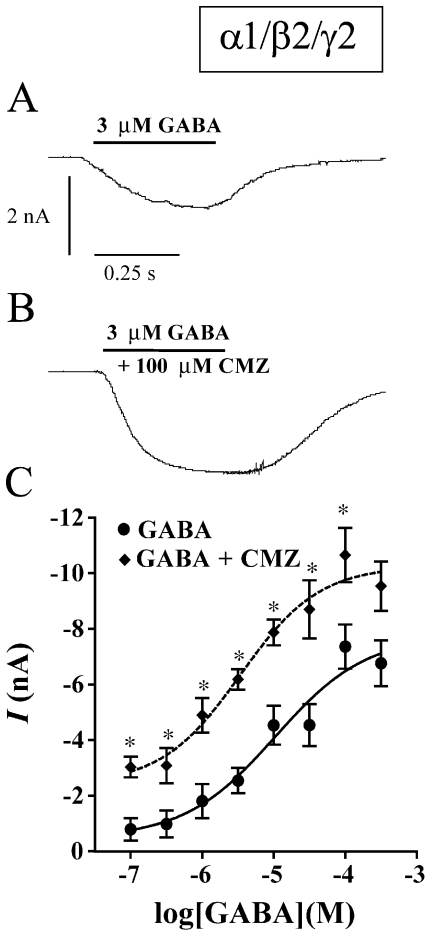

Fig. 6. Clomethiazole-potentiated GABA-activated currents in α1/β2/γ2-transfected cells. Currents evoked in response to application of GABA (3 μM, 500 ms) in the absence (A) or presence (B) of clomethiazole (CMZ; 100 μM); holding potential was −30 mV in each case. (C) Pooled concentration–response data. Points show mean ± S.E.M. of GABA-activated peak current (*I*) in the absence (circles, data from four cells) or presence (diamonds, data from four cells) of a low concentration of clomethiazole (CMZ; 30 μM). Asterisks mark GABA concentrations where co-application of clomethiazole produced significant potentiation. The Langmuir–Hill curves of best fit are shown for GABA alone (solid line) and GABA in the presence of clomethiazole (dashed line).

4. Discussion

4.1. General points

We studied the electrophysiological actions of GABA and clomethiazole on cell lines transfected with two different recombinant $GABA_A$ receptor subunit compositions: α1/β2/γ2, which is the most abundant combination in mammalian central nervous system (Fritschy et al., 1992; McKernan and Whiting, 1996) and α1/β1/γ2.

Brief application of GABA produced chloride-selective, bicuculline-sensitive currents in both cell types, with approximately 4-fold higher potency in the α1/β1/γ2 subunit-containing cells. It is possible that the $GABA_A$ currents recorded might have been carried by a heterogeneous population of receptor types (α/β, β, β/γ, etc.), but this seems unlikely for three reasons. First, the concentration–response relations for both cell types were fitted by single Langmuir–Hill curves for either GABA or clomethiazole. Second, for both drugs, these curves were well-separated for the two cell types, which differed only in the β subunit present. Finally, HEK293 cells transfected with α1, β2 and γ2 subunits were shown, using FRET analysis, to contain only $GABA_A$ receptors of stoichiometry $\alpha1_2\beta2_2\gamma2$ (Farrar et al., 1999). It also seems unlikely that the differences in responses to brief drug application, observed here, resulted from the different γ subunits present. The γ2L and γ2S subunits are splice variants, differing only in an eight amino acid insert in the cytoplasmic TM3–TM4 linker (see Mehta and Ticku, 1999).

4.2. Direct activation by clomethiazole

In the absence of GABA, clomethiazole (≥30 μM) directly activated $GABA_A$ currents in a bicuculline-sensitive manner. In an earlier study, direct current activation was observed in bovine chromaffin cells on pressure ejection of clomethiazole (3 mM), and virtually abolished in the presence of bicuculline (Hales and Lambert, 1992). These data were interpreted as evidence for an action of clomethiazole at the GABA-binding site (Hales and Lambert, 1992). Bicuculline has more recently been shown not only to compete for the GABA agonist binding site, but also to prevent channel activation once bound, by acting as an allosteric "inverse agonist" (Bianchi and Macdonald, 2001; Ueno et al., 1997). Thus, it is likely that direct activation by clomethiazole does not arise from action at the GABA recognition site. Indeed, clomethiazole (1 mM) did not displace labelled muscimol from rat cortical membranes, indicating that clomethiazole has negligible affinity for the GABA agonist binding site on native receptors (Green et al., 1996).

