British Journal of Pharmacology (1996) 117, 87–92

The influence of membrane cholesterol on the $GABA_A$ receptor

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1 Neurosteroids such as pregnanolone have been established as potent modulators of the $GABA_A$ receptor in both electrophysiological and binding studies. Since cholesterol is present in substantial amounts in the neuronal membranes, we have sought evidence for possible interactions of cholesterol with the neurosteroid site and more generally, with the $GABA_A$ channel.

2 Synaptosomal membranes were prepared from rat whole brain, cerebral cortex, cerebellum and spinal cord. These membranes were enriched with cholesterol to about double the original level by incubation with liposomes comprised of 50 phosphatidylcholine : 50 cholesterol in the presence of 1% BSA. The additional cholesterol formed a homogeneous mixture with the endogenous cholesterol.

3 The effects of cholesterol and modulatory drugs on the $GABA_A$ channel were assessed from the changes induced in [^{3}H]-flunitrazepam (FNZ) binding. Cholesterol enrichment did not affect FNZ binding itself; however, the enhancement of [^{3}H]-FNZ binding by pregnanolone was affected. In membranes from cerebral cortex, the potency of pregnanolone was reduced by a factor of 3.2 following cholesterol enrichment. By contrast, in membranes from spinal cord, the potency of pregnanolone was increased by a factor of 8.4 following cholesterol enrichment. In membranes from cerebellum, there was little overall change in pregnanolone potency although the effects of threshold concentrations were increased.

4 The enhancement of [^{3}H]-FNZ binding by propofol in whole brain membranes was reduced in cholesterol-enriched membranes, similar to the effects of pregnanolone. Experiments with muscimol resulted in an increase in its potency as a potentiator of [^{3}H]-FNZ binding, following cholesterol enrichment.

5 These results provide little evidence for a selective competition between cholesterol and pregnanolone at its binding site. Rather, they suggest an influence of membrane cholesterol on the functional coupling between the benzodiazepine site and the other specific drug sites on the $GABA_A$ channel. The detailed pattern of influence depended upon the region of CNS and may be related to the subunit composition of the $GABA_A$ channels present.

Keywords: Membrane cholesterol; pregnanolone; flunitrazepam; propofol; muscimol; neuronal membranes; $GABA_A$ channels

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter within the central nervous system acting upon its pentameric $GABA_A$ receptor (Nayeem *et al.*, 1994) selective for the conductance of Cl^- ions (Schofield *et al.*, 1987; Sieghart, 1992). As well as the GABA binding site, the receptor also contains modulatory sites for several other major classes of anxiolytic, hypnotic and convulsant compounds that include benzodiazepines (Sieghart, 1989), neuroactive steroids (Turner & Simmonds, 1989; Paul & Purdy, 1992) and propofol (Langley & Heel, 1988; Prince & Simmonds, 1992). The available evidence shows that neurosteroids are active in the nM range to potentiate GABA-evoked Cl^- currents and at μM concentrations they can directly activate the $GABA_A$ receptor-mediated Cl^- conductance (Puia *et al.*, 1990). The potentiation of $GABA_A$ receptor-mediated responses by neurosteroids also shows clear structure-activity requirements (Simmonds, 1991). All this is compatible with the idea that there are specific recognition sites for the neurosteroids on the $GABA_A$ channel protein and that perturbation of the lipid membrane by the neurosteroids is a less likely mechanism of action (Franks & Lieb, 1994).

The possibility of an interaction of the high concentration of cholesterol in the lipid membrane with the neurosteroid site or more generally with the $GABA_A$ receptor has not previously been investigated, although another member of the ligand-gated channel super-family, the nicotinic acetylcholine receptor, appears to have cholesterol closely associated with it (Jones & McNamee, 1988). We now describe a simple procedure for enriching neuronal membranes with cholesterol and the effects this has upon flunitrazepam (FNZ) binding to the $GABA_A$ channel protein and the potentiation of [^{3}H]-FNZ binding by pregnanolone, propofol and muscimol. Some of the data presented in this paper have been published previously in abstract form (Bennett & Simmonds, 1995).

