Biochemical Pharmacology, Vol. 28, pp. 91–95.

0006-2952/79/0101 0091 $02.00/0

THE INTERACTIONS BETWEEN PHOSPHATIDYLCHOLINES AND ANAESTHETIC STEROIDS

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(*Received* 13 *January* 1978; *accepted* 12 *May* 1978)

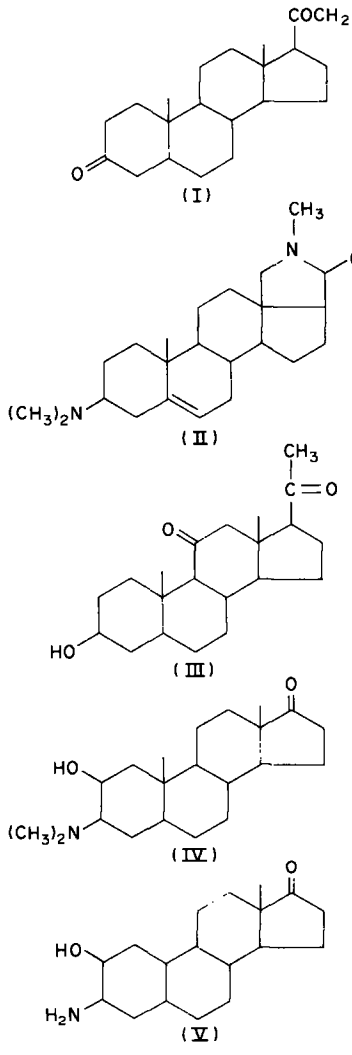
Abstract—The interactions between a number of anaesthetic steroids and phosphatidylcholines have been studied using chlorophyll A as a fluorescence probe. Aminosteroids Org NA13 and Org 6001 produce a decrease in phase transition temperature. Hydrocortisone, pregnanedione, pregnenolone, alphaxalone and Δ-16 alphaxalone have no effect on the phase transition temperature of dipalmitoyl phosphatidylcholine, although alphaxalone does have an effect on mixtures containing cholesterol. The results are consistent with the annular transition model for local anaesthesia.

In a recent paper [1] a particular model was presented for the mode of operation of local anaesthetics. The sodium channel in nerve was postulated to be surrounded by lipid in a rigid or gel-like state, and it was the rigidity of this lipid annulus which was postulated to keep open the actual slit in the sodium channel [2] through which the Na^+ ions move. Addition of local anaesthetics to the membrane was then postulated to lower the transition temperature for the annular lipid, which therefore transformed into a fluid, liquid crystalline-like state. When the lipid was in this fluid state, the sodium channel could collapse inwards, closing the slit and reducing the Na^+ conductance, as observed. The rigid lipid annulus around the sodium channel could originate either from the preferential selection of lipids with particularly saturated fatty acyl chains, or from a strong lipid–protein interaction [3].

The evidence in favour of the lipid component of the membrane as the binding site for local anaesthetics primarily follows from observations on the wide range of compounds that can act as local anaesthetics. Interaction directly involving membrane protein would be expected to show considerable stereochemical selectivity. Thus although the interaction between anticholinergic drugs and their receptors appears to depend largely on hydrophobic interactions [4], stereochemistry is still very important, as illustrated by the activity ratio of 60 for the optical isomers of the cyclohexylphenylglycollic acid ester of choline. In contrast, activity ratios for optical isomers of local anaesthetics are usually close to 1 [5], showing that hydrophobic interactions between local anaesthetics and their sites of action are sterically undemanding. The interaction also appears to be undemanding with respect to charge, since neutral, positively and negatively charged molecules are known which can act as local anaesthetics. Hille [6] and Strichartz [7] have suggested that positively charged amines act as local anaesthetics by binding within the channel, and Hille [6] has suggested that the binding site could include a negative charge in the region of the channel which he calls "the selectivity filter". The same charge is suggested to be part of the binding site for tetrodotoxin [6]. Interestingly, minor changes in the structure of tetrodotoxin are known to cause complete loss of activity [8].

