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Effects of membrane cholesterol on the sensitivity of the GABA_A receptor to GABA in acutely dissociated rat hippocampal neurones

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Abstract

The effects of membrane cholesterol on the GABA_A receptor were investigated in acutely dissociated rat hippocampal neurones, using the whole-cell patch clamp technique. Neuronal cholesterol was manipulated within the range 56–250% control by incubation with methyl- β -cyclodextrin for depletion and a complex of cholesterol and methyl- β -cyclodextrin for enrichment. Manipulation over a narrower range was achieved with cholesterol + phosphatidylcholine liposomes. A complex of epicholesterol and methyl- β -cyclodextrin was used to insert epicholesterol. Cholesterol enrichment reduced the potency of GABA, as did cholesterol depletion, with increases in EC₅₀ of up to 4-fold. Cholesterol enrichment reduced the potency of the competitive antagonist bicuculline but did not affect that of the non-competitive antagonist picrotoxinin. Cholesterol depletion did not affect the potencies of either antagonist. Epicholesterol substituted functionally for cholesterol with respect to the effects of enrichment. In cholesterol-depleted neurones, however, only incubation with cholesterol was able to restore GABA potency to normal. These results suggest a specific requirement for cholesterol at control levels to maintain optimal GABA potency, which may involve specific binding of cholesterol to the GABA_A receptor. The reduction in GABA potency by enrichment with cholesterol or epicholesterol is more likely to be due to reduced plasma membrane fluidity. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: GABA_A receptor; GABA; Cholesterol; Methyl- β -cyclodextrin; Patch-clamp; Hippocampal neurone

1. Introduction

Cholesterol is an essential component of mammalian cells. It is found in high concentration in the plasma membrane, and in low concentration in intracellular membranes (Yeagle, 1993). Several membrane receptor proteins have been reported to depend upon cholesterol for their functional activities, for example, the nicotinic acetylcholine receptor (Fernandez-Ballester et al., 1994), the oxytocin receptor and the brain cholecystokinin receptor (Gimpl et al., 1997), and rhodopsin (Albert et al., 1996). Other membrane proteins have also been reported to be modulated by cholesterol, such as Na⁺–K⁺ ATPase (Cornelius, 1995; Vemuri and Phillipson, 1989), adenylate cyclase (Whetton et al., 1983) and a transfer protein mediating γ -aminobutyric acid (GABA) uptake

(North and Fleischer, 1983). More than one mechanism of modulation by cholesterol is likely to be involved (Yeagle, 1993).

Until our own recent work (Bennett and Simmonds, 1996; Schlepper et al., 1998; Sooksawate and Simmonds, 1998), there had been no direct study of the influence of membrane cholesterol on the electrophysiological and pharmacological properties of the GABA_A receptor. This receptor is a member of a gene superfamily of ligand-gated ion channels characterised by a pentameric protein structure (Nayeem et al., 1994) which, for the GABA_A receptor, forms a chloride ion channel with many drug recognition sites. These include separate sites for GABA and several classes of modulators, including benzodiazepines, barbiturates, picrotoxinin, ethanol, volatile general anaesthetics, loreclezole and neurosteroids (Sieghart, 1995; Whiting et al., 1995).

In this report, we have focussed on the functional properties of the GABA recognition sites on hippocampal GABA_A receptors and their dependence on membrane cholesterol. Methyl- β -cyclodextrin has been used

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as the cholesterol carrier (Gimpl et al., 1997) in most experiments for both enrichment and depletion of membrane cholesterol. This has permitted more substantial manipulations of cholesterol than we were able to achieve using liposomes (Sooksawate and Simmonds, 1998). We have also examined the ability of epicholesterol, the 3 α -OH epimer of cholesterol, to substitute functionally for cholesterol.

2. Methods

2.1. Dissociation of hippocampal neurones

Dissociated hippocampal neurones were prepared from male Wistar rats aged 10–16 days as previously described (Sooksawate and Simmonds, 1998). In brief, brain slices in physiological salt solution (PSS), containing (mM): NaCl 140, KCl 4.7, CaCl₂ 2.5, MgCl₂ 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 was separated by centrifugation through a 5% solution of bovine serum albumin in PSS at 175g for 3 min to yield a loose pellet of neurones.