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Methods

Membrane preparation

Membranes were prepared either from the whole brain, or from cerebral cortex, cerebellum, and spinal cord of male Wistar rats (130–200 g). The 'buffy coat' fraction of the tissue homogenate was obtained by the method of Zukin *et al.* (1974). In brief, the tissue was homogenized in 0.32 M sucrose (at pH 7.4) with an Ultra Turrax T-25 homogenizer, then with eight strokes of a teflon homogenizer. The homogenate was centrifuged at 1520 *g* and 4°C for 10 min to pellet any debris and unbroken cells. The supernatant was then collected and centrifuged at 31000 *g* for 20 min. The resulting pellet was resuspended in 20 ml distilled H_2O and incubated for 30 min on ice. This suspension was centrifuged at 12900 *g* for 20 min and the buffy coat layer was resuspended in the supernatant. The suspended buffy coat was then centrifuged at 48400 *g* for 20 min to form a pellet of synaptic membranes. The pellet was resuspended in distilled H_2O and this washing step was re-

peated a further two times. The final pellet was resuspended in ice cold wash buffer (5 mM Tris-HCl, 1 mM EDTA, pH 7.4) and stored at −20°C until required.

Liposome preparation

Phosphatidylcholine (PC) and cholesterol (0.75 mg of each per ml of buffer added) were dissolved in a small amount of chloroform. The chloroform was evaporated under a stream of nitrogen to leave a thin film of lipids and prevent their oxidation. SET buffer (0.25 M sucrose, 1 mM EDTA, 50 mM Tris-Base, 0.02% sodium azide, pH 6.9 at 25°C) was added and the lipids allowed to hydrate for 1 h at room temperature. The lipids were then dispersed with a Branson Sonifier 250 fitted with a half inch tip on 80% duty cycle under a stream of nitrogen, whilst incubating on an ice bath. Three periods of 4 min sonication were carried out at 90 W allowing the same time in between each sonication for cooling. After sonication the translucent solution was centrifuged at 48400 *g* for 20 min at room temperature and the pellet discarded. This procedure generally incorporated about 80% of the cholesterol into liposomes. Pure PC liposomes were prepared by the same procedures.

Cholesterol enrichment of synaptic membranes

Previously prepared membranes were washed at 4°C with wash buffer and centrifuged at 8000 *g* for 25 min at 4°C and resuspended in SET buffer. Cholesterol-enrichment was achieved by incubating the membranes (0.4 mg protein ml^{-1}) in SET buffer containing 1% BSA, and approximately 0.5 mg total liposome lipids ml^{-1} at 37°C. In some preliminary studies, 5 $\mu g\ ml^{-1}$ of the lipid transfer protein pre-nsL-TP (Ossendorf *et al.*, 1992) was included to determine whether it enhanced the lipid transfer. The cholesterol transfer was terminated after 4 h by addition of an equal volume of ice cold assay buffer (50 mM Tris-base, 150 mM NaCl, pH 7.4 at 4°C) and the mixture centrifuged at 48400 *g* for 20 min. The pellets were resuspended in wash buffer. Essentially the same procedures were used to expose membranes to pure PC liposomes. For comparative purposes, a portion of the original membrane preparation was subjected to these same procedures but with the omission of the liposomes and these membranes were designated as 'unenriched'. All membranes were frozen until required.

To monitor the time course of cholesterol enrichment, experiments were performed in which the membranes were sampled at intervals during the incubation, for assay of membrane cholesterol (Sigma diagnostics kit) and protein (BioRad reagent). The stability of enrichment in membranes held at 4°C was assessed by repeated washing of the membranes with the wash buffer.

