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Modulation by general anaesthetics of rat $GABA_A$ receptors comprised of $\alpha1\beta3$ and $\beta3$ subunits expressed in human embryonic kidney 293 cells

Paul A. Davies, *Ewen F. Kirkness & [1]Tim G. Hales

Department of Anesthesiology, UCLA Medical Center, LA. CA. 90095 and *The Institute for Genomic Research, 9712 Medical Center Drive. Rockville, MD. 20850, U.S.A.

1 Radioligand binding and patch-clamp techniques were used to study the actions of γ-aminobutyric acid (GABA) and the general anaesthetics propofol (2,6-diisopropylphenol), pentobarbitone and 5α-pregnan-3α-ol-20-one on rat $\alpha1$ and $\beta3$ $GABA_A$ receptor subunits, expressed either alone or in combination.

2 Membranes from HEK293 cells after transfection with $\alpha1$ cDNA did not bind significant levels of [^{35}S]-tert-butyl bicyclophosphorothionate ([^{35}S]-TBPS) (<0.03 pmol mg^{-1} protein). GABA (100 μM) applied to whole-cells transfected with $\alpha1$ cDNA and clamped at -60 mV, also failed to activate discernible currents.

3 The membranes of cells expressing $\beta3$ cDNAs bound [^{35}S]-TBPS (~1 pmol mg^{-1} protein). However, the binding was not influenced by GABA (10 nM–100 μM). Neither GABA (100 μM) nor picrotoxin (10 μM) affected currents recorded from cells expressing $\beta3$ cDNA, suggesting that $\beta3$ subunits do not form functional $GABA_A$ receptors or spontaneously active ion channels.

4 GABA (10 nM–100 μM) modulated [^{35}S]-TBPS binding to the membranes of cells transfected with both $\alpha1$ and $\beta3$ cDNAs. GABA (0.1 μM–1 mM) also dose-dependently activated inward currents with an EC_{50} of 9 μM recorded from cells transfected with $\alpha1$ and $\beta3$ cDNAs, clamped at -60 mV.

5 Propofol (10 nM–100 μM), pentobarbitone (10 nM–100 μM) and 5α-pregnan-3α-ol-20-one (1 nM–30 μM) modulated [^{35}S]-TBPS binding to the membranes of cells expressing either $\alpha1\beta3$ or $\beta3$ receptors. Propofol (100 μM), pentobarbitone (1 mM) and 5α-pregnan-3α-ol-20-one (10 μM) also activated currents recorded from cells expressing $\alpha1\beta3$ receptors.

6 Propofol (1 μM–1 mM) and pentobarbitone (1 mM) both activated currents recorded from cells expressing $\beta3$ homomers. In contrast, application of 5α-pregnan-3α-ol-20-one (10 μM) failed to activate detectable currents.

7 Propofol (100 μM)-activated currents recorded from cells expressing either $\alpha1\beta3$ or $\beta3$ receptors reversed at the Cl^- equilibrium potential and were inhibited to $34\pm13\%$ and $39\pm10\%$ of control, respectively, by picrotoxin (10 μM). 5α-Pregnan-3α-ol-20-one (100 nM) enhanced propofol (100 μM)-evoked currents mediated by $\alpha1\beta3$ receptors to $1101\pm299\%$ of control. In contrast, even at high concentration 5α-pregnan-3α-ol-20-one (10 μM) caused only a modest facilitation (to $128\pm12\%$ of control) of propofol (100 μM)-evoked currents mediated by $\beta3$ homomers.

8 Propofol (3–100 μM) activated $\alpha1\beta3$ and $\beta3$ receptors in a concentration-dependent manner. For both receptor combinations, higher concentrations of propofol (300 μM and 1 mM) caused a decline in current amplitude. This inhibition of receptor function reversed rapidly during washout resulting in a 'surge' current on cessation of propofol (300 μM and 1 mM) application. Surge currents were also evident following pentobarbitone (1 mM) application to cells expressing either receptor combination. By contrast, this phenomenon was not apparent following applications of 5α-pregnan-3α-ol-20-one (10 μM) to cells expressing $\alpha1\beta3$ receptors.

9 These observations demonstrate that rat $\beta3$ subunits form homomeric receptors that are not spontaneously active, are insensitive to GABA and can be activated by some general anaesthetics. Taken together, these data also suggest similar sites on $GABA_A$ receptors for propofol and barbiturates, and a separate site for the anaesthetic steroids.

Keywords: $GABA_A$ receptors; propofol; pentobarbitone; neurosteroids; general anaesthetic; patch-clamp; radioligand binding

Introduction

At clinically relevant concentrations most intravenous general anaesthetics (IVGAs) including propofol, etomidate, propanidid and the anaesthetic barbiturates and steriods modulate $GABA_A$ receptors (Franks & Lieb, 1994; Hales & Olsen, 1994). $GABA_A$ receptor modulation by propofol and pentobarbitone has three components: (1) at low concentrations both agents potentiate γ-aminobutyric acid (GABA)-evoked responses; (2) at higher concentrations, in the absence of GABA, they directly activate $GABA_A$ receptors; (3) at even higher concentrations propofol and pentobarbitone inhibit receptor function (Peters *et al*., 1989; Robertson, 1989; Hara *et al*., 1993; 1994; Orser *et al*., 1994; Adora & Hales, 1995). Anxiolytic and anticonvulsant benzodiazepines do not directly activate $GABA_A$ receptors but, like IVGAs, the benzodiazepines potentiate GABA-evoked respones (Hales & Olsen, 1994).

By analogy with the nicotinic acetylcholine receptor, the $GABA_A$ receptor is postulated to have a pentameric structure (Burt & Kamatchi, 1991). To date, 17 subunit types have been cloned from mammalian sources (α1-6, β1-3, γ1, γ2S, γ2L, γ3, δ, ρ1,2 and 3), thus providing numerous possible combinations with the potential for different pharmacological profiles (Burt & Kamatchi, 1991; Ogurusu & Shingai, 1996). The benzodiazepines require the presence of α and γ subunits for their

[1]Author for correspondence at: Dept. Anesthesiology, UCLA Medical Center, 10833 Le Conte Ave, Los Angeles, California 90095-1778, U.S.A.

modulation of $GABA_A$ receptors (Pritchett *et al*., 1989). By contrast, modulation of $GABA_A$ receptors by IVGAs does not appear to show an 'all-or-none' dependence on specific subunits (Shingai *et al*., 1991; Horne *et al*., 1993; Jones *et al*., 1995). However, there is evidence for an influence of receptor subunit combination on the efficacy of IVGAs, relative to GABA, as $GABA_A$ receptor-activators (Jones *et al*., 1995; Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996).

$GABA_A$ receptor modulation can be investigated by use of radioligand binding and electrophysiological assays. However, comparisons between binding and electrophysiological data are complicated by the markedly different time courses of the two experimental approaches. IVGAs enhance [^{3}H]-muscimol binding to membranes containing $GABA_A$ receptors and this corresponds to their ability to enhance GABA-activated currents recorded from cells expressing receptors (Hales & Olsen, 1994; Peters *et al*., 1988; Turner *et al*., 1989). Additionally, the IVGAs also modulate the binding of the convulsant ligand [^{35}S]-TBPS to $GABA_A$ receptors (Concas *et al*., 1994; Hawkinson *et al*., 1994; Slany *et al*., 1995). This action may in part correspond to the ability of these agents to activate receptors directly and evoke Cl^- currents in the absence of GABA.

By transiently transfecting immortalized human embryonic kidney cells with $\alpha1$ and $\beta3$ cDNAs, either alone or in combination, we have investigated the ability of GABA, propofol, pentobarbitone and 5α-pregnan-3α-ol-20-one to activate recombinant $GABA_A$ receptors. Previous studies suggest that only certain homomeric GABA receptors form functional ion channels. These include the ρ (Shimada *et al*., 1992) and β subunits (Sigel *et al*., 1989; Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996). Most homomeric β receptors show spontaneous channel openings that are enhanced by some IVGAs (Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996). Whether these compounds directly activate homomeric β receptors or potentiate spontaneous channel activity remains undetermined. In addition, spontaneously active β homomers of some species are insensitive to GABA (Krishek *et al*., 1996; Cestari *et al*., 1996). It is unclear whether this lack of sensitivity is due to the absence of GABA recognition or to the receptor being already active. We investigated whether rat $\beta3$ subunits form homomeric receptors that bind [^{3}H]-muscimol and/or [^{35}S]-TBPS, and whether GABA and the IVGAs can modulate the binding of these compounds and/or activate Cl^- currents directly.

