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Pressure reversal of the action of octanol on postsynaptic membranes from *Torpedo*

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1 Octanol increases the binding of [^{3}H]-acetylcholine to the desensitized state of the nicotinic receptor in postsynaptic membranes prepared from *Torpedo californica*.

2 This increase in binding results from an increase in the affinity of [^{3}H]-acetylcholine for its receptor without any change in the number of sites or the shape of the acetylcholine binding curve.

3 High pressures of helium (300 atm) decrease [^{3}H]-acetylcholine binding by a mechanism that changes only the affinity of acetylcholine binding.

4 Helium pressure reverses the effect of octanol on the affinity of [^{3}H]-acetylcholine for its receptor.

5 This pressure reversal of the action of octanol at a postsynaptic membrane is consistent either with pressure counteracting an octanol-induced membrane expansion or with independent mechanisms for the actions of octanol and pressure.

6 The data do not conform with a mechanism in which pressure displaces octanol from a binding site on the receptor protein.

Introduction

The underlying mechanisms by which anaesthetics produce general anaesthesia remain largely undefined. Their site of action *in vivo* is thought to be in a non-polar region of a membrane, probably in the lipid bilayer or a suitable region of a protein. Indeed, the potencies of some two dozen structurally diverse general anaesthetics correlate highly over four orders of magnitude with their lipid bilayer partition coefficients (Janoff *et al.*, 1981). However, the very success of this correlation means that little physico-chemical information remains from which to draw mechanistic insights.

In the past decade, however, studies of the pressure reversal of general anaesthesia have led to the development of a number of theories of general anaesthesia based on the assumption that anaesthetics induce a membrane expansion that can be reversed by applying pressure. (For reviews see Miller & Miller, 1975; Roth, 1979; Janoff & Miller, 1982; Franks & Lieb, 1982.) In these theories, anaesthetics and pressure produce effects that oppose each other. Thus, the effect of the anaesthetic, although reversed by, is actually independent of, pressure. (For example, a given concentration of anaesthetic expands the membrane equally at 1 atm and at high pressure, but the *net* volume change relative to the 1 atm control is smaller at high pressure because of the action of mechanical compression.) These theories contrast to the predictions of those direct anaesthetic-protein interaction models in which pressure is assumed to act by displacing the anaesthetic from its binding site (Franks & Lieb, 1982). In these models it is not necessary to suppose that pressure has any effect in the absence of the anaesthetic. Resolution between such models using data derived *in vivo* is difficult, and a more profitable approach may lie in studying specific excitable proteins in their membrane environment.

Choosing a model system in which to perform such studies is somewhat arbitrary because of the ill-defined physiology of general anaesthesia (Richards, 1978). Furthermore, general anaesthetics are rather non-specific in their pharmacological actions. This increases both the chances of finding a suitable model system and the risk of encountering mechanisms related to secondary effects rather than to general anaesthesia *per se*.

One accessible excitable membrane upon which a

wide variety of general anaesthetics are known to act is the postsynaptic membrane of the neuromuscular junction. Volatile, barbiturate, steroid and amine general anaesthetics all reduce excitation, and it has been suggested that the actions of these volatile anaesthetics and long-chain alcohols are related to their lipid solubility and may be exerted via lipid fluidization (for a review see Gage & Hamill, 1981). In addition to their effects on permeability, general anaesthetics also potentiate the onset of desensitization of the acetylcholine receptor (Magazanik & Vyskocil, 1976). A biochemical analogue of desensitization may be found in the acetylcholine receptor-rich membranes isolated from electric fish, where agonists induce a slow increase in the affinity of acetylcholine binding, which follows the same time course as the desensitization of channel permeability (Neubig *et al.*, 1982). General anaesthetics also cause an increase in the affinity of acetylcholine binding (Cohen *et al.*, 1980; Sauter *et al.*, 1980a; Young & Sigman, 1980). Some workers have attempted to relate the ability of general anaesthetics and alcohols to cause such desensitization to their lipid solubility (Young & Sigman, 1981). In this study, we examine the ability of pressure to reverse the effects of octanol on acetylcholine binding to the desensitized state of the receptor. A preliminary account of some of the pressure work has been published previously (Miller, 1978).

