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Influence of membrane cholesterol on modulation of the $GABA_A$ receptor by neuroactive steroids and other potentiators

[1,2]T. Sooksawate & *,[1]M. A. Simmonds

[1]Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX

1 The influence of membrane cholesterol on some pharmacological properties of the $GABA_A$ receptor was investigated in acutely dissociated rat hippocampal neurones with whole cell patch clamp recording. The cholesterol levels were varied between 56% and 235% control using methyl-β-cyclodextrin as the cholesterol carrier.

2 Enrichment of neurones with cholesterol increased the effects of the non-steroidal GABA potentiators propofol, flunitrazepam and pentobarbitone. A similar result was obtained after pre-incubation of neurones with epicholesterol, the 3α-hydroxy isomer of cholesterol.

3 In contrast, the effects of the steroidal GABA potentiators pregnanolone and alfaxalone were reduced by cholesterol enrichment, but not by epicholesterol. Depletion of membrane cholesterol increased the potentiation of GABA by pregnanolone and alfaxalone but did not affect the non-steroidal potentiators.

4 The steroidal antagonist of GABA, pregnenolone sulphate, reduced the maximum response to GABA. This effect, also, was diminished in cholesterol-enriched neurones and enhanced in cholesterol-depleted neurones.

5 The effects of the cholesterol manipulations that were selective for the steroidal modulators of GABA are suggested to arise from direct interactions between membrane cholesterol and the $GABA_A$ receptor. The separate effects on the non-steroidal potentiators of GABA of cholesterol-enrichment or addition of epicholesterol to the neurones are suggested to be due to changes in membrane fluidity.

6 In view of the likely physiological modulation of $GABA_A$ receptors by endogenous neuroactive steroids and evidence of the *in vivo* lability of membrane cholesterol, the present observations may have physiological as well as pharmacological relevance.

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Abbreviations: DMSO, dimethyl sulphoxide; GABA, γ-aminobutyric acid; PSS, physiological salt solution

Introduction

Several classes of drug receptor have been shown to be influenced by the cholesterol content of the cell membranes in which they are located. They include the nicotinic acetylcholine receptor of *Torpedo* (Fong & McNamee, 1986; Fernandez-Ballester *et al*., 1994; Rankin *et al*., 1997), oxytocin and cholecystokinin receptors (Gimpl *et al*., 1997) and the $GABA_A$ receptor (Sooksawate & Simmonds, 1998; 2001). Two types of mechanism have been distinguished whereby cholesterol might modulate receptor function: (1) an effect on membrane fluidity, which would be relatively unselective with regard to the receptors affected, and independent of the means of changing membrane fluidity; (2) a specific interaction of cholesterol with a binding site on the receptor protein, which would be expected to show a more restricted occurrence and a more stringent structure-activity requirement. In a comprehensive exploration of this distinction for two G-protein linked receptors, Gimpl *et al.* (1997) have shown that ligand binding to the brain cholecystokinin receptor is influenced predominantly by membrane fluidity whereas ligand binding to the oxytocin receptor is influenced predominantly by a specific cholesterol-receptor interaction.

In studies on the $GABA_A$ receptor, we have reported that enrichment of dissociated hippocampal neurones with cholesterol by the phosphatidylcholine liposome method caused a small reduction in GABA potency (Sooksawate & Simmonds, 1998). The GABA potentiating effect of the neuroactive steroids, but not that of propofol, was also reduced. The maximum cholesterol enrichment that we could obtain with the liposome method was 26% and we were unable to achieve depletion of cholesterol at that time. Subsequently, the methyl-β-cyclodextrin carrier protocols of Gimpl *et al.* (1997) were used to obtain a wider range of cholesterol enrichments and depletions and, in these experiments, the effects on GABA potency were found to be more substantial and complex (Sooksawate & Simmonds, 2001). The reduction in GABA potency with cholesterol enrichment was confirmed and suggested to be due to a reduced membrane fluidity. Cholesterol depletion, however, also reduced GABA potency, revealing a requirement for cholesterol to maintain optimal GABA potency that

*Author for correspondence; E-mail: mike.simmonds@ulsop.ac.uk
[2]Current address: Department of Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

probably involves a specific binding site for cholesterol on the $GABA_A$ receptor. In the same series of experiments, some neuroactive steroids and other GABA potentiators were studied and it is this aspect of the work that we now report.

Methods

Dissociation of hippocampal neurones

Dissociated hippocampal neurones were prepared from male Wistar rats aged 10–16 days as previously described (Sooksawate & Simmonds, 1998). In brief, hippocampal slices in physiological salt solution (PSS), containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, Glucose 11, HEPES 10, adjusted to pH 7.4 with Tris-base, were subjected to enzyme treatment followed by trituration. The suspension of dissociated neurones and cell debris were separated by centrifugation through a 5% solution of bovine serum albumin in PSS at $175 \times g$ for 3 min to yield a loose pellet of neurones.

Manipulation of membrane cholesterol

A complex of cholesterol + methyl-β-cyclodextrin was prepared by stirring together 3 mM cholesterol with 30 mM methyl-β-cyclodextrin in PSS, under nitrogen, until a clear solution was obtained. This stock solution was filtered through a 0.22 μm Millipore filter and stored at -20°C.

To enrich hippocampal neurones with cholesterol, half of each preparation of dissociated neurones was incubated at 31°C in oxygen-saturated PSS containing either a 1 in 20 dilution or a 1 in 10 dilution of the cholesterol + methyl-β-cyclodextrin stock solution for 5–30 min, depending on the degree of enrichment required. The cholesterol transfer was terminated by centrifugation at $340 \times g$ for 3 min and two washes of the neurones with PSS. The other half of the neurones was subjected to the same procedure but without the cholesterol + methyl-β-cyclodextrin complex and these were designated control neurones.

To deplete membrane cholesterol, dissociated neurones were incubated with 10 mM methyl-β-cyclodextrin in PSS, using a similar protocol to that described for cholesterol enrichment, to obtain a range of depletions.