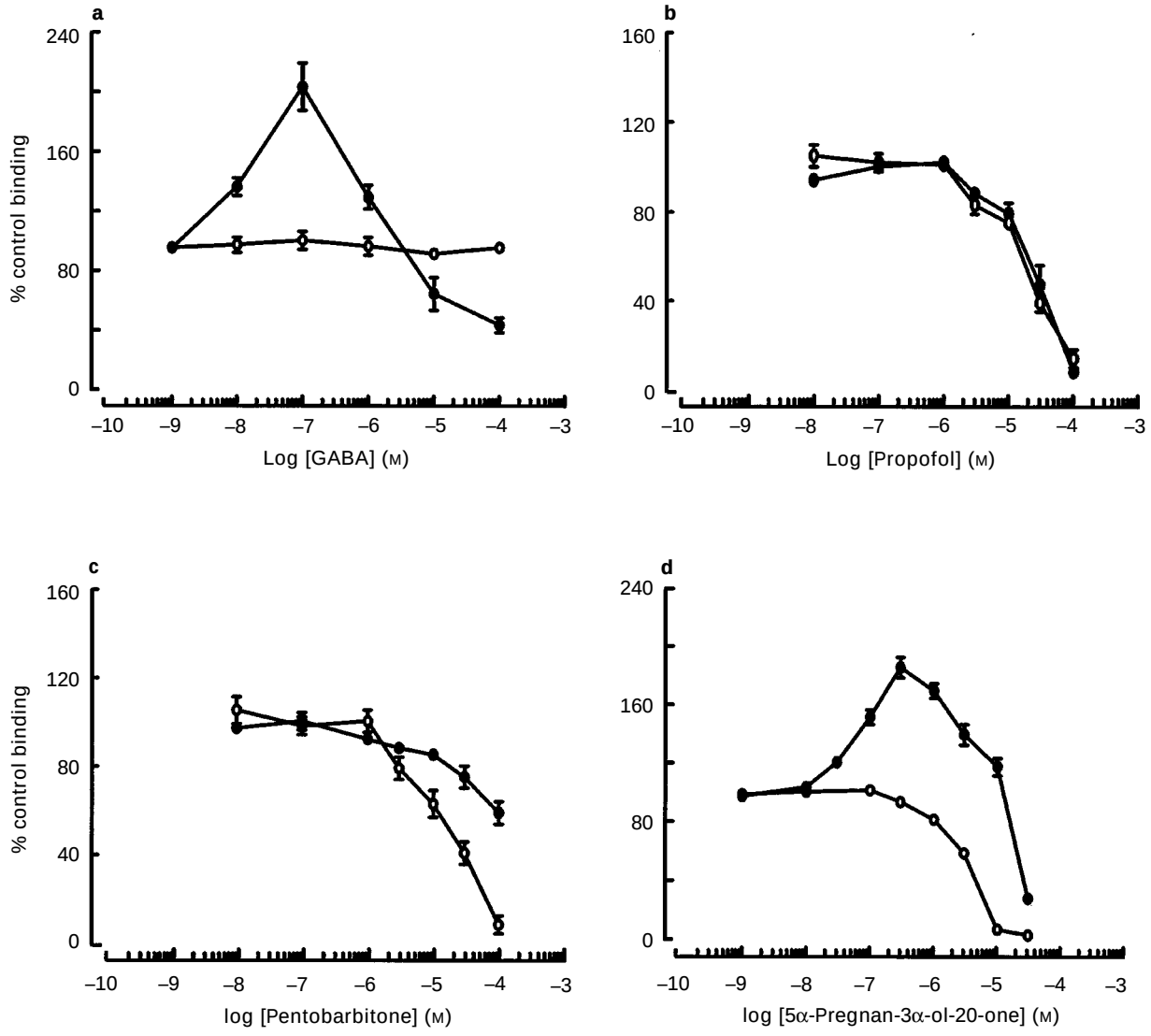

Figure 1 Modulation of [^{35}S]-TBPS binding to HEK293 cell membranes containing $\alpha1\beta3$ and $\beta3$ $GABA_A$ receptors. (a) GABA did not effect [^{35}S]-TBPS binding to membranes expressing $\beta3$ receptors. [^{35}S]-TBPS binding to $\alpha1\beta3$ containing membranes was enhanced and displaced by low and high doses of GABA, respectively. In all graphs (●) and (○) represent data points for $\alpha1\beta3$ and $\beta3$ receptors, respectively. (b) Propofol dose-dependently displaced [^{35}S]-TBPS bound to membranes containing either $\alpha1\beta3$ or $\beta3$ receptors. (c) Pentobarbitone also dose-dependently displaced [^{35}S]-TBPS from $\alpha1\beta3$ and $\beta3$ containing receptors. (d) The anaesthetic steroid 5α-pregnan-3α-ol-20-one had a biphasic effect on [^{35}S]-TBPS binding to membranes containing $\alpha1\beta3$ receptors, but only displaced binding from $\beta3$ expressing membranes. Points represent the mean of at least three independent experiments; vertical lines indicate s.d.

Methods

Cell cultures

Human embryonic kidney (HEK293) cells were maintained in growth medium comprised of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 iu ml^{-1} penicillin, and 50 $\mu g\ ml^{-1}$ streptomycin. Cultures were incubated in an atmosphere of 5% CO_2, 95% air, at 37°C and a relative humidity of 100%. Cells were harvested once each week by resuspending in a Ca^{2+}-, Mg^{2+}-, and bicarbonate-free phosphate buffered saline containing trypsin (500 $\mu g\ ml^{-1}$) and EDTA (200 $\mu g\ ml^{-1}$). After washing by centrifugation and resuspension in fresh growth medium, cells were seeded at 25–50% confluency. Transfections were performed 24 h after subculturing.

