Biochemical Pharmacology, Vol. 32, No. 10, pp. 1601–1608. 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00
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FORSKOLIN AND ETHANOL BOTH PERTURB THE STRUCTURE OF LIVER PLASMA MEMBRANES AND ACTIVATE ADENYLATE CYCLASE ACTIVITY

ANTHONY D. WHETTON,* LINDSEY NEEDHAM,† NICHOLAS J. F. DODD,* CLARE M. HEYWORTH† and MILES D. HOUSLAY†‡§

*Biophysical Chemistry Section, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.; †Department of Biochemistry and Applied Molecular Biology, UMIST, P.O. Box 88, Manchester M60 1QD, U.K.; ‡California Metabolic Research Foundation, P.O. Box 1556, La Jolla, CA 92038, U.S.A.

(*Received* 5 *November* 1982; *accepted* 24 *December* 1982)

Abstract—Both forskolin and ethanol elicit the activation of basal and ligand-stimulated adenylate cyclase activities in rat liver plasma membranes. Ethanol is most potent at activating the fluoride- and glucagon-stimulated activities whilst having little effect on basal activity. In contrast forskolin exerts its greatest effect on basal activity. Over the concentration range that ethanol activates adenylate cyclase, it also increases bilayer fluidity as indicated by a decrease in the values of the order parameters for an incorporated fatty acid spin probe. At high concentrations forskolin does increase bilayer fluidity. However, it only begins to do so at concentrations above those where forskolin has already exerted its maximal effect in activating adenylate cyclase. Forskolin can still activate, albeit to a reduced extent, detergent-solubilized adenylate cyclase whereas ethanol cannot. Forskolin elicits a pronounced rise in hepatocyte intracellular cyclic AMP concentrations, whereas ethanol does not. Both forskolin and ethanol reduce the temperature of onset of the lipid phase separation occurring in rat liver plasma membranes. This is detected in Arrhenius plots of both glucagon-stimulated adenylate cyclase activity and order parameters of an incorporated fatty acid spin probe, where we find that forskolin is particularly potent in decreasing the temperature at which this lipid phase separation occurs. Our results are consistent with the notion that forskolin exerts its effect on adenylate cyclase primarily by a direct action on the catalytic unit of the enzyme. However, as forskolin is a potent perturber of the organisation of the lipid bilayer it is possible that this could modulate its effect on adenylate cyclase and might be expected to affect the activity of other membrane enzymes.

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii*, is used as a powerful tool for the investigation of the mechanism of action of adenylate cyclase [1]. It, unlike other regulatory ligands which act through guanine nucleotide regulatory proteins [2–5], appears to act directly on the catalytic unit of the enzyme [3–6].

Adenylate cyclase is an integral membrane enzyme [3] whose activity is modulated by changes in membrane fluidity [5, 7, 8]. Increases in membrane fluidity elicited by, for example, local anaesthetics activate the enzyme [7–9] whereas decreases in membrane fluidity caused by cholesterol and other agents [7, 10] lead to inhibition of the enzyme. As forskolin is a highly apolar compound, soluble in organic solvents [1], it is not untoward to expect it to partition into the lipid bilayers of biological membranes and perturb them. In this study we have attempted to assess any contribution of a forskolin-induced perturbation of the lipid bilayer on liver adenylate cyclase activity. This was chosen as a model system as we have previously defined in some detail both the physical properties of the lipid bilayer of this membrane and the response of rat liver adenylate cyclase to changes in membrane fluidity [7–10]. As forskolin is added to assays as an ethanolic solution, which leads to correspondingly high ethanol concentrations in the assays, we have also investigated its effect on adenylate cyclase activity and on membrane fluidity.

§ Author to whom correspondence should be addressed at: Department of Biochemistry and Applied Molecular Biology, UMIST, P.O. Box 88, Manchester M60 1QD, U.K.