Methods

Preparation of receptor-rich membranes

The procedure used was an adaptation of the method of Cohen *et al.* (1972). All processes were carried out on ice and all solutions contained 0.02% sodium azide. Portions of skinned, fresh electroplaque tissue dissected from chilled *Torpedo californica* (Pacific Biomarine, Venice, CA) were added to 2 volumes of distilled water and homogenized in a Virtis Hi-speed 45 homogenizer (Virtis Company, Gardiner, NY) for 2 min at 95% maximum speed. After 30 s this process was repeated for 1 min. The homogenate was then centrifuged at 5,000 *g* for 10 min and the supernatant set aside. The pellet was resuspended by ten strokes of a Teflon pestle in a glass homogenizer tube and centrifuged as above. The combined supernatants were filtered through gauze and centrifuged at 15,000 *g* for 90 min. The pellets were resuspended as above. The yield from each 400 g of wet tissue was split into six 56 ml aliquots of water, each layered on 14 ml of 1.08 M sucrose and centrifuged at 80,000 *g* for 90 min. Each pellet was resuspended in 23 ml of water and placed on a density gradient formed by freezing and thawing 1.2 M sucrose twice. After centrifugation at 80,000 *g* for 4 h, 1 ml fractions were collected and assayed for acetylcholine binding sites and protein concentration. The pooled receptor-containing fractions from 400 *g* of wet tissue typically yielded 24 ml of suspension in 1.2 M sucrose, containing 3–10 μM acetylcholine binding sites with a specific activity of 1–2 μmol of receptor g^{-1} protein. This suspension was stored under nitrogen at 4°C.

Equilibrium binding of [^{3}H]-acetylcholine

An aliquot of stock receptor suspension was diluted with an equal volume of water, pelleted at 80,000 *g* for 70 min, and resuspended in *Torpedo* Ringer (containing m M: NaCl 250, KCl 5, $CaCl_2$ 3, $MgCl_2$ 2, and sodium phosphate 5, pH 7.0). For stock dilutions greater than 100 fold, equivalent results were obtained by direct addition to *Torpedo* Ringer. To this supension 100 μM diisopropylfluorophosphate was added. A 30 min incubation at room temperature proved sufficient to render acetylcholinesterase activity undetectable by the method of Ellman *et al.* (1961). Aliquots were then incubated with or without octanol for 15 min in sealed polycarbonate centrifuge tubes. The [^{3}H]-acetylcholine solution was added, and after a further 30 min, duplicate samples were withdrawn for scintillation counting. The membranes were separated either by centrifugation at 100,000 *g* for 90 min or by filtration using 25 mm diameter GF/F glass fibre filters (Whatman, Clifton, NJ). Two aliquots of filtrate or supernatant were withdrawn for counting. The bound concentration was determined as the difference between total and free counts. Non-specific binding was determined from control samples to which α-bungarotoxin (Miami Serpentarium Labs, Miami, FL) had been added in 10 fold excess at least 30 min before ligand addition.

Counting was performed in Biofluor (Beckman Instruments, Palo Alto, CA) or Liquiscint (National Diagnostics, Somerville, NJ) on a Beckman LS 8100 scintillation counter. Efficiency curves were obtained using the channels ratio method with tritiated water (New England Nuclear, Boston, MA) as standard.