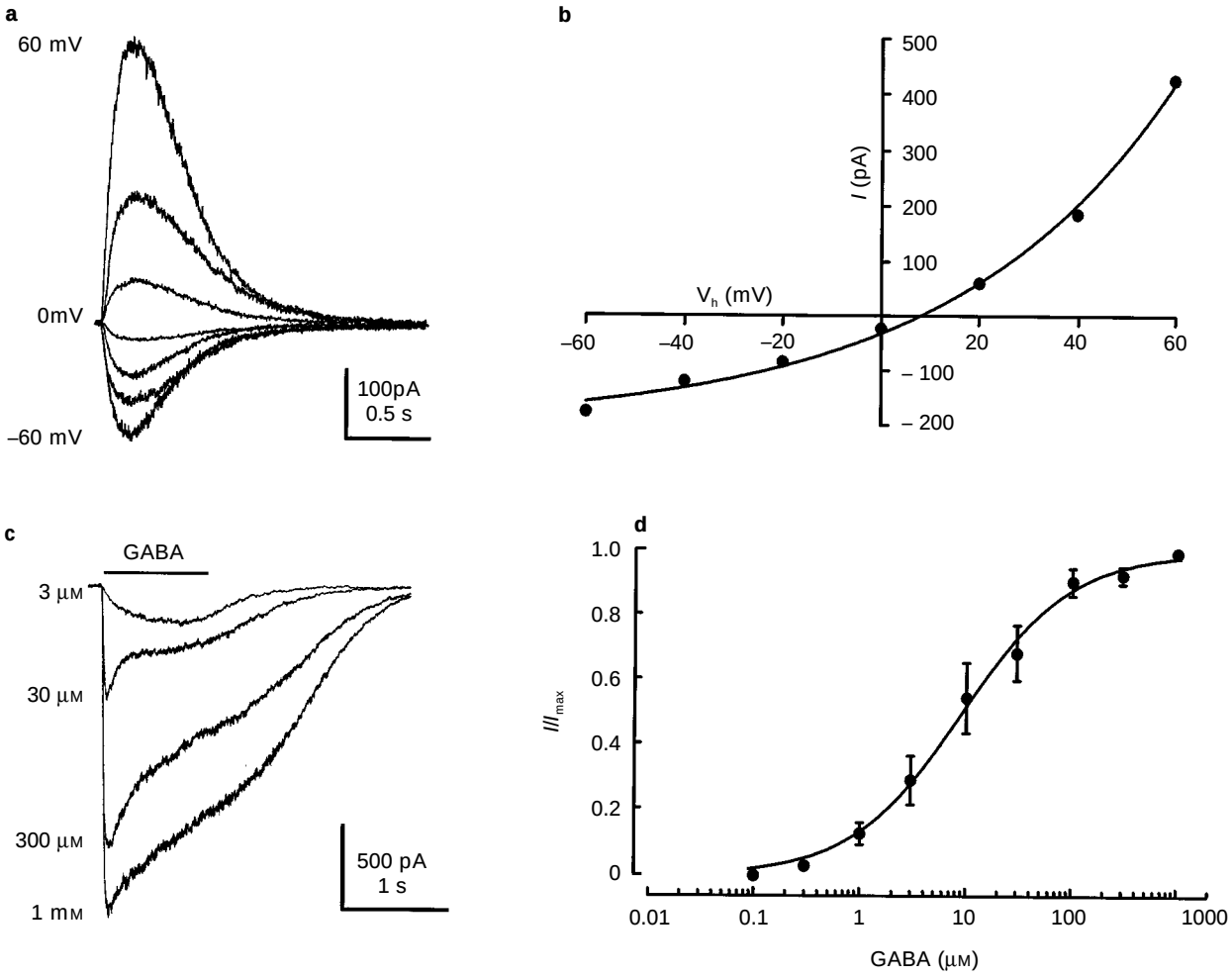

Figure 2 Activation of $\alpha1\beta3$ receptors by GABA. (a) GABA (100 μM)-activated currents recorded from a cell voltage-clamped between −60 and 60 mV transfected with $\alpha1$ and $\beta3$ cDNAs. Superimposed traces represent the mean of two currents recorded at each holding potential. (b) The plot of the current amplitude against holding potential (from the recordings illustrated in (a)) shows that GABA-evoked currents exhibit outward rectification and in this case reversed at approximately 7 mV. On average, GABA-evoked currents reversed at 0.5 ± 2.8 mV ($n=4$), close to the theoretical Cl^- equilibrium potential with the solutions described in the Methods section. The curve was fitted to the data points by an exponential function. (c) Currents activated by increasing concentrations of GABA recorded from an HEK293 cell expressing $\alpha1\beta3$ receptors. (d) A plot of the concentration-response relationship for GABA-activated currents. Data points represent the mean of at least four determinations and the vertical lines represent s.e.mean. From the logistic fit (Adora & Hales, 1995) to the data points, GABA activated currents with an EC_{50} of 9.1 ± 1.2 μM and a Hill slope of 0.84 ± 0.07.

Table 1 Activation of $\alpha1\beta3$ and $\beta3$ receptors by GABA and the general anaesthetics

	$\alpha1\beta3$		$\beta3$	
Agonist	*Fraction responding*	*Mean amplitude* (pA)	*Fraction responding*	*Mean amplitude* (pA)
GABA	54/90	741 ± 158 ($n=54$)	0/13	–
5α-Pregnan-3α-ol-20-one	16/29	117 ± 40.4 ($n=16$)	0/11	–
Propofol	49/104	514 ± 125 ($n=36$)	124/299	275 ± 48 ($n=54$)
Pentobarbitone	4/8	120 ± 61 ($n=4$)	7/16	52 ± 17 ($n=6$)

A summary of the compounds tested that do or do not activate $\alpha1\beta3$ and $\beta3$ receptors including mean current amplitudes. GABA (100 μM), 5α-pregnan-3α-ol-20-one (10 μM), propofol (100 μM) and pentobarbitone (1 mM) were pressure-applied to individual cells. Values represent the number of cells which responded out of the total number tested.

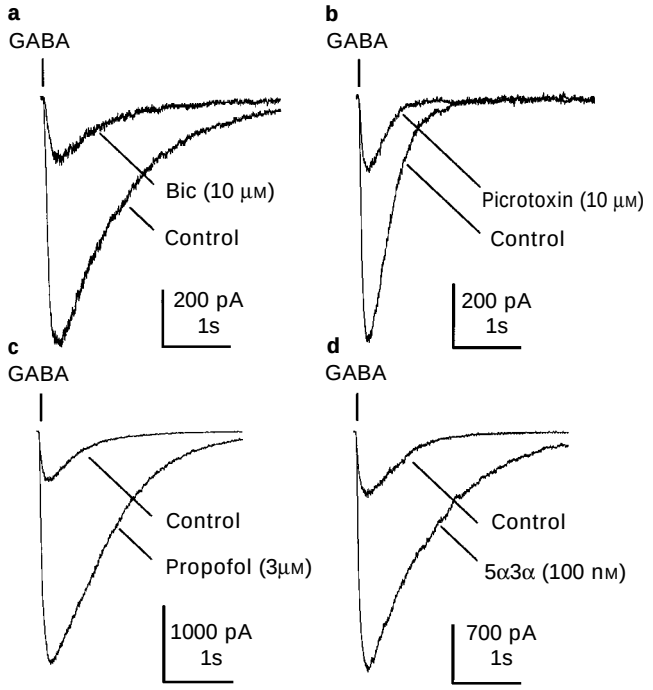

Figure 3 Pharmacology of GABA-evoked currents recorded from HEK293 cells expressing $\alpha1\beta3$ receptors. (a) The selective $GABA_A$ receptor antagonist bicuculline methiodide (Bic, 10 μM), when applied to the bath, inhibited GABA-activated currents. (b) Picrotoxin (10 μM), applied to the bath, also inhibited currents activated by pressure applied GABA (100 μM). (c) Bath application of propofol (3 μM) potentiated currents activated by brief pressure application of GABA (100 μM). (d) Bath application of 5α-pregnan-3α-ol-20-one also enhanced GABA-activated currents. Experiments were performed on separate cells voltage-clamped at -60 mV, superimposed traces represent averages of three individual currents in the presence and absence of drugs. All drug effects reversed during wash off.

Transfection

Cells were transfected with plasmids (pCDM8) containing cDNAs for rat α1 and β3 $GABA_A$ receptor subunits, either alone or in combination, by the calcium phosphate precipitation method. Complete open reading frames, encoding the rat $GABA_A$ receptor α1 (Khrestchatisky *et al*., 1989) and β3 (Lolait *et al*., 1989) subunits were subcloned into the pCDM8 vector (Invitrogen, San Diego, CA). Plasmid(s) containing cDNA for the $GABA_A$ receptor subunits (10 μg) were added to a solution contained double distilled H_2O, $CaCl_2$ (2.5 M) and HEPES buffer at a volumetric ration of 9:1:10. HEPES buffer contained (in mM): NaCl 280, Na_2HPO_4 1.5, HEPES 50 (pH 7.10). The solution formed a light precipitate (10 min, 22°C) and was then added to cultures (0.1 ml solution to 1 ml of growth medium). Cells were incubated (5% CO_2, 95% air, at 37°C) for 24 h, washed and incubated for a further 48–72h before experimentation.

