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Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors

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1 The pharmacology of the stable cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptor was investigated using whole-cell patch-clamp techniques.

2 $\alpha_4\beta_3\delta$ receptors exhibited increased sensitivity to GABA when compared to $\alpha_4\beta_3\gamma_2$ receptors, with EC_{50}'s of 0.50 (0.46, 0.53) μM and 2.6 (2.5, 2.6) μM respectively. Additionally, the GABA partial agonists piperidine-4-sulphonate (P4S) and 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (THIP) displayed markedly higher efficacy at $\alpha_4\beta_3\delta$ receptors, indeed THIP demonstrated greater efficacy than GABA at these receptors.

3 The δ subunit conferred slow desensitization to GABA, with rate constants of 4.8 ± 0.5 s for $\alpha_4\beta_3\delta$ and 2.5 ± 0.2 s for $\alpha_4\beta_3\gamma_2$. However, both P4S and THIP demonstrated similar levels of desensitization on both receptor subtypes suggesting this effect is agonist specific.

4 $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ demonstrated equal sensitivity to inhibition by the cation zinc (2–3 μM IC_{50}). However, $\alpha_4\beta_3\delta$ receptors demonstrated greater sensitivity to inhibition by lanthanum. The IC_{50} for GABA antagonists SR-95531 and picrotoxin, was similar for $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$. Likewise, inhibition was observed on both subtypes at high and low pH.

5 $\alpha_4\beta_3\delta$ receptors were insensitive to modulation by benzodiazepine ligands. In contrast Ro15-4513 and bretazenil potentiated GABA responses on $\alpha_4\beta_3\gamma_2$ cells, and the inverse agonist DMCM showed allosteric inhibition of $\alpha_4\beta_3\gamma_2$ receptors.

6 The efficacy of neurosteroids at $\alpha_4\beta_3\delta$ receptors was greatly enhanced over that observed at $\alpha_4\beta_3\gamma_2$ receptors. The greatest effect was observed using THDOC with $524\pm71.6\%$ potentiation at $\alpha_4\beta_3\delta$ and $297.9\pm49.7\%$ at $\alpha_4\beta_3\gamma_2$ receptors. Inhibition by the steroid pregnenolone sulphate however, showed no subtype selectivity. The efficacy of both pentobarbitone and propofol was slightly augmented and etomidate greatly enhanced at $\alpha_4\beta_3\delta$ receptors *versus* $\alpha_4\beta_3\gamma_2$ receptors.

7 We show that the $\alpha_4\beta_3\delta$ receptor has a distinct pharmacology and kinetic profile. With its restricted distribution within the brain and unique pharmacology this receptor may play an important role in the action of neurosteroids and anaesthetics.

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Abbreviations: Alphaxalone, 5α-pregnan-3α-ol-11,20-di-one; DMCM, Methyl-6,7,-dimethoxy-4-ethyl-beta-carboline-3-carboxylate; GABA, γ-aminobutyric acid; HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; P4S, piperidine-4-sulphonic acid; Pregnenolone sulphate, 5α-pregnen-3β-ol-20-one sulfate; THDOC, 5α-pregnane-3α,21-diol-20-one; THIP, tetrahydroisothiazolo-[5,4-c]pyridin-3-ol

Introduction

GABA (γ-Aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian central nervous system. Its primary action is through the $GABA_A$ receptor, which is composed of a family of functionally diverse subunits that assemble into a pentameric structure (McKernan & Whiting, 1996). To date there are 17 different subunits identified (α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-2}, δ, ε, θ). These subunits have discrete locations within the brain, but the most abundant receptor subtypes have been found to express α, β and γ subunits (Barnard, 1998; Sieghart, 1995). The $GABA_A$ receptor can be modulated by a number of therapeutic agents, including benzodiazepines, barbiturates, anaesthetics, ethanol and neuroactive steroids. The extent of this modulation is subunit specific. Recombinant studies have shown the α and γ subunits are responsible for benzodiazepine and zinc sensitivity (Pritchett *et al*., 1989; Draguhn *et al*., 1990). The role of subunits for other modulators remains the subject of investigation.

The α_4 and δ subunits have a very restricted distribution within the brain, but primarily co-localize in the thalamus and hippocampus (Sperk *et al*., 1997; Sur *et al*., 1999; Pirker *et al*., 2000). The α_4 subunit is most homologous to the α_6 subunit, both are insensitive to diazepam but have high affinity for the benzodiazepines Ro15-4513 and bretazenil (Wisden *et al*., 1991). The role of the δ subunit is currently still relatively unclear as this has proved to be very difficult to express in transient recombinant systems. In this study we have created a dexamethasone-inducible, stable cell line in mouse L(-tk) cells, which expresses the human $\alpha_4\beta_3\delta$ $GABA_A$

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subtype and the pharmacology of this δ subunit containing receptor is investigated and compared to a similar cell line expressing human $\alpha_4\beta_3\gamma_2$ $GABA_A$ receptors allowing a direct comparison of the δ subunit with γ_2 containing receptors, using the whole-cell patch-clamp technique.

Methods

Construction of the $\alpha_4\beta_3\delta$ cell line

Stable cell lines were produced using the mouse L(-tk) fibroblast cell line. Generation of the $\alpha_4\beta_3\gamma_2$ control cell line was described in Sur *et al*., 1999, where all subunits were under the control of a dexamethasone inducible promoter. However, for the stable cell line expressing $\alpha_4\beta_3\delta$ receptors, c-*myc* tagged δ was constitutively expressed from a human CMV promoter (pcDNA3.1Zeo, Invitrogen), and α_4 and β_3 expressed from a dexamethasone inducible promoter as before. An epitope-tagged δ subunit was constructed that contained nucleotides 224 to +99 (i.e., the 5′ untranslated region, the signal peptide, 6 amino acids of the mature protein) of bovine GABA-A receptor α_1 cDNA, a sequence encoding the c-*myc* epitope tag (EQKLISEEDL), a cloning site encoding the amino acids Asn-Ser-Gly, and DNA encoding amino acids 34–452 of the GABA-A receptor gene product. Constitutive expression of the subunit was demonstrated using Northern blotting (data not shown), however no cell surface expression of the *myc* tag was present until induction of α_4 and β_3. The ELISA-based assay for cell surface c-*myc* expression was performed essentially as described in Bonnert *et al*. (1999) and expressed as $A_{620\ nm}$ following 24 h stimulation with dexamethasone at a range of concentrations. An L(-tk) cell line expressing $\alpha_3\beta_3\gamma_2$ (not epitope tagged) was used as the negative control.