2.2. Manipulation of membrane cholesterol

A complex of cholesterol and 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 μ m Millipore filter and stored at –20°C.

2.2.1. Cholesterol enrichment

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 340g 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.

In a few experiments, liposomes prepared from phosphatidylcholine (PC, 99% from egg yolk) + cholesterol were used for cholesterol enrichment by the method previously described (Sooksawate and Simmonds, 1998).

2.2.2. Cholesterol depletion

Dissociated neurones were incubated with 10 mM methyl- β -cyclodextrin in PSS under the same conditions as described for cholesterol enrichment to obtain a range

of depletions. In a few experiments, pure PC liposomes were used for depletion by the method previously described (Sooksawate and Simmonds, 1998).

2.2.3. Restoration of cholesterol following depletion

Dissociated neurones were incubated at 31°C in oxygen-saturated PSS containing 10 mM methyl- β -cyclodextrin for 20 min. Following centrifugation at 340g 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 340g for 3 min and two washes of the neurones with PSS.

2.2.4. Incubation with epicholesterol

The preparation of a stock solution of epicholesterol complexed with methyl- β -cyclodextrin and dilutions for incubation with neurones, together with the incubation conditions, were exactly as described for cholesterol.

2.3. 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 homogenised (Ultra Turrax Homogeniser, Germany) in ice cold wash buffer containing (mM): Tris-base 5, EDTA 1, pH 7.4 at 4°C, centrifuged at 48,400g (J2-21M/E, Beckman, UK), and then resuspended in ice cold wash buffer. Cholesterol and protein assays were carried out on the suspensions using the Sigma cholesterol diagnostic kit and the BioRad Protein Assay, respectively.

2.4. Electrophysiological measurements

Patch pipettes prepared from thin wall borosilicate glass capillaries (Clark Electromedical Instrument, UK) were filled with a solution containing (mM): CsCl 140, MgCl₂ 4, Na₂ ATP 4, EGTA 11, CaCl₂ 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 M Ω .

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 (List-Electronic, Germany) and was monitored simultaneously on an oscilloscope (Gould, UK) and a pen recorder (Gould, France). During recording, neurones were superfused with PSS at a rate

of 3 ml/min. All experiments were performed at room temperature (~20°C).

2.5. Drugs and chemicals

GABA was dissolved in PSS. Bicuculline methochloride and picrotoxinin were dissolved in DMSO at concentrations of 10 mM and 20 mM, respectively, and then diluted into PSS. Rapid application of drugs close to the recorded neurone was performed with the “U-tube” method previously described (Sooksawate and Simmonds, 1998).

All drugs and chemicals were purchased from Sigma (USA) with the exception of the BioRad Protein Assay from Bio-Rad (Germany), methyl- β -cyclodextrin from Aldrich (UK), and CsCl, MgCl₂ and CaCl₂ from BDH (Poole, UK).

2.6. Data analysis

For concentration–response analysis, data from each neurone were plotted using GraphPad PRISM™ (GraphPad Software, USA) and fitted with a logistic equation in the form:

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

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 of the same neurone. Combined data are expressed as mean \pm SEM. Significance was tested by Student's t -test and a p value of less than 0.05 was considered to be significant.

3. Results

3.1. Cholesterol enrichment and depletion

Incubation of neurones with the complex of cholesterol and methyl- β -cyclodextrin yielded reproducible increases in the neuronal cholesterol levels that were related to the duration of incubation and the cholesterol concentration used (Table 1). The greatest enrichment achieved, that was compatible with stable whole-cell recording by patch electrode, was an increase in cholesterol to about 250% control.

