

Salicylidene salicylhydrazide, a selective inhibitor of $\beta 1$ -containing GABA_A receptors

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1 A high-throughput assay utilizing the voltage/ion probe reader (VIPR) technology identified salicylidene salicylhydrazide (SCS) as being a potent selective inhibitor of $\alpha 2\beta 1\gamma 1\theta$ GABA_A receptors with a maximum inhibition of $56 \pm 5\%$ and an IC₅₀ of 32 (23, 45) nM.

2 Evaluation of this compound using patch-clamp electrophysiological techniques demonstrated that the compound behaved in a manner selective for receptors containing the $\beta 1$ subunit (e.g. maximum inhibition of $68.1 \pm 2.7\%$ and IC₅₀ value of 5.3 (4.4, 6.5) nM on $\alpha 2\beta 1\gamma 1$ receptors). The presence of a $\beta 1$ subunit was paramount for the inhibition with changes between $\alpha 1$ and $\alpha 2$, $\gamma 1$ and $\gamma 2$, and the presence of a θ subunit having little effect.

3 On all subtypes, SCS produced incomplete inhibition with the greatest level of inhibition at $\alpha 1\beta 1\gamma 1\theta$ receptors ($74.3 \pm 1.4\%$). SCS displayed no use or voltage dependence, suggesting that it does not bind within the channel region. Concentration – response curves to GABA in the presence of SCS revealed a reduction in the maximum response with no change in the EC₅₀ or Hill coefficient. In addition, SCS inhibited pentobarbitone-induced currents.

4 Threonine 255, located within transmembrane domain (TM) 1, and isoleucine 308, located extracellularly just prior to TM3, were required for inhibition by SCS.

5 SCS did not compete with the known allosteric modulators, picrotoxin, pregnenolone sulphate, dehydroepiandrosterone 3-sulphate, bicuculline, loreclezole or mefenamic acid. Neither was the inhibition by SCS influenced by the benzodiazepine site antagonist flumazenil.

6 In conclusion, SCS is unique in selectively inhibiting GABA_A receptors containing the $\beta 1$ subunit *via* an allosteric mechanism. The importance of threonine 255 and isoleucine 308 within the $\beta 1$ subunit and the lack of interaction with a range of GABA_A receptor modulators suggests that SCS is interacting at a previously unidentified site.

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Keywords: GABA_A receptor; salicylidene salicylhydrazide; β subunit; ion channel; transmembrane domain; mutagenesis; allosteric; modulator

Abbreviations: DHEAS, dehydroepiandrosterone 3-sulphate; FRET, fluorescence resonance energy transfer; GABA, γ -aminobutyric acid; GABA_A, γ -aminobutyric acid type A; I, isoleucine; K, lysine; M, methionine; MBS, modified Barth's saline; N, asparagine; PS, pregnenolone sulphate; PTX, picrotoxin; R, arginine; S, serine; SCS, salicylidene salicylhydrazide; T, threonine; TM, transmembrane domain; VIPR, voltage/ion probe reader.

Introduction

The majority of fast inhibitory neurotransmission within the mammalian brain is mediated by the neurotransmitter GABA acting on postsynaptic GABA_A receptors. The GABA_A receptor, which belongs to the superfamily of ligand-gated ion channels, is formed by an assembly of five homologous subunits. These subunits have been subclassified according to their degree of amino-acid homology as $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , $\rho 1$ – $\rho 3$, θ , ϵ and π , with the majority of receptors composed of two α , two β and a γ subunit (Chang *et al.*, 1996; Farrar *et al.*, 1999). Each subunit has a large extracellular N-terminal

domain, four hydrophobic membrane-spanning domains that form the channel region and a small extracellular C-terminus (Korpi *et al.*, 2002).

A large number of clinically used psychoactive drugs, for example, benzodiazepines, general anaesthetic agents, anticonvulsants and ethanol, exert their effects mainly or exclusively *via* an interaction with GABA_A receptors. In addition, a number of other substances have been shown to interact with GABA_A receptors, for example, loreclezole, avermectin, furosemide, zinc, picrotoxin and lanthanum (for a recent review, see Korpi *et al.*, 2002). Electrophysiological and radioligand-binding studies using recombinant receptors have demonstrated that a number of these compounds display subunit selectivity. Meanwhile, the generation of chimeras and site-directed mutagenesis has enabled specific amino-acid residues that confer the selectivity to be identified, for

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example, selectivity of the anaesthetic etomidate for $\beta 2$ - and $\beta 3$ -containing receptors is due to an asparagine residue at positions 289 and 290, respectively (Belelli *et al.*, 1997). Note an alternative numbering for this residue is B2/3 N265.

Within the CNS, the total number of GABA_A receptor subtypes and the function of many of these are currently unknown (Sieghart & Sperk, 2002; Whiting, 2003). One way to dissect out the behavioural effects of the different GABA_A receptor subtypes is to use a subtype-selective compound. In order to identify novel subunit selective compounds, a library of some 10,000 compounds was screened against three GABA_A receptor subtypes ($\alpha 2\beta 1\gamma 1\theta$, $\alpha 3\beta 3\gamma 2s$ and $\alpha 4\beta 3\gamma 2s$). The subtypes were chosen from a large but not exhaustive repertoire of stable cell lines, with the initial aim of identifying compounds selective for as many subunits as possible. Herein we describe the identification and characterization of a salicylic acid derivative, salicylidene salicylhydrazide, with unique potency and selectivity for $\beta 1$ -containing receptors.

There are limited biological data with SCS; however, it is known to be a chelator of metal ions and *in vitro* has comparable cytotoxicity to cisplatin ($IC_{50} = 1.8 \mu M$) (Ainscough *et al.*, 1999). To our knowledge, this is the first report to describe its interaction with GABA_A receptors.

Methods

Voltage/ion probe reader assay (VIPR)

Primary screening data were generated using a Voltage/Ion Probe Reader assay (VIPRTM; Aurora Biosciences, CA, U.S.A.) as previously described (Adkins *et al.*, 2001; Smith & Simpson, 2003). Briefly, *Ltk*⁻ cells stably expressing GABA_A receptor subunit combinations were seeded into black-sided Porvair 96-well plates and receptor expression was induced 24 h prior to experiment with $1 \mu M$ dexamethasone (Sigma, U.K.). Cells were washed in low-Cl⁻ buffer (in mM sodium-D-gluconate 160, potassium-D-gluconate 4.5, CaCl₂ 2, MgCl₂ 1, D-glucose 10, HEPES 10, pH 7.4) and dye-loaded for 30 min to give final concentrations of $4 \mu M$ chlorocoumarin-2-dimethylphosphatidylethanolamine (CC2-DMPE; FRET donor) and $1 \mu M$ bis(1,3-diethyl-2-thiobarbiturate)trimethineoxonol (DiSBAC₂(3); FRET acceptor), with 0.5 mM tartrazine present extracellularly. Plates were then placed in a VIPR which performs automated additions using a Hamilton 2200 pipettor and records fluorescence emissions at 460 and 580 nm simultaneously from eight wells. A 400DF15 filter was used in the excitation pathway, and 460DF45 and 580DF60 filters in the respective emission pathways. Rapid ratiometric FRET measurements were made of GABA-evoked depolarizations in low-Cl⁻ buffer as previously described (Adkins *et al.*, 2001) and the ability of compounds to modulate an EC₅₀ response to GABA was examined.