Work at elevated pressures

This work was carried out mainly in an 8 inch internal diameter steel pressure chamber. Twelve filtrations could be performed in a given experiment using custom made filter units (Sauter *et al.*, 1980b). Membrane suspensions were either kept pre-mixed on a burstable plastic diaphragm above the filter, or in a bank of three syringes driven by remotely controlled stepping motors until required for filtration. The chamber could be pressurized to 300 atm with helium and filtration carried out *in situ*. Control experiments

in this apparatus were all performed at slightly elevated pressures in order to provide a driving force to burst the diaphragm and effect filtration.

A few experiments were performed in small stainless steel pressure bombs. The membrane suspension mixed with ligands was contained in a syringe which was connected through a high pressure sampling valve (Valco, Houston, TX) to ambient pressure. Small aliquots of pressurized suspension could thus be decompressed and filtered on the bench as desired.

Vapourization of octanol

The concentration of octanol was always determined by gas chromatography using a Poropak P column (Waters Associates, Milford, MA). Centrifugation experiments were carried out with full, capped polycarbonate tubes. Supernatants were sampled from well below the surface immediately on opening. This method involved essentially no loss of octanol. In filtration, sampling of the filtrate by GC showed a 10–15% loss of octanol. Some of this loss probably occurred after passage through the filter. Within our experimental errors, such small changes in concentration would not appreciably affect acetylcholine binding, and indeed, centrifugation and filtration experiments gave similar results with octanol (Table 1). Samples exposed to pressure could not be assayed for octanol because of bubbling during decompression. Such samples were kept in syringes until shortly before filtration. Controls on the bench showed no appreciable loss during this procedure.

Materials

[^{3}H]-acetylcholine was obtained in the acetate labelled form from Amersham-Searle (Arlington Heights, IL). Radiochemical purity was checked by paper chromatography and by precipitation of [^{3}H]-acetylcholine with excess receptor. [^{3}H]-acetylcholine was kept in small aliquots of anhydrous ethanol. A fresh aliquot was dried down and dissolved in *Torpedo* Ringer daily.

Results

Effects of anaesthetics on acetylcholine binding at equilibrium

1-Octanol increased the binding of [^{3}H]-acetylcholine. The concentration-dependence of this effect is illustrated in Figure 1, which shows that 2 mM octanol enhanced acetylcholine binding up to 2.4 fold, with the effect first becoming significant at about 400 μM octanol.

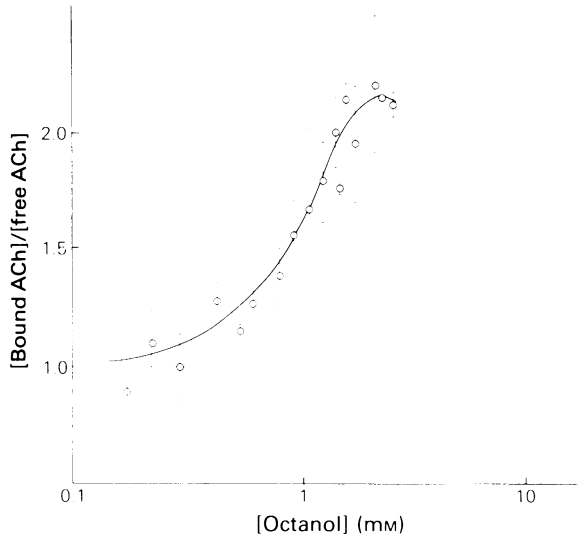

Figure 1 The effects of 1-octanol on [^{3}H]-acetylcholine (ACh) binding to receptor membranes from *Torpedo californica*. Membranes pretreated with diisopropylfluorophosphate were pre-incubated with or without anaesthetic for 15 min and then [^{3}H]-acetylcholine was added. After a further 30 min, membranes were separated by centrifugation or, in a few cases, by filtration. Each point shows the mean from several experiments and vertical lines represent s.d. Receptor concentration was usually 10–15 nM in acetylcholine sites, and total acetylcholine concentration was equal to this. These conditions yielded approximately 50% occupancy of acetylcholine sites and a 1:1 ratio, [bound acetylcholine]/[free acetylcholine]. Small variations from this condition in different preparations have been accounted for in the plot by normalizing the controls to a value of one. The bound acetylcholine concentration was corrected for non-specific binding using data from controls pre-incubated with α-bungarotoxin. The line was drawn by eye.