Whole-cell patch-clamp of L(-tk) cells

Experiments were performed on the stable L(-tk) cell lines expressing either $\alpha_4\beta_3\delta$ or $\alpha_4\beta_3\gamma_2$ $GABA_A$ receptors following 24 h induction with 25 nM dexamethasone. Glass coverslips containing a monolayer of cells were placed in a chamber on the stage of a Nikon Diaphot inverted microscope. Cells were perfused continuously with artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl 149, KCl 3.25, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, D-Glucose 11, D(+)-Sucrose 22, pH 7.4 and 350 mOSM, and observed with phase-contrast optics. Fire-polished patch pipettes were pulled on a WZ, DMZ-Universal puller using conventional 120TF-10 electrode glass. Pipette tip diameter was approximately 1.5–2.5 μm, with resistances around 4 MΩ. The intracellular solution contained (in mM): CsCl 130, HEPES 10, BAPTA.Cs 10, ATP.Mg 5, Leupeptin 0.1, $MgCl_2$ 1, $NaVO_3$ 100 μM, pH adjusted to 7.3 with CsOH and 320–340 mOsm. Cells were voltage-clamped at −60 mV *via* an Axon 200B amplifier (Axon Instr., Foster City, CA, U.S.A.). Drug solutions were applied to the cells *via* a multi-barrel drug delivery system, which could pivot the barrels into place using a stepping motor. This ensured rapid application and washout of the drug. Measured agonist exchange time using this system was approximately 20–30 ms. GABA agonists were applied to the cell for 5 s with a 30 s washout period between applications which is a sufficient time period to reverse any desensitization which may occur. Noncumulative concentration–response curves to agonists and modulators were constructed. Curves were fitted using a nonlinear least square–fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. EC_{50}'s and IC_{50}'s were calculated for individual cells and combined to be expressed as geometric means with 95% confidence intervals. Several modulators showed sharp reversal at high concentrations due to either direct inhibition or increased desensitization. In these cases curves were fitted to the available data excluding these points. Allosteric modulators were pre-applied for 30 s with the resulting modulation of GABA receptors measured relative to a GABA EC_{20} and antagonists investigated relative to a GABA EC_{50} individually determined for each cell to account for differences in GABA affinity. Data was recorded and analysed using P-clamp (version 8, Axon instruments, Foster City, CA, U.S.A.). For the experiments addressing receptor kinetics, agonists were applied for 10 s and the time to peak, desensitization and deactivation rate were fitted using P-Clamp software, and data were best fit by single exponential functions.

Drugs used

The following drugs were purchased from Sigma: 5α-pregnan-3α-ol-20-one, Alphaxalone, Flunitrazepam, GABA, Lanthanum chloride, P4S, Pentobarbitone, Picrotoxin, THDOC, and Zinc chloride. DMCM, Ro15-4513 and SR95531 were purchased from RBI. Etomidate was obtained from Janssen Pharmaceutica, Propofol from Aldrich and THIP was purchased from Tocris. Bretazenil was synthesized by Merck Sharp & Dohme chemistry department. All drugs were made as stock solutions (1 M–10^{-2} M) in either DMSO or sterile water. Drugs were diluted in aCSF to their final concentrations prior to use. The final concentration of DMSO did not exceed 0.3% and had no effect on current responses.

Data analysis

Arithmetic mean values or geometric mean values were calculated from a number (n) of different cells. The statistical significance of differences between mean values was assessed by Student's two-tailed t-test.

Results

Functional expression of α_4,β_3 and δ containing receptors

Recent evidence suggests that the $\alpha_4\beta\delta$ GABA subunit combination may be an important native receptor subtype with high levels in hippocampus and thalamus (Sur *et al*., 1999; Pirker *et al*., 2000). The $GABA_A$ δ subunit has proved notoriously difficult to express in recombinant systems, and as a consequence there have been relatively few accounts of the pharmacology of this receptor subtype. Here we have generated a novel cell line expressing the $\alpha_4\beta_3\delta$ receptor, and have studied the pharmacological properties of this $\alpha_4\beta_3\delta$ $GABA_A$ receptor, compared directly with that of the $\alpha_4\beta_3\gamma_2$

receptor in the same type of cell line. To ensure expression of the correct subunit assembly, an N-terminally c-*myc* epitope-tagged construct of the δ subunit was constitutively expressed in an L(-tk) cell line, together with the α_4 and β_3 subunits controlled under a dexamethasone inducible promoter. The constitutively expressed epitope tagged δ subunit was observed, using an ELISA-based assay, not to reach the cell surface unless the expression of both α_4 and β_3 subunits was first induced by dexamethasone, demonstrating that the δ present in receptors on the cell surface was co-assembled with α_4 and β_3 (data not shown).

Effects of GABA and GABA agonists

An overall comparison of the maximum current amplitudes generated from the two cell lines demonstrated a reduced maximum response to GABA (100 μM) from 4777 ± 378 pA for $\alpha_4\beta_3\gamma_2$ receptors compared to 1504 ± 171 pA for $\alpha_4\beta_3\delta$ receptors.

Concentration–response curves to GABA revealed $\alpha_4\beta_3\gamma_2$ receptors to have an EC_{50} of 2.57 (2.51, 2.63) μM whereas $\alpha_4\beta_3\delta$ receptors exhibited greater sensitivity to GABA with an EC_{50} of 0.50 (0.46, 0.53) μM (Figure 1a,b). Full concentration–response curves to the partial GABA agonists piperidine-4-sulphonic acid (P4S) (Figure 2a) and 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (THIP) (Figure 2b) showed that both these agonists have a lower EC_{50} value and greater maximally evoked current for $\alpha_4\beta_3\delta$ containing receptors compared to $\alpha_4\beta_3\gamma_2$ receptors. Efficacy measures were expressed as the percentage current amplitude relative to a subsequent high concentration of GABA (100 μM) and data are summarized in Table 1. While P4S behaves as a partial agonist on both $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, THIP shows partial agonist efficacy at $\alpha_4\beta_3\gamma_2$ receptors but behaves as a 'super'-agonist on $\alpha_4\beta_3\delta$ receptors. The maximum response to THIP was consistently larger than that of a maximum response to GABA on $\alpha_4\beta_3\delta$ receptors.

The kinetic parameters of the GABA response in both receptor subtypes were also investigated. 100 μM GABA was used to evoke maximum amplitude currents and application was maintained for 10 s to produce receptor desensitization. The time to peak, deactivation and desensitization for each current was calculated and fit using a single exponential function. Receptors differed significantly in their rate of desensitization, with $\alpha_4\beta_3\gamma_2$ receptors desensitizing faster ($\tau = 2.5 \pm 0.2$ s ($n = 5$)) than $\alpha_4\beta_3\delta$ receptors which exhibited much slower desensitization ($\tau = 4.8 \pm 0.5$ s ($n = 10$)) (Figure 3a). Whilst accepting that accurate measures of rise time and extremely fast components may be missed using this system due to the limitations imposed by the agonist application system, differences in receptor subtype could be measured. The GABA time to peak was faster for $\alpha_4\beta_3\gamma_2$ receptors with a τ of 51 ± 5.6 ms ($n = 5$) compared to 101 ± 8.8 ms ($n = 10$) on $\alpha_4\beta_3\delta$. The deactivation phase of the response was similar for both receptor subtypes with τ values of 401.5 ± 23.3 ($n = 10$) and 413.9 ± 35.9 ($n = 5$) ms respectively. The presence of the δ subunit appears to produce slow GABA desensitization as previously reported for $\alpha_6\beta_2\delta$ (Haas & MacDonald, 1999). We were interested in whether this was also the case for the other agonists, particularly THIP, which produced responses larger than GABA. Interestingly, a maximally effective concentration of P4S produced less desensitization