Depletion of neuronal cholesterol by incubation with methyl- β -cyclodextrin alone was similarly reproducible and dependent on the duration of incubation (Table 1). The greatest depletion achieved was to 56% control, in which state the neuronal membrane was perceptibly less

Table 1

Enrichment and depletion of membrane cholesterol in acutely dissociated hippocampal neurones using methyl- β -cyclodextrin

<i>Enrichment</i>			Neuronal cholesterol ($\mu\text{mol}\cdot\text{mg protein}^{-1}$)
Incubation			
Time (min)	m β cd (mM)	chol (mM)	
5–20	0	0	0.319 \pm 0.009 (12)
5	1.5	0.15	0.423 \pm 0.011 ^a (3)
10	1.5	0.15	0.513 \pm 0.019 ^a (4)
20	1.5	0.15	0.560 \pm 0.032 ^a (5)
30	3.0	0.30	0.809 \pm 0.029 ^a (3)
<i>Depletion</i>			Neuronal cholesterol ($\mu\text{mol}\cdot\text{mg protein}^{-1}$)
Incubation			
Time (min)	m β cd (mM)		
5–20	0		0.309 \pm 0.006 (14)
5	10		0.225 \pm 0.017 ^a (3)
10	10		0.218 \pm 0.005 ^a (4)
20	10		0.175 \pm 0.010 ^a (6)

^a Significantly different from control (m β cd=0) $p < 0.0001$. Numbers of experiments are shown in parentheses.

rigid than normal during the electrode patching procedure.

A more limited degree of cholesterol enrichment and depletion was achieved using liposomes, the maximum enrichment being to 130% control and the maximum depletion to 80% control.

3.2. Neuronal responses to GABA

The membrane current evoked by application of GABA rose rapidly to a plateau with the lower concentrations and to a peak followed by fade with the higher concentrations (Fig. 1). Good reproducibility of the log concentration–response relationships between neurones is shown by the small error bars for the combined data in Fig. 2. The EC_{50} values for GABA, calculated from individual neurones, were also highly reproducible, the mean value in control neurones being $5.15 \pm 0.14 \mu\text{M}$ ($n=9$).

3.3. Effect of membrane cholesterol on responses to GABA

Enrichment of membrane cholesterol reduced responsiveness to GABA (Fig. 1) and caused parallel shifts to the right of the GABA log concentration–response curves (Fig. 2), the extent of the shift being related to the degree of cholesterol enrichment. Depletion of membrane cholesterol also reduced responsiveness to GABA (Fig. 1) and caused parallel shifts to the right of the GABA log concentration–response curves (Fig. 2). The

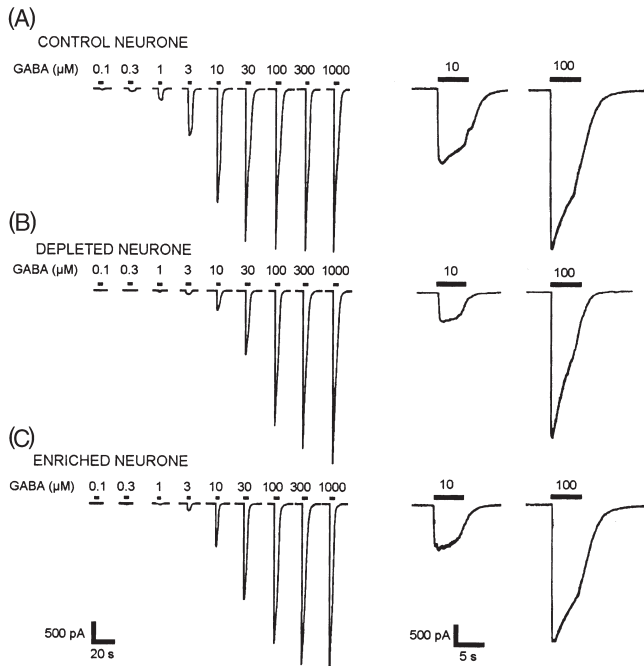


Fig. 1. Effects of changing membrane cholesterol on whole cell current responses to GABA in acutely dissociated hippocampal neurones. The cholesterol-depleted neurone (B) was pre-incubated with methyl- β -cyclodextrin to reduce its cholesterol content to 56% control. The cholesterol-enriched neurone (C) was pre-incubated with a complex of cholesterol and methyl- β -cyclodextrin to increase its cholesterol content to 182% control. Responses shown on the longer and shorter time bases are from different neurones.

resultant U-shaped relationship between GABA EC_{50} and membrane cholesterol level is shown in Fig. 3. Complementary results were obtained with methyl- β -cyclodextrin and with PC liposomes as the cholesterol carriers, the GABA EC_{50} values from both sets of experiments falling on the same curve. The reduced responsiveness to GABA in either cholesterol-enriched or cholesterol-depleted neurones was not associated with any consistent change in the current fade within responses (Fig. 1).