Effects of pressure on acetylcholine binding at equilibrium

Filtration assays were carried out in a high pressure chamber pressurized with helium. Helium pressure decreased acetylcholine binding, but the magnitude of the effect was not large. The ratio (bound acetylcholine]/[free acetylcholine] was determined in quadruplicate at 5 atm and at 300 atm. The results of six such experiments gave a mean decrease at high pressure to a value that was 73 ± 5.0 (s.d.) % of the low pressure value.

To examine the reversibility of the effect of helium pressure, two experiments were performed. In the big pressure chamber, membranes were exposed to 200 atm of helium, decompressed slowly to 10 atm,

Table 1 The effects of n-octanol and helium pressure on [^{3}H]-acetylcholine binding parameters

Conditions	K_l (nM)	n_H	R_T (nM)
Control[a]	9.9±1.45	1.6±0.29	27±2.5
Octanol 1.1 mM[a]	3.0±0.31	1.6±0.25	28±1.1
5 atm[b]	8.0±0.66	1.3±0.09	35±1.5
5 atm + octanol 1 mM[b]	3.1±0.23	1.6_5±0.16	32±1.1
275 atm[b]	11.0±1.02	1.4±0.12	33±1.7
300 atm + octanol 1 mM[b]	4.8±0.15	1.7±0.09	27±0.5

[a] Acetylcholine binding curves were determined by centrifugation assay, as described in the text, and fitted to the Hill equation by non-linear least squares analysis to yield the concentration at which half the binding sites are occupied, K_l, together with the slope, n_H, and the concentration of binding sites, R_T. The errors are standard deviations.
[b] Acetylcholine binding curves were determined by filtration assay in the high pressure chamber under various pressures of helium and analysed as in [a].

and then filtered. The amount of binding was within 1% of the value obtained in the same experiment at 10 atm during compression. In the small chamber, samples could be withdrawn without depressurizing the chamber and then filtered on the bench. Samples were withdrawn for assay at 1 and 5 atm, both before and after exposure to 300 atm of helium. Binding was identical in all these samples. The occupancy of the receptors was close to 50% in this experiment. Furthermore, we have shown previously that the number of acetylcholine binding sites is unaltered by similar pressure treatments (Sauter *et al.*, 1981).

The pK_2 of the phosphate buffer decreases 0.13 units during pressurization to 300 atm; however, with Tris-buffer, this effect is negligible (Disteche, 1972). Controls showed that substituting 5 mM Tris-HCl for 5 mM phosphate in the *Torpedo* Ringer had no effect on acetylcholine binding. In one experiment the (bound acetylcholine]/[free acetylcholine] ratio was 1.51±0.15 and 1.51±0.062 in the Tris and phosphate buffers (both 5 mM), respectively, at 5 atm. Upon pressurization to 300 atm, these values fell to 1.15±0.057 and 1.11±0.014, respectively. The latter figures are not significantly different ($P=0.25$). Thus, the small dependence of pH on pressure in the phosphate buffer does not appear to influence the results.

Origin of effects of anaesthetics and pressure on acetylcholine binding

To establish causes for the changes described above, [^{3}H]-acetylcholine binding was studied as a function of [^{3}H]-acetylcholine concentration. Binding curves were fitted to the Hill equation by a non-linear least squares method. Control results were essentially in agreement with those published previously (Cohen *et al.*, 1974). Binding showed a Hill coefficient of around 1.3 to 1.7 and half saturation (K_l) of 4–10 nM, where the range refers to the values encountered in different membrane preparations and with different samples of [^{3}H]-acetylcholine.