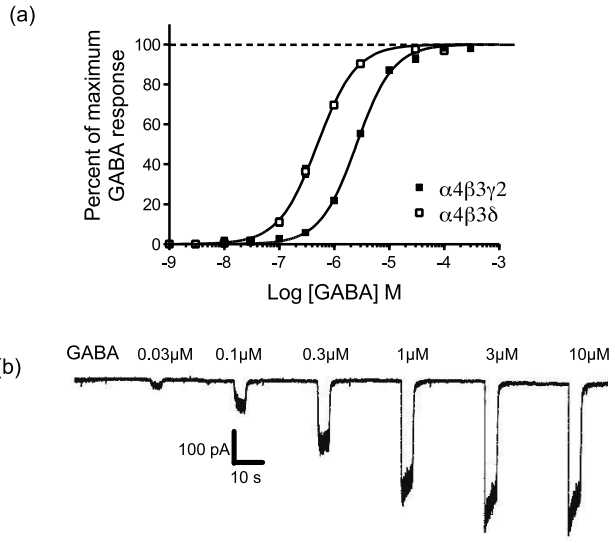

Figure 1 Comparison of GABA-gated currents on L(-tk) stable cell lines expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors. (a) Concentration–response curves for GABA on $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors. Data represent the mean ± s.e.mean of eight cells in each case. (b) An example recording of inward currents in response to increasing concentrations of GABA on $\alpha_4\beta_3\delta$ $GABA_A$ receptors.

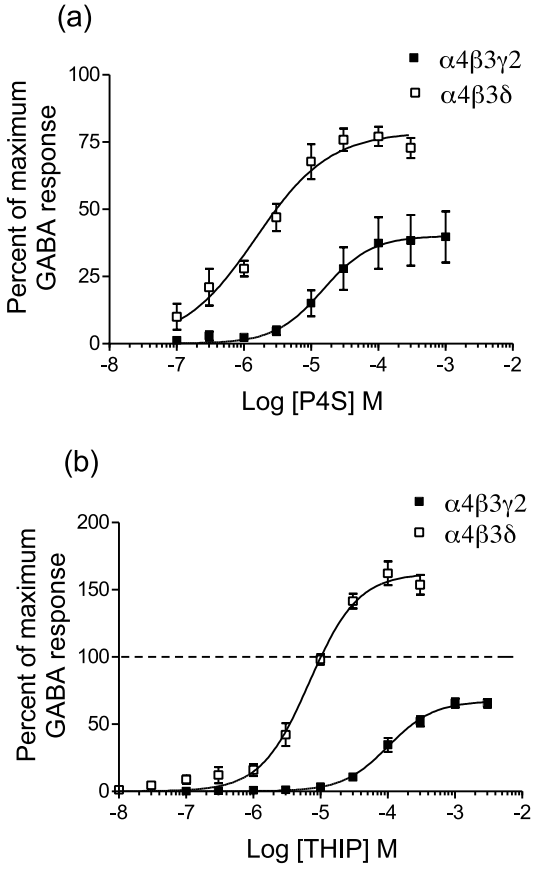

Figure 2 Concentration–response curves for the GABA agonists (a) P4S and (b) THIP on L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors. Dashed line represents maximal response to GABA. Data are normalized to the maximum response to GABA (100 μM) on each cell and represent the mean ± s.e.mean of six or more cells.

Table 1 EC_{50}, maximum efficacy relative to GABA, and Hill coefficient of GABA, THIP, and P4S in L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors

	Receptor	*EC_{50} (μM)*	*Maximum efficacy*	*Hill slope*	n
GABA	$\alpha_4\beta_3\gamma_2$	2.57 (2.51, 2.63)	100	1.3±0.07	8
	$\alpha_4\beta_3\delta$	0.49 (0.46, 0.53)	100	1.3±0.07	8
THIP	$\alpha_4\beta_3\gamma_2$	101.6 (85.8, 121.1)	66.8±3.0	1.4±0.03	6
	$\alpha_4\beta_3\delta$	6.3 (5.4, 7.3)	162.5±7.4	1.4±0.27	7
P4S	$\alpha_4\beta_3\gamma_2$	15.6 (14.0, 17.4)	39.5±9.7	1.3±0.06	6
	$\alpha_4\beta_3\delta$	0.9 (0.6, 1.4)	73.2±3.5	1.0±0.14	6

Values were calculated for each individual cell and represent the arithmetic mean±s.e.mean or geometric mean (−s.e.mean+s.e.mean) from a number (n) of different cells.

than GABA on $\alpha_4\beta_3\gamma_2$ receptors, but despite having greater efficacy on $\alpha_4\beta_3\delta$ receptors, the desensitization rate was not significantly different between the two subtypes, with a τ of 7.2±0.6 s (n=11) compared to 5.8±0.3 s (n=14) on $\alpha_4\beta_3\gamma_2$ (Figure 3b). THIP showed the least difference in desensitization rate with a τ value of 4.4±0.2 s (n=10) on $\alpha_4\beta_3\gamma_2$ (1 mM) compared to 4.8±0.3 s (n=10) on $\alpha_4\beta_3\delta$ (100 μM) (Figure 3c).

Finally, to assess the rectifying properties of the delta subunit, current/voltage relationships were determined on $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, by applying a current ramp from −70 to +60 mV. $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors both demonstrated relatively linear I/V relationships (Figure 4).

$GABA_A$ antagonists and allosteric inhibitors

The inhibitory effects of the GABA antagonists SR-95531 and picrotoxin were studied at an EC_{50} GABA concentration. The competitive antagonist SR-95531 showed similar potency on both cell types with IC_{50}'s of 196 (167, 231) nM and 224 (203, 246) nM on $\alpha_4\beta_3\gamma_2$ receptors and $\alpha_4\beta_3\delta$ receptors respectively. Similarly, the non-competitive antagonist picrotoxin showed no difference between the two receptor types with IC_{50}'s of 334 (300, 370) nM and 422 (375, 474) nM on $\alpha_4\beta_3\gamma_2$ receptors and $\alpha_4\beta_3\delta$ receptors respectively.

Inhibition of GABA induced currents by the cation zinc has been reported to vary, and depends particularly on the presence of a γ_2 subunit, which reduces sensitivity to zinc (Draguhn *et al*., 1990). The δ subunit has also been shown to reduce sensitivity to zinc when expressed with α_6 (Saxena & MacDonald, 1994). The effect of the δ subunit on zinc inhibition was investigated comparing concentration–response curves to zinc on an EC_{50} GABA response (Figure 5a). The IC_{50} for zinc on $\alpha_4\beta_3\delta$ was 2.9 (2.5, 3.4) μM compared to 2.0 (1.8, 2.2) μM at $\alpha_4\beta_3\gamma_2$ receptors, indicating that these receptor subtypes have similar sensitivity to zinc. Most $\alpha\beta$ combinations when expressed in the absence of a γ_2 exhibit IC_{50}'s close to 0.1 μM (Draguhn *et al*., 1990), however attempts to express $\alpha_4\beta_3$ in HEK cells for comparison, resulted in low expression levels, with current amplitudes too small to obtain meaningful data.