3.4. Effect of depletion and repletion of membrane cholesterol

The U-shaped relationship in Fig. 3 raised the possibility that the reductions in GABA potency might not be due to the changes in membrane cholesterol but to additional effects of the carrier systems, independent of whether or not cholesterol was combined with them. To examine this, the effect of cholesterol depletion followed by repletion was investigated with the methyl- β -cyclodextrin system. Fig. 4 shows that, at the end of the depletion and repletion, the GABA EC_{50} had returned to control values, strongly suggesting that the prolonged exposure to methyl- β -cyclodextrin had no additional effect that could alter GABA potency.

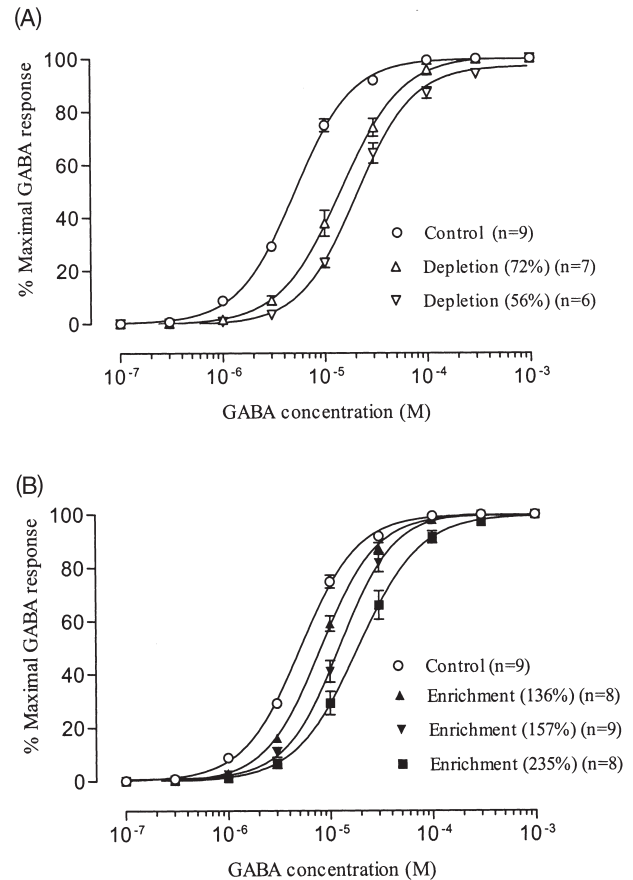


Fig. 2. Effects of cholesterol depletion (A) and enrichment (B), using methyl- β -cyclodextrin, on the GABA log concentration-whole cell current response relationship in dissociated hippocampal neurones. Each point is the mean \pm SEM of the current response, expressed as percent of the calculated maximal response.

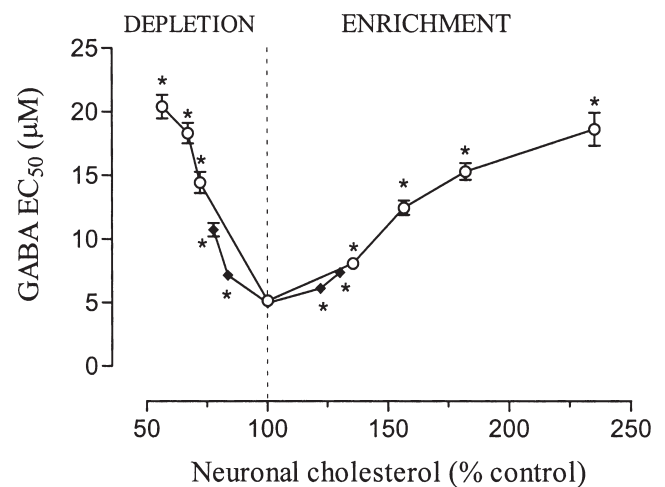


Fig. 3. Effects of cholesterol depletion and enrichment on the EC_{50} values derived from GABA log concentration-whole cell current response relationships. Open symbols show data from experiments with methyl- β -cyclodextrin and filled symbols show data from experiments with PC liposomes. Each point is the mean \pm SEM from 6–12 experiments. * EC_{50} value significantly different from that of control neurones (cholesterol=100%) $p < 0.0001$.