Octanol increased the affinity of [^{3}H]-acetylcholine binding without altering the Hill coefficient or the number of binding sites (Table 1). The shift in the half saturation concentration of acetylcholine was 3.3±0.59 fold at 1.1 mM octanol (Table 1).

High pressures of helium decreased the affinity of [^{3}H]-acetylcholine binding without altering the Hill coefficient or number of sites. Five separate binding curves on different preparations were determined in the range of 275–300 atm helium, each with a control at 5–10 atm helium (Table 2). Since the binding parameters varied somewhat from one preparation to another, each experiment was compared to its own control. The ratio of half staturation concentration at high pressure to that at low pressure was 1.32±0.074 ($P=0.006$), and the equivalent ratio for the Hill coefficient was 0.99±0.16 ($P=0.9$). (The low value of the dissociation constants in experiment three probably reflects a misassigned specific activity for

Table 2 The effects of helium pressure on [^{3}H]-acetylcholine binding parameters

Experiment Number	Low pressure		High pressure		Ratio	
	K_l^1 (μM)	n_H^1	K_l^h (μM)	n_H^h	K_l^h/K_l^1	n_H^h/n_H^l
1	7.6±0.80	1.6±0.21	9.5±1.05	1.4±0.17	1.24±0.19	0.88±0.16
2[a]	8.0±0.66	1.3±0.09	11.0±1.02	1.4±0.12	1.37±0.17	1.11±0.12
3	4.0±0.19	1.6±0.12	5.6±0.32	1.9±0.22	1.42±0.11	1.20±0.17
4	10.1±0.36	1.5±0.09	12.9±1.23	1.4±0.17	1.28±0.13	0.92±0.12
5	10.1±0.62	1.7±0.13	13.0±0.82	1.4±0.07	1.28±0.11	0.83±0.08
mean±s.d.					1.32±0.074	0.99±0.16

[a] As in Table 1.

the sample of [^{3}H]-acetylcholine used in this experiment.)

A set of four binding curves comparing the effects of 1.0 mM octanol at low and high pressures of helium were performed as a series. Controls were run in the same apparatus in the pressure chamber at a slightly elevated pressure in order to provide a driving force for filtration. Each binding curve consisted of 24 filtrations and required two separate experiments. Corrections for non-specific binding were determined from a further experiment with α-bungarotoxin pre-incubated membranes. The results of the Hill analysis are shown in Table 1. The effect of 1.0 mM octanol was partially reversed by 300 atm of helium. Thus, in this experiment octanol shifted the K_I (concentration at which half the binding sites are occupied) from 8.0 to 3.1 nM, and the application of pressure shifted it back to 4.8 nM. The shift in K_I produced by octanol is the same within error at both high and low pressure. Conversely, the effects of pressure application are independent of the presence of the anaesthetic.

The slopes of the binding curves, n_H or Hill coefficient, were difficult to obtain with high precision. This is well illustrated by the range of values obtained in Table 2. The estimate of this slope is much more sensitive than is the half binding concentration to errors in assigning the total number of sites. Nonetheless, while it is clear that no major systematic shift in slope occurs (such as a shift to the relationship to be expected for mass action binding to a single site), we cannot rule out smaller effects.

Discussion

Our work shows that exposure to octanol and high helium pressures causes changes in opposite directions of the dissociation constant of [^{3}H]-acetylcholine for its receptor. These effects are independent, so that exposure to appropriate quantities of either octanol or helium pressure in the presence of the other causes shifts similar to those seen in the presence of either agent alone (Tables 1 and 2).