In experiments to investigate the disposition of the cholesterol enrichment, whole brain membranes were incubated with PC : cholesterol liposomes as detailed above but including [^{3}H]-cholesterol (specific activity 1.86 mCi $mmol^{-1}$). Following enrichment, the membranes were incubated for a further 4 h with or without PC liposomes (0.25 mg PC ml^{-1}) at 37°C in SET buffer containing 1% BSA to achieve a substantial reversal of the cholesterol enrichment. Aliquots were removed for assay of [^{3}H]-cholesterol, total cholesterol and protein after 2 min and 4 h.

Binding experiments

Experiments were performed to determine the effects of FNZ, pregnanolone, propofol and muscimol on [^{3}H]-FNZ binding to cholesterol-enriched, PC-incubated and unenriched membranes. On the day of assay the membranes were thawed and centrifuged at 48400 *g* for 20 min at 4°C and the pellets resuspended in assay buffer to give a final protein concentration of about 1 mg ml^{-1}. Aliquots of membranes (100 μl) were preincubated with FNZ 10 pM − 1 μM, pregnanolone 30 nM − 10 μM, propofol 10 μM − 3 mM, or muscimol 1 nM − 0.1 mM in assay buffer at 37°C for 10 min. [^{3}H]-FNZ was added to give a 1 nM concentration in a final volume of 0.5 ml and the samples were then incubated on ice for 60 min. The reaction was terminated with addition of 2 ml ice cold wash buffer, followed by rapid filtration through Whatman GF-C filters using a Brandell Cell Harvester. The filter mat was washed a further four times with 2 ml wash buffer and the radioactivity determined by standard liquid scintillation techniques (Beckman 5801). Non-specific binding was determined by use of 10 μM FNZ and was in the region of 5 − 10%. FNZ was dissolved initially in ethanol, pregnanolone was dissolved in dimethylsulphoxide (DMSO) and propofol was dissolved in acetone. The final concentration of ethanol was less than 0.4%, DMSO was 0.45% and acetone was 0.1%. Since DMSO caused a consistent reduction in [^{3}H]-FNZ binding by about 30%, it was present in all assay tubes for experiments involving pregnanolone. [^{3}H]-FNZ (1 nM) was used as a trace concentration which should have no functional effect on the $GABA_A$ receptor.

All chemicals and drugs were obtained from Sigma with the exception of [^{3}H]–FNZ (82.5 Ci $mmol^{-1}$ specific activity) and [^{3}H]-cholesterol (51.1 Ci $mmol^{-1}$ specific activity) which were obtained from Amersham International. The pre-nsL-TP was obtained from Prof. K.W.A. Wirtz, Utrecht University. Data were fitted using the Inplot Package (Graphpad Software). Statistical comparisons were made by Student's *t* test for independent samples.

Results

Cholesterol enrichment

Preliminary studies using pre-nsL-TP for the cholesterol enrichment of rat whole brain membranes yielded an enhancement no greater than that of spontaneous cholesterol enrichment (196% and 208% increase above basal, respectively). Therefore, in subsequent studies, spontaneous cholesterol transfer was routinely used.

A time course study of the spontaneous enrichment of whole brain membranes with cholesterol from liposomes comprised of 50 PC : 50 cholesterol showed that the enrichment reached equilibrium after 3 h (Figure 1). On the basis of these results all subsequent cholesterol enrichment studies involved 4 h incubations. Washing of the membranes to remove liposomes and a further 4 washes at 4°C with wash buffer resulted in a loss of about 20% of the cholesterol enrichment.

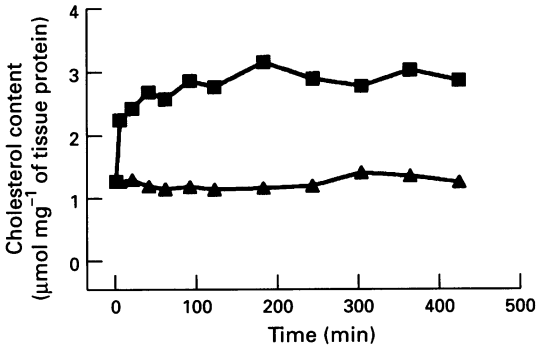

Figure 1 The time course of a cholesterol enrichment of rat whole brain membranes after incubating with 50 PC : 50 cholesterol liposomes (■) or in their absence (▲). Cholesterol enrichment was rapid at first but plateaued after 180 min. The control incubation showed no change in cholesterol content. Similar results were obtained in 3 other experiments.