For each time point at each fluorescence emission wavelength, background fluorescence was subtracted (recorded from wells without cells in the same plate) and the ratio of fluorescence at 460–580 nm was calculated. GABA-evoked depolarizations were then expressed as a fractional change in this ratio. Algorithms written as Excel 97 (Microsoft Corp.) macros were used for automated calculation of fluorescence ratio and GABA responses.

Whole-cell patch clamp in mammalian cells

Whole-cell patch-clamp experiments were performed on the stable *Ltk*⁻ cell lines expressing $\alpha 1\beta 1\gamma 2s$, $\alpha 1\beta 2\gamma 2s$, $\alpha 2\beta 1\gamma 1$, $\alpha 2\beta 3\gamma 2s$ and $\alpha 2\beta 1\gamma 1\theta$ GABA_A receptors as described elsewhere (Brown *et al.*, 2002). In brief, cells were patch clamped using a pipette with a tip diameter of approximately 1.5–2.5 μm and a resistance of around 4 M Ω . The intracellular solution contained (in mM): CsCl 130, HEPES 10, BAPTA.Cs 10, ATP.Mg 5, leupeptin 0.1, MgCl₂ 1, NaVO₃ 0.1 (pH adjusted to 7.3) with CsOH and 320–340 mOsm by adding sucrose. Cells were voltage-clamped at -20 mV *via* an Axon 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.) and perfused with artificial cerebrospinal fluid (aCSF) consisting of (in mM) NaCl 149, KCl 3.25, HEPES 10, MgCl₂ 2, CaCl₂ 2, D-glucose 11, sucrose 22, at pH 7.4. 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. The measured agonist exchange time using this system was approximately 20–30 ms. GABA (1 mM and $1 \mu M$) was applied to the cell (5 s on, 30 s washout) and the amplitude of the currents used to calculate an approximate EC₂₀ concentration (individually determined for each cell and ranging from 8 to 35% of the response to 1 mM GABA). Noncumulative concentration–response curves examining the modulatory effects of SCS were constructed with SCS being applied for 30 s prior to co-application with the GABA EC₂₀. Data were recorded and analysed using P-clamp (Version 8, Axon Instruments, Foster City, CA, U.S.A.).

Two-electrode voltage clamp in *Xenopus oocytes*

The cloning and sequencing of human $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 2s$ and the generation of the $\beta 1/\beta 2$ chimeras $\beta \Delta 1.1$, $\beta \Delta 1.2$ and $\beta \Delta 1.4$ (hereafter referred to as $\beta 2_{1-237}\beta 1_{238-474}$, $\beta 1_{1-236}\beta 2_{237-474}$, and $\beta 1_{1-236}\beta 2_{237-334}\beta 1_{335-474}$, respectively) and the point mutants $\beta 1^{S290N}$, $\beta 2^{N289S}$, $\beta 1^{T255I}$, $\beta 1^{I308M}$ have been described elsewhere (Wingrove *et al.*, 1994, Hadingham *et al.*, 1993a, b). Site-directed mutagenesis, using standard techniques incorporating a diagnostic restriction site, was performed to generate the point mutants $\beta 1^{K334R}$, $\beta 2^{R333K}$, $\beta 2^{I254T}$ and $\beta 2^{M307I}$ (Wingrove *et al.*, 1994). Oligonucleotide primers were synthesized by Sigma-Genosys (Cambridge, U.K.). Mutants were identified by the presence of the diagnostic site and this was confirmed by DNA sequencing using a CEQ 2000 Genetic Analysis System (Beckman-Coulter). The amino-acid numbering starts with the initiating methionine.

All aspects of animal care and use were conducted in accordance with the Animal (Scientific Procedures) Act 1986 and its associated guidelines. The isolation and injection of oocytes by this laboratory has been described in detail elsewhere (Whiting *et al.*, 1995). In brief, stage V and VI oocytes were manually isolated, treated with collagenase (Type IA, 0.5 mg ml⁻¹ for 6 min) and then injected with 10–20 nl of injection buffer (in mM NaCl 88, KCl 1, HEPES 15, at pH 7, filtered through nitro-cellulose 0.2 μm) containing different combinations of human GABA_A subunit cDNAs engineered into the expression vector pCDM8 or pCDNA1.1Amp. The ratio of $\alpha 2:\beta y:\gamma 2s$ (where y equals $\beta 1$, $\beta 2$ or chimeras or single-point mutations thereof) in these stock solutions was 1:1:1 or 1:0.1:1 with 1 corresponding to 6.6 ng μl^{-1} .

Following incubation for 24–72 h, oocytes were placed in a 50 μ l bath and perfused at 4–6 ml min⁻¹ with modified Barth's medium (MBS) consisting of (in mM) NaCl 88, KCl 1, HEPES 10, MgSO₄ 0.82, Ca(NO₃)₂ 0.33, CaCl₂ 0.91, NaHCO₃ 2.4, at pH 7.5. Cells were impaled with two 1–3 M Ω electrodes containing 2 M KCl, voltage-clamped at –70 mV using a Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA, U.S.A.) and recordings visualized on a Gould Oscilloscope 1602, a Gould Windrograf chart recorder (Gould Medical Products Group, Valley View, OH, U.S.A.) and a Compaq Deskpro IBM compatible computer containing the software package 'Oocyte' (Digitimer Ltd, Welwyn Garden City, Hertfordshire, U.K.).

After a period of stabilization (5–10 min), a maximal concentration of GABA (3 mM) was applied followed by a GABA EC₂₀ concentration (individually determined for each oocyte and ranging from 15 to 30% of the response to 3 mM GABA). Noncumulative concentration–response curves examining the modulatory effects of SCS were constructed with SCS being applied for 45 s prior to co-application with the GABA concentration. Further experiments were designed to examine the interaction of SCS with other known GABA_A receptor modulators. Experiments using GABA_A receptor inhibitors were designed as follows: a GABA EC₂₀ was applied and the response allowed to reach a plateau, a concentration of picrotoxin (PTX), pregnenolone sulphate (PS), dehydroepiandrosterone sulphate (DHEAS) or bicuculline, which was previously shown to inhibit a GABA EC₂₀ response by approximately 70%, was then co-applied. Finally, when this inhibition reached a plateau, SCS 1 μ M was co-applied. The effect of SCS on the GABA mimetic effect of pentobarbital and the modulatory effect of loreclezole and mefenamic acid were also examined. *IV* curves were constructed in a stepwise manner of 20 mV increments to determine the reversal potential for a GABA EC₂₀ concentration in the absence and presence of SCS. Finally, to evaluate a possible use-dependent block, SCS or picrotoxin, for comparison, was continuously applied during repeated coapplications of a GABA EC₂₀ concentration.