Additionally, it has been reported that the δ subunit renders the receptor more sensitive to inhibition by the cation lanthanum (Saxena *et al*., 1997). Comparing the effects of lanthanum on the $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ subtypes revealed inhibition by this cation on both receptors, the $\alpha_4\beta_3\delta$ receptors being inhibited to a greater maximal extent than the $\alpha_4\beta_3\gamma_2$ receptors (Figure 5b) with an IC_{50} of 11.8 (9.6, 14.9) μM at $\alpha_4\beta_3\delta$ and a maximal inhibition of 34% at 1 mM at $\alpha_4\beta_3\gamma_2$ receptors.

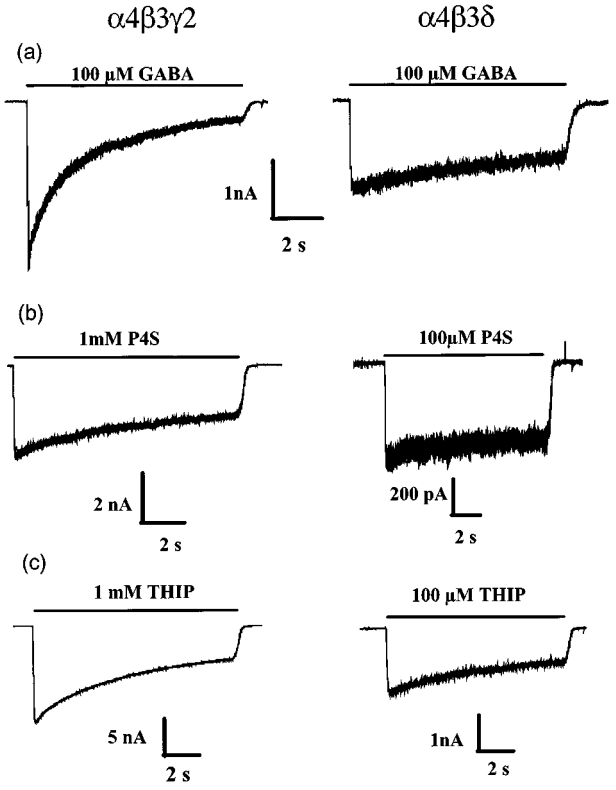

Figure 3 Effect of the δ subunit on receptor kinetics. Individual recordings showing the desensitization following a 10 s application of a maximally effective concentration of (a) GABA (100 μM) (b) P4S (100 μM on $\alpha_4\beta_3\delta$ and 1 mM on $\alpha_4\beta_3\gamma_2$) and (c) THIP, (100 μM on $\alpha_4\beta_3\delta$ and 1 mM on $\alpha_4\beta_3\gamma_2$). Data are all from individual cells and current amplitudes are indicated by the scale bars.

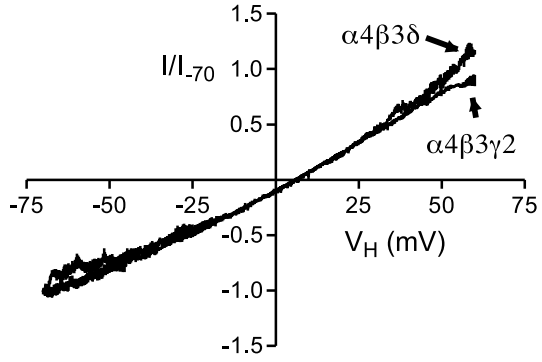

Figure 4 Current-voltage relationship to GABA for $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$. Currents were evoked by ramping the cell holding potential from −70 to 60 mV using a 100 ms pulse in the presence and absence of 100 μM GABA. The difference current, obtained by subtracting control from current in the presence of GABA was normalized to the current at −70 mV, and the data from five cells averaged to produce the mean current-voltage response for each receptor. Data shown are mean and s.e.mean for each receptor subtype.

As changes in extracellular pH can regulate $GABA_A$ receptor function, dependent upon subunit composition, the effect of H^+ ions was also investigated on $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors. Constant responses to an EC_{50} GABA concentration (5 s application) were applied and compared over a pH range of 4.4–10.4. The data was expressed relative to the current at pH 7.4 and inhibition of GABA currents was observed for both acid and alkali pH conditions with little difference between the two receptor subtypes (Figure 6).

Effects of benzodiazepines and anaesthetics

Receptors containing either α_4 or α_6 have a unique pharmacology in response to benzodiazepines, however, the γ subunit appears to be required to form the benzodiazepine binding site (Yang *et al*., 1995; Saxena & MacDonald, 1996; Sur *et al*., 1999). We looked to see if any of the benzodiazepine ligands active at $\alpha_4\beta_3\gamma_2$ showed any effect on $\alpha_4\beta_3\delta$. Full concentration–response curves for flunitrazepam (Figure 7a) showed no modulation of an EC_{20} GABA response up to 3 μM for either receptor subtype with values of $-3.1\pm5.7\%$ and $0.75\pm2.8\%$ at $\alpha_4\beta_3\gamma_2$ receptors and $\alpha_4\beta_3\delta$ receptors respectively, at a concentration of 3 μM. Ro15-4513 and bretazenil have previously been shown to have efficacy at α_4 and α_6 containing receptors (Wafford *et al*., 1996, Knoflach *et al*., 1996) and a concentration-response curve to Ro15-4513 showed a maximum of $39.5\pm3.1\%$ modulation with EC_{50} 61 (54, 68) nM on $\alpha_4\beta_3\gamma_2$ receptors, whereas $\alpha_4\beta_3\delta$ receptors were unaffected ($-3.3\pm1.9\%$ at 3 μM) (Figure 7b). Likewise, for bretazenil (Figure 7c) no effect was seen on $\alpha_4\beta_3\delta$ receptors, but a maximum of $76.3\pm4.6\%$ potentiation was recorded for $\alpha_4\beta_3\gamma_2$ receptors. Lastly, for the β-carboline inverse agonist DMCM (methyl-6,7,-dimethoxy-4-ethyl-beta-carboline-3-carboxylate), no modulation of an EC_{20} GABA response was observed at $\alpha_4\beta_3\delta$ receptors, however, for $\alpha_4\beta_3\gamma_2$ receptors a maximum inhibition of $-37.9\pm3.4\%$ modulation was noted (Figure 7d). Thus, the δ subunit appears not to confer sensitivity to benzodiazepine ligands, even with those compounds that are active at $\alpha_4\beta_3\gamma_2$ receptors.