To deplete and then restore membrane cholesterol, dissociated neurones were incubated at 31°C in oxygen-saturated PSS containing 10 mM methyl-β-cyclodextrin for 20 min. Following centrifugation at $340 \times g$ for 3 min, the pellet was resuspended in oxygen-saturated PSS containing a 1 in 30 dilution of the cholesterol + methyl-β-cyclodextrin stock solution and incubated at 31°C for 1–10 min. The cholesterol transfer was terminated by centrifugation at $340 \times g$ for 3 min and two washes of the neurones with PSS.

To incorporate epicholesterol into the neuronal membranes, a stock solution of epicholesterol complexed with methyl-β-cyclodextrin was prepared exactly as described for cholesterol. Dissociated neurones were incubated with a 1 in 20 dilution of the stock solution for 20 min, using the same protocol as for enrichment with cholesterol.

The batches of hippocampal neurones subjected to these procedures were the same as those reported in Sooksawate & Simmonds (2001) where quantitative details of the cholesterol levels achieved can be found.

Cholesterol and protein assays in dissociated neurones

After plating samples of the pretreated neurones for electrophysiological recording, the remaining neurones were frozen for later assays of cholesterol and protein. For the assays, the thawed neurones were homogenized in ice-cold wash buffer containing (mM): Tris-base 5, EDTA 1, pH 7.4 at 4°C. The homogenate was centrifuged at $48{,}400 \times g$ and the pellet resuspended in ice cold wash buffer. Cholesterol and protein assays were carried out on the suspensions using the Sigma cholesterol diagnostic kit (Sigma Diagnostics, St Louis MO, U.S.A.) and the BioRad Protein Assay (Bio-Rad Laboratories, Munich, Germany), respectively.

Electrophysiological measurements

Patch pipettes prepared from thin wall borosilicate glass capillaries (Clark Electromedical Instrument, U.K.) were filled with a solution containing (mM): CsCl 140, $MgCl_2$ 4, Na_2 ATP 4, EGTA 11, $CaCl_2$ 1, and HEPES 10, adjusted to pH 7.2 with Tris-base. The resistance between the patch-pipette and a reference electrode in the external solution (PSS) ranged from 3–5 MΩ.

Neurones plated onto coverslips were allowed to adhere for at least 30 min before attempting to attach a patch electrode for whole-cell patch clamp recording (Hamil *et al*., 1981). Membrane currents were recorded from the somata of the neurones, voltage-clamped at −20 mV. Membrane current was measured with a List EPC-7 patch clamp amplifier and was recorded with a Gould pen recorder. During recording, neurones were superfused with PSS at a rate of 3 ml min^{-1}. All experiments were performed at room temperature (~20°C).

Drugs and chemicals

GABA was dissolved in PSS and applied close to the recorded neurone for 3 s, once a minute, using the rapid application 'U-tube' method previously described (Sooksawate and Simmonds, 1998). Pentobarbitone sodium was dissolved in PSS; pregnenolone (5β-pregnan-3α-ol-20-one), alfaxalone (5α-pregnan-3α-ol-11,20-dione), propofol, flunitrazepam and pregnenolone sulphate (5-pregnen-3β-ol-20-one sulphate) were dissolved in DMSO and then diluted into PSS. These drugs were applied simultaneously with GABA in the same solution. The final concentration of DMSO applied to the neurones was less than 0.1%, which was shown to have no effect of its own on the responses to GABA.

All drugs and chemicals were purchased from Sigma with the following exceptions: alfaxalone was a gift from Glaxo Group Research (Greenford, U.K.); epicholesterol was purchased from Steraloids (U.S.A.); and methyl-β-cyclodextrin from Aldrich (U.K.). CsCl, $MgCl_2$ and $CaCl_2$ were from BDH (Poole, U.K.).

Data analysis

For concentration-response analysis data from each neurone were plotted using GraphPad PRISM (GraphPad Software, U.S.A.) and fitted with a logistic equation in the form:

$$I = I_{min} + (I_{max} - I_{min})/(1 + (EC_{50}/[X])^{H}) \quad (1)$$

where I is the GABA current at GABA concentration [X]. I_{min} and I_{max} are the minimal and the maximal responses, EC_{50} is the concentration of GABA eliciting 50% of the maximal response and H is the Hill coefficient. To combine data from different neurones, the GABA responses on each neurone were expressed as percentages of the derived maximum response to GABA on the same neurone. Combined data are expressed as mean ± s.e.mean and differences were tested for significance by ANOVA and Dunnett's test, $P<0.05$ being considered significant.

The maximum current evoked by GABA, although reproducible in individual neurones, differed considerably between neurones. Consequently, the effects of membrane cholesterol on the maximum response to GABA could not be determined with any confidence because different neurones were recorded for each cholesterol condition. In contrast, EC_{50} values for GABA are highly reproducible across different neurones (Sooksawate & Simmonds, 2001).

Results

Membrane cholesterol and the potentiation of GABA by neuroactive steroids

In control neurones, not subjected to manipulation of membrane cholesterol, 1 μM pregnanolone caused the expected increase in submaximal responses to GABA (Figure 1) which was quantified as a decrease in GABA EC_{50} (Table 1). The potentiation was not associated with any obvious