If binding to the selectivity filter were to be important for the positively charged local anaesthetics, other sites of action would have to be postulated for negatively charged and neutral anaesthetic molecules (and presumably for positively charged anaesthetics with shapes very different from those postulated to act at the selectivity filter). The possibility that the sodium channel would contain a whole series of different binding sites for the man-made anaesthetic molecules seems unlikely. Many of these problems can be overcome if a lipid binding site is assumed for the local anaesthetics. As one test for the particular model proposed earlier, a correlation has been sought between the concentrations of compound that produce a significant (3–4°) drop in lipid transition temperature and the concentration that blocks the sodium conductance in nerve. For neutral and negatively charged molecules, the concentrations of compound that produce a 3–4° drop in transition temperature for dipalmitoyl phosphatidylcholine equal the concentrations that block the sodium conductance in nerve. For positively charged molecules, possible charge interactions with negatively charged lipids have to be considered, but taking this into account, a good correlation is again obtained. So far, the following compounds or groups of compounds have been tested: alcohols [9–11], amines [12, 13], barbiturates [14], chlorpromazine [15], β-blockers [15] and trihexyphenidyl and benztropine [16].

Here we extend these studies to steroid molecules, of which some show local anaesthetic activity. From time to time, a number of steroids have been used as anaesthetics or have been shown to possess anaesthetic activity: these include hydroxydione sodium succinate [I] [17], conessine [II] [18] alphaxalone [III] [19] and the amino steroids Org. NA13 [IV] [20] and Org. 6001 [V] [21]. The only one of these



which appears to be at present in clinical use is alphaxalone, the main active component in the general anaesthetic Althesin [19].

METHODS

Dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine, pregnanedione and pregnenolone were obtained from Koch–Light, myristic acid from Sigma, hydrocortisone from Organon and cholesterol from B.D.H. Chlorophyll A was prepared as previously reported [14]. Samples were prepared by dissolving lipid plus chlorophyll A and anaesthetic (if water insoluble) in chloroform in 10 ml stoppered flasks and evaporating to dryness under a stream of nitrogen. Buffer (0.01 M Tris–HCl pH 7.2; NaCl 0.1 M) was added and the mixture shaken on a Vortex mixer. Water soluble anaesthetics were added in the buffer.

Fluorescence measurements were made on an Aminco Bowman SPF Fluorimeter, exciting at 420 nm and recording at 670 nm. Temperature was continuously monitored with a thermocouple inserted directly into the fluorescence cell.

As described elsewhere [22] the effects of charged drugs on phase transition temperatures can be fitted to a theoretical model involving Langmuir adsorption isotherms for binding of both the charged and uncharged forms of the drug. Three parameters are introduced, the maximum number of drug binding sites per unit area of membrane (σ^{max}), the change in drug pK on binding (ΔpK) and a dissociation constant to describe the binding of the uncharged form of the drug (K^{unch}):

$$\sigma^{unch} = \frac{1}{K^{unch}}(\sigma^{max} - \sigma^{unch} - \sigma^{ch})[A]^{unch}_{x=0}$$

$$\sigma^{ch} = \frac{1}{K^{ch}}(\sigma^{max} - \sigma^{unch} - \sigma^{ch})[A]^{ch}_{x=0}$$

where σ^{unch} and σ^{ch} are respectively the number of uncharged and charged drug molecules adsorbed to the membrane per unit area and $[A]^{unch}_{x=0}$ and $[A]^{ch}_{x=0}$ are respectively the concentrations of uncharged and charged species at the membrane–solution interface. The binding constants K for the uncharged and charged species can be shown [22] to be related by

$$K^{unch}\,K^{ch} = \exp(2.303/\Delta pK).$$

The concentration of the uncharged form of the drug at the membrane–solution interface will be equal to its bulk concentration but the concentration of the charged form will be less as a result of charge effects, as described by the Gouy-Chapman theory:

$$[A]^{ch}_{x=0} = [A]\exp(-F\psi_0/RT)$$

$$\sinh(F\psi_0/2RT) = 136.6\sigma^{ch}/\sqrt{c}$$

where F is the Faraday and c is the electrolyte concentration (assuming that only a monovalent electrolyte is present). The depression ΔT of the transition temperature can then be calculated from the mole fraction x_{drug} of drug in the membrane,

$$\Delta T = \frac{RT^2}{\Delta H}x_{drug}$$

where T is the transition temperature and ΔH is the enthalpy of the transition.