Analysis of data

Concentration–response curves were fitted using Prism (Version 3.03, Graph Pad Software Incorporated, San Diego, CA, U.S.A.) to a nonlinear least-square-fitting function represented by the equation $y = \min + [\max - \min] / [1 + 10^{(\text{Log}[\text{IC}_{50} - \text{Log}x] / n_H)}]$, where y is the response, min and max are the minimum and maximum responses, x is the drug concentration, IC₅₀ is the concentration of drug eliciting a half-maximal inhibition of the response and n_H is the Hill coefficient. The current–voltage plots were fitted to a second-order polynomial fit and the reversal potentials calculated (Prism). Data for the IC₅₀ values are shown as the geometric mean (–s.e.m., +s.e.m.), whereas the Hill coefficients, maximum inhibition values and reversal potentials are shown as the arithmetic mean \pm s.e.m. Data were analysed for statistical significance using the Students *t*-test or a one-way ANOVA followed by the Tukey–Kramer *post hoc* test for multiple comparisons. A *P*-value less than 0.05 was considered significant.

Drugs and reagents

Electrophysiology GABA, flumazenil, PTX, PS, DHEAS, bicuculline and mefenamic acid were purchased from Sigma

Chemical Company (St Louis, MO, U.S.A.). SCS was initially synthesised by the Merck Sharp and Dohme Chemistry Department and later purchased from Lancaster Synthesis Ltd (Morecambe, Lancashire, U.K.). Pentobarbitone sodium (Sagatal) was purchased from Rhone Merieux Inc (Athens, GA, U.S.A.) and loreclezole from Tocris (Avonmouth, Bristol, U.K.). General salts and DMSO were from either VWR International Ltd (Lutterworth, Leicestershire, U.K.) or Sigma Chemical Company. Stock solutions of GABA (1 M in deionized H₂O), flumazenil, PTX, bicuculline, loreclezole (10⁻² M in DMSO) and PS, DHEAS and mefenamic acid (10⁻¹ M in DMSO) were prepared and stored at –20°C. When using *Xenopus* oocytes, inhibition to SCS was only apparent if the stock solution (10⁻² M in DMSO) was made fresh each day and dilutions in MBS made immediately prior to application.

VIPR CC2-DMPE was obtained from Aurora Biosciences Corporation (CA, U.S.A.) and DiSBAC₂(3) was from Molecular Probes Inc. (OR, U.S.A.) Tartrazine, gluconate salts and all other GABA_A receptor modulators were obtained from Sigma. Compounds 1–5 were synthesized by the Merck Sharp and Dohme Chemistry Department, but are all commercially available from various sources.

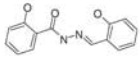
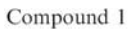
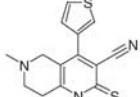
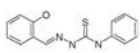
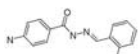
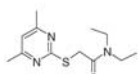
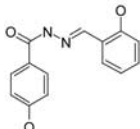
Results

VIPR screening

Approximately 10,000 compounds from a structurally diverse screening library were screened for activity on $\alpha 2\beta 1\gamma 1\theta$, $\alpha 3\beta 3\gamma 2s$ and $\alpha 4\beta 3\gamma 2s$ GABA_A receptors at a single concentration (8 μ M). The VIPR provides the opportunity to measure, with high throughput in 96-well format, changes in membrane potential elicited by activation of GABA_A receptors. The ability of compounds to modulate the half-maximal response to GABA was examined. This screen identified structurally novel compounds with activity at each of these subtypes and, from this, active and apparently subtype-selective compounds were selected for confirmation and potency titrations. One of these, SCS, was confirmed in the follow-up experiments as being a partial inhibitor of GABA responses at $\alpha 2\beta 1\gamma 1\theta$ and $\alpha 2\beta 1\gamma 1$ receptors stably expressed in *Ltk*⁻ cells. It was not, however, found to inhibit the responses at $\alpha 1\beta 3\gamma 2s$, $\alpha 2\beta 3\gamma 2s$, $\alpha 3\beta 3\gamma 2s$, or $\alpha 4\beta 3\gamma 2s$ receptor subunit combinations.

In follow-up investigations, 150 compounds with structural similarity to SCS were tested in a single point screen for their ability to modulate GABA responses mediated by $\alpha 2\beta 1\gamma 1\theta$ receptors. Several active compounds were identified and tested at a range of concentrations using cells expressing $\alpha 2\beta 1\gamma 1\theta$ or $\alpha 3\beta 3\gamma 2s$ receptors to confirm their activity and selectivity for $\alpha 2\beta 1\gamma 1\theta$, notably compounds 1 (IC₅₀ 67 nM), 2 (54 nM) and 3 (207 nM). All of these had very modest maximal effects on GABA responses however. Two further compounds from this set, compounds 4 and 5, exhibited more pronounced maximal inhibition of GABA responses, although they were less potent and showed less selectivity (four-fold) than SCS or compounds 1–3. SCS was considered to possess a novel selectivity and activity profile deserving further evaluation (Table 1).

Table 1 IC₅₀ and maximum inhibition values for SCS and a range of structurally related compounds using FRET techniques and VIPR measurements at $\alpha 2\beta 1\gamma 1\theta$ and $\alpha 3\beta 3\gamma 2s$ GABA_A receptors expressed in *Ltk*⁻ cells

Compound	$\alpha 2\beta 1\gamma 1\theta$		$\alpha 3\beta 3\gamma 2s$	
	IC ₅₀ (nM)	% modulation of GABA EC ₅₀	IC ₅₀ (nM)	% modulation of GABA EC ₅₀
SCS 	32 (23; 45)	-56 ± 5	ND	12 ± 11 at 3 μM
Compound 1 	67 (64; 70)	-15 ± 8	ND	-10 ± 3 at 3 μM
Compound 2 	54 (39; 76)	-20 ± 3	3355 (2699; 4169)	-33 ± 4
Compound 3 	207 (175; 245)	-19 ± 4	ND	-0.3 ± 7 at 3 μM
Compound 4 	998 (922; 1080)	-59 ± 6	3969 (3384; 4656)	-37 ± 10
Compound 5 	342 (179; 655)	-38 ± 3	1660 (1185; 2326)	-15 ± 3
				

Data represent modulation of the response to an EC₅₀ concentration of GABA, previously determined for each receptor subunit combination. Data for the IC₅₀ values are presented as the geometric mean (-s.e.m., +s.e.m.) and the maximum inhibition as the arithmetic mean ± s.e.m. of three to eight determinations. ND = not determined.

SCS selectivity for $\beta 1$ -containing receptors using whole-cell patch clamp

Further characterization of SCS was performed using electrophysiological techniques. SCS (0.1 nM–3 μM) produced a concentration-dependent inhibition of GABA EC₂₀ currents recorded from *Ltk*⁻ cells expressing $\alpha 2\beta 1\gamma 1\theta$, $\alpha 2\beta 1\gamma 1$ and $\alpha 1\beta 1\gamma 2s$ receptors compared with $\alpha 2\beta 3\gamma 2s$ and $\alpha 1\beta 2\gamma 2s$ receptors upon which SCS had no effect (Figure 1 and Table 2). SCS was less potent, produced less inhibition and had a steeper Hill coefficient on $\alpha 1\beta 1\gamma 2s$ receptors than $\alpha 2\beta 1\gamma 1$ and $\alpha 1\beta 1\gamma 1\theta$ ($P < 0.05$). Whether this difference was due to the presence of the $\gamma 1$ subunit within both receptor subtypes or the individual presence of a $\alpha 2$ and a θ subunit within each receptor subtype was not investigated.