[^{35}S]-TBPS binding experiments

Transfected cells were washed twice with phosphate-buffered saline and harvested by scraping into an ice-cold TEN solution comprised of (in mM): Tris-HCl 10, EDTA 1, NaCl 100 (pH 7.5). The cells were collected by centrifugation (5000 *g*, 10 min), hand-homogenized in TEN, and a crude membrane fraction was obtained by centrifugation (30,000 *g*, 30 min). The membrane pellet was washed twice with TEN and resuspended in TEN at a protein concentration of $\sim$3 mg ml^{-1}. Membranes were used for binding studies either immediately after preparation, or after storage at -20°C. A single freeze-thaw cycle had little effect on [^{35}S]-TBPS binding activity. The [^{35}S]-TBPS binding activity of membranes (50–100 ng protein) was assayed by incubation for 90 min at 25°C in 200 μl of a solution containing 5 nM [^{35}S]-TBPS, 20 mM Tris-HCl, 1 M NaCl (pH 7.5). Non-specific binding was determined in the presence of 100 μM picrotoxinin, and was equal to the binding of mock-transfected cell membranes ($<$0.03 pmol mg^{-1} protein). Following incubation, 5 ml of ice-cold buffer (20 mM Tris-HCl, 1 M NaCl; pH 7.5) was added, and the membranes filtered through Whatman GF/C filters. The filters were washed twice with 5 ml of the same buffer before scintillation counting. Binding data are expressed as the mean $\pm$ s.d.

Electrophysiology

The patch-clamp technique was used to record whole-cell currents from HEK293 cells voltage-clamped at -60 mV (unless otherwise stated). The recording chamber was perfused (5 ml min^{-1}) with an extracellular solution consisting of (in mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11 and HEPES 10 (pH 7.4). The electrode solution contained (in mM): KCl 140, $MgCl_2$ 2.0, EGTA 11 and HEPES 10 (pH 7.4). In several experiments examining β3 homomers Mg-ATP (3 mM) was included in the electrode solution in an attempt to minimize current run-down. Membrane currents were monitored by an Axopatch-200A (Axon Instruments Inc.) patch-clamp amplifier. Currents were low-pass filtered at a cutoff frequency of 2 KHz (Bessel characteristics), digitized with a digital audio processor (Sony PCM-501ES) and recorded onto VCR tapes for subsequent analysis. Currents were simultaneously recorded (Gould 2200) onto chart paper.

In experiments examining activation of $GABA_A$ receptors, GABA and anaesthetics were applied locally by pressure ejection (General Valve Picospritzer II) at a pressure of 70 kPa, from glass micropipettes positioned approximately 50 μm from the cell under investigation. To minimize $GABA_A$ receptor desensitization cells were continuously superfused with recording solution. In dose-response experiments randomized doses of GABA or propofol were applied via different pipettes with similar resistances (2.7 $\pm$ 0.2 MΩ) positioned, with the aid of an eye piece graticule, in the same location (Adodra & Hales, 1995). During concentration-response experiments agonists were applied for 1 s in order to achieve equilibrium concentrations. It is possible that dilution may occur at some receptor sites, therefore concentrations represent maximum estimates of true agonist concentrations. A period of at least 4 min wash was allowed between each application to prevent desensitization. All other drugs were bath applied, as were the anaesthetics in experiments investigating their GABA-potentiating effects. For modulation experiments, in which antagonists or anaesthetics were bath applied, GABA (100 μM) was applied briefly at a duration (5–30 ms) that activated $<$10% of the maximum GABA (100 μM)-evoked current amplitude.

Concentration-response relationships were fitted with the logistic equation as previously described (Adodra & Hales, 1995). Consistent with previous studies (Bormann *et al*., 1987; Peters *et al*., 1989; Robertson, 1989; Hales & Lambert, 1991; Adodra & Hales, 1995), $GABA_A$-receptor-mediated current-voltage relationships displayed outward rectification and were well fitted with an exponential function (Bormann *et al*., 1987). All electrophysiology data are expressed as the arithmetic mean $\pm$ s.e.mean.

Drugs and reagents used

The [^{35}S]-TBPS used in binding experiments had a specific activity of 115 Ci mmol^{-1} (DuPont-New England Nuclear Boston, MA). The anaesthetically active compounds used were 2,6-diisopropylphenol (propofol, from Aldrich, Milwaukee, WI), pentobarbitone and 5α-pregnan-3α-ol-20-one (both from Sigma, St. Louis, MO). The inactive progesterone metabolite

5α-pregnan-3β-ol-20-one (Sigma, St. Louis, MO) was used as a control in binding experiments. The GABA$_A$ receptor inhibitors, bicuculline methiodide and picrotoxin (Sigma, St. Louis, MO) were used to characterize GABA- and anaesthetic-activated currents. Picrotoxinin was used in binding assays. Stock solutions of propofol, the steroids, picrotoxin and picrotoxinin, in ethanol, were diluted to achieve an ethanol concentration of $<0.1\%$. This concentration has no significant effect on recombinant GABA$_A$ receptors lacking γ2L subunits (Wafford *et al*., 1991). Tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD) and all other reagents were from Sigma (St. Louis, MO).

Results

Modulation of [^{35}S]-TBPS binding to HEK293 membranes by general anaesthetics

Membranes that had been prepared from HEK293 cells following mock-transfection, transfection with the pCDM8 vector, or transfection with the α1 cDNA alone, did not bind significant levels of [^{35}S]-TBPS (<0.03 pmol mg^{-1} protein). In contrast, cells that had been transfected with the β3 subunit, either alone or in combination with the α1 subunit, bound [^{35}S]-TBPS at $\sim$1 pmol mg^{-1} protein. The binding of [^{35}S]-TBPS to $\alpha1\beta3$ receptors was modulated by GABA (Figure 1a). Low concentrations of GABA (10 nM – 1 μM) enhanced binding, whereas higher concentrations (10 – 100 μM) had an inhibitory effect. In contrast, the TBPS binding site of β3 receptors was insensitive to GABA. A lack of high-affinity GABA-binding sites on β3 receptors was confirmed by their inability to bind [^{3}H]-muscimol (1 – 500 nM; results not shown). In common with GABA, propofol, pentobarbitone and the anaesthetic steroid 5α-pregnan-3α-ol-20-one modulated [^{35}S]-TBPS binding to $\alpha1\beta3$ receptors (Figure 1). However, unlike GABA, the anaesthetics also displaced the ligand from membranes containing only β3 subunits. 5α-Pregnan-3β-ol-20-one (0.1 – 10 μM), a stereoisomer of 5α-pregnan-3α-ol-20-one which lacks anaesthetic activity, did not significantly affect ligand binding to either $\alpha1\beta3$ or β3 receptors (results not shown).

Activation of recombination GABA$_A$ receptors by GABA

Local application of GABA (100 μM) to HEK293 cells expressing $\alpha1\beta3$ GABA$_A$ receptors activated inward whole-cell currents recorded by the patch-clamp technique with a holding