MATERIALS AND METHODS

Liver plasma membranes were isolated from male Sprague–Dawley rats weighing between 200 and 300 g as before [11, 12]. Adenylate cyclase activity was measured as previously described by us, taking initial rates from linear time courses under all assay conditions [11]. Briefly, membranes were added to a cocktail at final pH 7.4 containing final concentrations of 25 mM triethanolamine–HCl, 1 mM EDTA, 1 mM theophylline, 5 mM $MgSO_4$, 7.4 mg/ml creatine phosphate and 1 mg/ml creatine kinase. Arrhenius plot data were analysed by a least-squares minimalisation procedure as used previously by us [13] to identify break-points. Protein determinations were carried out as before [13]. Adenylate cyclase was rendered soluble using the non-toxic detergent Lubrol 12A9 as in [14]. Only the fluoride-preactivated state was stable [3, 14].

Isolated hepatocytes were prepared from 225–

250 g male Sprague–Dawley rats [15] and incubated as described previously [16]. Cells (4–5 mg dry wt/ml) were pre-incubated for 20 min at 37° prior to the addition of the phosphodiesterase inhibitor, IBMX (final concentration 10^{-3} M). The pre-incubation was continued for a further 10 min before the addition of the required ligand. After incubation the cell suspension was spun through bromododecane into a PCA (0.62 M) and sucrose (0.25 M) mixture [17]. The PCA precipitate was resuspended and recentrifuged at 4000 *g* for 5 min. The supernatant was decanted and the PCA neutralized with ethanolamine [0.5 M and KOH (2 M)]. This supernatant was then stored at −20° prior to assay.

ATP was determined using the luciferase reaction [18]. Cyclic AMP was measured as previously described [11] using bovine heart muscle binding protein [14].

ESR measurements were made using a Varian E-9 Xband spectrometer equipped with variable temperature accessory (Varian E-257). The temperature was calibrated from 0° to 45° with a thermocouple placed in the resonant cavity. Measurements were made at a 2.0 G modulation amplitude, 0.30 sec time constant, 10 mW microwave power on a scan range of 100 G at a receiver gain of 5×10^3 or less. Sample spectra were compared with a standard manganese (Mn^{2+}) sample in a microwave cavity operating in the HO14 mode. The spectrometer was used in conjunction with a Nicolet 1020A signal averager from which the inner and outer hyperfine splittings $2T_{\parallel}$ and $2T_{-}$ were measured in terms of channels on the averager. These measurements were calibrated by comparison with the observed separation of the manganese marker peaks on the signal averager. The order parameters S and $S(T_{\parallel})$ of rat liver plasma membranes were calculated as previously detailed [19]. The values of S and $S(T_{\parallel})$ reflect the membrane fluidity. S may assume a value between 0 and 1 representing a free fluid or immobilized environment, respectively.

The method of preparation for ESR studies of rat liver plasma membranes was essentially as described earlier [19, 20]. Samples (50 μl) of membranes (4.2 mg of protein/ml in 50 mM Tris–HCl, pH 7.2) were added to the $I(12,3)$ spin label that had initially been deposited on the side of the tube by evaporation from aqueous ethanol in a nitrogen gas stream and were gently vortexed to yield a ratio of 9 μg of probe/mg of protein. All samples were pre-incubated at 4° for 15 min before placing the sample in a capillary tube which was taken for measurement in the ESR spectrometer. Forskolin was added to the plasma membranes as a solution in 95% aqueous ethanol or in an analogous fashion to the addition of spin label to the membranes. In the latter instance, incubation for periods of up to several hours with forskolin did not elicit further changes in S, $S(T_{\parallel})$ or the depression of the break-point. It would appear, on this basis, that under these conditions forskolin rapidly equilibrated with sites in the membrane.

The $I(12,3)$ spin label, *N*-oxyl-4′,4′-dimethyl oxazolidine derivative of 5-oxo-stearic acid, was obtained from Syva Co. (Palo Alto, CA). Forskolin (7β - acetoxy - 9,13 - epoxy - 1α,6*N*,9α - trihydroxy-labd-14-ene-11-one) was from Calbiochem. Phosphocreatine was from Sigma Chemical Co. (St. Louis, MO). Creatine kinase, collagenase, ATP, GTP and triethanolamine–HCl were from Boerhinger. Radiochemicals were from Amersham International. Glucagon was a gift from Dr. W. W. Bromer (Lilly Research Lab., IN). All other chemicals were of A.R. grade from BDH Chemicals (Dorset, U.K.).