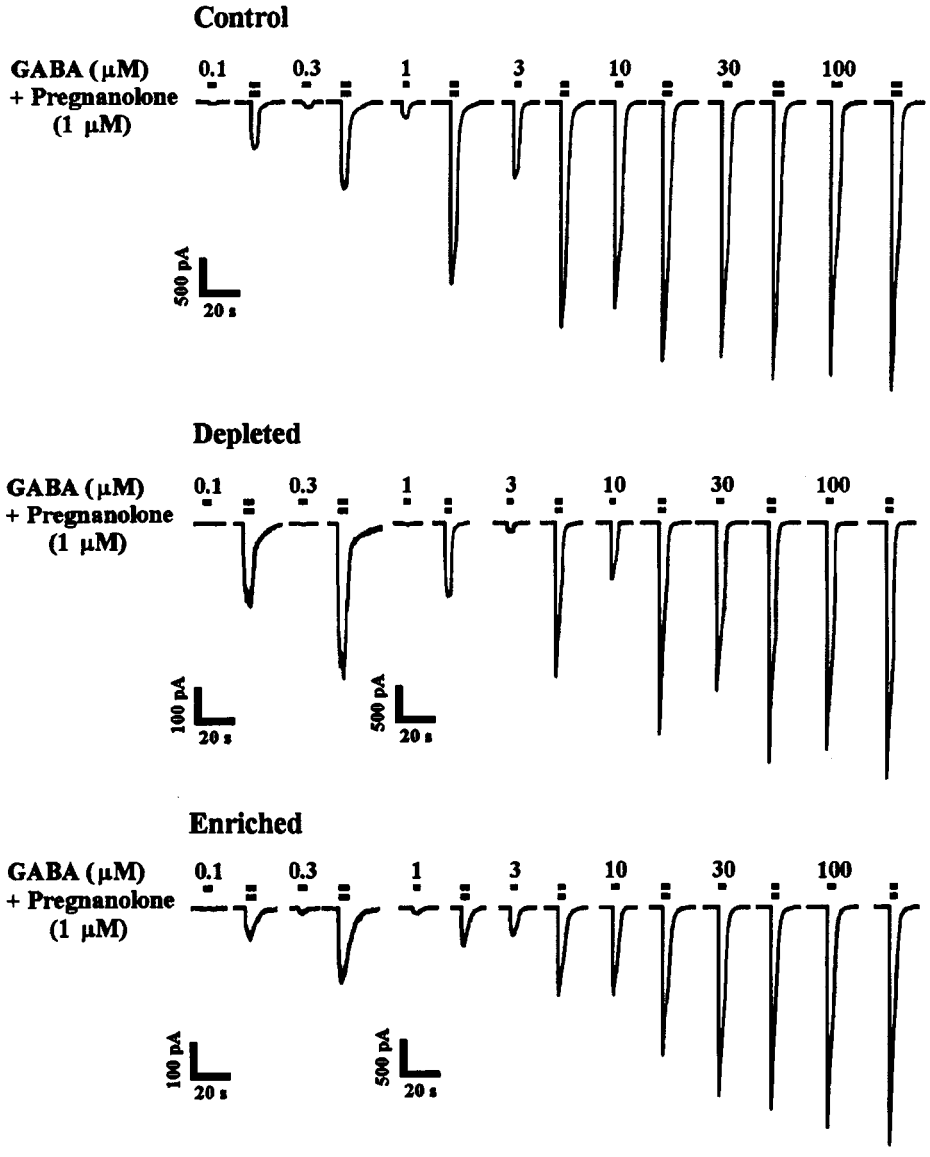

Figure 1 Examples of whole cell current responses to GABA alone and GABA + 1 μM pregnanolone in a control neurone, a neurone depleted of cholesterol to 56% control and a neurone enriched with cholesterol to 235% control. Applications of pregnanolone alone gave no response (data not shown). For display purposes, the responses to GABA alone and GABA + pregnanolone are shown alternately, with increasing GABA concentration. In the actual experiments, the responses to GABA alone were all obtained first, with increasing concentrations of GABA, followed by all the responses to GABA + pregnanolone.

Table 1 Membrane cholesterol depletion and enrichment, and the incorporation of epicholesterol into control membranes, influences the potentiation of GABA by various drugs

		GABA EC_{50} ratio		
Potentiator	*Control*	*Cholesterol-depleted* (56% control)	*Cholesterol-enriched* (182% control)	*Epicholesterol incorporated*
1 μM pregnanolone	0.197±0.014 (9)	0.115±0.011 **(6)	0.293±0.024 §§ (9)	0.151±0.014 (9)
1 μM alfaxalone	0.275±0.015 (9)	0.185±0.008 ** (7)	0.358±0.022 §§ (7)	ND
5 μM propofol	0.228±0.014 (10)	0.196±0.023 (9)	0.122±0.008 ** (9)	0.131±0.021 ** (8)
10 μM flunitrazepam	0.477±0.018 (10)	0.397±0.039 (10)	0.276±0.017 ** (8)	0.297±0.010 ** (8)
20 μM pentobarbitone	0.563±0.023 (8)	0.518±0.033 (8)	0.437±0.013 ** (10)	0.477±0.021 * (8)

Potentiation of GABA is expressed as GABA EC_{50} ratio, i.e. (GABA EC_{50} in the presence of a potentiator)/ (GABA EC_{50} prior to addition of the potentiator); thus, the smaller the value, the greater is the potentiation. Each value is the mean±s.e.mean from the numbers of neurons shown in brackets. Increases in GABA EC_{50} ratio, compared with control, are indicated as §§ ($P<0.01$), and decreases as * ($P<0.05$) and ** ($P<0.01$) (ANOVA + Dunnett's test). ND = not done. Typical EC_{50} values for GABA alone, taken from the pregnanolone series of experiments, were 5.15±0.14 μM ($n=9$) for control neurones; 20.4±0.93 μM ($n=6$) for cholesterol-depleted neurones; 15.3±0.66 μM ($n=9$) for cholesterol-enriched neurones; 15.8±0.56 μM ($n=9$) for epicholesterol-incubated neurones.

reduction in the fade of the response during the application of GABA. The maximum response to GABA, determined from the fitted curve (Figure 2), showed only a marginal increase of 6.3±1.89% ($P<0.05$, $n=9$) due to pregnanolone. The Hill slope was significantly decreased by pregnanolone (Table 2).

A similar overall pattern of effect of pregnanolone was seen with neurones that had been depleted of cholesterol to 56% control and with neurones that had been enriched with cholesterol to 235% control (Figures 1 and 2), except that the effect of pregnanolone on GABA EC_{50} was enhanced by cholesterol depletion and reduced by cholesterol enrichment (Table 1). Cholesterol depletion and enrichment did not change the Hill slope of the GABA concentration-response relationship nor the extent of its reduction by pregnanolone (Table 2). The marginal increase in maximum response to GABA due to pregnanolone persisted unchanged in the cholesterol-depleted neurones as a 4.6±1.99% increase ($P<0.05$, $n=6$) and in the cholesterol-enriched neurones as a 1.9±0.81% increase ($P<0.05$, $n=9$). It can also be seen in Figure 2 that both cholesterol depletion and enrichment increased the EC_{50} for GABA alone (see also the footnote to Table 1), an aspect of the data from these experiments that has already been reported in detail (Sooksawate & Simmonds, 2001).