Inhibition by SCS is not voltage or use dependent

The current–voltage relationship was examined for a GABA EC₂₀ in the absence and presence of SCS (300 nM) in *Xenopus* oocytes expressing $\alpha 2\beta 1\gamma 2s$ receptors. Comparable degrees of inhibition were observed at positive and negative holding

potentials suggesting no voltage dependence (Figure 2a). The reversal potentials were not significantly different from one another (-24.4 ± 3.5 (GABA) and -22.0 ± 3.6 mV (GABA + SCS)), and are close to the predicted reversal potential for Cl⁻ ions of -25.4 mV in *Xenopus* oocytes, with an external Cl⁻ concentration of 89.91 mM (MBS used in this study) and an internal Cl⁻ concentration of 33.4 mM (Barish, 1983). Picrotoxin, which is known to bind within the channel, displayed clear use dependence with successive GABA EC₂₀ responses showing greater inhibition. Identical studies with SCS showed no use dependence, with successive GABA EC₂₀ responses showing similar or slightly less inhibition (Figure 2b).

Structural determinants necessary for the inhibition of GABA_A receptors by SCS are located within the region arginine 238 and glycine 335 of the $\beta 1$ subunit

The unique selectivity for $\beta 1$ -containing receptors prompted mutagenesis studies in *Xenopus* oocytes to identify the amino-acid residues responsible for this selectivity. Concentration–response curves to SCS on *Xenopus* oocytes expressing $\alpha 2\beta 1\gamma 2s$ and $\alpha 2\beta 2\gamma 2s$ receptors confirmed the data obtained with *Ltk*⁻

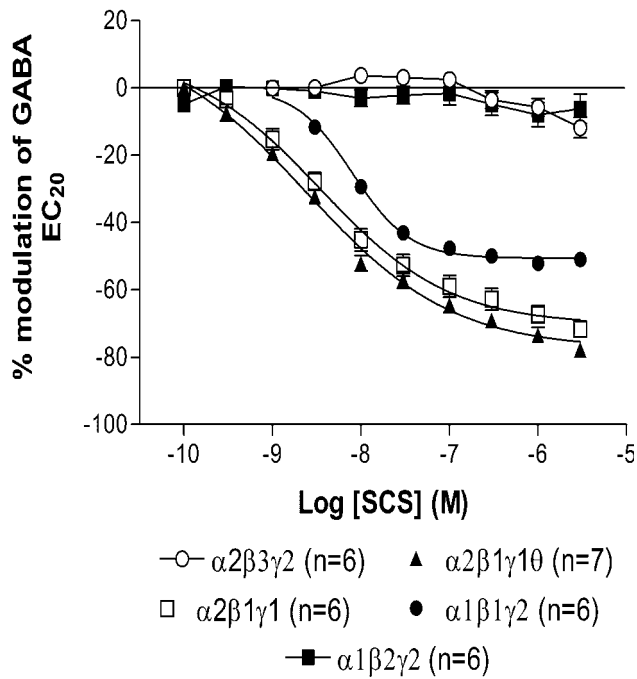


Figure 1 Concentration–response curves to SCS on *Ltk*⁻ cells expressing $\alpha 2\beta 3\gamma 2s$, $\alpha 2\beta 1\gamma 10$, $\alpha 2\beta 1\gamma 1$, $\alpha 1\beta 1\gamma 2s$ and $\alpha 1\beta 2\gamma 2s$ receptors. Inhibition of the GABA EC₂₀ response by SCS was only observed on $\alpha 2\beta 1\gamma 10$, $\alpha 2\beta 1\gamma 1$, $\alpha 1\beta 1\gamma 2s$ receptors. Data are normalized to the control GABA EC₂₀ response immediately prior to construction of the concentration–response curve. Data are mean \pm s.e.m. with the number of cells in each group indicated in brackets.

Table 2 IC₅₀, maximum inhibition and Hill coefficient values for SCS inhibition curves using whole-cell patch clamp on different GABA_A receptors expressed in *Ltk*⁻ cells

Receptor	IC ₅₀ (nM)	Maximum inhibition (%)	Hill coefficient
$\alpha 2\beta 3\gamma 2s$	ND	11.8 \pm 3.0 at 3 μ M	ND
$\alpha 2\beta 1\gamma 10$	4.5 (3.9, 5.1)	74.3 \pm 1.4	-0.75 \pm 0.02
$\alpha 2\beta 1\gamma 1$	5.3 (4.4, 6.5)	68.1 \pm 2.7	-0.81 \pm 0.04
$\alpha 1\beta 1\gamma 2s$	7.9 (7.6, 12.6)*	50.7 \pm 0.9*	-1.36 \pm 0.05*
$\alpha 1\beta 2\gamma 2s$	ND	6.2 \pm 4.8 at 3 μ M	ND

Data represent modulation of the response to an EC₂₀ concentration of GABA, individually determined for each cell. Data for the IC₅₀ values are presented as the geometric mean (-s.e.m., +s.e.m.) and the maximum inhibition as the arithmetic mean \pm s.e.m. of six to seven cells. ND = not determined. **P* < 0.05 vs $\alpha 2\beta 1\gamma 1$ or $\alpha 2\beta 1\gamma 10$ (one-way ANOVA followed by a Tukey–Kramer *post hoc* test).

cells. On $\alpha 2\beta 2\gamma 2s$ receptors SCS, up to 3 μ M, had no effect on the GABA EC₂₀ response compared with a maximum inhibition of 32.8 \pm 4.9%, an IC₅₀ of 4.36 (3.27, 5.81) nM and a Hill coefficient of -1.29 \pm 0.25 (*n* = 4) on $\alpha 2\beta 1\gamma 2s$ receptors (Figure 3b).

A series of chimeras between $\beta 1$ and $\beta 2$ were previously constructed (Wingrove *et al.*, 1994) and used here to delineate the region of the $\beta 1$ subunit that conferred inhibition by SCS (Figure 3a). Each chimera was co-expressed in *Xenopus* oocytes with $\alpha 2$ and $\gamma 2s$ cDNAs and inhibition curves to SCS constructed (Figure 3b). $\alpha 2\beta 2_{1-237}\beta 1_{238-474}\gamma 2s$ gave similar results to wild type $\beta 1$ -containing receptors (maximum inhibition of 44.3 \pm 7.5%, an IC₅₀ of 20.51 (15.70, 26.79) nM