Evidence was sought to distinguish between the possibilities that the exogenous cholesterol mixed entirely with the endogenous membrane cholesterol, and the alternative possibility that the exogenous cholesterol was associated with the membrane as a separate pool. Whole brain membranes were enriched with [^{3}H]-cholesterol and the specific activity (Sp.Act.) of the [^{3}H]-cholesterol in the membranes was measured at intervals during subsequent washing and depletion of cholesterol from the membranes at 37°C with a buffer containing 1% BSA. It was predicted that depletion of [^{3}H]-cholesterol should result in no change in Sp.Act. of the [^{3}H]-cholesterol remaining in the membranes if depletion was from a homogeneous mixed pool of exogenous and endogenous cholesterol. In contrast, depletion from a separate exogenous pool having the same Sp.Act as the [^{3}H]-cholesterol in the liposomes, should result in a clear reduction in the whole membrane Sp.Act. of the remaining [^{3}H]-cholesterol. When this was tested experimentally the results obtained were as shown in Table 1. In the wash period following enrichment with [^{3}H]-cholesterol, a 28–34% depletion of total cholesterol was associated with no change in Sp.Act. of [^{3}H]-cholesterol remaining in the membranes. This contrasts with a predicted reduction in Sp.Act. of 14143 ± 1439 d.p.m. μmol^{-1} cholesterol (68%) or 10757 ± 732 d.p.m. μmol^{-1} cholesterol (52%), depending on the presence or absence of PC liposomes, if the depleted cholesterol had come from a separate exogenous pool.

In many of the experiments involving the binding of [^{3}H]-FNZ, membranes from discrete areas of CNS were used. The cholesterol enrichments in membranes from cerebral cortex, cerebellum and spinal cord following a 4 h incubation with 50 PC : 50 cholesterol liposomes are shown in Table 2.

Influence of cholesterol enrichment on [^{3}H]-FNZ binding and its potentiation by pregnanolone

In initial experiments to determine the effect of cholesterol enrichment on FNZ binding, displacement of [^{3}H]-FNZ by unlabelled FNZ (10 pM – 1 μM) was measured in rat whole brain membranes. FNZ binding was found to be unaffected by cholesterol enrichment (Figure 2).

The foregoing result allowed us to investigate the influence of cholesterol enrichment upon the modulatory action of pregnanolone on the $GABA_A$ receptor by determining its effect on [^{3}H]-FNZ binding. These experiments were performed on membranes from discrete areas of the CNS. In unenriched membranes from cerebral cortex, cerebellum and spinal cord, the threshold concentrations of pregnanolone for enhancement of [^{3}H]-FNZ binding were about 0.3 μM (Figure 3). The effects of pregnanolone increased with concentration up to 3 μM. These concentration-effect relationships were compared with analogous data from enriched membranes. Where cholesterol enrichment caused lateral shifts of the concentration-response

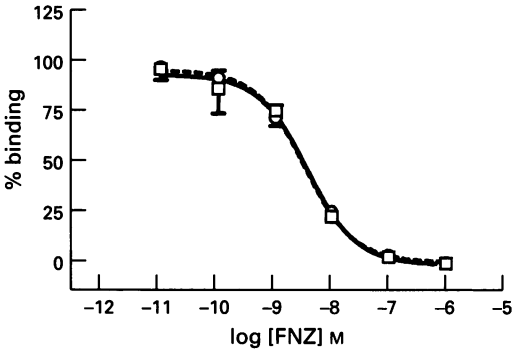

Figure 2 [^{3}H]-FNZ displacement by unlabelled FNZ in cholesterol enriched (□) and unenriched (○) whole brain membranes. The cholesterol content was 3.247 and 2.724 μmol cholesterol mg^{-1} protein for cholesterol enriched and unenriched membranes, respectively. Data are the means of 4 experiments ± s.e.mean.

