THE LOCAL ANAESTHETIC AND BILAYER FLUIDISING AGENT, BENZYL ALCOHOL DECREASES THE THERMOSTABILITY OF THE INTEGRAL MEMBRANE PROTEIN ADENYLATE CYCLASE

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1. Introduction

Benzyl alcohol is a neutral, water-soluble molecule that readily partitions into lipid bilayers where it increases their fluidity and decreases the temperature at which lipid phase transitions or separations occur [1,2]. This ability to rapidly and reversibly increase bilayer fluidity using a neutral molecule, which precludes any charge interactions, has meant that benzyl alcohol has been an extremely useful molecule with which to define the effects of increased bilayer fluidity on the activity of a wide variety of integral membrane enzymes [2–8]. Increases in bilayer fluidity has led to the augmentation of enzyme activity, presumably by increasing the conformational flexibility of the protein. However, the sensitivity of different enzymes to changes in bilayer fluidity, effected by benzyl alcohol, varies considerably with some enzymes being inhibited by high concentrations of this agent [2].

The thermostability of enzymes is markedly dependent upon changes in environmental conditions. Thus changes in cation concentration, the polarity of environment and modifications of the degree of conformational flexibility of the protein that can, for example, be effected by intramolecular cross-linking agents [9] will all affect the stability of the protein. This study examines the effect of benzyl alcohol on the thermostability of the fluoride-stimulated adenylate cyclase activity of rat liver plasma membranes which is an integral membrane protein [10–12].

2. Materials and methods

Rat liver plasma membranes were isolated from male (200–300 g) Sprague Dawley rats as in [13]. Membranes were stored in 1 mM $KHCO_3$ (pH 7.2) at 10 mg protein/ml in liquid nitrogen.

Adenylate cyclase was assayed at 30°C as in [14] taking initial rates from linear timecourses.

Spin-label studies were carried out as in [2]. The *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid [I(12,3)] was used as a spin-probe at experimentally determined 'low-probe' concentrations. The outer and inner hyperfine splittings, 2 $T_{\parallel}$ and 2 $T_{\perp}$, were measured and used to obtain the order parameters $S_{T_{\parallel}}$ and S. These order parameters each assume values between 0 and 1, where the extreme values indicate that the probe samples fluid and immobilised environments, respectively. The order parameter S is corrected for changes in polarity that may occur whereas $S_{T_{\parallel}}$ is not. However, both have been shown to be reliable indicators of changes in membrane fluidity in these membranes [2,15,16].

For heat treatment studies, membranes at 2 mg protein/ml final conc. were incubated in 500 μl 1 mM $KHCO_3$ (pH 7.2) with and without various concentrations of benzyl alcohol. After determined intervals of incubation at 35°C samples (20 μl) were taken and immediately cooled in 1.5 ml microfuge tubes on ice. Aliquots (10 μl) were then taken for determination of fluoride-stimulated adenylate cyclase activity in 100 μl total assay vol.

I(12,3) was from Syva Co. (Palo Alto CA); benzyl alcohol was from Hopkin and Williams (Essex); all biochemicals were from Sigma (London) Chemical Co.

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(Sussex); radiochemicals were from the Radiochemical Centre (Amersham, Bucks.) and all other reagents were of AR grade from BDH Chemicals (Dorset).

3. Results and discussion

Preincubation of rat liver plasma membranes at 35°C under the conditions described, and in either the presence or absence of benzyl alcohol, led to an apparent first order decay of fluoride-stimulated adenylate cyclase activity over the range of inactivation studied (fig.1). The fluoride-stimulated activity is believed to be dependent upon the interaction of 2 distinct protein species, namely the guanine-nucleotide (G) coupling protein and the catalytic unit of adenylate cyclase [11]. Our study makes no attempt to ascertain whether the apparent first order loss in activity was due to either a more-labile G protein or more labile catalytic unit. The available evidence suggests that the G-protein is much more stable than the catalytic unit [17] and is in excess over the catalytic units [18] which suggests that, over the range examined, we are looking at the rate of decay of the catalytic units. However, a complex denaturation of both species with similar thermostabilities could yield apparently linear first order plots. Increasing benzyl alcohol concentrations led to a dramatic decrease in the thermostability of the fluoride-stimulated adenylate cyclase activity (fig.1), yielding first order decay plots.