RESULTS

Ethanol at concentrations greater than 100 mM led to the activation of basal, fluoride- and glucagon-stimulated adenylate cyclase activities (Fig. 1). This effect was most apparent for the fluoride-stimulated activity which was increased 3-fold, and was smallest for basal activity which was increased by about 1.5-fold (Fig. 1). At high ethanol concentrations (>0.9 M) there was an inhibition of both the fluoride- and glucagon-stimulated activities (Fig. 1). Ethanol also achieved a corresponding increase in bilayer fluidity as detected by the progressive decrease in the value of the order parameters ΔS, $\Delta S(T_{\parallel})$ for the incorporated $I(12,3)$ fatty acid spin probe (Fig. 1d). At ethanol concentrations that elicited optimal activation of both the fluoride- and glucagon-stimulated activities (0.8 M ethanol), the order parameters S and $S(T_{\parallel})$ were decreased by 2.8 and 1.7%, respectively (Fig. 1).

Forskolin began to activate adenylate cyclase activities at concentrations as low as 10^{-9}–10^{-7} M and exerted its maximum effect at around 10^{-4} M. At concentrations above this it appeared to inhibit activity slightly (Fig. 2). Whilst both the fluoride- and glucagon-stimulated activities were increased some 3 to 5-fold, basal activity was increased by as much as 50-fold by 10^{-4} M forskolin (Fig. 2). Forskolin, like ethanol, elicited an increase in bilayer fluidity as detected by a decrease in the order parameters S, $S(T_{\parallel})$ of the incorporated $I(12,3)$ spin probe (Fig. 2d). In contrast to ethanol, it did not appear to be as efficacious in increasing bilayer fluidity but interestingly exerted larger effects on the order parameter $S(T_{\parallel})$ than on the polarity-corrected order parameter S (Figs. 1 and 2; Table 1).

All of the effects of both ethanol and forskolin on adenylate cyclase activity were fully reversible (>90% recovery of activity) upon either dilution or washing the membranes by centrifugation (15,000 *g* × 10 min at 4°) in 1 mM $KHCO_3$ buffer, pH 7.2.

The detergent-solubilized enzyme was, in contrast to the membrane-bound enzyme, inhibited by increasing ethanol concentrations (Fig. 1b). However, forskolin elicited an activation of this enzyme (Fig. 2c) although its effect was somewhat reduced.

Both ethanol and forskolin perturbed intracellular cyclic AMP concentrations in intact hepatocytes under conditions where >90% of the cyclic AMP phosphodiesterase activity was inhibited by isobutylmethylxanthine (Fig. 3). Forskolin (Fig. 3b) caused a dramatic increase in cyclic AMP concentrations, exhibiting a dose dependency which paralleled its ability to activate basal adenylate cyclase activity (Fig. 2b). In marked contrast, ethanol exerted very little effect on intracellular cyclic AMP concentrations (Fig. 3b), which again reflected its