Table 1 Changes in cholesterol and in [^{3}H]-cholesterol specific activity after a 4 h [^{3}H]-cholesterol enrichment and a further 4 h incubation with or without PC liposomes

	Membrane cholesterol (μmol mg^{-1} protein)	*% change from 2 min wash*	*Specific activity of membrane [^{3}H]-cholesterol* (d.p.m. μmol^{-1} cholesterol)	*% change from 2 min wash*
Before incubation	2.10 ± 0.10		0	
After 4 h incubation with [^{3}H]-cholesterol PC: liposomes				
2 min wash	3.22 ± 0.12		20887 ± 1197	
4 h wash with PC liposomes	2.17 ± 0.07	–34%	20914 ± 504	–0.1%
4 h wash without PC liposomes	2.33 ± 0.16	–28%	20852 ± 476	0.2%

Values shown are the mean ± s.e.mean of 4 experiments.

Table 2 Total membrane cholesterol following a 4 h incubation at 37°C with 50 PC : 50 cholesterol liposomes (enriched) and without liposomes (unenriched)

	Cholesterol content (μmol cholesterol mg^{-1} protein)	
CNS region	*Unenriched*	*Enriched*
Cortex	2.25 ± 0.04	4.54 ± 0.09
Cerebellum	1.96 ± 0.10	3.63 ± 0.13
Spinal cord	2.64 ± 0.13	4.23 ± 0.17

A single batch of membranes was prepared for each area of CNS. Values shown are mean ± s.e.mean of 3 readings.

curves, the log shifts were calculated to determine changes in the potency of pregnanolone. In cerebral cortex membranes, enrichment with cholesterol was associated with a 3.2 fold reduction in pregnanolone potency (Figure 3). In cerebellar membranes, enrichment with cholesterol was associated with no change in pregnanolone potency over most of the concentration-range. However, at the threshold of the concentration-effect curve in the enriched cerebellar membranes, concentrations of 0.1 and 0.3 μM pregnanolone gave a significant enhancement of [^{3}H]-FNZ binding to 108.2±1.4% of control compared with no enhancement in unenriched membranes (mean±s.e.mean, $n=4$) (Figure 3). In spinal cord membranes, enrichment with cholesterol was, by contrast, associated with an 8.4 fold increase in pregnanolone potency (Figure 3).

To ascertain whether the changes in pregnanolone potency were due to cholesterol alone or to possible changes in the PC content of the membranes, experiments were performed with membranes preincubated with pure PC liposomes (0.25 mg ml^{-1}). In membranes from the cerebral cortex, pregnanolone (30 nM–10 μM) potentiated [^{3}H]-FNZ binding to a similar extent in membranes preincubated with PC liposomes and membranes not exposed to PC (Figure 4). Likewise, in cerebellar membranes, potentiation of [^{3}H]-FNZ binding by 1 μM pregnanolone was found to be 116.7±4.28% of control binding for the membranes pre-incubated with PC liposomes and 110.1±4.46% of control binding for membranes not exposed to PC (mean±s.e.mean, $n=4$). A similar experiment in spinal cord membranes showed 1 μM pregnanolone potentiation of [^{3}H]-FNZ to be 140.1±15.92% of control binding and 128.3±12.75% of control binding, respectively, for membranes pre-incubated with PC liposomes and membranes not exposed to PC liposomes (mean±s.e.mean, $n=7$). In neither case was the difference statistically significant.

Influence of cholesterol enrichment on the potentiation of [^{3}H]-FNZ binding by propofol and muscimol

To investigate if cholesterol-enrichment would affect other binding sites on the $GABA_A$ receptor protein, the potentiating effects of propofol and muscimol on [^{3}H]-FNZ binding were determined.