RESULTS

It has been shown in previous publications that changes in the fluorescence of chlorophyll A incorporated into liposomes can be used to measure the temperature of lipid phase transitions [14]. Figure 1 shows the effects of addition of Org. 6001 and Org. NA13 on the mid-point transition temperature of the main gel to liquid–crystalline phase transition of dipalmitoyl phosphatidylcholine at pH 7.2. Figure 2 shows the effect of 3 mM Org. 6001 as a function of pH up to pH 8.2 beyond which it precipitates out. As will be discussed later, the non-linearity of the concentration plots in Fig. 1 can be attributed to the build-up of positive charge on the liposomes as a result of drug binding. It would therefore be expected that incorporation of negative charge into the liposomes should increase the effect of the drugs. This is indeed so: incorporation of 11 mole per cent myristic acid into the liposomes increases the temperature drop caused by Org. 6001 by about 1 .

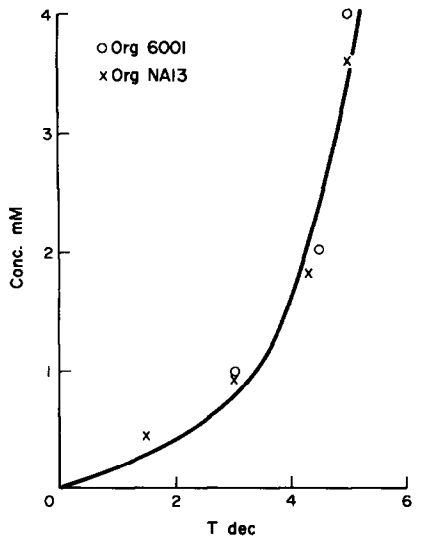


Fig. 1. The decrease in transition temperature for dipalmitoyl phosphatidylcholine caused by addition of (○) Org. 6001 and (×) Org. NA13 at pH 7.2. The solid line is the best fit to theory, with $K = 5 \times 10^{-4}$.

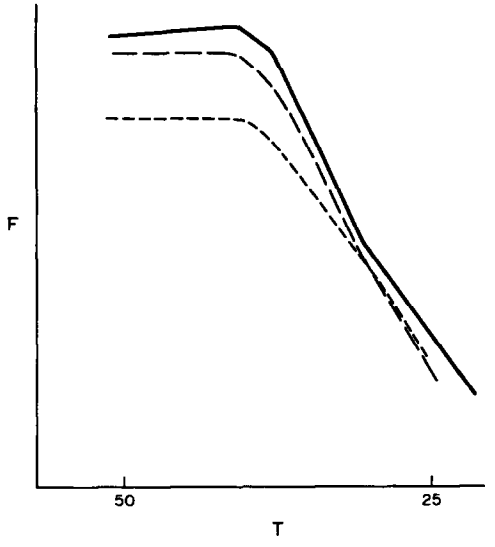


Fig. 3. The fluorescence intensity (arbitrary units) of chlorophyll A in liposomes of dipalmitoyl phosphatidylcholine as a function of temperature: — in pure dipalmitoyl phosphatidylcholine; — — — in the lipid containing 10 mole per cent pregnandione, and — — — — in lipid containing 20 mole per cent pregnandione.

In contrast to these results, incorporation of pregnan-3-20-dione into liposomes of dipalmitoyl phosphatidylcholine had no effect on the transition temperature of the lipid, although at high concentrations it caused a broadening of the transition and a loss of the pre-transition normally appearing at *ca.* 29° (Fig. 3). Very similar results were obtained with pregnenolone, alphaxalone and Δ-16 alphaxalone. The effect of alphaxalone was also tested on dimyristoyl phosphatidylcholine and found to be identical to those on dipalmitoyl phosphatidylcholine. The effect of alphaxalone is shown in Fig. 4 on mixtures containing dipalmitoyl phosphatidylcholine, dioleoyl phosphatidylcholine and cholesterol in a molar ratio of 8:2:5. In the absence of drug, breaks are seen in the fluorescence plots at *ca.* 25° and 58°. Addition of alphaxalone produces a decrease of the upper transition temperature to 50°, together with a marked decrease in fluorescence intensity at high temperatures. Δ-16 Alphaxalone has no effect on the transition temperatures although it does also produce a slight decrease in fluorescence intensity at high temperature. Hydrocortisone up to 0.8 mM (the limit of aqueous solubility) produces at most a 1° decrease in transition temperature for dipalmitoyl phosphatidylcholine.