Figure 3 shows that progressive decreases in membrane cholesterol were associated with progressive increases in the potentiation of GABA by pregnanolone, the increases in potentiation being statistically significant for cholesterol depletions to 67 and 56% control. Enrichment of membrane cholesterol to 156% control was associated with a statistically significant decrease in the potentiation of GABA by pregnanolone, and no further progression of this effect with cholesterol enrichments up to 235% control.

Alfaxalone 1 μM caused less potentiation of GABA than did 1 μM pregnanolone but the same dependency of the potentiation on membrane cholesterol was seen (Table 1).

Further experiments were made to check that the increase in effect of the neuroactive steroid associated with the depletion of cholesterol did, in fact, result from the loss of cholesterol, rather than from some other influence of the incubation with a high concentration of methyl-β-cyclodextrin. Figure 4 shows that the specific repletion of cholesterol, following its depletion, fully reversed the increase in effect of pregnanolone.

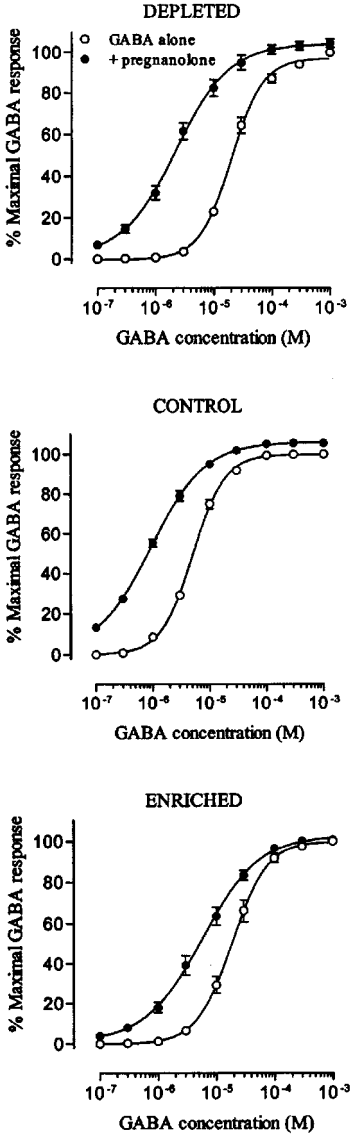

Figure 2 Influence of membrane cholesterol on the potentiation of GABA by 1 μM pregnanolone. In each panel, the responses to GABA alone (○) and to GABA in the presence of pregnanolone (●) are expressed as per cent of the calculated maximum response to GABA alone. Each data point is the mean±s.e. mean of six (depleted), nine (control) and eight (enriched) experiments. Cholesterol depletion was to 56% of control and enrichment to 235% control.

Table 2 Effect of 1 μM pregnanolone on the Hill slope of the GABA concentration–response relationship and the influence of membrane cholesterol

	Hill slope (mean ± s.e.mean)		
	Control	*Cholesterol-depleted* (56% control)	*Cholesterol-enriched* (182% control)
GABA alone	1.614 ± 0.126 (9)	1.627 ± 0.123 (6)	1.557 ± 0.073 (8)
GABA + 1 μM pregnanolone	1.016 ± 0.104 ** (9)	0.954 ± 0.055 ** (6)	0.961 ± 0.064 ** (8)

** $P<0.005$ (Student's t-test) compared with corresponding value for GABA alone.

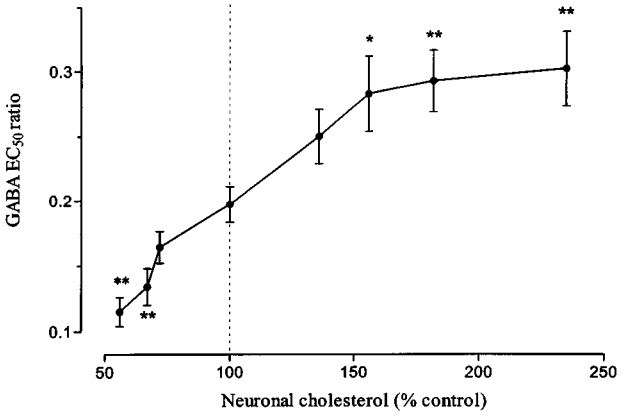

Figure 3 Potentiation of GABA by 1 μM pregnanolone, expressed as GABA EC_{50} ratios, i.e. (GABA EC_{50} in the presence of pregnanolone)/(GABA EC_{50} prior to addition of the pregnanolone). Each point is the mean ± s.e.mean from 6–9 neurones. Depletion of membrane cholesterol enhanced the potentiation (reduced EC_{50} ratio) and enrichment of cholesterol decreased the potentiation. Significant differences from control neurones are indicated by $*P<0.05$ and $**P<0.01$ (ANOVA + Dunnett's test). The cholesterol content of control neurones was 0.319 ± 0.009 μmoles mg protein^{-1} ($n=12$). Details of the cholesterol contents at each level of depletion and enrichment, and the protocol used to achieve them, are reported in Sooksawate & Simmonds (2001).

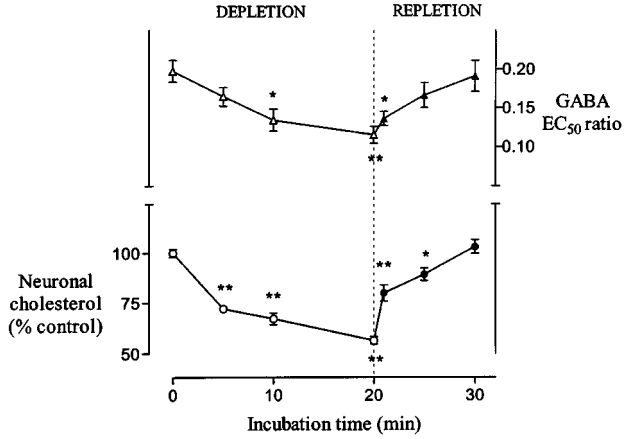

Figure 4 Effect of cholesterol depletion and repletion on the potentiation of GABA by 1 μM pregnanolone, expressed as GABA EC_{50} ratios. Reduction in the EC_{50} ratio represents an enhanced potentiation. Each point is the mean ± s.e.mean from 6–9 neurones. The cholesterol depletion was achieved by incubation with 10 mM methyl-β-cyclodextrin at 31°C and repletion by incubation with a complex of 0.1 mM cholesterol + 1 mM methyl-β-cyclodextrin at 31°C. Significant differences from the value at time 0 are indicated by $*P<0.05$ and $**P<0.01$ (ANOVA + Dunnett's test).