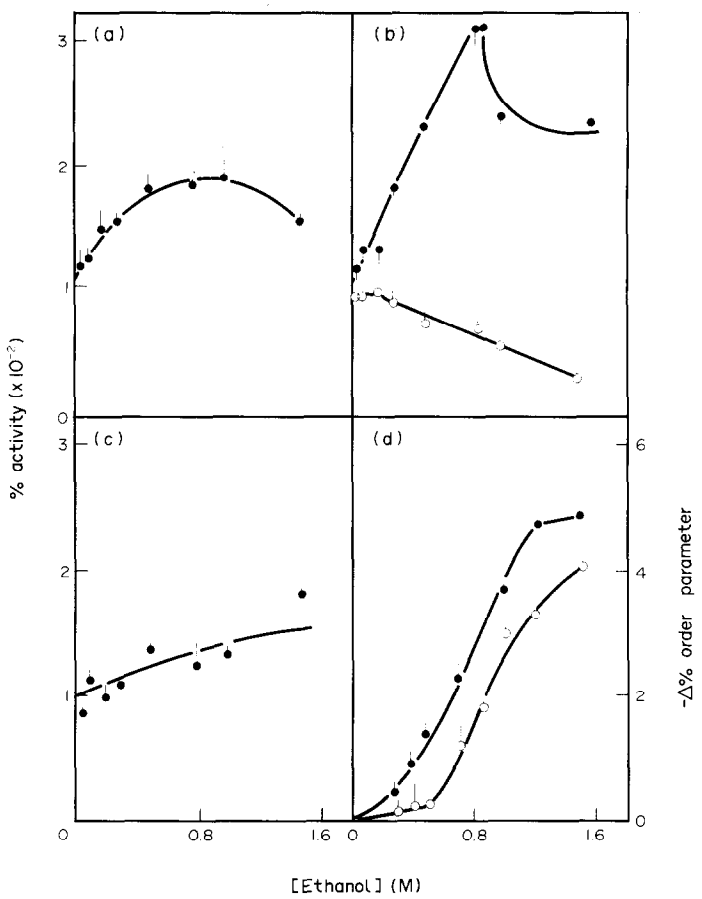

Fig. 1. Effect of ethanol on adenylate cyclase activity and the mobility of a spin probe in liver plasma membranes. (a) Glucagon-stimulated adenylate cyclase activity; (b) fluoride-stimulated adenylate cyclase activity in native (●) and solubilized membranes (○); (c) basal activity and (d) order parameters S (●) and $S(T_{\|})$ (○) for $I(12,3)$-labelled membranes. Experiments were carried out at 30° using three membrane preparations with errors ± S.D.

Table 1. Forskolin, ethanol and benzyl alcohol increase the mobility of a fatty acid spin probe incorporated into liver plasma membranes

Condition	% Change in order parameter $\Delta S(T_{\|})$	ΔS
1 mM forskolin*	−2.13 ± 0.07	−0.72 ± 0.10
850 mM ethanol*	−1.76 ± 0.20	−2.48 ± 0.14
50 mM benzyl alcohol†	−6.64 ± 0.65	−3.40 ± 0.46
Forskolin + ethanol (observed)*	−4.91 ± 0.19	−3.14 ± 0.18
Forskolin + ethanol (calculated)‡	−3.89	−3.2
Forskolin + benzyl alcohol (observed)†	−6.23 ± 0.17	−3.91 ± 0.29
Forskolin + benzyl alcohol (calculated)‡	−8.77	−4.12
Ethanol + benzyl alcohol (observed)‡	−9.17 ± 0.41	−6.08 ± 0.28
Ethanol + benzyl alcohol (calculated)‡	−8.4	−5.88

All experiments were carried out at 30°. Errors are S.E.M. and data are the average of either ten experiments (*) or six experiments (†). Calculated order parameters (‡) for mixtures of compounds are the sum of the respective changes in order parameters elicited in the presence of each ligand presented alone to the membranes.

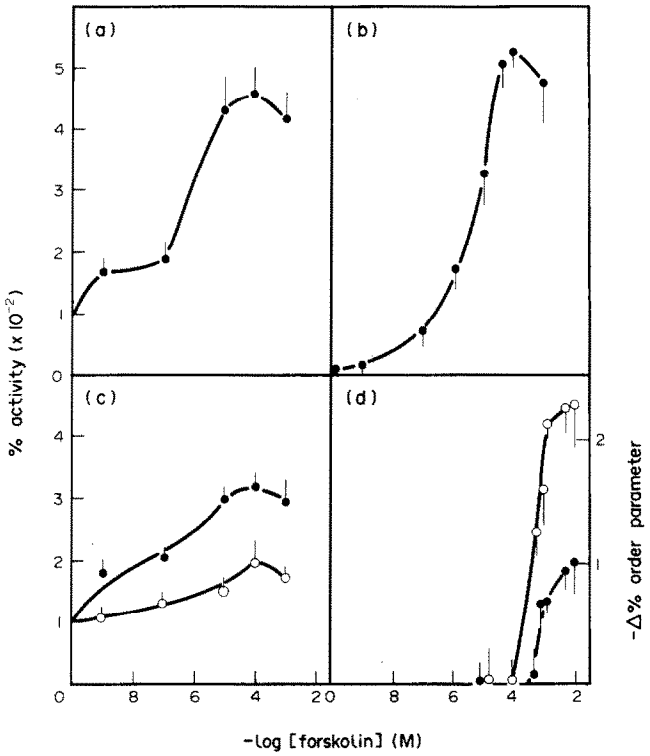

Fig. 2. Effect of forskolin on adenylate cyclase activity and the mobility of a spin probe in liver plasma membranes. (a) Glucagon-stimulated activity; (b) basal activity; (c) fluoride-stimulated activity in native (●) and solubilized (○) membranes and (d) the order parameters S (●) and $S(T_{\|})$ (○) for $I(12,3)$-labelled membranes. Experiments were carried out at 30° in the presence of 0.85 M ethanol using three membrane preparations with errors ± S.D.