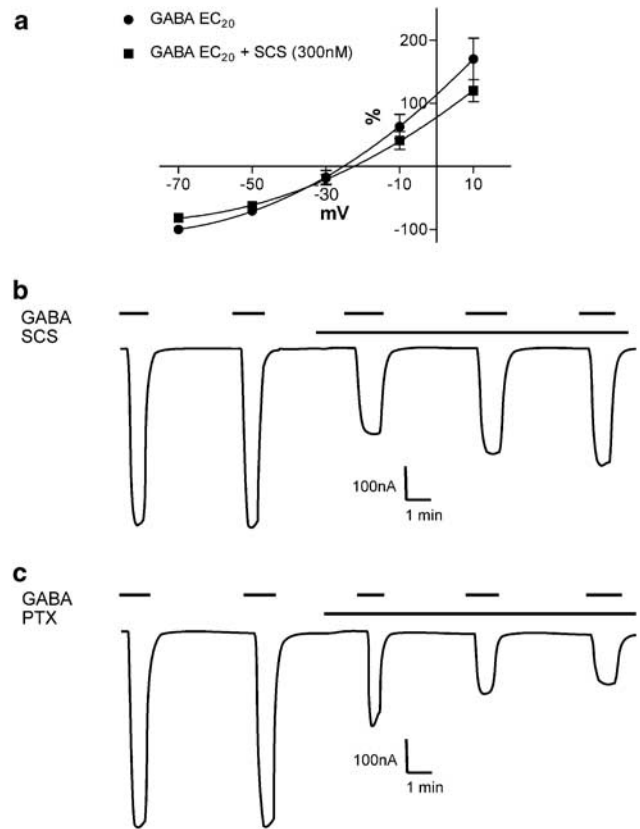


Figure 2 (a) Current–voltage relationship for a GABA EC₂₀ concentration in the absence and presence of SCS (300 nM) on *Xenopus* oocytes expressing $\alpha 2\beta 1\gamma 2s$ receptors. Both *IV* curves (absence and presence of SCS) were performed in one oocyte and replicated four times. Data are normalized to the inward current obtained with the GABA EC₂₀ concentration at -70 mV (= -100%). The inhibition by SCS showed no voltage dependence. Data are mean \pm s.e.m. (b) A representative trace illustrating the lack of use dependence of SCS on a *Xenopus* oocyte expressing $\alpha 2\beta 1\gamma 2s$ receptors. Note the successive decrease in the degree of inhibition of the GABA EC₂₀ from 52 to 41 to 35%. (c) A representative trace illustrating the use dependence of picrotoxin (PTX) on a *Xenopus* oocyte expressing $\alpha 2\beta 1\gamma 2s$ receptors. Note the successive increase in degree of inhibition of the GABA EC₂₀ from 53 to 69 to 75%.

and a Hill coefficient of -1.26 \pm 0.15 (*n* = 4)), whereas $\alpha 2\beta 1_{236}\beta 2_{237-474}\gamma 2s$ and $\alpha 2\beta 1_{1-236}\beta 2_{237-334}\beta 1_{335-474}\gamma 2s$ gave similar results to $\beta 2$ wild type, that is, no inhibition by SCS up to 3 μ M. These results indicated that this small region between K237 and G334 that included TM1–TM3 was responsible for the selectivity of SCS (Figure 3c).

T255 and I308 of the $\beta 1$ subunit are required for inhibition by SCS

Alignment of the predicted amino-acid sequences of the $\beta 1$, $\beta 2$ and $\beta 3$ subunits between amino acids 237 and 334 revealed four amino acids that were different in $\beta 1$ compared to $\beta 2$ and $\beta 3$ ($\beta 1$ T255, S290, I308, K334). These four residues were individually mutated in $\beta 1$ to the $\beta 2/3$ equivalent and in $\beta 2$ to the $\beta 1$ equivalent and co-expressed with $\alpha 2\gamma 2s$. Similar to wild-type receptors, the single point mutations $\beta 1S290N$ and $\beta 1K334R$ were inhibited by SCS; IC₅₀ = 39.5 (33.5, 46.5) nM, maximum inhibition = 32.3 \pm 5.9%, Hill coefficient = -1.13 \pm 0.14