If the influences of the cholesterol manipulations on GABA potency and the potentiation of GABA by pregnanolone are taken as a whole, the EC_{50} for GABA in the presence of 1 μM pregnanolone in control neurones (0.97 μM) is significantly increased ($P<0.01$) by both cholesterol depletion (to 2.30 μM) and enrichment (to 5.68 μM). The influence of cholesterol depletion is a dominant reduction in GABA potency overiding an enhanced potentiation by pregnanolone, whereas the influence of enrichment is the additive effect of a reduction in GABA potency and a reduced potentiation by pregnanolone.

Membrane cholesterol and the potentiation of GABA by propofol, flunitrazepam and pentobarbitone

The GABA log. concentration-response curve was shifted to the left by each of the potentiators 5 μM propofol, 10 μM flunitrazepam and 20 μM pentobarbitone (Table 1) with no statistically significant effect on the maximum response to GABA (data not shown). Thus, in these respects, the potentiations of GABA closely resembled those due to neuroactive steroids. The influence of membrane cholesterol was, however, different. Depletion of cholesterol to 56% control had no significant effect on the potentiation of GABA by either propofol or flunitrazepam or pentobarbitone (Table 1). Enrichment with cholesterol to 182% control caused small, but statistically significant, enhancements in the potentiations by each of these three drugs, in contrast to the decrease in potentiation due to neuroactive steroids (Table 1).

Modulation of GABA potentiations by epicholesterol incorporated into the membrane

Methyl-β-cyclodextrin has previously been shown to be as efficient at adding epicholesterol, the 3α-OH isomer of cholesterol, to cell membranes as it is at adding cholesterol (Gimpl *et al*., 1997). The same authors have also shown that epicholesterol has similar effects to cholesterol on membrane fluidity. Experiments were, therefore, performed to determine whether addition of epicholesterol to the neuronal membranes would mimic the effects of enrichment with cholesterol on the GABA potentiators. It was established that incubation of neurones with a complex of epicholesterol and methyl-β-cyclodextrin caused no coincident loss of membrane cholesterol, which remained at 98.3 ± 2.47% control (mean ± s.e.mean, $n=3$).

The incubation with epicholesterol had similar effects to enrichment with cholesterol on the potentiation of GABA by propofol, flunitrazepam and pentobarbital (Table 1). In

contrast, epicholesterol failed to mimic the effect of cholesterol enrichment on the potentiation of GABA by pregnanolone. Rather than the reduction in effect of pregnanolone, there was a tendency towards an increase in effect, corresponding with the increased effects of the other GABA potentiators (Table 1).

Membrane cholesterol and the antagonism of GABA by pregnenolone sulphate

The selective interaction of membrane cholesterol with the GABA potentiating neuroactive steroids prompted a similar investigation with pregnenolone sulphate, a neurosteroid antagonist of GABA. In control neurones, 20 μM pregnenolone sulphate doubled the EC_{50} for GABA and reduced the maximum response (Figures 5 and 6, Table 3). Both effects were statistically significant ($P>0.001$). The antagonism of GABA was associated with a marked increase in the fade of the response during the application of GABA (Figure 5). Depletion of membrane cholesterol to 56% control significantly enhanced the effect of pregnenolone sulphate in reducing the GABA maximum, whilst enrichment with cholesterol to 182% control significantly decreased the effect of pregnenolone sulphate (Table 3). The GABA EC_{50} ratio for pregnenolone sulphate remained unchanged.

Discussion

The non-steroidal potentiators of GABA examined in this study, propofol, flunitrazepam and pentobarbitone, each interact with their own unique recognition sites on the