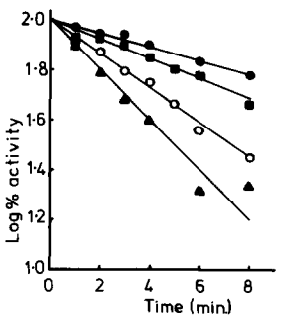

Fig.1. Benzyl alcohol decreases the thermostability of fluoride-stimulated adenylate cyclase activity in liver plasma membranes. Prior to assay membranes were pre-incubated at 35°C in the absence (●) or presence of various benzyl alcohol concentrations. This plot shows typical curves for a selected range of concentrations of benzyl alcohol at 0 (●), 20 mM (■), 80 mM (○) and 100 mM (▲).

The decreased thermostability of adenylate cyclase is presumably due to a change in the structure of the enzyme caused by benzyl alcohol. This could be effected either by benzyl alcohol partitioning directly into domains of the protein(s) or by virtue of the ability of benzyl alcohol to increase the fluidity of the lipid bilayer and increase the conformational flexibility of the protein. Indeed we already have an indication that benzyl alcohol can presumably affect the conformational flexibility of the protein by perturbing membrane fluidity as the membrane-bound enzyme, but not the solubilised one, is activated by benzyl alcohol over a concentration range where bilayer fluidity is increasing [2,3].

If the decreased thermostability of the enzyme were simply related to the partition of benzyl alcohol into either the membrane itself or some domain in the protein(s) then we might expect that the half-life for denaturation ($T_{0.5}$) would be inversely related to the benzyl alcohol concentration. This is because the concentration of benzyl alcohol in the membrane/protein domain (X_d) is linearly related to the total benzyl alcohol concentration (X_{tot}) by the expression:

$$[X_d] = \frac{[X_{tot}]}{1 + (1/K)}$$

where K is the partition coefficient. We can presume that the rate constant for thermal inactivation of the protein, k, is directly proportional to the total benzyl alcohol concentration. Thus as $k = 1/T_{0.5}$ then it follows that $X_{tot} \propto 1/T_{0.5}$ and if this hypothesis is correct a plot of $1/T_{0.5}$ *vs* benzyl alcohol concentration should be linear. However, such a transformation (fig.2a) yielded a gentle upwardly curving plot correlation coefficient, $r = 0.974$ for a curve of the form $y = me^{nx}$, although a straight line could be drawn through the data with a much poorer correlation coefficient, $r = 0.933$. This suggests that the $T_{0.5}$ is not simply related to benzyl alcohol concentration, but that greater values of benzyl alcohol have progressively less effect on the half-life.

It is possible that increases in lipid fluidity could decrease the thermostability of the protein by virtue of increasing its conformation flexibility. To assess this the fluidity of the membranes was measured using a fatty acid spin label I(12,3), yielding the order parameters S and $S_{T_{\|}}$. These measure the flexing of the fatty acyl chain region of the membrane and have been found to be good indicators of bilayer fluidity

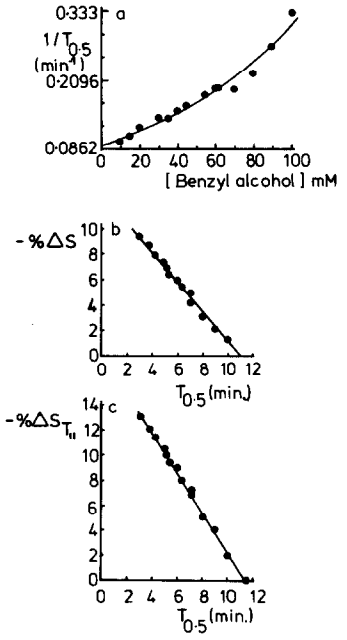

Fig.2. The relationship between the half-lives for thermal denaturation of adenylate cyclase and either benzyl alcohol concentration or membrane fluidity. (a) Plot of the reciprocal of the half-life for thermal denaturation of fluoride-stimulated adenylate cyclase at 35°C against total benzyl alcohol concentration. (b) As for (c) but the polarity corrected order parameter, S is used as an indicator of membrane fluidity at 35°C. (c) Plot of the order parameter $S_{T_{||}}$ for I(12,3)-labelled plasma membranes *vs* half-life for thermal denaturation of fluoride-stimulated adenylate cyclase at 35°C. The values of these parameters were determined at identical benzyl alcohol concentrations at 35°C. Thus $S_{T_{||}}$ reflects a change in membrane fluidity, elicited by benzyl alcohol, which is plotted against the thermostability of fluoride-stimulated adenylate cyclase in such membrane samples.