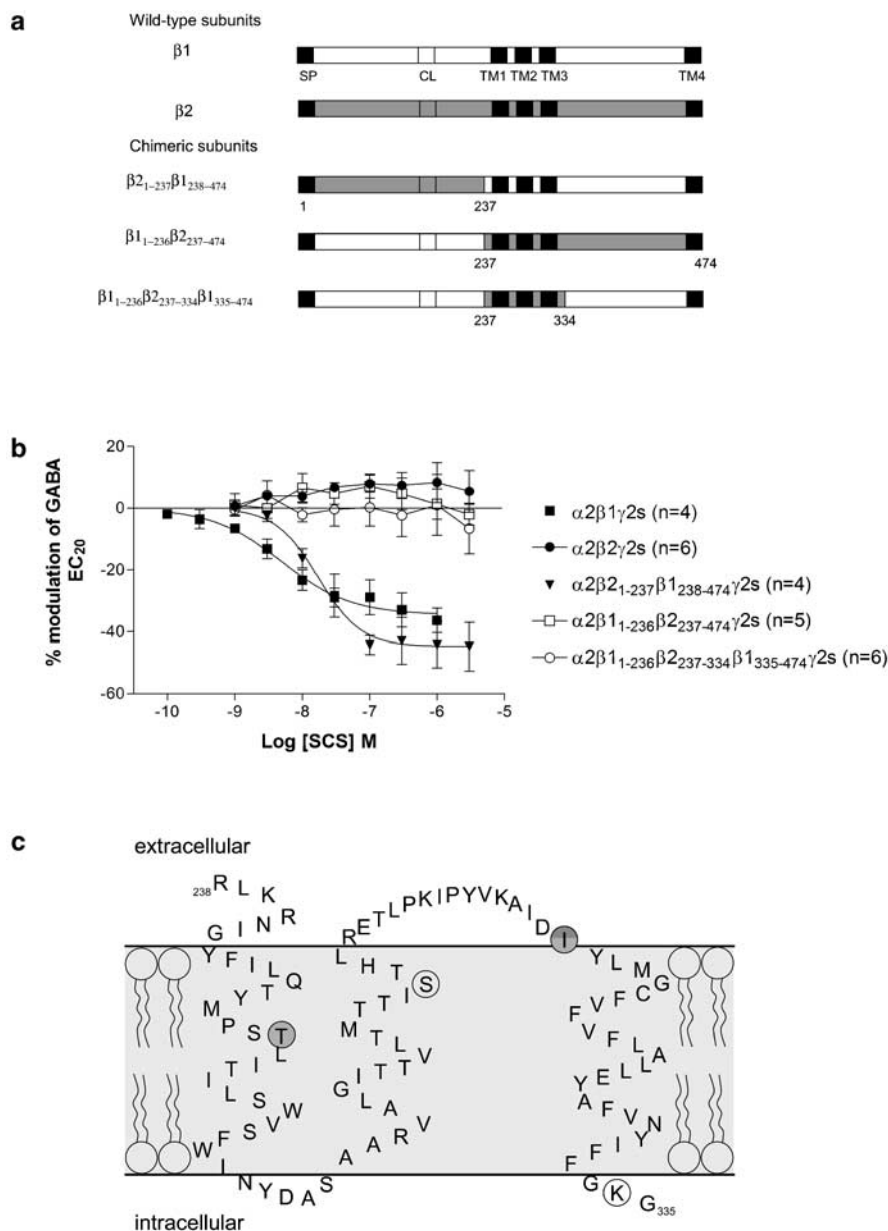


Figure 3 Effect of SCS on the $\beta 1/\beta 2$ chimeras. (a) Diagrammatic illustration of the $\beta 1/\beta 2$ chimeras. $\beta 1$ sequence is represented by clear boxes and $\beta 2$ sequence by shaded boxes. SP = signal peptide, CL = cysteine link, TM = transmembrane. (b) Concentration-response curves to SCS on *Xenopus* oocytes expressing $\alpha 2\beta 1\gamma 2s$, $\alpha 2\beta 2\gamma 2s$, $\alpha 2\beta 2_{1-237}\beta 1_{238-474}\gamma 2s$, $\alpha 2\beta 1_{1-236}\beta 2_{237-474}\gamma 2s$ and $\alpha 2\beta 1_{1-236}\beta 2_{237-334}\beta 1_{335-474}\gamma 2s$ receptors. Data are normalized to the control GABA_{EC20} response immediately prior to construction of the concentration-response curve. Data are mean \pm s.e.m. with the number of cells in each group indicated in brackets. Inhibition by SCS is abolished when a small region encompassing TM1, TM2 and TM3 is replaced in $\beta 1$ with the corresponding $\beta 2$ sequence. (c) Schematic diagram illustrating the critical region responsible for SCS inhibition within the human $\beta 1$ subunit as identified from the $\beta 1/2$ chimeras. The TM regions are illustrated within the light grey area, and are in agreement with those proposed by Korpi *et al.* (2002). The four amino-acid residues that are different between $\beta 1$ and $\beta 2/3$ are circled. The darker two circles correspond to threonine 255 and isoleucine 308, the two residues critical for inhibition by SCS.

and $IC_{50} = 15.8$ (12.2, 20.6) nM, maximum inhibition = $34.2 \pm 5.6\%$, Hill coefficient = -1.29 ± 0.15 , respectively. The maximum inhibition and Hill coefficient for $\alpha 2\beta 1S290N\gamma 2s$ and $\alpha 2\beta 1K334R\gamma 2s$ were not significantly different from wild type, whereas the IC_{50} values were significantly greater than wild type ($P < 0.05$). Conversely $\beta 1T255I$, $\beta 1I308M$ and all the four single point mutations within the $\beta 2$ subunit (I254T, N289S, M307I and R333K) were unaffected by SCS similar to wild-type $\alpha 2\beta 2\gamma 2s$ (Figure 4). These data demonstrate that both T255 and I308 within the $\beta 1$ subunit confer the inhibition

observed with SCS and mutation of either residue abolishes this inhibition. However, when these individual residues were mutated within $\beta 2$ to the $\beta 1$ counterpart inhibition was not conferred. Construction of a $\beta 2$ cDNA containing both these point mutations was unsuccessful, so we were unable to determine if both residues could confer inhibition to $\beta 2$.

White *et al.* (1995) have shown that the EC_{50} value generated from GABA concentration-response curves was not different in oocytes expressing $\alpha 2\beta 1\gamma 2L$ and $\alpha 2\beta 2\gamma 2L$ receptors. In agreement with this, our EC_{20} concentrations used for the two

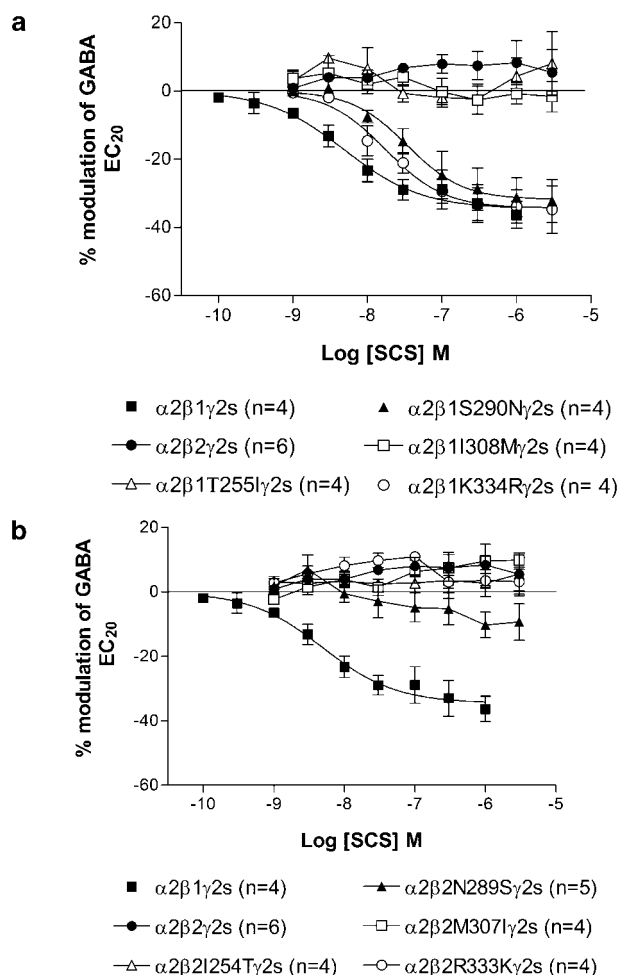


Figure 4 Effect of SCS on the $\beta 1$ (a) and $\beta 2$ (b) single point mutations co-expressed with $\alpha 2$ and $\gamma 2s$ in *Xenopus* oocytes. Data are normalized to the control GABA EC_{20} response immediately prior to construction of the concentration–response curve. Mutation of threonine 255 or isoleucine 308 in $\beta 1$ to the $\beta 2$ equivalent amino-acid was sufficient to abolish inhibition. Data are mean \pm s.e.m. with the number of cells in each group indicated in brackets.

wild-type receptors were not significantly different. Wingrove *et al.* (1994) who expressed the $\beta 1/2$ chimeras and some of the point mutations with $\alpha 1$ and $\gamma 2s$ reported that the affinities for GABA were unchanged. In this study, the EC_{20} values of most of the chimeras and point mutations were not significantly different from the wild-type receptors (the exceptions being $\alpha 2\beta 1\gamma 2s$ vs $\alpha 2\beta 2_{1-237}\beta 1_{238-474}\gamma 2s$ and $\alpha 2\beta 1\gamma 2s$ vs $\alpha 2\beta 2N289S\gamma 2s$ one-way ANOVA with Tukey Kramer *post hoc* test). Caution has to be applied to any significance obtained, since firstly the number of cells expressing the various chimeras and point mutations were low (between 4 and 6) and secondly the EC_{20} concentration used was within the range of EC_{15} – EC_{30} and not determined from a concentration–response curve.