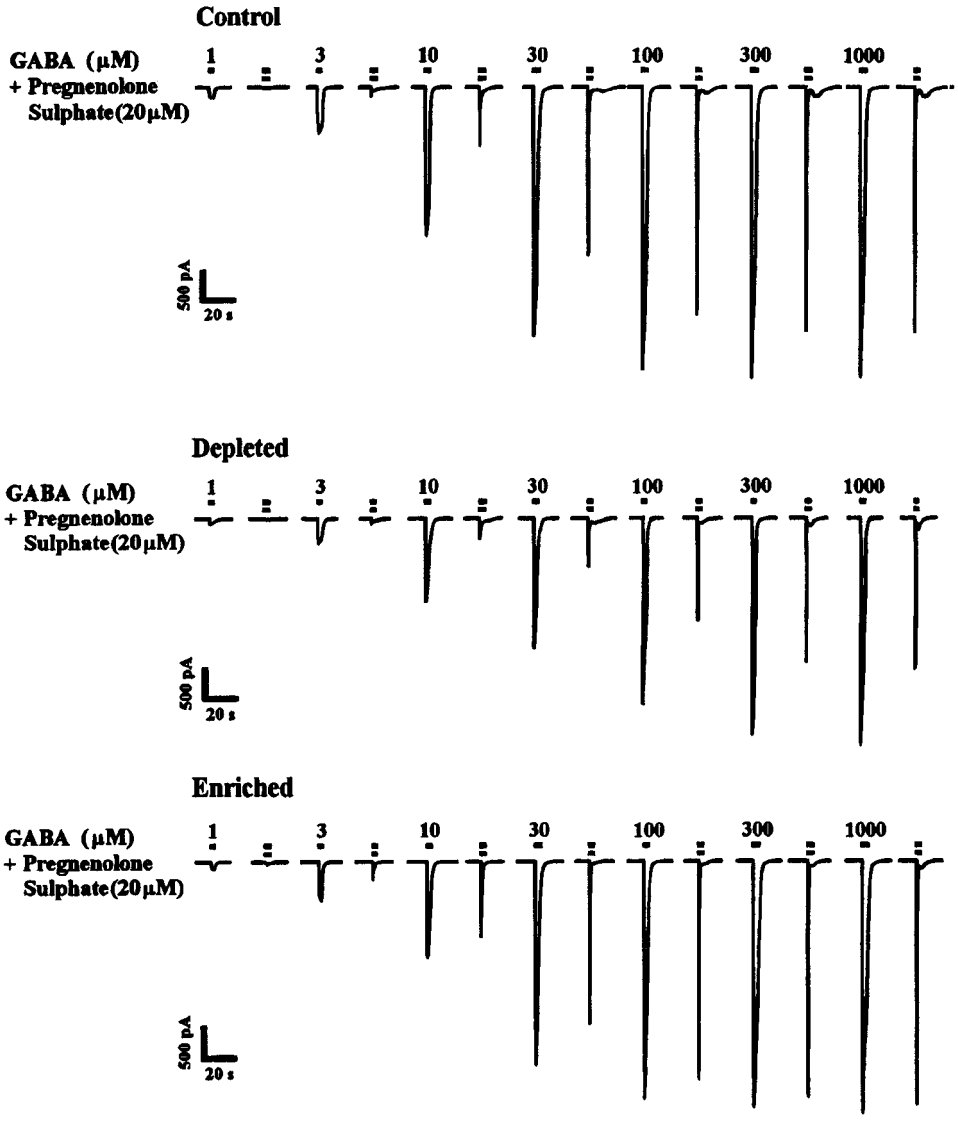

Figure 5 Examples of whole cell current responses to GABA alone and GABA + 20 μM pregnenolone sulphate in a control neurone, a neurone depleted of cholesterol to 56% control and a neurone enriched with cholesterol to 182% control. Applications of pregnenolone sulphate alone gave no response (data not shown). For display purposes, the responses to GABA alone and GABA + pregnenolone sulphate are shown alternately, with increasing GABA concentration. In the actual experiments, the responses to GABA alone were all obtained first, with increasing concentrations of GABA, followed by all the responses to GABA + pregnenolone sulphate.

$GABA_A$ receptor protein (see Sieghart, 1995 for a review). Despite this distinction, all three drugs were similarly affected by neuronal cholesterol manipulations. Depletion of cholesterol had no effect whilst enrichment with cholesterol, or the addition of epicholesterol, increased the potentiation of GABA. It is also apparent in the results that the increased potentiations were superimposed on simultaneous reductions in the potency of GABA due to the cholesterol or epicholesterol enrichments, which we have previously reported (Sooksawate & Simmonds, 2001). We attributed this latter phenomenon to a reduced plasma membrane fluidity, on the basis that epicholesterol had the same effect as cholesterol not only on GABA potency but on membrane fluidity as well (Gimpl *et al*., 1997). The same line of reasoning would attribute the cholesterol-induced increases in potentiation of GABA by propofol, flunitrazepam and pentobarbitone to a reduced membrane fluidity. A rather general effect of such a mechanism is not unexpected; indeed, it might be anticipated that the steroidal potentiators of GABA should be similarly affected, and it is possible that they were. When an over-riding, more selective interaction between cholesterol enrichment and pregnanolone was avoided, by adding epicholesterol to the membrane rather than cholesterol, the effect of pregnanolone tended to increase like that of the non-steroidal potentiators. Although our current evidence cannot exclude other mechanisms, a membrane fluidity effect does seem to offer the most plausible explanation for the increases in potentiator action with cholesterol enrichment.

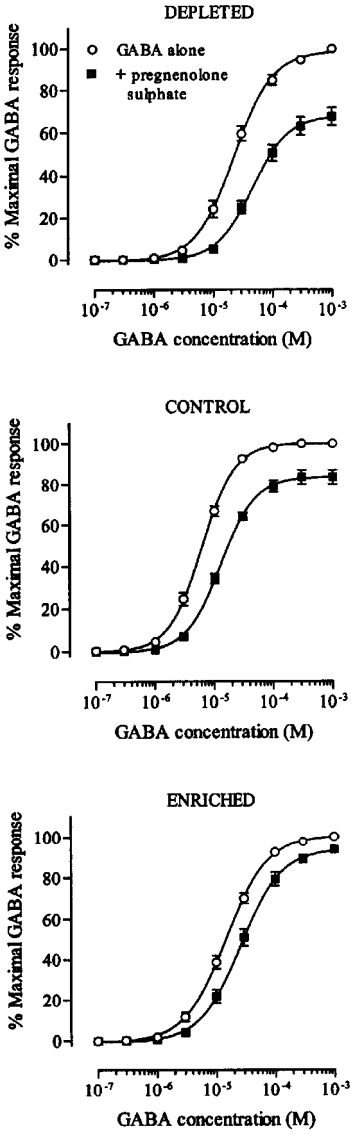

Figure 6 Influence of membrane cholesterol on the antagonism of GABA by 20 μM pregnenolone sulphate. In each panel, the responses to GABA alone (○) and to GABA in the presence of pregnenolone sulphate (■) are expressed as per cent of the calculated maximum response to GABA alone. Each data point is the mean ± s.e.mean of 10 (depleted), 10 (control) and eight (enriched) experiments. Cholesterol depletion was to 56% of control and enrichment to 182% control.

A distinctly different pattern and type of dependency on membrane cholesterol was found to influence, selectively, the steroidal potentiators of GABA. Pregnanolone and alfaxalone each showed a reduced effectiveness with cholesterol enrichment, confirming our earlier observations (Sooksawate & Simmonds, 1998). This effect of cholesterol was not mimicked by addition of epicholesterol to the membrane. Even control levels of cholesterol appeared to be exerting some restraint on the steroidal potentiators since depletion of membrane cholesterol increased their effectiveness. In considering the overall picture (Figure 3), however, it is possible that the effects of cholesterol enrichment on the neuroactive steroids included a component analogous to the interaction of cholesterol with the other GABA potentiators. Such a component would contribute an enhancement in steroidal effectiveness and would, therefore, appear as a limitation on the specific reduction in neuroactive steroid effect in cholesterol-enriched neurones. Perhaps this is the reason why the relationship shown in Figure 3 tends to plateau at cholesterol enrichments above 156% control. Allowing for this, the specific interaction between the neurosteroid potentiators and membrane cholesterol would operate over a wide range of cholesterol levels, from about half to about double the control value.