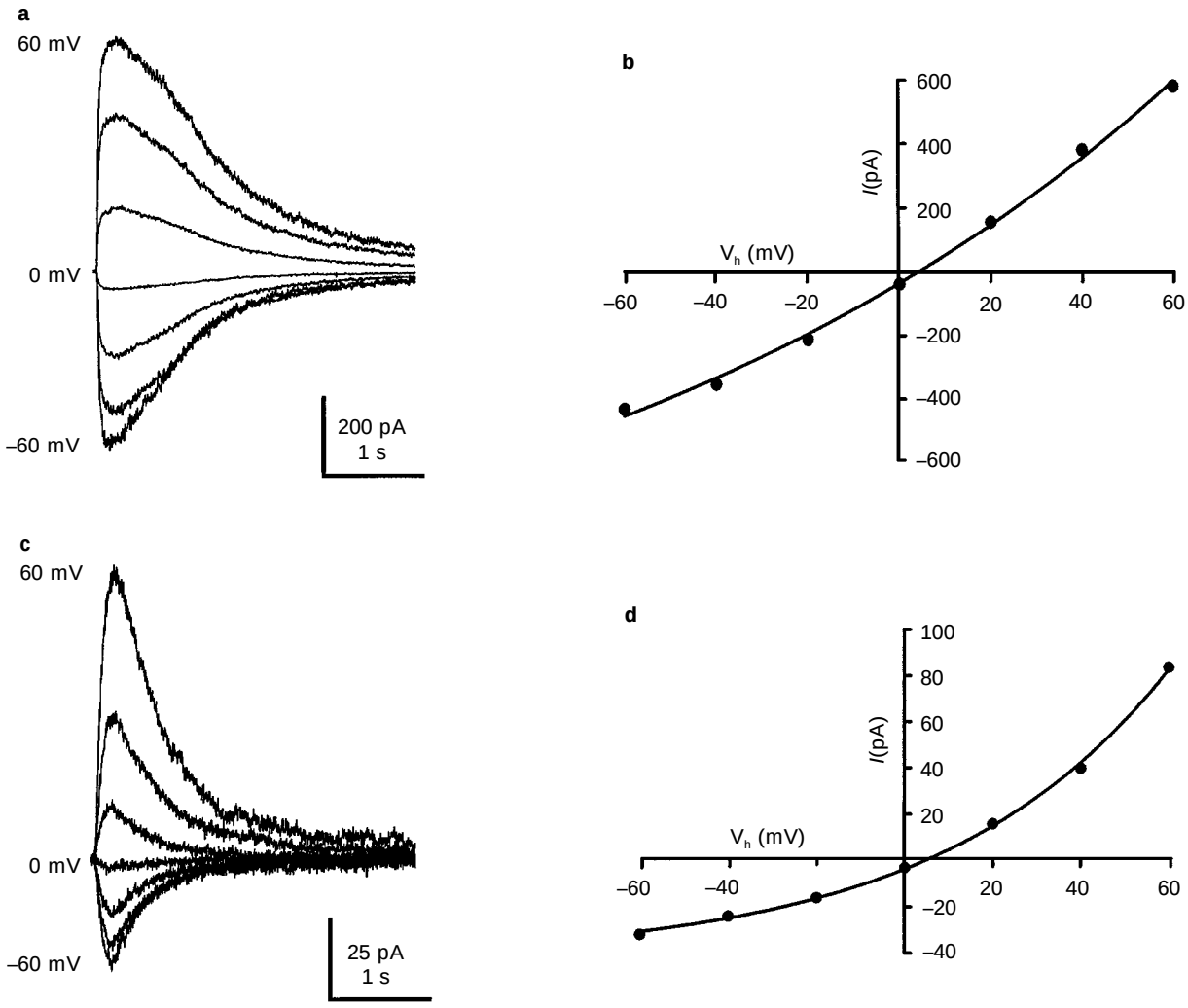

Figure 4 Activation of $\alpha1\beta3$ and β3 receptors by propofol. (a) Propofol (100 μM)-activated currents recorded from an HEK293 cell expressing $\alpha1\beta3$ receptors, voltage-clamped between -60 and 60 mV. (b) The plot of the current amplitude against holding potential shows that propofol-evoked currents in this cell reversed at 4 mV. (c) Propofol (100 μM)-activated currents recorded from a cell expressing β3 receptors, voltage-clamped between -60 and 60 mV. (d) The graph of current amplitude versus voltage shows that propofol-activated currents mediated by β3 homomers exhibit a similar relationship to holding potential and reverse at the same potential to those mediated by $\alpha1\beta3$ receptors. Superimposed traces are averages of two currents recorded at each potential.

potential of −60 mV ($n=54$). In order to establish the current-voltage relationship of the GABA-elicited currents of the α1β3 receptor, cells were held at potentials from −60 to 60 mV. With intracellular and extracellular Cl^- concentrations of 144 mM and 152 mM, respectively, the GABA-elicited currents reversed at 0.5±2.8 mV ($n=4$), close to the theoretical Cl^- equilibrium potential under these recording conditions (Figure 2a, b). Currents were larger at positive potentials compared to their amplitudes at corresponding negative potentials indicative of outward rectification. The outward rectification seen in the present study constrasts with the linear current-voltage relationship observed by Valeyev *et al*. (1993) under similar ionic conditions.

The amplitude of GABA-activated currents, recorded from cells clamped at −60 mV, increased with increasing concentrations of GABA (0.1 μM–1 mM) applied for a duration of 1 s (Figure 2c). By fitting the concentration-response data with a curve described by the logistic equation (Adodra & Hales, 1995) the Hill coefficient and EC_{50} were calculated to be 0.84 and 9.1 μM, respectively. This concentration-response relationship is similar to that from a previous study of the actions of GABA on recombinant α1β3 receptors (Valeyev *et al*., 1993). GABA (100 μM) application to control HEK293 cells ($n=10$) and cells transfected with α1 ($n=6$) or β3 ($n=13$) subunit cDNAs alone failed to activate discernible currents (Table 1).

Pharmacological properties of α1β3 $GABA_A$ receptors

$GABA_A$ receptors can be identified on the basis of their sensitivity to the selective inhibitors bicuculline and picrotoxin. GABA (100 μM)-evoked currents recorded from HEK293 cells expressing α1β3 $GABA_A$ receptors were inhibited to 16±9% ($n=5$) of their control amplitude by bath applied bicuculline methiodide (10 μM) (Figure 3a), the inhibition was reversed on washout of the antagonist. GABA-evoked currents were also reversibly inhibited to 28±6% ($n=5$) of control by superfusion of picrotoxin (10 μM) (Figure 3b).

Many compounds with central depressant activity including propofol (Hales & Lambert, 1991), the anaesthetic barbiturate pentobarbitone (Schulz & Macdonald, 1981) and the anaesthetic steroid 5α-pregnan-3α-ol-one (Majewska *et al*., 1986) substantially enhance GABA-evoked responses recorded from neurones. Currents activated by brief GABA application to HEK293 cells expressing α1β3 receptors were potentiated by bath applied propofol and 5α-pregnan-3α-ol-20-one (Figure 3c,d). The mean potentiations of GABA-induced currents by