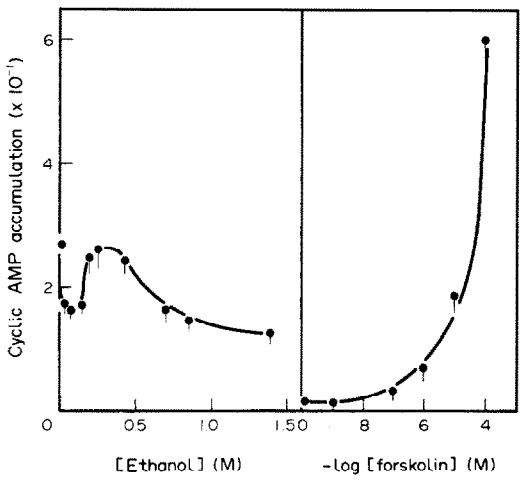

Fig. 3. Effect of forskolin and ethanol on hepatocyte intracellular cyclic AMP accumulation. Hepatocytes were preincubated for 10 min at 37° with IBMX to block cyclic AMP phosphodiesterase activity. They were then compared to ethanol or forskolin (+0.25 M EtOH) for 5 min prior to determination of intracellular cyclic AMP. Results are ± S.D.

action on basal adenylate cyclase activity. Although ethanol concentrations of 200–500 mM appeared to increase cyclic AMP concentrations, any further increase in ethanol actually led to a diminution of intracellular cyclic AMP concentrations (Fig. 3a). In the presence of forskolin, intracellular ATP concentrations remained normal (9.4 nmol/mg dry wt) and, with ethanol, they also remained normal except at the highest concentration used, when they fell. This presumably accounts for the drop in intracellular cyclic AMP concentrations observed using high ethanol concentrations.

Ethanol (0.85 M) caused a depression of the break-point in Arrhenius plots of glucagon-stimulated adenylate cyclase from 28°, seen in native membranes [7, 8, 11] to around 24°. When both ethanol (0.85 M) and forskolin (10^{-3} M) were added to the assays, the break was decreased to around 15° (Fig. 4; Table 2). Ethanol (0.85 M) decreased the temperature of the lipid phase separation, identified by Arrhenius-type plots of the order parameters S and $S(T_{\|})$, in $I(12,3)$-labelled membranes from 28° (see [7, 11, 19, 20]) in native membranes to around 24° (Fig. 5, a and b; Table 2). The addition of forskolin (10^{-3} M) alone caused a dramatic depression of the

Table 2. Effect of ethanol and forskolin on Arrhenius plots of glucagon-stimulated adenylate cyclase activity and order parameters of a membrane-incorporated spin probe

Ligand	Probe	Break-point ($T_s°$)	Activation energy for adenylate cyclase reaction Ea (kJ/mole) $T° > T_s°$	$T° < T_s°$
EtOH (0.85 M)	Glucagon-stimulated adenylate cyclase	23.7 ± 0.6	20.3 ± 5.1	105.6 ± 7.8
EtOH (0.85 M)	Fatty acid spin probe $I(12,3)$ order parameters $[S, S(T_\parallel)]$	24.4 ± 0.8	—	—
EtOH (0.85 M) + forskolin (10^{-3} M)	Glucagon-stimulated adenylate cyclase	15.5 ± 1.7	30.8 ± 10.7	82.8 ± 7.7
EtOH (0.85 M) + forskolin (10^{-3} M)	Fatty acid spin probe $I(12,3)$ order parameters $[S, S(T_\parallel)]$	15.0 ± 2.0	—	—
Forskolin (10^{-3} M)	Fatty acid spin probe $I(12,3)$ order parameters $[S, S(T_\parallel)]$	19.5 ± 0.7	—	—

Results reflect average values (± S.D.) of three separate experiments. Break-points were determined by a least-squares minimalisation procedure as described before [13].

lipid phase separation to around 19° (Fig. 5, c and d; Table 2). When both forskolin and ethanol were used together, they achieved additive effects in depressing the lipid phase separation to around 15° (Fig. 5, e and f; Table 2). In native membranes [19] and membranes with either forskolin alone (Fig. 5, e and f) or ethanol alone (Fig. 5, a and b), the slope of these plots was greater above the break than below for $S(T_\parallel)$ whereas the converse was true for S. However, when both forskolin and ethanol were present together then this situation was entirely reversed with the slope above the break being smaller than that below for $S(T_\parallel)$, and greater above the break for S (Fig. 5, c and d).