The effect of a number of anaesthetic agents on the properties of $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ $GABA_A$ receptors was explored, and as with the benzodiazepines, allosteric modulators were studied using an EC_{20} concentration of GABA. Concentration–response curves to modulation by the barbiturate pentobarbitone (Figure 8a) demonstrated a maximum of $260\pm63\%$ potentiation with an EC_{50} of 23 (20, 26) μM for $\alpha_4\beta_3\gamma_2$ receptors and $390\pm41\%$ modulation with EC_{50} 29 (27, 32) μM for $\alpha_4\beta_3\delta$ receptors. While slightly more potentiation was observed on $\alpha_4\beta_3\delta$ this did not reach statistical significance. Likewise propofol (Figure 8b) elicited $326\pm46\%$ potentiation with an EC_{50} of 3.3 (3.1, 3.6) μM on $\alpha_4\beta_3\gamma_2$ receptors compared to $458\pm98\%$ maximum modulation with an EC_{50} of 4.5 (3.4, 5.8) μM for $\alpha_4\beta_3\delta$ receptors. At 30 μM there was a significantly greater efficacy on $\alpha_4\beta_3\delta$ receptors ($P\leqslant0.05$), however at this concentration propofol produced a direct effect of $28.4\pm2.8\%$ when expressed as a percentage of the maximum GABA response on $\alpha_4\beta_3\delta$ cells, but only $3.3\pm1.0\%$ on $\alpha_4\beta_3\gamma_2$ cells, which may account for this difference (data not shown). The anaesthetic etomidate elicited $532\pm118\%$ potentiation with an EC_{50} of 1.45 (1.08, 1.97) μM on $\alpha_4\beta_3\delta$ receptors, but only $58\pm9\%$ potentiation was seen at 100 μM for $\alpha_4\beta_3\gamma_2$ receptors (Figure 8c). The presence of the δ subunit appeared to increase the efficacy of some anaesthetic modulators, particularly that of etomidate.

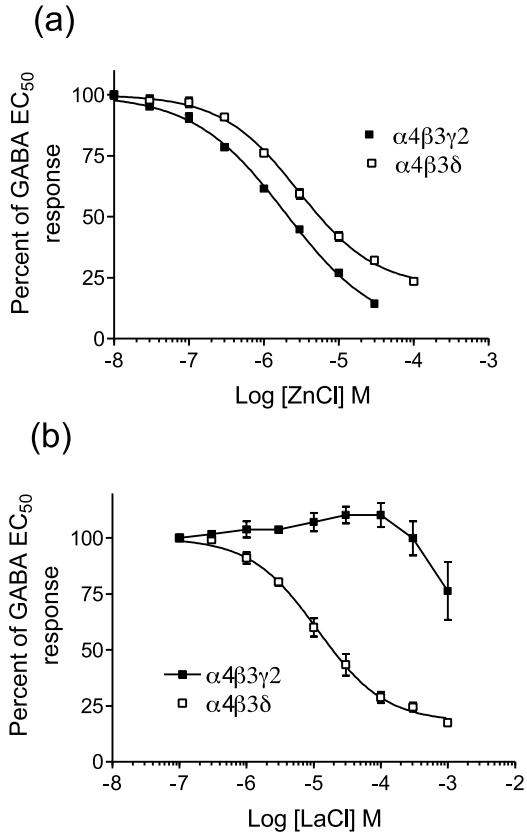

Figure 5 Effect of the δ subunit on sensitivity to inorganic cations. Inhibition of a GABA EC_{50} current from L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors by increasing concentrations of (a) zinc chloride and (b) lanthanum chloride. Data represent the mean±s.e.mean of at least five cells.

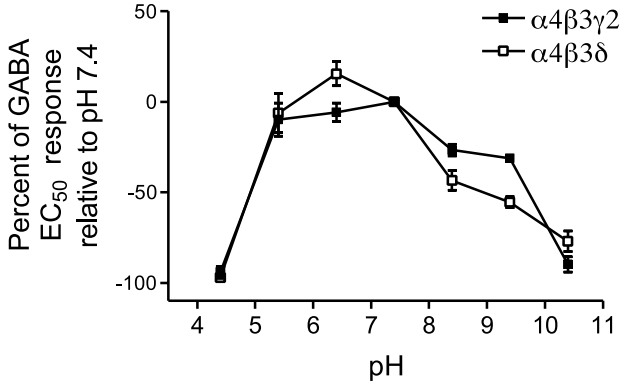

Figure 6 The effect of pH on $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors. EC_{50} GABA currents elicited at different pH's were normalized to the EC_{50} current at pH 7.4. Cells were voltage-clamped at -60 mV, and data set represents mean±s.e.mean of six cells.

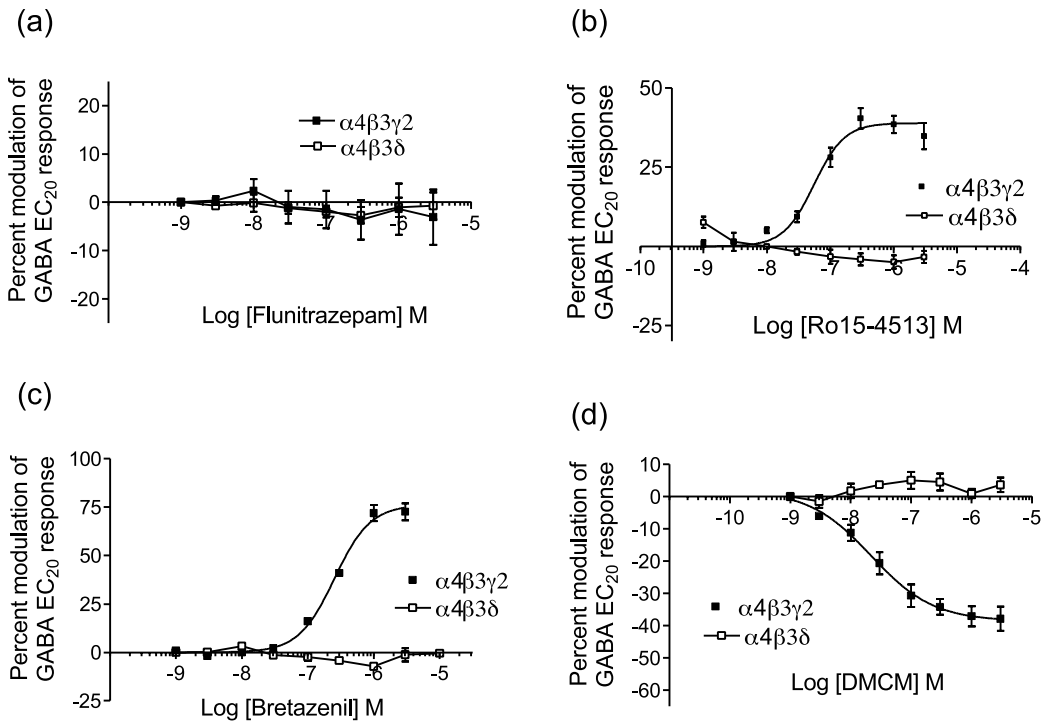

Figure 7 Effect of the δ subunit on benzodiazepine sensitivity. Modulation of control GABA EC_{20} responses in L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors by the benzodiazepines (a) flunitrazepam (b) Ro15-4513 (c) bretazenil and (d) DMCM. The data shown are the mean $\pm$ s.e.mean of at least four cells.