Table 3 Effects of depletion and enrichment of membrane cholesterol on the antagonism of GABA by 20 μM pregnenolone sulphate (PS)

	Control	*Cholesterol-depleted* (56% control)	*Cholesterol-enriched* (182% control)
GABA EC_{50} ratio	2.11 ± 0.141 (10)	2.00 ± 0.082 (10)	1.87 ± 0.172 (8)
Maximum response to GABA + PS (per cent of maximum to GABA alone)	83.8 ± 1.55 (10)	68.0 ± 2.54 ** (10)	93.8 ± 2.15§§ (8)

GABA EC_{50} ratio = (GABA EC_{50} in the presence of PS)/(GABA EC_{50} prior to addition of PS). Each value is the mean ± s.e.mean from the numbers of neurones shown in brackets. Significant changes in maximum response to GABA + PS, due to cholesterol manipulation, are indicated as ** for a decrease ($P<0.01$) and §§ for an increase ($P<0.01$) (ANOVA + Dunnett's test).

These conclusions raise the question of the mechanism by which membrane cholesterol selectively reduces the action of the steroidal potentiators of GABA. A simple view would be that cholesterol normally occupies the neuroactive steroid binding site resulting in competition between these steroids, but structure-activity considerations do not rest easily with this view. An absolute requirement for GABA potentiator activity is a 3-OH in the α-configuration (Harrison *et al*., 1987; Purdy *et al*., 1990) and cholesterol has a 3β-OH. Furthermore, epicholesterol which does have a 3α-OH clearly does not compete with the steroidal potentiators and there is no doubt that epicholesterol entered the cell membrane since it reduced the potency of GABA in these same experiments as effectively as did enrichment with cholesterol (Sooksawate & Simmonds, 2001). An alternative view, therefore, is that cholesterol operates through an allosteric site on the $GABA_A$ receptor.

A further consideration is the analogous dependency on cholesterol of the action of pregnenolone sulphate in reducing the maximum response to GABA, an effect not seen with picrotoxinin which also reduces the maximum response to GABA (Sooksawate & Simmonds, 2001). This suggests that pregnenolone sulphate and picrotoxinin have separate sites of action on the $GABA_A$ receptor, a conclusion already reached by others on evidence of discrimination in receptors containing a γ2 subunit mutation (Shen *et al*., 1999). The mechanisms of antagonism may also differ, with the suggestion that exacerbation of GABA desensitization by pregnenolone sulphate is an important feature of its mode of action (Shen *et al*., 2000), although some caution has been expressed (Akk *et al*., 2001). Certainly, our results show the more rapid fade of the responses to GABA to be a prominent feature of the action of pregnenolone sulphate. There is also evidence that the site of action of pregnenolone sulphate is distinct from that of the steroidal potentiators of GABA; the potentiators remain fully effective in the presence of pregnenolone sulphate and, conversely, pregnenolone sulphate remains fully effective in the presence of the potentiators (Zaman *et al*., 1992; Park-Chung *et al*., 1999). The cholesterol dependency of pregnenolone sulphate, therefore, is likely to be separate from that of the GABA potentiating steroids, even though the actions of both classes of neuroactive steroid are negatively modulated in proportion to the membrane concentration of cholesterol over a wide range. We have no evidence at present that would distinguish a competitive from an allosteric interaction between cholesterol and pregnenolone sulphate.

The wider implications of all these observations will depend on how labile the cholesterol content of plasma membranes is *in vivo* and on the functional importance of the regulatory sites affected. For well-perfused tissues outside the central nervous system, it has been estimated that the free cholesterol content would exchange completely with circulating free cholesterol within about 1 day (Phillips *et al*., 1987). Not surprisingly, therefore, lowering of circulating cholesterol levels in hypercholesterolaemic patients by treatment with pravastatin has been shown to result in decreases in erythrocyte and platelet membrane cholesterol contents (Lijnen *et al*., 1996). Moreover, these changes were associated with an increase in Na^+ pump activity in these membranes.

With regard to the central nervous system, whole brain levels of cholesterol are found to be quite stable. The turnover is very low, the source of new cholesterol being synthesis in the brain rather than circulating plasma cholesterol, for which a blood/brain barrier may operate (Jurevics & Morell, 1995; Lütjohann *et al*., 1996). Myelin contains a substantial proportion of the brain cholesterol and may account for the stable level overall. It is possible, however, that the fraction of cholesterol present in grey matter may be more labile. For example, mice fed a diet containing 5% cholesterol for 8 weeks, which resulted in a 200% increase in serum cholesterol, were found to have no significant increase in whole brain cholesterol but they did show a significant 20% increase in cholesterol measured in frontal cortex, which has less white matter than the brain as a whole (Howland *et al*., 1998). Increases in brain cholesterol as a result of atherogenic diets have also been reported for rabbits (Sparks, 1997) and for rats, in which there was an associated fall in brain Ca^{2+}-ATPase activity (Oner *et al*., 1991).

Overall, the results presented here and in our previous papers (Sooksawate & Simmonds, 1998; 2001) show at least some of the ways in which the functioning of the $GABA_A$ receptor may be modulated by its lipid environment. It is clear that the various regulatory sites on the $GABA_A$ receptor protein are differentially affected, with consequences for comparisons of drug potency. For example, the present results show that 5 μM propofol and 1 μM alfaxalone were equi-effective as GABA potentiators in cholesterol-depleted neurones whereas 5 μM propofol caused a 3 fold greater potentiation of GABA than did 1 μM alfaxalone in cholesterol-enriched neurones. The dominant contribution to this outcome was the selective inverse relationship between membrane cholesterol levels and neuroactive steroid effectiveness. In addition to the pharmacological significance of these observations, the likely physiological role of some neuroactive steroids at the $GABA_A$ receptor (Compagnone & Mellon, 2000) points to the possibility that dietary or therapeutically-induced changes in circulating cholesterol could have a neuroactive steroid-dependent influence on $GABA_A$ receptor-mediated inhibition. Such an influence would be in addition to the more direct modulation of GABA potency by cholesterol that we have previously reported (Sooksawate & Simmonds, 2001).