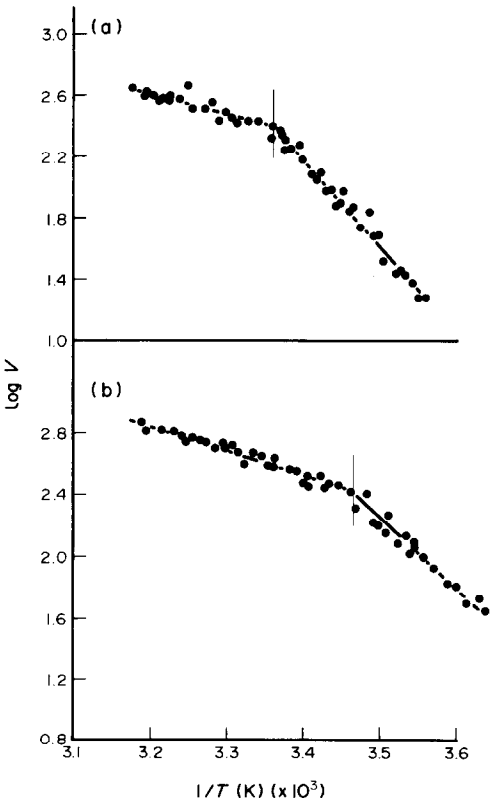

Fig. 4. Arrhenius plots of glucagon-stimulated adenylate cyclase activity (a) in the presence of 0.85 M ethanol; (b) in the presence of 10^{-3} M forskolin plus 0.85 M ethanol. Activities are pmol/min per mg protein. Vertical lines signify break-points.

When forskolin and ethanol were added together to spin-labelled membranes, they depressed the values of S and $S(T_\parallel)$ by an amount which was equal to or greater than that expected by summing their individual effects on these parameters when added alone to the membranes. A similar effect was observed (Table 1) for mixtures of ethanol and benzyl alcohol, which can also increase membrane fluidity [7, 19]. In contrast, when both forskolin and benzyl alcohol were added together, their combined effect was far from being additive on $S(T_\parallel)$, whilst it was slightly less than additive for S (Table 1).

DISCUSSION

Forskolin is believed to exert its action at the level of the catalytic unit of adenylate cyclase, rather than being mediated through the guanine nucleotide regulatory protein [1, 6]. It is possible therefore that forskolin acts either directly on the catalytic unit of adenylate cyclase itself or, because of the apolar nature of this diterpene, it could potentiate the activity of this enzyme by perturbing the lipid bilayer.

The activity of adenylate cyclase has been demonstrated to be highly sensitive to perturbations of its membrane environment ([8, 19]; see [7] for review). Agents that increase bilayer fluidity activate the enzyme, whereas those that decrease bilayer fluidity inhibit activity [7, 10]. Forskolin, being sparingly soluble in water, is usually added to assays as an ethanolic solution, where concentrations of ethanol can attain *ca* 0.85 M if high concentrations of forskolin are to be added ([1]; this work). We