Effects of neuroactive steroids

Several steroids, including the endogenous metabolite of progesterone, 5α-pregnan-3α-ol-20-one, have been shown to potentiate the function of $GABA_A$ receptors (Callachan *et al*., 1987). A previous study has demonstrated a reduction in neurosteroid potentiation on $\alpha_6\beta_3\delta$ receptors (Zhu *et al*., 1996). A number of different neuroactive steroids were tested to establish the effect of the δ subunit on neurosteroid modulation of a submaximal GABA response (Figure 9). The steroids 5α-pregnan-3α-ol-20-one, alphaxalone and THDOC (all positive modulators of GABA) were studied on the two cell lines. Similar EC_{50}'s were observed for 5α-pregnan-3α-ol-20-one on $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors (12 (10, 14) nM and 48 (31, 75) nM respectively), however, much greater potentiation was observed on the $\alpha_4\beta_3\delta$ cell line with a maximum of $61 \pm 4\%$ potentiation on $\alpha_4\beta_3\gamma_2$ compared to $313 \pm 37\%$ potentiation on $\alpha_4\beta_3\delta$ (Figure 9a). Similarly, alphaxalone (5α-pregnan-3α-ol-11,20-di-one) on $\alpha_4\beta_3\gamma_2$ receptors showed $128 \pm 23\%$ modulation with an EC_{50} of 145 (122, 171) nM compared to $372 \pm 43\%$ modulation with an EC_{50} 341 (276, 417) nM for $\alpha_4\beta_3\delta$ (Figure 9b). Likewise, THDOC (5α-pregnane-3α,21-diol-20-one) which had the greatest efficacy of all, showed a marked increase in efficacy with inclusion of the δ subunit, eliciting $298 \pm 50\%$ modulation and an EC_{50} of 121 (106, 139) nM on $\alpha_4\beta_3\gamma_2$ receptors but an increased $469 \pm 28\%$ modulation and EC_{50} of 186 (169, 206) nM on $\alpha_4\beta_3\delta$ receptors (Figure 9c). Like other modulators THDOC produced apparent inhibition of responses at high concentrations, this effect has been reported previously for compounds such as pentobarbitone and could be possibly be accounted for by direct receptor inhibition through channel block (Krampfl *et al*., 2002). Pregnenolone sulphate (5α-pregnen-3β-ol-20-one sulphate) has been shown to exert an opposite effect on $GABA_A$ receptors, inhibiting the currents produced by GABA (Majewska *et al*., 1988). The effects of this inhibitory steroid were studied by performing complete concentration-response curves at an EC_{50} concentration of GABA. Both receptor subtypes were fully inhibited by pregnenolone sulphate, with IC_{50}'s of 1.2 (1.1, 1.3) μM at $\alpha_4\beta_3\delta$ and 0.50 (0.48,0.53) μM at $\alpha_4\beta_3\gamma_2$ (Figure 9d), being significantly more potent at $\alpha_4\beta_3\gamma_2$ ($P \leqslant 0.0001$). These results demonstrate that $\alpha_4\beta_3\delta$ receptors show a marked increase in the maximum potentiation by neurosteroids relative to $\alpha_4\beta_3\gamma_2$, and are both inhibited by pregnenolone sulphate.

Discussion

This study describes the first detailed characterization of the human $GABA_A$ $\alpha_4\beta_3\delta$ receptor subtype, with a direct comparison to the $\alpha_4\beta_3\gamma_2$ subtype, using two stably expressing cell lines. In order to show that the δ-subunit was incorporated into the receptors, a novel system of receptor expression was utilized whereby the c-*myc*-tagged δ subunit was constitutively expressed, but the α_4 and β_3 were under the control of a dexamethasone inducible promoter. The c-*myc*-tag could only be detected on the cell surface following induced expression of the α_4 and β_3 subunits, demonstrating that these proteins are required to incorporate the δ subunit into a functional receptor.

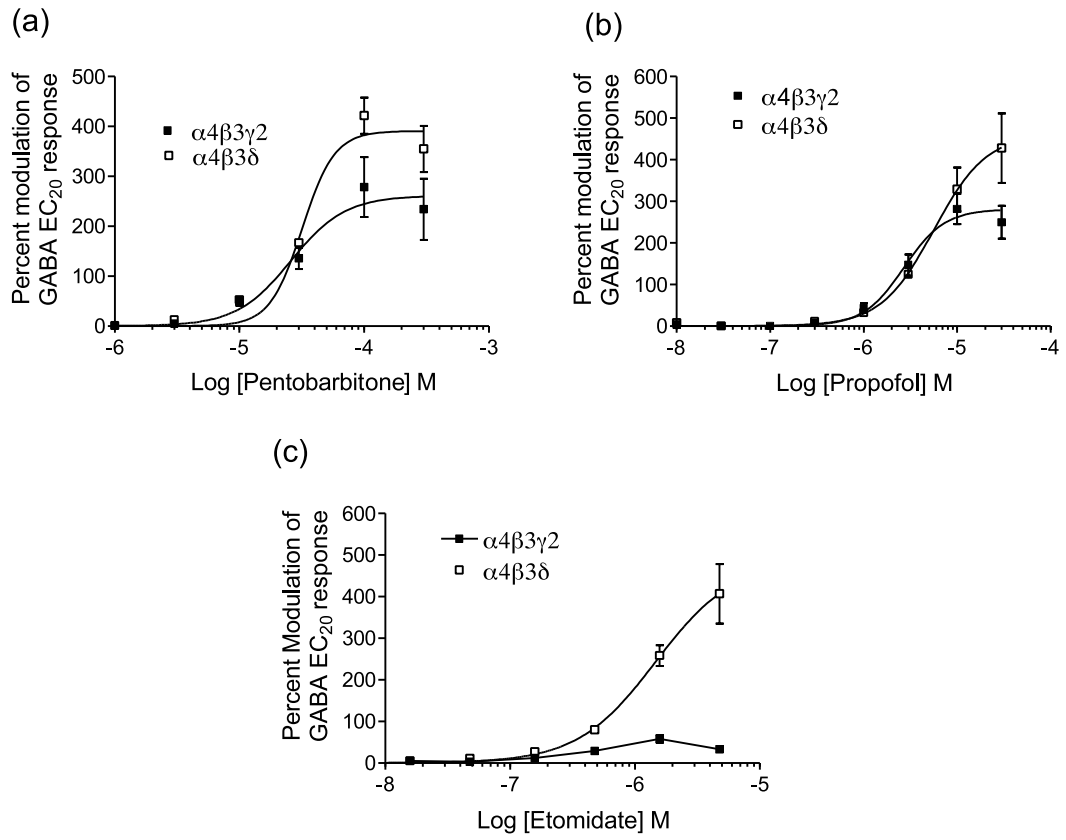

Figure 8 Effect of the δ subunit on anaesthetic sensitivity. Potentiation of control GABA EC_{20} responses in L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors by (a) pentobarbitone (b) propofol and (c) etomidate. Data shown are the mean $\pm$ s.e.mean of at least five cells.