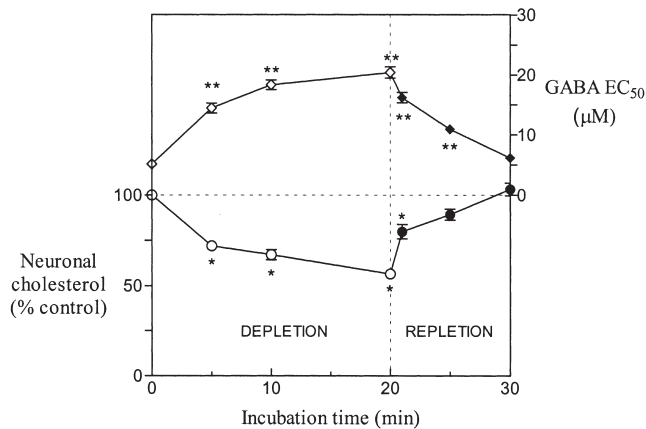


Fig. 4. Effects of cholesterol depletion (open symbols) followed by repletion (filled symbols) on the EC₅₀ values derived from GABA log concentration–whole cell current response relationships. **EC₅₀ value significantly ($p < 0.0001$) different from that of control neurones, shown at time 0 ($n = 6-9$). *Neuronal cholesterol significantly ($p < 0.05$) lower than control ($n = 3-6$).

3.5. Effect of membrane cholesterol on GABA antagonists

Co-perfusion of neurones with the competitive antagonist 5 μM bicuculline methochloride and GABA resulted in a parallel shift to the right of the GABA log concentration–response curve with no change in maximum response (Fig. 5). The EC₅₀ ratio in control neurones was 7.57 ± 0.55 ($n = 8$). In neurones enriched with cholesterol to 182% of control, the potency of bicuculline was significantly reduced ($p < 0.001$), the EC₅₀ ratio for GABA antagonism being 4.51 ± 0.46 ($n = 8$). In neurones depleted of cholesterol to 56% of control, however, the potency of bicuculline did not differ from control, the EC₅₀ ratio for GABA antagonism being 6.54 ± 0.67 ($n = 8$).

Similar experiments performed with the non-competitive antagonist 10 μM picrotoxinin resulted in a non-parallel shift to the right and depression of the maximum of the GABA log concentration–response curve. There were no changes in picrotoxinin effect with either cholesterol enrichment (Fig. 6) or depletion (data not shown).

3.6. Effect of epicholesterol on responses to GABA

Incubation of neurones with a complex of epicholesterol and methyl-β-cyclodextrin, in an exactly analogous way to the incubation with cholesterol and methyl-β-cyclodextrin, resulted in closely similar shifts to the right of the GABA log concentration–response curves. Thus, incubation conditions that, with cholesterol, resulted in enrichment to 176% of control and increased the GABA EC₅₀ from 5.15 ± 0.14 μM ($n = 9$) to 15.30 ± 0.66 μM ($n = 9$) (Fig. 3) also, with epicholesterol, increased the GABA EC₅₀ to 15.83 ± 0.56 μM ($n = 9$). Following the incubation