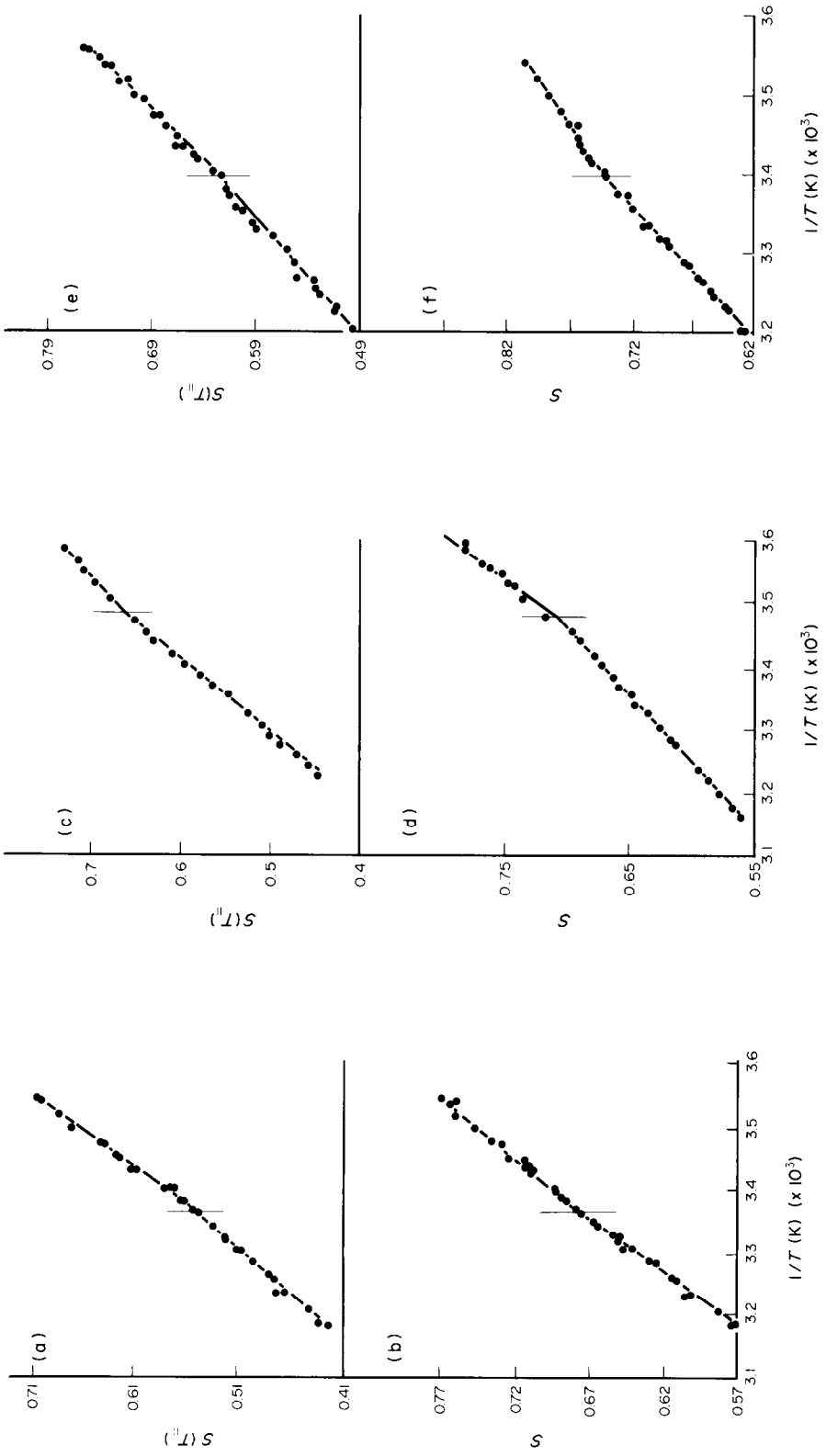

Fig. 5. Arrhenius plots of the order parameters S, $S(T_{\parallel})$ for $I(12,3)$-labelled liver plasma membranes. In the presence of 0.85 M ethanol for (a) $S(T_{\parallel})$ and (b) S; in the presence of 0.85 M ethanol plus 10^{-3} M forskolin for (c) $S(T_{\parallel})$ and (d) S; and in the presence of 10^{-3} M forskolin alone for (e) $S(T_{\parallel})$ and (f) S. Break-points are delineated by vertical lines.

thus need to identify alterations in adenylate cyclase activity due to perturbations of the membrane induced by either forskolin or ethanol.