Interaction with other GABA_A receptor ligands

Pentobarbitone is known to directly activate GABA_A receptors *via* a site distinct from the GABA-binding site (Thompson *et al.*, 1996; Pistis *et al.*, 1999). Similar to its effects on GABA-activated currents, SCS (1 μM) inhibited currents induced by a submaximal concentration of pentobarbitone (100 μM) on

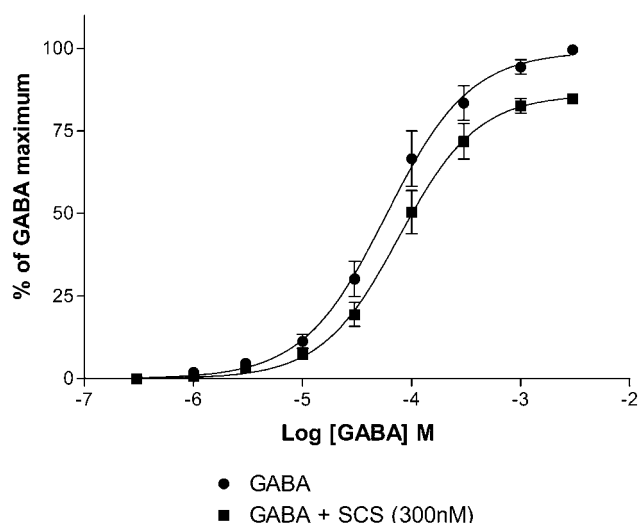


Figure 5 Concentration–response curve to GABA in the absence and presence of SCS on *Xenopus* oocytes expressing $\alpha 2\beta 2\gamma 2s$ receptors. Both concentration–response curves (absence and presence of SCS) were performed in one oocyte and replicated four times. Data were normalized to the current obtained with 3 mM GABA in the absence of SCS. The GABA pEC_{50} and Hill coefficient values were not significantly different (-4.2 vs -4.1 M and 1.1 vs 1.2, respectively), whereas the maximum response was significantly reduced in the presence of SCS (99.1 vs 85.9%, $P = 0.002$). Data are mean \pm s.e.m.

$\alpha 2\beta 1\gamma 2s$ receptors. The degree of inhibition ($39.3 \pm 3.4\%$, $n = 5$) was comparable to that observed with GABA, suggesting that the inhibition is not competitive with GABA but allosteric in nature. To further confirm this hypothesis, a concentration–response curve to GABA in the absence and presence of SCS (300 nM) was constructed. As illustrated in Figure 5, SCS caused a significant reduction in the maximum response with no change in the pEC_{50} or Hill coefficient indicative of a noncompetitive mechanism. To verify that this reduction in maximum was a real effect and not simply run down, a maximum concentration of GABA (3 mM) was applied until reproducible responses were obtained and then repeated in the presence of SCS. As expected, the response to 3 mM GABA was reduced by $33.8 \pm 3.6\%$ ($n = 3$).

Experiments were performed with the benzodiazepine site antagonist flumazenil to investigate a possible interaction of SCS *via* the benzodiazepine-binding site. Flumazenil (1 μM) alone elicited a small degree of inhibition of the GABA EC_{20} response ($8.4 \pm 2.1\%$, $n = 3$). This inhibition was further increased in the presence of SCS (1 μM) to $30 \pm 1.5\%$ ($n = 3$), demonstrating that SCS does not bind to the benzodiazepine-binding site.

Since SCS exhibits only partial block of the GABA response ($36.3 \pm 3.9\%$ at 1 μM), we hypothesized that if SCS interacted *via* the same binding site as a known antagonist, a reduction in the degree of inhibition would be observed. Conversely, if the two compounds interacted at separate sites, the degree of inhibition would be increased (additive effect). Competition experiments with SCS and a number of known GABA_A receptor inhibitors (PTX, PS, DHEAS and bicuculline) were performed. Concentrations of each inhibitor that produced approximately 70% inhibition of a GABA EC_{20} response were determined. As shown in Figure 6, the degree of inhibition

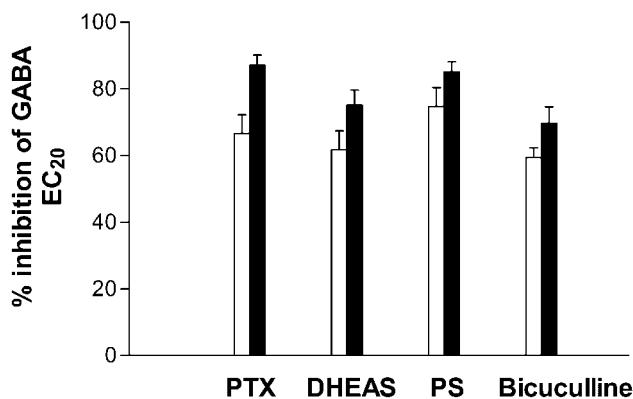


Figure 6 Competition of SCS with PTX, DHEAS, PS and bicuculline on *Xenopus* oocytes expressing $\alpha 2\beta 1\gamma 2s$ receptors. The white bars represent the inhibition of a GABA EC₂₀ by PTX (200 nM), DHEAS (10 μ M), PS (10 μ M) and bicuculline (6 μ M), whereas the black bars represent the inhibition observed upon co-application of SCS (1 μ M). Data are mean \pm s.e.m. of four oocytes for each inhibitor.

with all the four inhibitors was further increased upon co-application of SCS, suggesting additive effects.

A number of allosteric modulators demonstrate β -subunit selectivity, for example, loreclezole, etomidate, furosemide (Wafford *et al.*, 1994; Hill-Venning *et al.*, 1997; Thompson *et al.*, 1999). Regardless of whether the compound inhibits (e.g. furosemide) or potentiates (e.g. loreclezole) the GABA response, the selectivity reported has been for $\beta 2/3$ -containing receptors vs $\beta 1$ -containing receptors. More recently, Halliwell *et al.* (1999) have described the selectivity profile for the nonsteroidal anti-inflammatory agent mefenamic acid. Similar to loreclezole, this compound potentiated GABA-activated currents on $\alpha 1\beta 2\gamma 2s$ receptors but not on $\alpha 1\beta 1\gamma 2s$. More relevant to this study was the observation that on $\alpha 1\beta 1$ receptors, GABA-activated currents were inhibited by mefenamic acid compared with potentiation on $\alpha 1\beta 2$. Experiments were performed to determine whether SCS influenced the functional response to loreclezole on $\alpha 2\beta 2\gamma 2s$ receptors and mefenamic acid on $\alpha 1\beta 1$ receptors. As illustrated in Figure 7, SCS (1 μ M) had no significant effect on the potentiation of a GABA EC₂₀ by loreclezole (10 μ M) (256 ± 40 vs $222 \pm 26\%$, $n = 4$) or the inhibition of a GABA EC₂₀ by mefenamic acid. SCS (1 μ M) produced $57.5 \pm 1.9\%$ inhibition at $\alpha 1\beta 1$ receptors, and as previously reported; 300 μ M mefenamic acid produced $82.7 \pm 2.9\%$ inhibition. When combined, 300 μ M mefenamic acid plus 1 μ M SCS elicited $90.7 \pm 1.2\%$ inhibition ($n = 4$) slightly greater than mefenamic acid alone, again suggesting a purely additive effect.

Discussion

SCS was initially identified from a high-throughput VIPR screen as being a potent partial subtype-selective inhibitor of GABA_A receptors. This selectivity was not specific to SCS since a number of structurally similar compounds also displayed $\beta 1$ selectivity. Detailed electrophysiological experiments identified SCS as having a unique pharmacology producing selective partial inhibition of $\beta 1$ -containing receptors.