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References

AKK, G., BRACAMONTES, J. & STEINBACH, J.H. (2001). Pregnenolone sulfate block of $GABA_A$ receptors: mechanism and involvement of a residue in the M2 region of the α subunit. *J. Physiol.,* **532,** 673–684.

COMPAGNONE, N.A. & MELLON, S.H. (2000). Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front. Neuroendocrinol.,* **21,** 1–56.

FERNANDEZ-BALLESTER, G., CASTRESANA, J., FERNANDEZ, A.M., ARRONDO, J.L., FERRAGUT, J.A. & GONZALEZ-ROS, J.M. (1994). A role for cholesterol as a structural effector of the nicotinic acetylcholine receptor. *Biochemistry,* **33,** 4065–4071.

FONG, T.M. & McNAMEE, M.G. (1986). Correlation between acetylcholine receptor function and structural properties of membranes. *Biochemistry,* **25,** 830–840.

GIMPL, G., BURGER, K. & FAHRENHOLZ, F. (1997). Cholesterol as modulator of receptor function. *Biochemistry,* **36,** 10959–10974.

HAMIL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.,* **391,** 85–100.

HARRISON, N.L., MAJEWSKA, M.D., HARRINGTON, J.W. & BARKER, J.L. (1987). Structure-activity relationships for steroid interaction with the γ-aminobutyric acid$_A$ receptor complex. *J. Pharmacol. Exp. Ther.,* **241,** 346–353.

HOWLAND, D.S., TRUSKO, S.P., SAVAGE, M.J., REAUME, A.G., LANG, D.M., HIRSCH, J.D., MAEDA, N., SIMAN, R., GREENBERG, B.D., SCOTT, R.M. & FLOOD, D.G. (1998). Modulation of secreted beta-amyloid precursor protein and amyloid beta peptide in brain by cholesterol. *J. Biol. Chem.,* **273,** 16576–16582.

JUREVICS, H. & MORELL, P. (1995). Cholesterol for synthesis of myelin is made locally, not imported into brain. *J. Neurochem.,* **64,** 895–901.

LIJNEN, P., ECHEVARIA-VAZQUEZ, D. & PETROV, V. (1996). Influence of cholesterol-lowering on plasma membrane lipids and function. *Methods Find. Exp. Clin. Pharmacol.,* **18,** 123–136.

LÜTJOHANN, D., BREUER, O., AHLBORG, G., NENNESMO, I., SIDÉN, Å., DICZFALUSY, U. & BJÖRKHEM, I. (1996). Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. *Proc. Natl Acad. Sci. U.S.A.,* **93,** 9799–9804.

ONER, P., BEKPINAR, S. & OZ, B. (1991). Alterations in some lipid components and Ca^{2+}-ATPase activity in brain of rats fed an atherogenic diet. *Res. Comm. Chem. Pathol. Pharmacol.,* **72,** 337–345.

PARK-CHUNG, M., MALAYEV, A., PURDY, R.H., GIBBS, T.T. & FARB, D.H. (1999). Sulfated and unsulfated steroids modulate γ-aminobutyric acid$_A$ receptor function through distinct sites. *Brain Res.,* **830,** 72–87.

PHILLIPS, M.C., JOHNSON, W.J. & ROTHBLAT, G.H. (1987). Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.,* **906,** 223–276.

PURDY, R.H., MORROW, A.L., BLINN, J.R. & PAUL, S.M. (1990). Synthesis, metabolism, and pharmacological activity of 3α-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. *J. Med. Chem.,* **33,** 1572–1581.

RANKIN, S.E., ADDONA, G.H., KLOCZEWIAK, M.A., BUGGE, B. & MILLER, K.W. (1997). The cholesterol dependence of activation and fast desensitisation of the nicotinic acetylcholine receptor. *Biophys. J.,* **73,** 2446–2455.

SHEN, W., MENNERICK, S., COVEY, D.F. & ZORUMSKI, C.F. (2000). Pregnenolone sulfate modulates inhibitory synaptic transmission by enhancing GABA$_A$ receptor desensitization. *J. Neurosci.,* **20,** 3571–3579.

SHEN, W., MENNERICK, S., ZORUMSKI, E.C., COVEY, D.F. & ZORUMSKI, C.F. (1999). Pregnenolone sulfate and dehydroepiandrosterone sulfate inhibit GABA-gated chloride currents in *Xenopus* oocytes expressing picrotoxin-insensitive GABA$_A$ receptors. *Neuropharmacology,* **38,** 267–271.

SIEGHART, W. (1995). Structure and pharmacology of γ-aminobutyric acid$_A$ receptor subtypes. *Pharmacol. Rev.,* **47,** 181–234.

SOOKSAWATE, T. & SIMMONDS, M.A. (1998). Increased membrane cholesterol reduces the potentiation of GABA$_A$ currents by neurosteroids in dissociated hippocampal neurones. *Neuropharmacology,* **37,** 1103–1110.

SOOKSAWATE, T. & SIMMONDS, M.A. (2001). Effects of membrane cholesterol on the sensitivity of the GABA$_A$ receptor to GABA in acutely dissociated rat hippocampal neurones. *Neuropharmacology,* **40,** 178–184.

SPARKS, D.L. (1997). Dietary cholesterol induces Alzheimer-like β-amyloid immunoreactivity in rabbit brain. *Nutr. Metab. Cardiovasc. Dis.,* **7,** 255–266.

ZAMAN, S.H., SHINGAI, R., HARVEY, R.J., DARLISON, M.G. & BARNARD, E.A. (1992). Effects of subunit types of the recombinant GABA$_A$ receptor on the response to a neurosteroid. *Eur. J. Pharmacol. Mol. Pharmacol.,* **225,** 321–330.

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