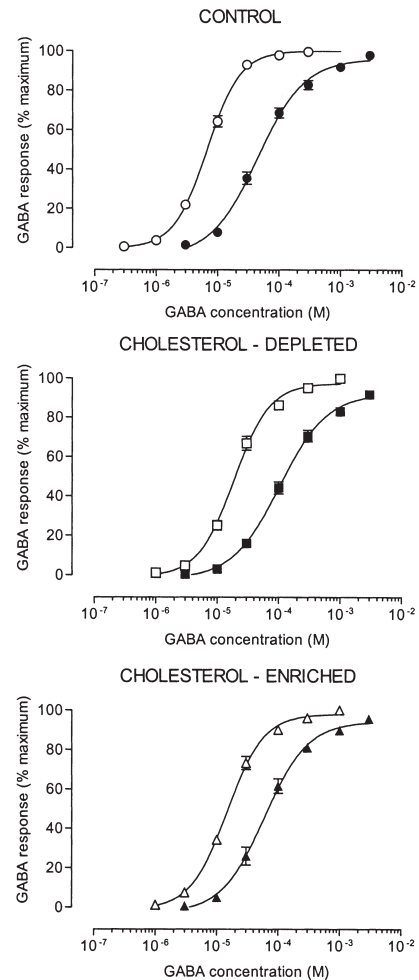


Fig. 5. Effects of cholesterol depletion to 56% control and enrichment to 182% control on the antagonism of GABA by 5 μM bicuculline methochloride (filled symbols). The EC₅₀ ratio for GABA antagonism by bicuculline was not affected by cholesterol depletion but was significantly reduced by cholesterol enrichment (see text).

with epicholesterol, the neuronal cholesterol content remained unchanged at $98.3 \pm 2.5\%$ of control. Since it has been shown that methyl-β-cyclodextrin can transfer epicholesterol as efficiently as cholesterol into membranes (Gimpl et al., 1997), it would appear that epicholesterol enrichment can substitute functionally for cholesterol enrichment to reduce the potency of GABA.

In contrast, in experiments analogous to those shown in Fig. 4, epicholesterol did not substitute functionally for cholesterol with regard to restoring GABA potency. Thus, when neurones were depleted of cholesterol to 56% control and then incubated for up to 20 min with a complex of epicholesterol and methyl-β-cyclodextrin, the GABA EC₅₀ remained elevated at 18.50 ± 0.87 μM ($n = 8$) compared with the value of 20.40 ± 0.93 μM ($n = 6$) in cholesterol-depleted neurones not exposed to epicholesterol. The incubation with epicholesterol and methyl-β-cyclodextrin had no further effect on membrane chol-

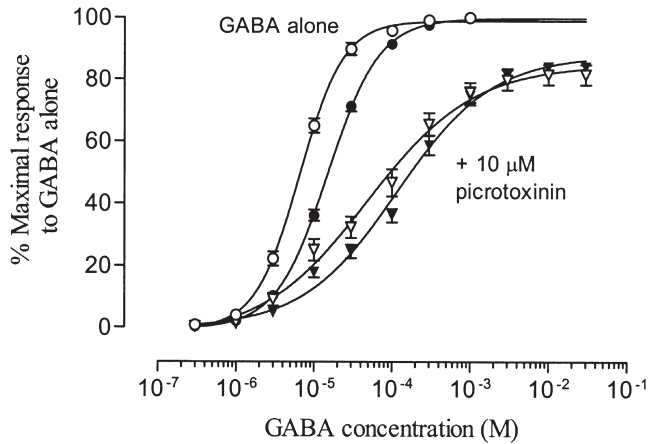


Fig. 6. Antagonism of GABA by 10 μ M picrotoxinin in control neurones (open symbols) and neurones enriched with cholesterol (filled symbols) to 182% control. Each point is the mean \pm SEM of the current response, expressed as percent of the calculated maximal response to GABA alone on the same neurone. The action of picrotoxinin was unaffected by the cholesterol level. The EC_{50} ratios were 9.18 ± 2.28 ($n=9$) in control neurones and 8.44 ± 1.14 ($n=9$) in cholesterol-enriched neurones and were not significantly different. The depressions of the maximum by picrotoxinin were $15.2 \pm 2.77\%$ ($n=9$) in control neurones and $12.1 \pm 1.94\%$ ($n=9$) in cholesterol-enriched neurones and were not significantly different.

esterol, the depletion remaining unchanged at 55% control.

4. Discussion

4.1. Manipulation of membrane cholesterol

Methyl- β -cyclodextrin proved to be a very effective carrier of cholesterol into and out of acutely dissociated neurones without disrupting their electrophysiological integrity. We were able to obtain much greater enrichments and depletions than with PC liposomes as a carrier system. Our use of methyl- β -cyclodextrin was prompted by the study by Gimpl et al. (1997) on the cholesterol dependence of the G-protein linked receptors for oxytocin and cholecystokinin expressed in HEK293 cells. We have now shown that the cholesterol dependence of a ligand-gated ion channel in neurones can be studied in the same way.