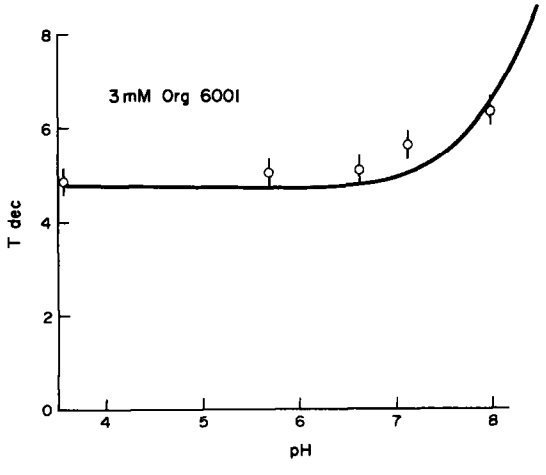


Fig. 2. The decrease in transition temperature for dipalmitoyl phosphatidylcholine caused by addition of 3 mM Org. 6001 as a function of pH. The solid line is the best fit to theory, with $K = 5 \times 10^{-4}$.

DISCUSSION

The results presented here show clearly that the amino steroids Org. NA13 and Org. 6001 interact with dipalmitoyl phosphatidylcholine and produce a decrease in the phase transition temperature. As shown for other positively charged molecules [12, 13, 15, 16] build-up of positive charge on the liposomes tends to limit binding of the drug, and incorporation of negatively charged lipid therefore increases the effects of the drugs. This conclusion is at variance with the suggestion of Ueda *et al.* [23] that only the neutral form of the anaesthetic binds to bilayers of phosphatidylcholines.

However, as discussed elsewhere [22] we believe that the suggestion of Ueda *et al.* [23] is thermodynamically inconsistent. Certainly, Fig. 2 shows that Org. 6001 causes a large decrease in transition temperature at pH values at which it is presumably predominantly in the charged form, so that the charged form seems able to bind to the membrane. In short there seems to be no reason for supposing that both charged and uncharged forms of a drug should not bind to a membrane, and, indeed, there is considerable evidence that both forms can indeed bind [22].

Making these assumptions it is then possible to

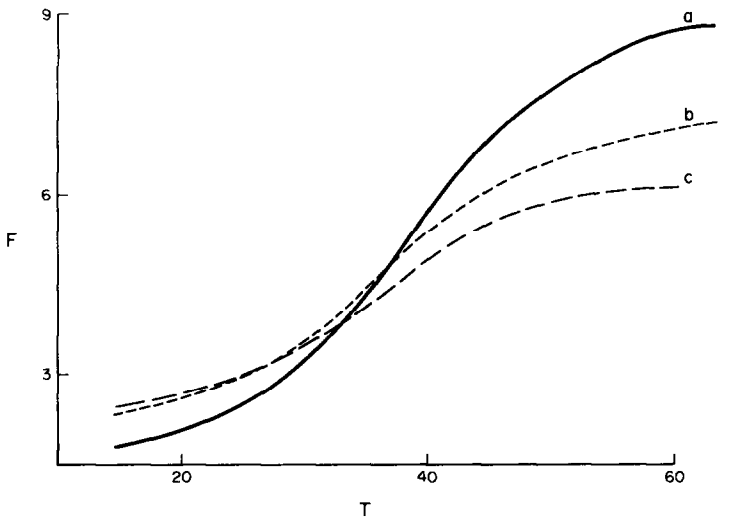


Fig. 4. The fluorescence intensity (arbitrary units) of chlorophyll A in liposomes of dipalmitoyl phosphatidylcholine dioleoyl phosphatidylcholine and cholesterol at a molar ratio of 8:2:5:a, alone; b plus 50 mole per cent Δ-16 alphaxalone; c, plus 50 mole per cent alphaxalone.

calculate the effect of drug binding on the transition temperatures of lipids.