In unenriched membranes, the threshold concentration of propofol for enhancement of [^{3}H]-FNZ binding was 10 μM (Figure 5). The enhancement increased with propofol concentration up to 300 μM and then declined at higher concentrations. In the cholesterol-enriched membranes, [^{3}H]-FNZ binding was depressed at propofol concentrations of 300 μM and above.

Muscimol enhanced [^{3}H]-FNZ binding in unenriched membranes in a simple concentration-dependent manner between 0.1 μM and 10 μM muscimol. In cholesterol-enriched membranes, the potency of muscimol was increased 3.0 fold (Figure 6) and the maximal effect of muscimol was also significantly increased to 150.0±2.24% control [^{3}H]-FNZ binding compared with the unenriched membranes 140.3±3.83% (mean±s.e.mean, $n=6$, $P=0.05$).

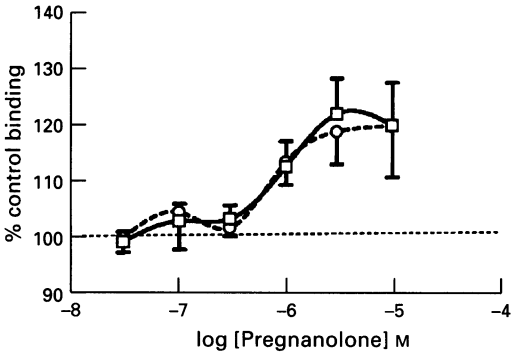

Figure 4 Potentiation of [^{3}H]-FNZ binding by pregnanolone in phosphatidylcholine incubated (□) and control (○) cerebral cortex membranes. Data are the means of 5 experiments±s.e.mean.

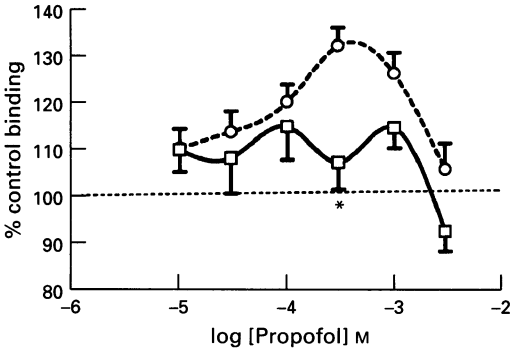

Figure 5 Potentiation of [^{3}H]-FNZ binding by propofol in cholesterol enriched (□) and unenriched (○) whole brain membranes. The cholesterol content was 4.168 and 1.764 μmol cholesterol mg^{-1} protein for cholesterol enriched and unenriched membranes, respectively. Data are the means of 5 experiments±s.e.mean. *Significant difference between enriched and unenriched by Student's t test, $P<0.05$.

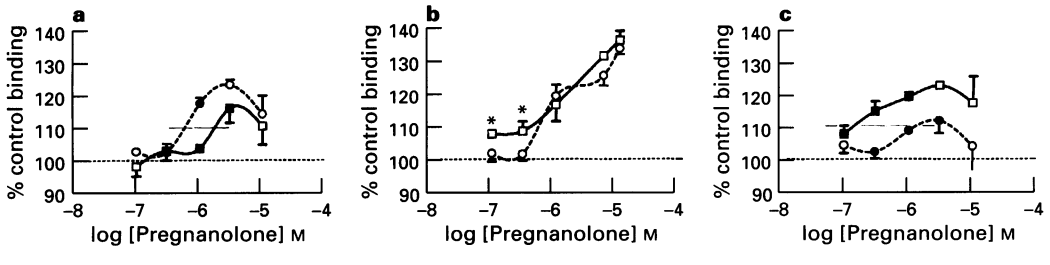

Figure 3 Potentiation of [^{3}H]-FNZ binding by pregnanolone in cholesterol enriched (□■) and unenriched (○, ●) membranes from (a) cerebral cortex, (b) cerebellum and (c) spinal cord. Cholesterol contents of the membranes are shown in Table 2. Data are the means of 3 experiments±s.e.mean except for the cortex where $n=4$. Solid symbols are those used to determine the lateral displacement of the curves at the levels indicated by the horizontal line. For cerebral cortex membranes (a), cholesterol enrichment gave a shift of 0.506±0.028 log units to the right (mean±s.e.mean) and for spinal cord membranes (c), cholesterol enrichment gave a shift of 0.926±0.052 log units to the left (mean±s.e.mean). *Significant difference between enriched and unenriched by Student's t test, $P<0.05$.