Previous reports expressing δ with α_6 have demonstrated a high affinity for GABA (Saxena & MacDonald, 1996) and this was also observed for the $\alpha_4\beta_3\delta$ receptor with a 5 fold increase in GABA EC_{50} over $\alpha_4\beta_3\gamma_2$ receptors. This was also paralleled with the other GABA agonists studied, with the largest shift observed for THIP, which showed a 17 fold increase in EC_{50} for $\alpha_4\beta_3\delta$ *versus* $\alpha_4\beta_3\gamma_2$. The relatively low current amplitude reported here is also similar to that reported for $\alpha_6\beta_3\delta$ receptors (Saxena & MacDonald, 1996) and a subsequent study has demonstrated that δ-containing receptors exhibit a similar single-channel conductance to $\alpha\beta\gamma_2$ but different gating kinetics, resulting in the lower whole cell currents measured (Fisher & MacDonald, 1997). These properties combined with the high GABA affinity and localization in granule cells suggest that $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ receptors may have similar functional roles. A large difference in GABA induced desensitization was also observed between the two subtypes with $\alpha_4\beta_3\delta$ showing a markedly slower desensitization rate than the equivalent γ_2 containing receptor. This agrees with the previously observed effects of δ when expressed with $\alpha_1\beta_2$ (Haas & MacDonald, 1999). Interestingly the difference in desensitization was less marked with other agonists. Responses to both P4S and THIP exhibited less desensitization on $\alpha_4\beta_3\gamma_2$ and this was not significantly reduced on $\alpha_4\beta_3\delta$ receptors. While the low efficacy of the partial agonist P4S may explain the reduced desensitization of this compound relative to GABA, THIP was unusual in that it elicited a response amplitude larger than GABA on $\alpha_4\beta_3\delta$ receptors, this is the first report of an agonist with greater efficacy than GABA, raising the possibility that GABA behaves as a partial agonist at this subtype. The extent of desensitization observed with THIP, suggests that the desensitization rate is not necessarily related to absolute efficacy but governed more by the nature of the agonist used. Indeed mutations at the AMPA receptor agonist binding site have been shown to have large effects on receptor desensitization (Stern-Bach *et al*., 1998). In addition to THIP, allosteric modulators are able to potentiate GABA responses to 2–3 times the maximum current achievable with GABA, which is most unusual and again suggests that the maximum response induced by GABA alone is relatively low. It would appear that while the α and β subunits are critical for forming the GABA-gated ion channel, the nature of the third GABA subunit (γ, δ or ε), plays a large role in GABA-induced desensitization. In addition to these and previous studies with δ, the ε-subunit has also been reported to confer increased receptor desensitization (Whiting *et al*., 1997). The linear nature of the current-voltage relationship observed here is similar to that reported in rat dentate gyrus granule cells an area which is rich in $\alpha_4\beta\delta$ containing GABA$_A$ receptors (Kapur *et al*., 1999).

The effects of a competitive antagonist, SR-95531 and the non-competitive antagonist picrotoxin appeared to be independent of the nature of the δ or γ_2 subunit. Interestingly, inhibition by zinc was also not significantly different between the two subtypes. Previous reports have shown δ-containing receptors to be slightly more sensitive to zinc than γ_2 containing receptors (Saxena & MacDonald, 1996), however, the nature of the α subunit is also a determinant of zinc modulation, confounding a direct

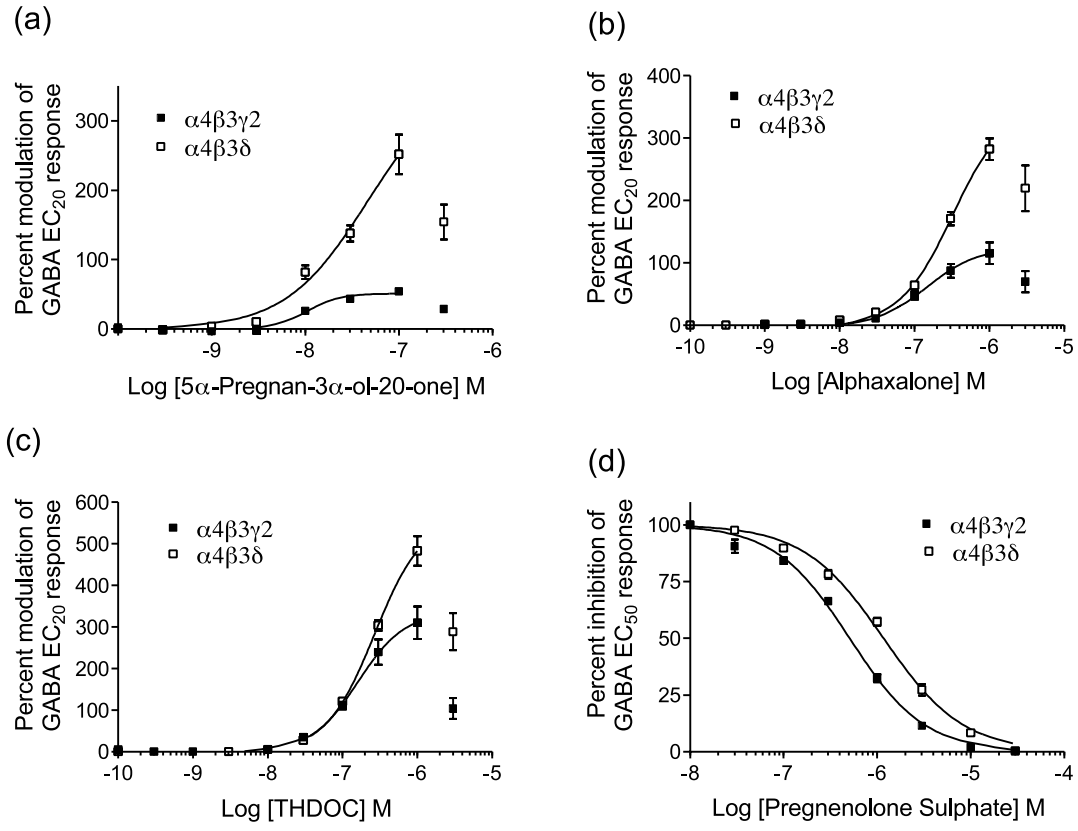

Figure 9 Effect of the δ subunit on neurosteroid sensitivity. The modulatory effects of the neurosteroids on GABA currents in L(-tk) cells expressing $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ receptors (a) 5α-pregnan-3α-ol-20-one (b) alphaxalone (c) THDOC and (d) pregnenolone sulphate. The data reported represent the mean ± s.e.mean of at least six cells.

comparison of these data. Draguhn *et al*. (1990) demonstrated that $\alpha\beta$ combinations exhibited very high sensitivity to zinc in the order of 100 nM, however, attempts to transiently express $\alpha_4\beta_3$ for direct comparison resulted in GABA-mediated currents of insufficient amplitude for the evaluation of zinc. The selective inhibition by lanthanum of $\alpha_4\beta_3\delta$ receptors is very similar to that observed for $\alpha_6\beta_3\delta$ and $\alpha_6\beta_3\gamma_2$ suggesting that the δ subunit is a major determinant in the lanthanum sensitivity of these receptor subtypes (Saxena *et al*., 1997). Both subtypes were inhibited by acid and alkaline conditions. A previous report demonstrated that $\alpha_1\beta_1\delta$ receptors were selectively potentiated under acid conditions whereas $\alpha_1\beta_1\gamma_2\delta$ were inhibited by both acid and alkali (Krishek *et al*., 1996). This study however makes use of different α and β subunits that may account for the different results observed here.