in these membranes [2,15,16]. They relate to viscosity in that they have values between 0 and 1, which measure the probability of *trans–gauche* isomerisation in the bilayer. When $S = 1$ the bilayer is in the solid state and the acyl chains are fully extended in the all-*trans* position and when $S = 0$ the bilayer is in a totally fluid state [2,21]. Thus the viscosity of the bilayer, $\gamma \propto S$ or $\gamma \propto S_{T_{||}}$. Hence the rate constant for thermal inactivation, k will be directly proportional to either S or $S_{T_{||}}$ and we can expect that $T_{0.5} \propto S_{T_{||}}$ and $T_{0.5} \propto S$. Such plots (fig.2b,c) are linear and show excellent correlations between the half-lives for thermostability at different membrane fluidities assessed by the order parameters $S_{T_{||}}$ (correlation coefficient, $r = 0.998$) and the polarity corrected order parameter S (correlation coefficient, $r = 0.993$). Indeed the goodness of fit against fluidity shows a great improvement over that against simply benzyl alcohol concentration. As one might expect that the increase in fluidity of the bilayer should, by similar arguments, be proportional to the total benzyl alcohol concentration, i.e., $[\text{benzyl alcohol}]_{\text{total}} \propto (1/S_{T_{||}})$ then the improvement in fit would seem difficult to rationalise. However examination of the dependence of fluidity on benzyl alcohol concentration (fig.3a) shows clearly that this is a curve. In model membranes the transition between the crystalline (solid) and liquid–crystalline (fluid) states of the bilayer is a highly co-operative event [19,20]. We would like to propose that the perturbation of bilayer fluidity by benzyl alcohol and its ability to disrupt lipid clusters in the bilayer [2] is also a co-operative event. Thus we would expect that, $S_{T_{||}} \propto (1/[\text{benzyl alcohol}]^n_{\text{tot}})$ where n is the index of co-operativity. Thus:

$$S_{T_{||}} = C/[\text{benzyl alcohol}]^n_{\text{tot}}$$

where C is a constant. Re-arranging and taking logarithms yields:

$$\log S_{T_{||}} = \log C - n \,.\, \log\,[\text{benzyl alcohol}]_{\text{tot}}$$

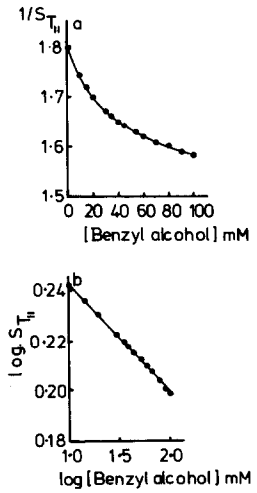

Fig.3..The relationship between the order parameter $S_{T_{||}}$ for the liver plasma membrane incorporated spin probe, I(12,3) and total benzyl alcohol concentration: (a) $1/S_{T_{||}}$ *vs* total benzyl alcohol concentration; (b) $\log S_{T_{||}}$ *vs* log [benzyl alcohol]$_{\text{total}}$. Similar results were obtained using S instead of $S_{T_{||}}$.

thus a plot of $\log S_{T_{\parallel}}$ *vs* log [benzyl alcohol] should yield a straight line if this model is correct. Such a plot (fig.3b) yields an excellent straight line of correlation coefficient, $r = 0.992$. Indeed, such a co-operative dependence allows us to explain our observations (fig.2a) that progressively increasing benzyl alcohol concentrations have proportionately less effect on the half-life for thermal denaturation.

Our data support the concept that the fluidity of the lipid bilayer can alter the tertiary structure of integral membrane proteins [21]. This would be expected to affect both the activity [3] and the thermostability [9] of such proteins, presumably by altering their conformational flexibility. The thermostability of cells in culture is directly related to their plasma membrane cholesterol content [22]. As cholesterol acts to increase the rigidity of cell membranes [21] then this would be consistent with the concept that lipid fluidity can affect the thermal sensitivity of membrane enzymes. However, our study cannot exclude the possibility that benzyl alcohol also exerts significant effects on the thermostability of this enzyme by direct interaction with the protein.

The sensitivity of the thermostability of this enzyme to benzyl alcohol requires that caution be exercised when attempting to interpret changes in activity of membrane proteins exposed to this drug. Indeed it makes it essential that any inhibitory effects seen with benzyl alcohol [2,3] are closely assessed in order to ascertain whether they are either freely reversible or due to irreversible thermal denaturation. As benzyl alcohol is used clinically [6,23] and like many other compounds that increase bilayer fluidity [21], is a local anaesthetic then the possible effects that these compounds might have in decreasing the thermostability of proteins in tissues that they contact should be carefully considered.

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