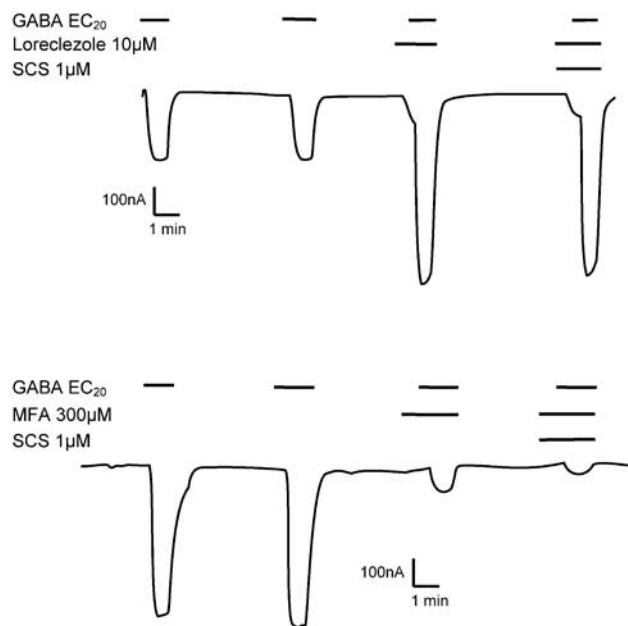


Figure 7 (a) A representative trace illustrating the effect of SCS on the potentiation of a GABA EC₂₀ by the $\beta 2/3$ selective modulator loreclezole on an oocyte expressing $\alpha 2\beta 2\gamma 2s$ receptors. (b) A representative trace illustrating the effect of SCS on the inhibition of a GABA EC₂₀ by mefenamic acid on an oocyte expressing $\alpha 1\beta 1$ receptors. Loreclezole, mefenamic acid and SCS were applied 45 s before coapplication of GABA EC₂₀. Current amplitude and time are indicated by the scale bars. A reduction in the amount of potentiation to loreclezole or inhibition to mefenamic acid by SCS would indicate competition and hence commonality of the two modulators at the binding or transduction level.

Comparison with other β -selective compounds

In recent years, a number of modulators of the GABA_A receptor have been reported that demonstrate $\beta 2/3$ selectivity over $\beta 1$, for example, loreclezole, etomidate, tracazolol, mefenamic acid, furosemide (Wafford *et al.*, 1994; Hill-Venning *et al.*, 1997; Halliwell *et al.*, 1999; Thompson *et al.*, 1999; 2002). In all cases, the potency of the modulator was reduced or abolished when serine was introduced into $\beta 2$ or $\beta 3$ (position 289 in human $\beta 2$ and 290 in human $\beta 3$) and increased or conferred when asparagine was introduced into $\beta 1$ (position 290). SCS differed from these other modulators since although it inhibited $\alpha 2\beta 1S290N\gamma 2s$ receptors with reduced potency maximum efficacy was unaffected, and SCS had no effect on $\alpha 1\beta 2N289S\gamma 2s$.

Mefenamic acid, loreclezole and SCS are broadly structurally similar in that they are composed of two aromatic or heteroaromatic rings linked by spacers of varying length. At physiological pH, the majority of SCS molecules will be nonionized, suggesting that the binding site is likely to exist in a lipid environment (the calculated pK_a for deprotonation of the phenolic groups in SCS is 8.4–8.5), compared with mefenamic acid which exists mainly as the deprotonated carboxylic acid (pK_a = 3.7), suggesting an aqueous environment for the binding site and loreclezole which is nonionized (for protonation of the triazole pK_a = 1.8).

Importance of threonine 255 and isoleucine 308

Mutation of either threonine 255 or isoleucine 308 within the $\beta 1$ subunit to the $\beta 2$ counterpart was sufficient to abolish the

inhibition; however, the converse individual mutations within $\beta 2$ did not introduce any inhibition. To our knowledge, these residues have not been implicated in the action of other GABA_A receptor modulators. A minor alteration to a compound's binding site or transduction pathway is often sufficient to abolish activity. It is much more difficult, however, to confer activity since this effect is often the net result of a number of processes involving many different regions of the protein. Although it is not certain if isoleucine 308 forms part of the TM2–TM3 loop or the TM3 domain itself (Bera *et al.*, 2002; Korpi *et al.*, 2002), the recent work of Bera *et al.* (2002) showed that in the $\alpha 1$ subunit the corresponding residue when mutated to cysteine was not accessible to MTS reagents, indicating that it is either tightly packed in the protein structure or that the covalent modification was functionally silent. Similarly, the residue corresponding to that of threonine 255 in the torpedo nicotinic acetylcholine receptor has been labelled with a hydrophobic probe (Blanton & Cohen, 1994).

From the data obtained, we suggest that SCS binds to a novel site on the GABA_A receptor which may incorporate the residues threonine 255 and isoleucine 308. SCS may share a similar transduction mechanism to loreclezole and mefenamic acid, etc., involving serine/asparagine 290.

Possible role of metal chelation

An alternative possible mechanism of action could involve the ability of SCS to chelate metal ions, as recently demonstrated by Wilkins & Smart (2002) for the potentiating effect of dithiothreitol. The assumption here is that a contaminating metal ion within the buffers used could potentiate GABA responses to $\beta 1$ -containing receptors but not to $\beta 2$ - or $\beta 3$ -containing receptors. Chelation of this metal ion by SCS thereby removes this potentiation, which is revealed by an apparent inhibition. For this suggestion to be feasible, the metal ion would need to be more potent than SCS, be selective for $\beta 1$ -containing receptors and induce potentiation. Of the metal ions that interact with GABA_A receptors,

most have been shown to inhibit GABA responses with no β selectivity.

Experiments to probe the binding site and role of $\beta 1$ subunits

We have demonstrated that SCS did not interact with any of the known allosteric modulators investigated in this study, suggesting an interaction at a novel binding site. The lack of voltage and use dependence would indicate that the binding site is not within the ion channel, but residues close to the channel are important for SCS inhibition. Although a tritiated form of SCS was synthesized, experiments designed to probe the binding site of SCS could not be performed due to very high nonspecific binding.

The $\beta 1$ subunit is widely distributed in the brain (Pirker *et al.*, 2000), making it difficult to speculate as to what therapeutic use a $\beta 1$ -selective inhibitor would have. Two recent reports using 'knock-in' mouse lines have demonstrated that the clinical effects of the anaesthetic agents etomidate and propofol can be attributed to either the $\beta 2$ (sedative-hypnotic effect) or the $\beta 3$ (anaesthetic effect) subunit (Jurd *et al.*, 2003; Reynolds *et al.*, 2003). In the absence of $\beta 1$ knockout mice, it was hoped that having identified a $\beta 1$ -selective compound we would be able to perform *in vivo* experiments in order to understand the role of $\beta 1$ -containing GABA_A receptors. Unfortunately, these studies were precluded by poor *in vivo* pharmacokinetics, making it difficult to utilize this compound to determine the role of $\beta 1$ -containing GABA_A receptors.

In conclusion, the present study describes the identification and characterization of SCS as a selective partial inhibitor of $\beta 1$ -containing GABA_A receptors. SCS showed no interaction with any of the GABA_A receptor modulators tested or any voltage or use dependence. Mutagenesis studies revealed the importance of threonine 255, located within TM1, and isoleucine 308, located just prior to TM3, as critical residues within $\beta 1$ for conferring the inhibition. We hypothesize that SCS may interact at a previously unidentified site and exerts its effects *via* an allosteric mechanism.

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