The α_4 subunit has a modified benzodiazepine binding site due to the presence of an arginine residue at position 102 (Wieland *et al*., 1992). $\alpha_4\beta\gamma_2$ receptors expressed in oocytes show little sensitivity to classical agonists such as flunitrazepam, but maintain affinity for Ro15-4513 and bretazenil which potentiate submaximal GABA responses (Wafford *et al*., 1996; Knoflach *et al*., 1996). In this study we have shown that while $\alpha_4\beta_3\gamma_2$ receptors exhibit sensitivity to Ro15-4513, bretazenil and DMCM, $\alpha_4\beta_3\delta$ are not modulated by any of these compounds, confirming that unlike γ subunits, δ cannot confer benzodiazepine sensitivity.

An area of particular interest is that of the modulation of δ-containing receptors by neuroactive steroids. This site on $GABA_A$ receptors may well confer allosteric modulation *via* endogenously produced steroid metabolites, and has been linked to physiological changes during stress (Serra *et al*., 2000) and hormonal changes during the estrus cycle (Finn & Gee, 1994), as well as being the target for the anaesthetic steroid alphaxalone (Harrison & Simmonds, 1984). A study expressing δ with $\alpha_6\beta_3$ and $\alpha_1\beta_3$ in HEK cells concluded that receptors containing δ exhibited reduced allosteric potentiation by the neurosteroid THDOC (Zhu *et al*., 1996). Similarly, the inhibitory neurosteroid pregnenolone sulphate showed reduced inhibition of GABA currents. In this study we demonstrate that $\alpha_4\beta_3\delta$ receptors show enhanced potentiation by several allosteric neurosteroids including THDOC. Interestingly the EC_{50} for potentiation by neurosteroids of $\alpha_4\beta_3\delta$ receptors was similar to that on $\alpha_4\beta_3\gamma_2$, however, the maximum efficacy was much greater for THDOC, alphaxalone and 5α-pregnan-3α-ol-20-one. Pregnenolone sulphate completely inhibited both $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$ but was significantly weaker on $\alpha_4\beta_3\delta$ receptors. It is currently unknown what may account for these differences in modulation by neurosteroids. Steroids have also been reported to directly activate the receptor at high concentrations, however, this was not observed in this study. This correlates well with previous studies using $\alpha_4\beta\gamma_2$ receptors expressed in oocytes, where no direct effect of anaesthetics were observed on this receptor subtype (Wafford *et al*., 1996), suggesting that this property is conferred by the α_4 subunit. A δ knock-out mouse has also been generated which shows reduced sensitivity to neuroactive steroids (Mihalek *et al*.,

1999). This result would be consistent with the δ subunit conferring enhanced sensitivity to potentiation by steroids as reported here. Interestingly, the sensitivity of the knock-out mice to other anaesthetics such as pentobarbitone and propofol were unchanged. In this study we demonstrate similar levels of efficacy and potency for pentobarbitone on both $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ and slightly greater efficacy for propofol on $\alpha_4\beta_3\delta$ which is consistent with these findings. An additional finding in the paper was an increase in the decay time of mISPC's recorded in hippocampal slices, suggesting that the slowly desensitizing δ-containing receptors may contribute to the synaptic currents in hippocampus (Mihalek *et al*., 1999). While pentobarbitone, and propofol appear to show little differences between $\alpha_4\beta_3\gamma_2$ and $\alpha_4\beta_3\delta$, etomidate was similar to the steroids in potentiating $\alpha_4\beta_3\delta$ to a much greater extent. While the location of the binding sites for these compounds is unknown, etomidate appears to selectively effect $\beta_{2/3}$ containing receptors (Belelli *et al*., 1997) and this data further differentiates etomidate from other clinically used anaesthetics, suggesting some additional selectivity for δ. In the δ k.o. mouse, etomidate appeared to show a larger difference than pentobarbitone and propofol but this did not reach significance (Mihalek *et al*., 1999).

The α_4 and δ subunit exhibit marked co-localization in the thalamus and dentate gyrus of the hippocampus (Pirker *et al*., 2000) and have been shown by immunoprecipitation to coassociate in the thalamus, indeed $\alpha_4\beta\delta$ is the major α_4 containing receptor subtype (Sur *et al*., 1999). The other major δ-containing receptor is $\alpha_6\beta_\delta$ which is expressed in cerebellar granule cells and appears to form an extrasynaptic receptor which is tonically activated by low concentrations of GABA and modifies the general excitability of granule cells (Nusser *et al*., 1998; Brickley *et al*., 1996; Wall & Usowicz, 1997). The properties of $\alpha_4\beta_3\delta$ suggest that this may be playing a similar role in the dentate gyrus of the hippocampus where the receptor is highly expressed on granule cells (Pirker *et al*., 2000). Recent evidence has demonstrated that this receptor subtype is upregulated on development of epilepsy (Brooks-Kayal *et al*., 1999) corresponding with a down-regulation of α_1, and the higher sensitivity to zinc has implicated the receptor subtype in the pathogenesis of temporal lobe epilepsy (Coulter, 2001). α_4-containing receptors have also been associated with neurosteroid withdrawal properties. Rapid fluctuations in circulating levels of the progesterone metabolite 3α-OH-pregnan-5α-ol-20-one can be observed during menstrual and pregnancy cycles and have been shown to result in upregulation of the α_4 subunit and corresponding changes in the properties of hippocampal $GABA_A$ receptors (Smith *et al*., 1998). The high efficacy of these steroids at $\alpha_4\beta_3\delta$ may elicit a resting chronic stimulation of GABA activity which results in upregulation of receptor when this is disturbed. Clearly the physiological and pharmacological properties of $\alpha_4\beta_3\delta$ $GABA_A$ receptors make this a unique receptor in the brain, and evidence suggests that this subtype may be primarily extrasynaptic, playing an important role in the control of neuronal excitability in the thalamus and hippocampus. The plastic nature of this subtype also suggests possible involvement in a number of pathological conditions such as drug withdrawal and epilepsy, making it an interesting and attractive target for novel therapeutic agents.

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