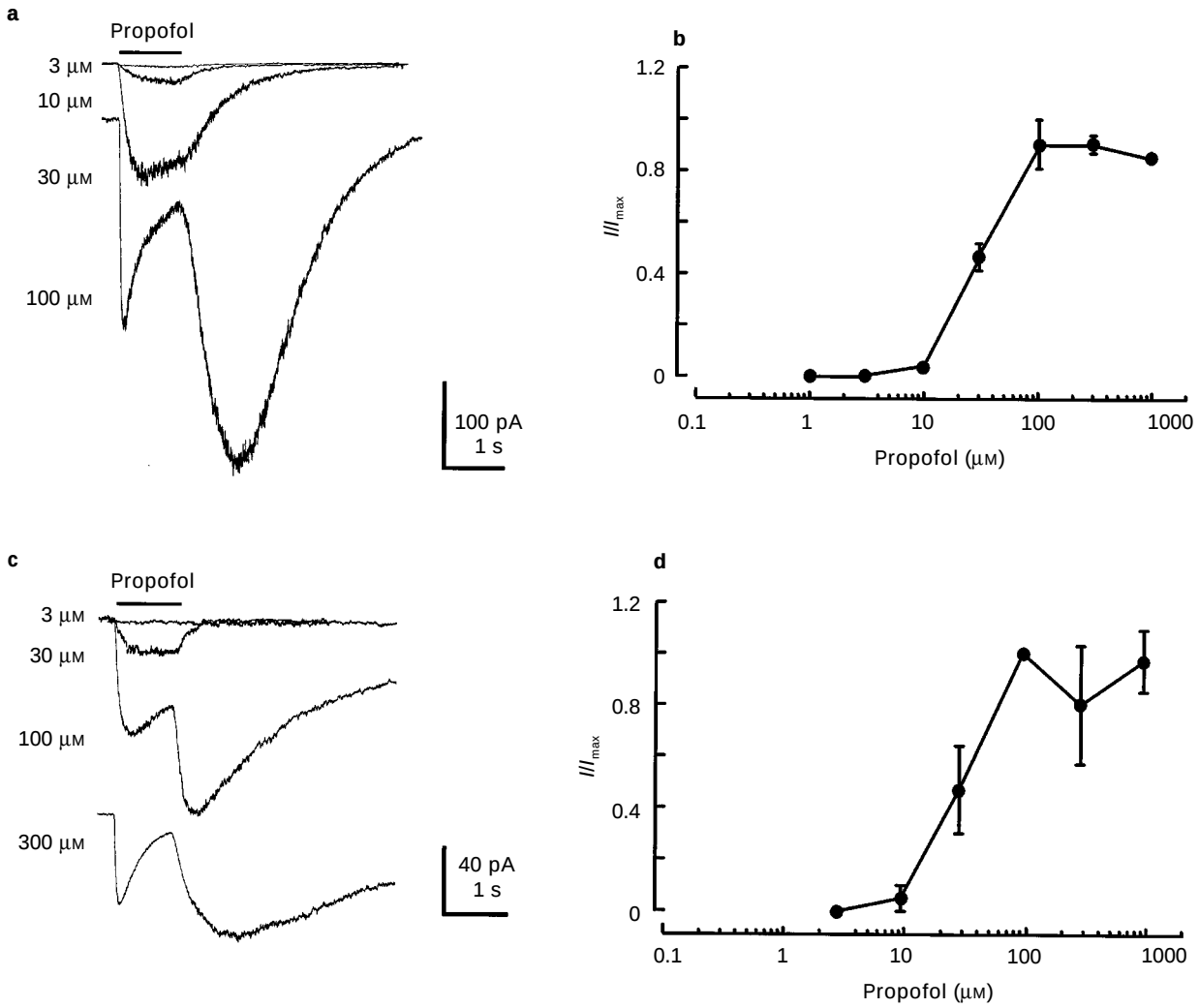

Figure 5 Propofol activated and inhibited α1β3 and β3 receptors. (a) Currents activated by propofol (3–100 μM) recorded from the same HEK293 cell expressing α1β3 receptors. On cessation of application of 100 μM propofol a pronounced surge of current was apparent. (b) The concentration-response relationship showed a decline in current amplitude in response to concentration greater than 100 μM propofol. (c) Propofol (3–300 μM)-evoked currents recorded from a cell expressing β3 homomers. (d) The graph of current amplitude against propofol concentration for β3 receptors was similar to that for α1β3 receptors. The EC_{50} for propofol's activation of both α1β3 and β3 receptors was approximately 40 μM. Data points represent mean data from at least four cells. Where larger than the symbols, vertical lines represent s.e.mean.

propofol (3 μM) and 5α-pregnan-3α-ol-20-one (100 nM) were 549±157% ($n=7$) and 206±45% ($n=5$) of control, respectively.

Activation of α1β3 and β3 receptors by the anaesthetics

In addition to enhancing GABA-activated currents, many general anaesthetics also directly activate $GABA_A$ receptors (Schulz & Macdonald, 1981; Hales & Lambert, 1991; Jones *et al*., 1995). The ability of propofol, pentobarbitone and 5α-pregnan-3α-ol-20-one to activate α1β3 $GABA_A$ receptors was investigated by locally applying these agents in the absence of GABA. Pressure application of propofol (100 μM) elicited inward currents recorded from cells voltage-clamped at −60 mV ($n=49$). By contrast, untransfected cells did not respond to the anaesthetic ($n=5$). The reversal potential of the propofol-elicited currents (4 mV, Figure 4a,b) was similar to that of GABA-activated currents (Figure 2a,b).

Cells transfected with the α1 cDNA alone failed to respond to propofol ($n=6$). However, propofol (100 μM) elicited inward currents in cells expressing homomeric β3 $GABA_A$ receptors voltage-clamped at −60 mV ($n=124$). The current-voltage relationship for propofol-activated currents mediated by the β3 receptor was similar to that of α1β3 receptors; currents exhibited outward rectification and reversed at 3.5±2.2 mV ($n=4$) (Figure 4c,d).

The amplitude of currents, seen in response to propofol's activation of α1β3 and β3 receptors, increased with increasing concentrations of propofol (3–100 μM) applied for 1 s (Figure 5). Currents activated by propofol concentrations greater than 100 μM tended to have a reduced amplitude and were associated with a surge current seen during propofol washout. This phenomenon has been observed in previous studies on cessation of application of high concentrations of propofol to embryonic hippocampal neurones (Orser *et al*., 1994) and immortalized hypothalamic neurones

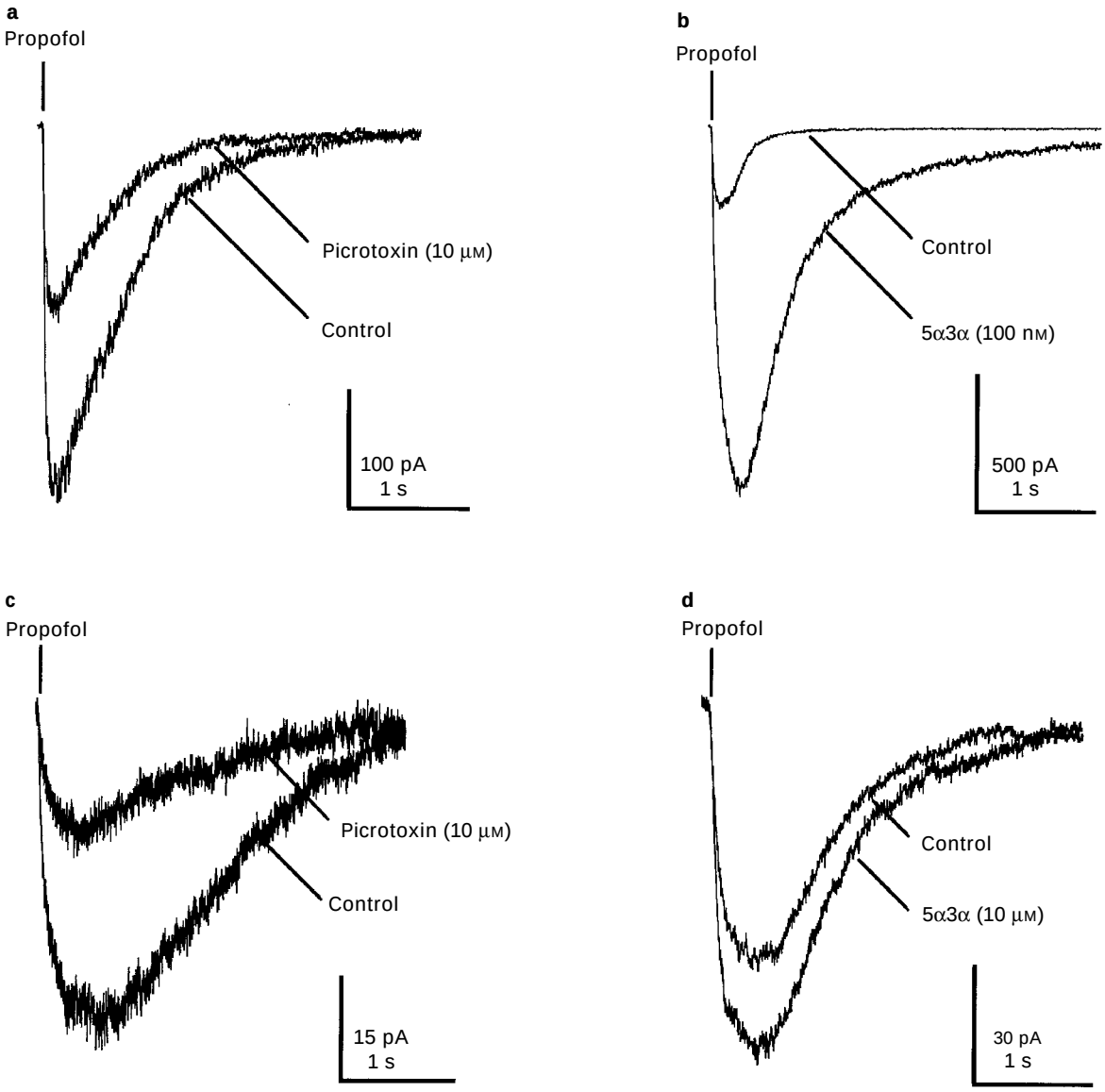

Figure 6 Pharmacology of propofol-evoked currents. (a) In cells expressing α1β3 receptors picrotoxin (10 μM) inhibited propofol (100 μM)-evoked currents. (b) The anaesthetic steroid 5α-pregnan-3α-ol-20-one (5α3α) (100 nM) enhanced propofol-activated currents mediated by α1β3 receptors. (c) Propofol (100 μM)-activated currents recorded from cells expressing β3 homomers were also inhibited by picrotoxin. (d) Propofol-activated currents mediated by β3 receptors were only slightly enhanced by 5α3α (10 μM). Experiments were performed on separate cells voltage-clamped at −60 mV, superimposed traces represent averages of three individual currents in the presence and absence of drugs. All drug effects reversed during wash off.