Full details of these calculations will be given elsewhere, but Figs. 1 and 2 show the excellent fits that can be obtained assuming that $\sigma^{max} = 1/60Å^2$, ΔpK is zero, $K^{unch} = 5 \times 10^{-4}$ and that the drug pK value is *ca.* 9.5. Unfortunately, the bulk pK values of Org. NA13 and Org. 6001 do not appear to have been determined although that for 3β-aminocholestane has been reported as *ca.* 10 [24]. The value of K^{unch} is comparable to those for dibucaine and propranolol [Lee, unpublished observations]. The concentrations of both amino steroids required to produce conduction block in desheathed sciatic nerve are reported to be similar to that for lignocaine [25, 26], and so are probably in the millimolar range. The concentration required to produce a 3° drop in transition temperature is about 1 mM. These results therefore fit the annular transition model for local anaesthesia [1].

The experiments with alphaxalone suggest that alphaxalone has no effect on the transition temperature of dipalmitoyl phosphatidylcholine. This is in contrast with the results of Conner *et al.* [27] which suggest that alphaxalone does reduce the phase transition temperature in sonicated aqueous dispersions of dipalmitoyl phosphatidylcholine, as measured using differential scanning calorimetry. The contradiction might perhaps be attributable to the effects of sonication. We also note that the results of other studies using differential scanning calorimetry on similar systems can be surprising. Thus Monniot and Lussan [28] have reported on the basis of such studies that cholesterol causes a decrease in transition temperature for dipalmitoyl phosphatidylcholine whereas it appears to be generally accepted that cholesterol actually causes an increase in transition temperature [29]. Nevertheless, the lack of effect found in our studies suggests that either alphaxalone is not a local anaesthetic, or that if it is, then its mechanism of action must be different to that of other local anaesthetics. In fact, alphaxalone has been reported to show no local anaesthetic action (L. S. Rao and H. H. Wang, personal communications) and alphaxalone has also been reported to have no effect on the compound action potentials due to the fibres of the lateral olfactory tract in isolated olfactory cortex of the guinea pig [30, 31].

The general anaesthetic action of alphaxalone remains unexplained but could be due to either a decrease in the amount of transmitter released by a nerve impulse at a synapse or due to a decrease in the sensitivity of the post-synaptic membrane to the transmitter. Certainly, alphaxalone does seem to have some effect on membranes containing cholesterol, and brings about a decrease in the upper transition temperature in this system (Fig. 4). Interestingly, Δ-16 alphaxalone has no general anaesthetic effect and appears to have no effect on the bilayers containing cholesterol. Lawrence and Gill [32] have previously shown that alphaxalone produces a decrease in order in bilayers containing cholesterol, whilst Δ-16 alphaxalone has no effect. How these observations relate to a mechanism for general anaesthesia is unclear.

It has also been shown here that hydrocortisone has no effect on the phase transition temperature of dipalmitoyl phosphatidylcholine. It has been shown to have no effect on nerve action potentials at concentrations up to 0.3 mM [33]. Munck [34] has suggested that a steroid such as hydrocortisone will lie "on edge" at a water–heptane interface, in order to permit entry into the water phase of all its polar groups, including the C11-OH. If it adopted a similar orientation at the lipid–water interface, then there would be little interaction with the lipid fatty acyl chains, and so little effect on the temperature of the phase transition, as observed. A rather similar orientation could be

expected for alphaxalone since a molecular model shows that the hydrophobic oxygen containing groups are spaced along one side of the molecule.

The other steroids tested, pregnanedione and pregnenolone, also have no effect on temperatures of lipid phase transitions. Although these compounds have been reported to have general anaesthetic properties [35] no studies appear to have been made of any local anaesthetic activity. The lack of effect of these steroids on phase transition temperatures is not due to a failure to incorporate into the bilayers, since they abolish the lipid pre-transition (Fig. 3). Hydrocortisone has also been reported to insert into monolayers of lipids in the fluid state at the air–water interface, although they are excluded from lipids when tightly packed [36].

Acknowledgements—I thank Dr. Sugrue, Organon Laboratories Ltd., for the samples of Org. NA13 and Org. 6001 and Dr. Rao of Glaxo Research Ltd., for the samples of alphaxalone and Δ-16 alphaxalone and for further details of their experiments.

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