The plasma membranes of cells contain as much as 90% of the cholesterol present in the cells (Yeagle, 1993) and more than 85% of the plasma membrane content is in the inner (cytofacial) leaflet (Igbavboa et al., 1996; Wood et al., 1999). Cholesterol moves freely between the inner and outer leaflets with a $t_{1/2}$ of 1–6 min (Schroeder et al., 1991). It may be concluded, therefore, that both the enrichments and depletions of neuronal cholesterol achieved in the present experiments most probably affected both leaflets of the plasma membrane.

There are two principal types of mechanism through

which cholesterol may modulate membrane protein function: (1) alteration of the membrane's physical properties whereby increased cholesterol, for example, would reduce membrane fluidity and consequently affect protein function by impeding conformational changes; and (2) direct binding of cholesterol to sites on the trans-membrane regions of a protein to modulate certain aspects of its function. The latter mechanism is likely to be much more specific than the former for particular proteins and certain of their functions.

4.2. Modulation of GABA responses

With regard to the present results with GABA at the $GABA_A$ receptor, the U-shaped relationship between GABA EC_{50} and membrane cholesterol may be explained by different mechanisms for the enrichment-induced and the depletion-induced increases in EC_{50} . This is supported by the differential effects of enrichment and depletion on the potency of bicuculline and by the ability of epicholesterol to substitute for cholesterol only in the effects of enrichment and not in reversal of the effects of depletion. The binding of GABA to the $GABA_A$ receptor is an energy-requiring event that depends on receptor structure and presumably results in a conformational change (Jones et al., 1998). Likewise, the binding of bicuculline is also an energy-requiring event which, again, presumably results in a conformational change. Conformational changes may be impeded by cholesterol enrichment due to a reduced membrane fluidity. The similar reduction in GABA potency in membranes pre-incubated with epicholesterol is compatible with this interpretation since epicholesterol has been shown to decrease membrane fluidity as effectively as cholesterol (Gimpl et al., 1997).

In contrast, the reduction in GABA potency consequent upon depletion of membrane cholesterol, with no effect on bicuculline potency, suggests the possibility of a requirement for cholesterol to maintain optimal agonist potency. By analogy, membrane cholesterol has been found necessary to support agonist-induced opening of the nicotinic acetylcholine receptor and stabilise the secondary structure of that receptor's transmembrane domains (Fernandez-Ballester et al., 1994; Fong and McNamee, 1986). Other membrane proteins such as Na^+/K^+ -ATPase and the Na^+ - Ca^{2+} exchanger (Vemuri and Phillipson, 1989), the GABA transporter (Shouffani and Kanner, 1990) and the oxytocin receptor (Gimpl et al., 1997) also have a requirement for cholesterol that is highly specific to cholesterol. In the present study, the failure of epicholesterol to substitute for the depleted cholesterol points to a specific binding site for cholesterol rather than a fluidity related effect in supporting the action of GABA.

4.3. Implications in vivo

It is interesting to note that the optimum level of membrane cholesterol for the action of GABA at the GABA_A receptor, under the experimental conditions employed in this study, was the level present in control neurones. Whether sufficient fluctuations in plasma membrane cholesterol occur in vivo to affect GABA potency is not known. Most of the cholesterol in the CNS is synthesised there (Lütjohann et al., 1996) and circulating levels of cholesterol are thought not to affect the total cholesterol content of the CNS, but the neuronal plasma membrane fraction has not been separately studied. It cannot be excluded, therefore, that central neurones may be affected in the same way as erythrocytes and platelets which show decreases in plasma membrane cholesterol when serum levels of cholesterol are lowered by pravastatin in hypercholesterolaemic patients (Lijnen and Petrov, 1995). In the absence of direct evidence, we can only speculate whether the effect of lowered membrane cholesterol on GABA_A receptors described in this paper, and the effects on other membrane proteins cited above, contribute to the adverse changes in behaviour and mood reported with lowered serum cholesterol (Kaplan et al., 1997; Morgan et al., 1993).

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