(Adodra & Hales, 1995). It has been suggested that the decline in current amplitude caused by high propofol concentrations is due to hindrance of $GABA_A$ receptor function and that the current surge represents reversal of the inhibition (Adodra & Hales, 1995). Due to the reduced peak current activated by high propofol concentrations no attempt was made to fit the data points. However, from the graphs of the peak current amplitude obtained in the presence of propofol at each concentration, the EC_{50} values for propofol's activation of α1β3 and β3 receptors are estimated to be approximately 40 μM in both cases. This estimate represents a lower limit and is included for comparison of the two concentration-response relationships.

Propofol-evoked currents recorded from β3 expressing cells often showed substantial run-down not seen in recordings from cells expressing α1β3 receptors. The current amplitude declined by 50% within 5 min after the initial propofol application. The rate of run-down declined after this period of time, the current after 10 min was approximately 30% of the initial response. Inclusion of ATP (3 mM) in the intracellular solution had no discernible effect on the rate of current decline. All experiments were conducted 10 min after the first propofol application. In order to overcome run-down when investigating the concentration-response relationship for propofol's activation of β3 receptors, each application was bracketed by applications of 100 μM propofol. Current amplitudes were normalized to the mean amplitudes achieved by bracketing the propofol (100 μM) applications.

Bath applied pentobarbitone (100 μM) potentiated GABA-evoked currents recorded from HEK293 cells expressing α1β3 receptors. At a higher concentration, pentobarbitone (1 mM) also activated currents when applied locally to cells expressing either α1β3 ($n=4$) or β3 ($n=7$) receptors (Table 1). Surge currents similar to those seen following propofol applications were also apparent on cessation of pentobarbitone application to cells expressing either receptor type (data not shown). Unlike propofol and pentobarbitone, 5α-pregnan-3α-ol-20-one (10 μM) did not activate discernible currents in HEK293 cells expressing β3 receptors ($n=11$; Table 1), even in those cells that had previously responded to propofol ($n=7$). By contrast, 5α-pregnan-3α-ol-20-one (10 μM) activated α1β3 receptors (Table 1). No reduction in the peak amplitude of currents occurred with high concentrations of the steroid and termination of its application was not associated with a surge current (data not shown). Therefore, unlike propofol and pentobarbitone, 5α-pregnan-3α-ol-20-one neither activates homomeric β3 receptors nor inhibits α1β3 $GABA_A$ receptor activity at high concentrations.

Pharmacology of propofol-activated receptors

Superfusion of picrotoxin (10 μM) reversibly inhibited currents evoked by locally applied propofol (100 μM) to $34\pm13\%$ ($n=4$) of control in cells expressing α1β3 receptors (Figure 6a).

Similar to its action on GABA-activated currents recorded from cells containing α1β3 receptors (Figure 3d), 5α-pregnan-3α-ol-20-one (100 nM) enhanced currents activated by brief propofol (100 μM) application to $1101\pm299\%$ ($n=9$) of control (Figure 6b). Picrotoxin (10 μM) also inhibited propofol (100 μM)-evoked currents recorded from cells expressing homomeric β3 receptors to $39\pm10\%$ ($n=7$) of control (Figure 6c). By contrast to its action on α1β3 receptors (Figures 3d and 6b), 5α-pregnan-3α-ol-20-one, even at a high concentration (10 μM), caused only a modest enhancement ($128\pm12\%$ of control, $n=7$) of propofol-activated currents when applied to cells expressing β3 homomers (Figure 6d).

Interestingly, application of picrotoxin (10 μM) did not cause a change in base-line current in the absence of propofol. Picrotoxin does alter base-line currents in recordings from cells expressing β1 homomers (Sanna *et al*., 1995., Krishek *et al*., 1996; Cestari *et al*., 1996). This phenomenon is caused by the blockade of spontaneous channel openings. No spontaneous currents were observed in recordings from α1β3 or β3 expressing cells.

Discussion

There are conflicting data regarding the subunit requirements for the formation of functional recombinant $GABA_A$ receptors in cell lines (Angelotti & Macdonald, 1993; Im *et al*., 1995) and *Xenopus* oocytes (Blair *et al*., 1988; Sigel *et al*., 1990). In general, studies performed soon after the initial cloning of $GABA_A$ receptor subunits suggested that α and β polypeptides, either alone or in combination, form GABA-activated receptors. However, more recent findings suggest that only certain homomeric receptors, in particular those formed by β subunits, are functional (Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996). In the present study we investigated whether α1 and β3 subunits form functional receptors when transiently expressed either alone or in combination in HEK293 cells. We examined whether the homomeric and/or heteromeric receptor combinations bind [^{35}S]-TBPS or are activated by GABA and the anaesthetic compounds propofol, pentobarbitone and 5α-pregnan-3α-ol-20-one.

[^{35}S]-TBPS bound to the membranes of HEK293 cells containing either the α1β3 combination or β3 homomers, suggesting that these subunits form receptors. By contrast, the lack of [^{35}S]-TBPS binding to the membranes of cells transfected with α1 cDNA suggests that this subunit alone does not form receptors. In agreement with this, cells transfected with α1 cDNAs do not respond to GABA application. Recent immunofluoresence studies examining the cellular distribution of α1 subunits demonstrate that when expressed alone in HEK293 cells, the subunit remains associated with internal membranes. By contrast, when the α1 and β2 subunits are expressed together both can be found in the plasma membrane where they form functional $GABA_A$ receptors (Connolly *et al*., 1996). Differential targeting of α1 and β1 subunits also occurs in epithelial cells in which, when expressed alone, these subunits are localized in the basolateral and apical membranes, respectively (Perez-Valazquez & Angelides, 1993). However, when expressed together both subunits appear in the apical membrane.

It is well established that α1β3 receptors form functional GABA-activated channels (Lolait *et al*., 1989; Ymer *et al*., 1989; Valeyev *et al*., 1993) and this is confirmed by our observations. These receptors also have binding sites for the anaesthetically active compounds propofol, pentobarbitone and 5α-pregnan-3α-ol-20-one. The IVGAs modulate [^{35}S]-TBPS binding to cell membranes containing α1β3 receptors and potentiate whole-cell GABA-evoked currents mediated by these receptors. At higher concentrations all three IVGAs also activate α1β3 receptors.

The β3 subunit, unlike the β2 subunit is able to access the plasma membranes of HEK293 cells even when expressed alone. This is evident from our observations of [^{35}S]-TBPS binding to β3 homomers and from a previous study (Slany *et al*., 1995). However, [^{35}S]-TBPS binding to membranes containing β3 homomers is insensitive to GABA. In addition, GABA fails to activate Cl^- currents recorded from cells expressing β3 receptors. These data suggest that β3 homomers are insensitive to GABA. Such an observation could lead to the assumption that β3 homomers are not functional. However, our data and those of Slany *et al*. (1995) show that propofol, pentobarbitone and anaesthetic steroids displace [^{35}S]-TBPS binding from membranes containing β3 receptors. Furthermore, propofol and pentobarbitone also activate whole-cell currents when applied to cells expressing β3 homomers. These observations demonstrate that in HEK293 cells the β3 subunit alone forms functional receptors that do not respond to GABA, but are activated by some IVGAs. It is possible that HEK293 cells could modify the properties of recombinant $GABA_A$ receptors by contributing a protein from

their own genome. Perhaps the most obvious candidate would be a $GABA_A$ receptor subunit. Indeed, detectable levels of β3 subunit mRNA are present in untransfected HEK293 cells (Kirkness & Fraser, 1993). It is unlikely that this subunit contributes to the properties of recombinant receptors in these cells because, prior to transfection, they do not respond to propofol or pentobarbitone, and cells transfected with α1 subunits neither respond to GABA nor do they bind either [^{3}H]-muscimol or [^{35}S]-TBPS. The possibility that there is an unidentified protein interacting with the recombinant receptors cannot be ruled out. However, it is unlikely that such an HEK293 cell-specific factor would be necessary for the formation of recombinant homomeric β subunits since these have also been observed in studies in which *Xenopus* oocytes were utilized (Sigel *et al*., 1989; Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996). Additionally, actinomycin D has been used to abolish transcription in oocytes before the injection of β1 cRNA and this did not prevent the expression of functional homomeric receptors (Krishek *et al*., 1996).