Forskolin only began to increase bilayer fluidity at concentrations close to those at which it had already exerted a near maximally-activating effect on adenylate cyclase (Fig. 2). These results imply that forskolin exerts its primary effect by acting directly on the catalytic unit of adenylate cyclase. In contrast, ethanol activated the enzyme over a similar concentration range to which it increased bilayer fluidity (Fig. 1; Table 1). We suggest that this activation, which is of a much smaller magnitude to that elicited by forskolin (Fig. 2), occurs as a result of the increase in bilayer fluidity. Further support for this contention comes from our observations using the fluoride-preactivated solubilized enzyme which, as it has been removed from its membrane environment, is insensitive to alterations in membrane fluidity [8,20]. Here forskolin can still elicit activation, albeit to a reduced degree (Fig. 2), whereas ethanol cannot (Fig. 1).

In rat liver plasma membranes there is a well-determined lipid phase separation occurring at 28°, detected by us and others using a wide variety of physical techniques (see [7, 22] for reviews), which is responsible for the occurrence of a break at this temperature in Arrhenius plots of glucagon-stimulated adenylate cyclase activity [7–11]. The temperature at which this lipid phase separation, and corresponding break in the Arrhenius plots of glucagon-stimulated adenylate cyclase activity, occurs can be decreased in the presence of various local anaesthetics which partition into the bilayer and increase its fluidity [7–10, 19]; increased by agents which decrease bilayer fluidity [7]; shifted by the insertion of defined lipids [7, 23] and abolished upon elevation of the cholesterol content of the membranes [7, 10]. As such, the temperature at which this lipid phase separation occurs acts as a sensitive indicator of perturbation of the liver plasma membrane lipid bilayer by extrinsic agents. The addition of ethanol (0.85 M) elicited a decrease in the break temperature in Arrhenius plots of glucagon-stimulated adenylate cyclase activity by approximately 4° (Table 2; Fig. 4). In contrast, when forskolin (10^{-3} M) was present together with ethanol the break-point was decreased dramatically by some 13° (Table 2; Fig. 4). These effects were corroborated by spin label studies which identified similar depressions in the lipid phase separation temperatures (Table 2; Fig. 5).

Although forskolin (10^{-3} M) has a relatively small effect in increasing bilayer fluidity (Table 1), it nevertheless acts as a potent perturber of the lipid phase separation occurring in these membranes (Table 2). This may be because the lipid phase separation is believed to be caused by a redistribution of membrane cholesterol to form quasi-crystalline clusters [20]. The diterpene nature of forskolin could make it peculiarly effective at disrupting specific cholesterol–phospholipid adducts (see [10, 24]) as is benzyl alcohol [7, 10, 19]. Indeed benzyl alchol would appear to act similarly to forskolin in perturbing the bilayer as they exert less than additive effects in decreasing the value of the order parameters (Table 1). The converse would appear to be true of ethanol (Tables 1 and 2), implying that these agents interact with the bilayer membrane in a rather different fashion. This might explain why both ethanol and forskolin achieve additive (or synergistic) effects on increasing bilayer fluidity (decreasing S, $S(T_{\|})$; Table 1) and decreasing the temperature at which the lipid phase transition occurs (Table 2). A degree of interaction between these two agents, in perturbing the bilayer, is implied from the form of the Arrhenius-type plots of the order parameters S, $S(T_{\|})$ for $I(12,3)$-labelled membranes (Fig. 5). For native membranes alone or in the presence of either ethanol or forskolin, the slope of the plot for $S(T_{\|})$ was greater above the break than below whereas the converse was true for plots of S. However, this situation is entirely reversed when both ethanol and forskolin are added together to the membranes.

Our evidence is consistent with the notion [1] that forskolin exerts its effect primarily by direct action on the catalytic unit of adenylate cyclase. However, as forskolin is a potent perturber of lipid organisation in the bilayer it is possible that this might, to some extent, modulate its effect on adenylate cyclase. Although forskolin is a useful tool for elevating intracellular cyclic AMP concentrations, our results suggest caution in using high concentrations of forskolin which, by perturbing membrane structure, could non-specifically influence the activity of a variety of membrane proteins.

Acknowledgements—This work was supported by grants from the MRC (to M.D.H.) and also the CRC (to N.J.F.D.).

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Note added in proof—Since acceptance of this paper, studies [25, 26] have suggested that forskolin can also exert effects on adenylate cyclase activity through protein components other than the catalytic unit. This in no way affects our conclusion, that the alterations in membrane fluidity achieved by forskolin are not responsible for its large activating effect on adenylate cyclase.