Clomethiazole displaced [^{35}S]-labelled TBPS-binding, indicative of channel activation, in HEK293 cells transfected either with β3 subunits alone, or with β3 together with α1, γ2 or α1/γ2, in each case with IC_{50} of about 90 μM (range 84–99 μM) (Zezula et al., 1996). This value is very close to that obtained in both rat cortical membrane preparations (Cross et al., 1989; Moody and Skolnick, 1989; Vincens et al., 1989) and cerebellar membrane preparations (Zezula et al., 1996). In contrast to clomethiazole, a range of compounds—including GABA, alphaxolone, (+)-etomidate, pentobarbital and propofol—exhibited different potencies for [^{35}S]TBPS displacement from cells transfected with β3, α1/β3, β3/γ2, or α1/β3/γ2 (Zezula et al., 1996). One interpretation of this study is that clomethiazole binds to the β subunit, in a fashion insensitive to the other subunits present. An alternative interpretation is that clomethiazole is completely promiscuous, and binds to α, β and γ subunits equally well. Our findings of 5-fold difference in potency between β1 and β2 containing cells favour the former view, that clomethiazole acts via the β subunit, at least for direct channel activation. The Hill coefficient for clomethiazole data suggests multiple clomethiazole binding sites per channel (see Table 1).

4.3. Allosteric action of clomethiazole

At concentrations where negligible direct current activation was observed, clomethiazole (30 μM) enhanced currents evoked by GABA, equivalent to ~3-fold increase in potency, in both cell types. In previous electrophysiological studies, clomethiazole (100 μM) also enhanced currents evoked by brief pressure ejections of GABA, both in CHO cells transfected with α1/β1 $GABA_A$ subunits (Hill-Venning et al., 1992) and in bovine chromaffin cells (Hales and Lambert, 1992; Lambert et al., 1991), known to contain predominantly α1, γ2 and a β subunit (see McKernan and Whiting, 1996). This potentiating effect of clomethiazole is believed to result from an allosteric action on the $GABA_A$ receptor complex, in common with the actions of several other GABAmimetics, including pentobarbitone, propofol and loreclezole, e.g. (Hales and Lambert, 1992; Hill-Venning et al., 1992; Lambert et al., 1991; Thompson et al., 1996, 1999; Wafford et al., 1994; Zhong and Simmonds, 1997). The allosteric action of clomethiazole at concentrations $<10^{-4}$ M could result from increased single-channel density, conductance, or open probability. Previous data indicated an increase in channel open probability, probably resulting from prolongation of burst length by clomethiazole at a concentration of 100 μM (Hales and Lambert, 1992).

4.4. Conclusion

We conclude that clomethiazole can directly activate $GABA_A$ receptors at low concentrations (≥100 μM), in addition to potentiating the agonist action of GABA. Clinically, the plasma concentration of clomethiazole can reach 100–200 μM at hypnotic doses (Kim and Khanna, 1983) and studies in rats have shown that brain tissue concentrations of clomethiazole are around 40% greater than the plasma concentration (Green et al., 2000b). Thus, brain clomethiazole concentrations of around 100 μM in patients are likely to occur at clinically relevant doses. The concentration observed in rat brain following an acute dose of clomethiazole, which produces sedative, anticonvulsant and neuroprotective activity, has also been shown to be in the 100–200 μM range (Green et al., 2000b). Thus, both mechanisms of action considered here, direct activation and allosteric potentiation, may be relevant in vivo. Direct activation is likely to be a potent tonic

inhibitory influence at extra-synaptic $GABA_A$ receptors (Mody, 2001), while the allosteric effect is likely to be more relevant at synaptic sites, potentiating the neurotransmitter action of GABA. In pathological states where ambient levels of GABA are low (for example, following acute cerebral ischaemia), clomethiazole could activate $GABA_A$ receptors, leading to restoration of GABAergic function (Green et al., 2000a). This proposal is supported by recent in vitro studies using ischaemic rat cortical tissue (Nelson et al., 2000, 2001b).

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