Therefore, it is possible to demonstrate in a single postsynaptic membrane that an effect of an anaesthetic and pressure may oppose each other. Such an observation is consistent with the assumption of many theories of anaesthesia, that pressure reverses anaesthesia *in vivo* by a direct effect on the target of the anaesthetic action rather than by an indirect excitation of a separate compensatory mechanism in, for example, some neural network. This conclusion is also supported by observations of pressure reversal of anaesthetic action on axonal conduction (Roth *et al.*, 1976; Kendig, 1980). However, electrophysiological studies at nicotinic synapses show that pressure enhances anaesthetic blockade, possibly by adding a presynaptic depressant effect to the postsynaptic effect of the anaesthetic (Kendig, 1980; Parmentier & Bennett, 1980; Parmentier *et al.*, 1981). Thus, although it is unlikely that central nicotinic synapses themselves are involved in the pressure reversal of anaesthesia, desensitization of the acetylcholine receptor does provide a model system in which the mechanism of anaesthetic-pressure interactions may be studied.

More detailed studies will be required to elucidate fully the underlying mechanisms, but the overall behaviour of acetylcholine binding enables some simple mechanistic models to be tested *A priori* octanol might act on the acetylcholine receptor either by binding directly to the protein or by perturbing its surrounding lipids.

If octanol acts by binding directly to a site or sites on the receptor protein, then, if pressure directly reverses this effect, it might do so by displacing octanol as is supposed in some theories of anaesthesia (Franks & Lieb, 1982). In fact, the shift in the acetylcholine dissociation constant produced by octanol at 5 atm is essentially the same as that produced at 275–300 atm. Thus the ability of octanol to bind to its hypothetical site does not depend strongly on pressure and the displaced anaesthetic model is not consistent with this data. It follows that if octanol does act directly on the protein, the effect of helium pressure must be independent of that of octanol.

On the other hand, if octanol acts by dissolving in the lipid bilayer surrounding the acetylcholine receptor, then the fact that the effect of octanol on the binding affinity of acetylcholine is independent of the pressure at which it is applied simply implies that its lipid to buffer partition coefficient is independent of pressure. This prediction is consistent with recent partial molar volume measurements in both lipid bilayers and red cell ghosts, which show that the partition coefficient of octanol would only decrease about 15% over the pressure range used here (Kita & Miller, 1982). This lipid perturbation model is consistent both with that proposed by Gage *et al.* (1981) to explain the effects of volatile anaesthetics and long chain alcohols on miniature endplate curents and with the correlation noted between the lipid solubility of volatile general anaesthetics and alcohols and their actions on the acetylcholine receptor (Young & Sigman, 1981). However, this model will require extensive quantitative testing by spectroscopic means before it can be accepted.

An alternative model that should be consistent with our octanol-pressure data is one in which octanol and pressure exert their opposing actions independently. If this is correct, an independent mechanism of action for both octanol and pressure must be proposed. Octanol might act directly on the protein,

binding to some hydrophobic cleft (see above). Pressure might then exert its effect by a number of mechanisms; for example, by acting directly on the ligand binding step. Thus, charge-charge neutralization upon binding acetylcholine would probably lead to a volume increase through a release of electrostricted water. This possibility might be tested by examining a wider range of cholinergic ligands. Alternatively, pressure might effect conformational changes subsequent to binding or alter the distribution of receptors between the various conformations available, even before ligand binding. The underlying mechanisms that might be involved have been recently reviewed (Heremans, 1982).

The binding of acetylcholine to its receptor is a complex process. The receptor pre-exists in at least two states of different affinity, and rapid kinetic studies suggest the existence of several other states (Heidmann & Changeux, 1979; Boyd & Cohen, 1980). Thus, the equilibrium dissociation constant characterized by half occupancy on the Hill plot (Table 1) is a composite quantity related to a number of underlying dissociation constants. The possibility that the actions of pressure and anaesthetics might be opposing each other independently thus remains probable.

One way of assessing the likelihood of this mechanism is to study the kinetics of acetylcholine binding. We have carried out preliminary studies under helium pressure alone (Sauter *et al.*, 1981), but problems associated with the volatility of octanol, while aging reaction mixtures in the chamber, have prevented the necessary detailed studies of pressure reversal using our current apparatus.

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