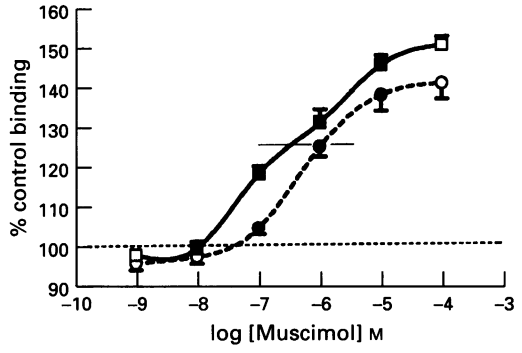

Figure 6 Potentiation of [^{3}H]-FNZ binding by muscimol in cholesterol enriched (■, □) and unenriched (●, ○) whole brain membranes. The cholesterol content was 5.037 and 2.211 μmol cholesterol mg^{-1} protein for cholesterol enriched and unenriched whole brain membranes, respectively. Data are the means of 6 experiments ± s.e.mean. Solid symbols are those used to determine the lateral displacement of the curve at the level indicated by the horizontal line. Cholesterol-enrichment gave a shift of 0.470 ± 0.190 log units to the left (mean ± s.e.mean) ($P<0.001$).

Discussion

Our primary purpose in undertaking these experiments was to investigate the possibility that the cholesterol in neuronal membranes might compete with neurosteroids for their site of action on the $GABA_A$ channel protein. Because cholesterol constitutes over 20% of the membrane lipids, even a low affinity for the neurosteroid site would be expected to result in effective competition. There could be some difficulty in discerning such an interaction, however, if there was an additional, more general influence of cholesterol on the properties of $GABA_A$ channels. No direct evidence for effects of cholesterol on the $GABA_A$ channel has been produced before although effects on other plasma membrane proteins have been described. For example, a critical concentration of cholesterol was found necessary for maximum catalytic activity of adenylate cyclase (Whetton *et al.*, 1983). For the GABA transporter protein, depletion of membrane cholesterol resulted in a loss of GABA uptake (North & Fleisher, 1983) and reconstitution of the solubilized transporter protein into liposomes required the inclusion of a substantial proportion of cholesterol for optimal transport function (Shouffani & Kanner, 1990). Reconstitution of purified nicotinic acetylcholine receptors from *Torpedo* into membranes of various compositions revealed a clear influence of lipid environment, including a requirement for cholesterol, on the ability of the receptor to undergo low- to high-affinity state transitions that may be relevant to channel activation and desensitization (Fong & McNamee, 1986).

In manipulating the lipid composition of membranes, it cannot be assumed that the various components are homogeneously distributed. Membrane proteins have specific affinities for different lipids, thus creating an intimate shell of lipids that has a different composition from the bulk phase. Nevertheless, the composition of the shell is likely to be influenced by the availability of specific lipids in the bulk phase (Benga, 1985). The nicotinic acetylcholine receptor is reported to interact strongly with sterol molecules but they are absent from the lipid shell. The physical chemical evidence suggests that cholesterol may bind in the axial clefts between the protein subunits (Jones & McNamee, 1988). The axial clefts may also be the locus of the binding sites for certain drugs (Changeux, 1990). Since the $GABA_A$ receptor is structurally related to the nicotinic acetylcholine receptor, we have an analogous model in mind for the neurosteroid sites on the $GABA_A$ channel protein.