Currents evoked by propofol and pentobarbitone recorded from HEK293 cells expressing β3 subunits were consistently smaller than were α1β3-mediated currents activated by the IVGAs or GABA (Table 1). There are a number of possible explanations for this observation. Homomeric β3 receptors may be less efficiently expressed than receptors comprised of α1 and β3 subunits. Alternatively, channels formed by β3 subunits alone may less effectively flux Cl^- than those formed by α1 and β3 subunits together. This could occur either as a result of differences in the single channel open probabilities (i.e. more frequent or longer openings with α1β3 than β3), or because of a difference in the conductance of these channels (i.e. larger conductance for α1β3 than β3). A comparison of recordings of single channels formed by homomeric β3 receptors and α1β3 receptors will be required to resolve this issue. However, it is interesting to note that β1 subunits form channels that have similar conductances to those formed when α1 and β1 subunits are expressed together (Blair *et al*., 1988; Krishek *et al*., 1996). This suggests that smaller currents are seen with homomeric β3 receptors in the present study because of less efficient expression, or due to a lower probability of channels opening when the α subunit is absent.

A comparison of our characterization of rat β3 homomeric receptors and previous studies of the properties of β subunits from various species, suggests that there are interesting similarities and important anomalies between the different β subtypes, that may in part be species-specific. Human and bovine β1 subunits expressed in oocytes form homomeric receptors that can be activated by GABA (Sanna *et al*., 1995; Krishek *et al*., 1996). By contrast, rodent β1 and murine β2 and β3 homomers cannot be activated by GABA (Sigel *et al*., 1989; Krishek *et al*., 1996; Cestari *et al*., 1996). Homomeric receptors comprised of rat and human β1 and murine β1 and β3 subunits show spontaneous picrotoxin-sensitive channel openings that are not apparent in recordings from oocytes expressing bovine β1 subunits. In addition, spontaneously active rodent β1 and murine β3 receptors cannot be further activated by GABA, while human β1 homomers can. It is not surprising perhaps that already active rodent β1 and murine β3 receptors cannot be further activated by GABA, and it remains undetermined whether this is caused by a lack of the GABA recognition site. However, rat β3 subunits form GABA-insensitive ion channels that are not spontaneously active and do not bind [^{3}H]-muscimol, clearly demonstrating that these homomeric receptors do not have a site for GABA-mediated activation.

Propofol and pentobarbitone elicit currents in oocytes expressing murine and human β1, or murine β3 subunits. This could either be due to potentiation of spontaneous channel openings or direct receptor activation by these IVGAs (Krishek *et al*., 1996; Cestari *et al*., 1996). Pentobarbitone potentiates GABA-activated currents recorded from cells expressing bovine β1 subunits, suggesting that a potentiating site is present on these homomers (Krishek *et al*., 1996). Therefore, whether spontaneously active β homomers possess an anaesthetic activation site remains unresolved. However, both propofol and pentobarbitone directly activate quiescent rat β3 homomeric receptors. Thus, sites for activation by these agents are clearly present on this β3 subunit and are distinct from the GABA activation site.

From the binding and the electrophysiological data the modulation of $GABA_A$ receptors by propofol and pentobarbitone appears similar. These compounds cause displacement of [^{35}S]-TBPS binding from α1β3 and β3 receptors. They also activate both $GABA_A$ receptor combinations and, at higher concentrations, cause surge currents during wash off. The surge currents, seen on cessation of application of pentobarbitone (Peters *et al*., 1989; Robertson, 1989) and propofol (Orser *et al*., 1994; Adodra & Hales, 1995), have been observed in recordings from other preparations and are thought to be caused by a rapid reversal of receptor block by high concentrations of IVGAs. By contrast to pentobarbitone and propofol, 5α-pregnan-3α-ol-20-one causes dissimilar modulation of [^{35}S]-TBPS binding to α1β3 and β3 receptors. At low concentrations 5α-pregnan-3α-ol-20-one enhances [^{35}S]-TBPS binding to α1β3 receptors, while at higher concentrations it displaces [^{35}S]-TBPS binding. Only the latter action of 5α-pregnan-3α-ol-20-one is evident in experiments with membranes containing β3 homomers. Even at high concentrations the steroid did not activate discernible currents when applied to HEK293 cells expressing β3 receptors. The disparity between the binding data and the electrophysiology suggests that displacement of [^{35}S]-TBPS binding by anaesthetic steroids may not be a reliable measure of their ability to activate $GABA_A$ receptors. Enhancement of [^{35}S]-TBPS binding to α1β3 receptors by 5α-pregnan-3α-ol-20-one correlates with the ability of this steroid to activate this $GABA_A$ receptor combination. However, the relationship between the modulation of $GABA_A$ receptor binding and the electrophysiological actions of the IVGAs is complicated. Correlations between these two experimental approaches should be made with caution, particularly in view of their markedly different time courses. It is likely that with higher agonist concentrations [^{35}S]-TBPS binding will be influenced by receptor desensitization and also, in the cases of propofol and pentobarbitone, by receptor block.

One interpretation of our data, and the results of previous studies (Amin & Weiss, 1993; Sanna *et al*., 1995; Krishek *et al*., 1996; Cestari *et al*., 1996), is that propofol and pentobarbitone can bind to and activate any combination of $GABA_A$ subunits that form receptors in the plasma membrane. At present it is not known whether the binding site is only on the β subunit, or common to many (perhaps all) subunits. The types of subunit may well be unimportant with the exception of their ability to form functional receptors.

A number of previous binding and electrophysiological studies support the notion that anaesthetic steriods act through a different site on the $GABA_A$ receptor from that of the anaesthetic barbiturates and propofol (Peters *et al*., 1998; Turner *et al*., 1989; Hales & Lambert, 1991; Belelli *et al*., 1996). [^{3}H]-muscimol binding, maximally enhanced by the barbiturate secobarbitone, can be enhanced further by the addition of the anaesthetic steroid 5β-pregnan-3α-ol-20-one (Peters *et al*., 1988). In addition, the anaesthetic steroids dramatically enhance currents activated by high concentrations of anaesthetic barbiturates, suggesting that these agents act at different sites on the $GABA_A$ receptor complex (Peters *et al*., 1988; Hales & Lambert, 1991; Belelli *et al*., 1996). By contrast, propofol causes only a small enhancement of currents activated by pentobarbitone suggesting that these agents may compete for a similar site of action (Hales & Lambert, 1991).

Like propofol and pentobarbitone, 5α-pregnan-3α-ol-20-one binds to the β3 subunit leading to the displacement of [^{35}S]-TBPS. However, the steroid cannot activate the receptor and has a greatly reduced capacity to potentiate currents activated by propofol. The anaesthetic steriod alphaxalone is also unable to active human β1 homomers directly, but does potentiate GABA-activated currents recorded from the same oocytes

(Sanna *et al*., 1995). These observations suggest that some β subunits may possess the steriod potentiation site, but lack their site of direct receptor activation.

In summary, our data and the results of previous studies on β subunits suggest that there are some properties common to all subunits of this class that have so far been examined. They form homomeric receptors that are inserted into cell surface membranes. These receptors can be activated by propofol and pentobarbitone, but not by the anaesthetic steroids. There are also surprising differences between the properties of these receptors even when comparing β subunits of the same subtype between species. For example, murine β3 subunits show spontaneous activity, while those of the rat do not. Also, rodent β1 subunits cannot be activated by GABA, while human and bovine β1 homomers can. By examining the differences between the sequences of these subunits it may be possible to identify the determinants of these important receptor properties.

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