If cholesterol in the membrane was competing with pregnanolone for its site of action on the $GABA_A$ receptor, enrichment of the membrane with cholesterol would be expected to reduce the potency of pregnanolone as a potentiator of [^{3}H]-FNZ binding. The results obtained on membranes from cerebral cortex were in accord with this but the results from cerebellum and spinal cord were not. Furthermore, the potentiating effect of propofol was reduced in whole brain membranes following enrichment with cholesterol, a similar result having been obtained with pregnanolone in whole brain membranes (data not shown). Other evidence suggests that propofol and the neurosteroids act at separate sites on the $GABA_A$ protein (Prince & Simmonds, 1992). We cannot, therefore, interpret the observations with pregnanolone on cerebral cortex membranes as a competitive displacement of pregnanolone by cholesterol, although such a mechanism has not been excluded. Furthermore, we have no evidence at present to distinguish between a reduction in [^{3}H]-FNZ affinity by pregnanolone and a reduction in the number of FNZ binding sites.

The substantial increase in pregnanolone potency following cholesterol enrichment of spinal cord membranes presumably arose from an allosteric interaction on the $GABA_A$ protein. A similar but smaller effect of cholesterol was seen on the potency of muscimol in whole brain membranes. These opposite effects seen in spinal cord and cerebral cortex membranes with respect to the interactions between cholesterol and pregnanolone are difficult to interpret. Conceivably, they were the net results of two opposing effects which were operating to different exents in both preparations. Detailed inspection of the results from cerebellar membranes tend to support this idea, assuming a more even balance between the two effects in this tissue. A further complication in the cerebellum is the biphasic nature of the unenriched curve (Figure 3), which suggests more than one component of pregnanolone binding.

Overall, the results are compatible with a predominant effect of cholesterol on the functional coupling between various sites on the $GABA_A$ channel. This includes the functional coupling between muscimol and FNZ, since cholesterol enrichment has no direct effect on the binding of either of these compounds but does influence the enhancement of [^{3}H]-FNZ binding by muscimol (Figure 6). Whatever the detailed mechanisms may be, it is clear that the interactions between cholesterol and pregnanolone, at least, are different in cerebral cortex, cerebellum and spinal cord. It is tempting to relate this to the known differences in the range and proportions of subunits that make up the $GABA_A$ channels in these regions (Persohn *et al.*, 1991; 1992; Wisden *et al.*, 1991; 1992; Laurie *et al.*, 1992). Studies with recombinant receptors are needed to address this question.

Cholesterol enrichment of synaptic membranes was achieved by cholesterol partitioning between liposomes and synaptic membranes. We found that the lipid transfer protein pre-nsL-TP did not enhance cholesterol transfer above the spontaneous enrichment obtained in its absence, in agreement with Nichols & Pagano (1983). The rapid spontaneous transfer required the presence of 1% BSA at 37°C, the BSA presumably acting as a carrier for the cholesterol (Bartholow & Geyer, 1982). Both enrichment and de-enrichment could be achieved under these conditions. Although the enrichment thus achieved was stable at 4°C in the absence of BSA and shown to mix with the endogenous lipids, we were unable to deplete cholesterol below the levels found in the membranes at the end of the membrane isolation procedure (data not shown). This suggests that there is a basal level of cholesterol in plasma membranes. With the lability of higher levels of cholesterol, it is not known whether the membranes *in vivo* contained cholesterol above the basal level, the extra cholesterol having been lost during the isolation procedure.

Further work is required to determine whether the rapid enrichment and de-enrichment of neuronal membrane fragments with cholesterol also occurs in the membranes of intact neurones *in vivo*. If it does, then our results suggest that fluctuations in circulating cholesterol could cause changes in the responsiveness of the $GABA_A$ channels to various agents in-

cluding the neurosteroids, which are present physiologically, and to GABA itself, as indicated by the result with muscimol. Since the changes in responsiveness differ between regions of the CNS, it would seem that not only the subunit composition of the $GABA_A$ receptor but also the membrane lipids in which it is embedded may be important determinants of $GABA_A$ function.

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(Received June 23, 1995
Revised September 14, 1995
Accepted September 18, 1995)