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Effects of temperature and benzyl alcohol on the structure and adenylate cyclase activity of plasma membranes from bovine adrenal cortex

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Adenylate cyclase activation by corticotropin (ACTH), fluoride and forskolin was studied as a function of membrane structure in plasma membranes from bovine adrenal cortex. The composition of these membranes was characterized by a very low cholesterol and sphingomyelin content and a high protein content. The fluorescent probes 1,6-diphenylhexa-1,3,5-triene (DPH) and a cationic analogue 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) were, respectively, used to probe the hydrophobic and polar head regions of the bilayer. When both probes were embedded either in the plasma membranes or in liposomes obtained from their lipid extracts, they exhibited lifetime heterogeneity, and in terms of the order parameter *S*, hindered motion. Under all the experimental conditions tested, *S* was higher for TMA-DPH than for DPH but both *S* values decreased linearly with temperature within the range of 10 to 40 °C, in the plasma membranes and the liposomes. This indicated the absence of lipid phase transition and phase separation. Addition to the membranes of up to 100 mM benzyl alcohol at 20 °C also resulted in a linear decrease in *S* values. Membrane perturbations by temperature changes or benzyl alcohol treatment made it possible to distinguish between the characteristics of adenylate cyclase activation with each of the three effectors used. Linear Arrhenius plots showed that when adenylate cyclase activity was stimulated by forskolin or NaF, the activation energy was similar (70 kJ · mol^{-1}). Fluidification of the membrane with benzyl alcohol concentrations of up to 100 mM at 12 or 24 °C produced a linear decrease in the forskolin-stimulated activity, that led to its inhibition by 50%. By contrast, NaF stabilized adenylate cyclase activity against the perturbations induced by benzyl alcohol at both temperatures. In the presence of ACTH, biphasic Arrhenius plots were characterized by a well-defined break at 18 °C, which shifted at 12.5 °C in the presence of 40 mM benzyl alcohol. These plots suggested that ACTH-sensitive adenylate cyclase exists in two different states. This hypothesis was supported by the striking difference in the effects of benzyl alcohol perturbation when experiments were performed below and above the break temperature. The present results are consistent with the possibility that clusters of ACTH receptors form in the membrane as a function of temperature and/or lipid phase fluidity. Furthermore, the difference observed between the adenylate cyclase activity resulting from stimulation by NaF and ACTH, respectively, implies that the active state of this enzyme is different in the presence of each of these effectors.

Abbreviations: ACTH, corticotropin; DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; PMSF, phenylmethylsulfonyl fluoride.

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Introduction

Adenylate cyclase is a good example of a membrane-bound enzyme complex whose proper functioning greatly depends on membrane structure, because its enzymatic activity requires the interaction of several integral membrane proteins [1,2]. This complex consists of three protein components embedded in the cell plasma membrane: the receptors, which bind hormones or neurotransmitters to the extracellular surface of the plasma membrane, the stimulatory and inhibitory GTP binding proteins, which bind and hydrolyze GTP on the intracellular surface of the membrane, and the catalytic unit whose active site is also located on the membrane's intracellular surface. The regulatory stimulatory and inhibitory GTP-binding proteins have been shown to be the intermediaries between the receptor and the catalytic unit required for hormone action on adenylate cyclase [3,4], and they are also the site of action of this enzyme's nonphysiological effectors such as fluoride [5] and the cholera and pertussis toxins [6,7]. Adenylate cyclase activation or inhibition by hormones therefore implies transmembrane coupling of membrane proteins, which is prevented by membrane solubilization, and lateral diffusion of some and perhaps all the protein components in the lipid bilayer. Different models of activation have been proposed from studies on native membrane systems [2,8,9]. Recently, the purified regulatory stimulatory and inhibitory GTP-binding proteins were shown to consist of three different protein subunits, α, β and γ. Studies of these proteins in detergent solutions provided a model showing that an association-dissociation equilibrium between their subunits might play a key role in adenylate cyclase activation [10–12]. However, all the experimental data do not fit this model, as critically reviewed by Levitski [13] and further experiments in this field are still necessary.

In the present paper, we examined, in purified plasma membranes from bovine adrenal cortex, the interaction between the three components of adenylate cyclase (the receptor, the catalytic unit and the stimulatory GTP-binding protein) by studying its activation by three effectors as a function of membrane structure. The chemical composition of the membrane fraction was characterized, including: the protein/phospholipid ratio, the phospholipid and fatty acyl chain composition, and the cholesterol content. The fluidity of this fraction was also determined by fluorescence anisotropy. Two fluorescent probes, DPH and the cationic analogue TMA-DPH, were used to probe the hydrophobic and polar head regions, respectively, of the bilayer [14,15]. Adenylate cyclase activity was determined in the presence of three stimulators acting via the different protein components of the complex. These stimulators were firstly ACTH 1–24, a fully active fragment of the physiological hormone corticotropin which stimulates steroidogenesis in the adrenals by binding to its receptors [16], secondly fluoride, which acts via the stimulatory GTP-binding protein [5], and thirdly forskolin which directly stimulates the catalytic unit while it also may enhance activation of the catalytic unit by the stimulatory GTP-binding protein [17]. These studies were performed after membrane perturbation, either by varying the temperature or by treatment with the local anaesthetic benzyl alcohol, previously shown to fluidify both model and biological membranes [18–20].

Our results are consistent with the idea that in addition to the interaction between the receptor, the stimulatory GTP-binding protein and the catalytic unit, there is a protein-protein interaction in the membrane that might be important for adenylate cyclase activity, namely, the interaction between receptors. Furthermore, the diffusion of the receptor in the membrane seems to be a necessary feature of the activation of adenylate cyclase by ACTH. Lastly, in agreement with previous results [21], the state of the activated portion of this enzyme may differ depending on whether it is stimulated by ACTH or by fluoride. Part of this work has already been presented as a preliminary report [22].

Materials and Methods

Chemicals. ATP, GTP, phospho(*enol*)pyruvate, pyruvate kinase, phenylmethylsulfonyl fluoride (PMSF), aprotinin, pepstatin and phospholipid standards were from Sigma. Forskolin was purchased from Calbiochem and NaF from Merck (Darmstadt, F.R.G.). ACTH 1–24 was a kind gift

from Drs. K. Scheibli and R. Andreatta (Ciba-Geigy, Basel). Dimethylsulfoxide was from Fluka and tetrahydrofuran, fluorescence spectroscopic grade, from Merck. Benzyl alcohol was purchased from Aldrich. Cholesterol (analytical grade) was from Serva (Heidelberg, F.R.G.). DPH and tetraphenylbutadiene were from Koch-Light (Colnbrook Bucks, U.K.). TMA-DPH was purchased from Molecular Probes (Junction City, OR). Hexane was from Farmitalia Carlo Erba, HPLC grade (Milan, Italy). Isopropanol, methanol and chloroform were from Merck, Lichrosolv grade and acetonitrile was from J.T. Baker (U.K.). Other chemicals were of analytical grade.

Plasma membrane preparations. Bovine adrenal cortex plasma membranes were prepared as previously described [21]. Briefly, the adrenal cortex was homogenized essentially according to Schlegel and Schwyzer [23], in a 1 mM $NaHCO_3$, 1 mM DTE, 0.25 M sucrose buffer (pH 7.5) containing the protease inhibitors: 0.1 mM PMSF, and aprotinin and pepstatin at 0.2 mg per g of tissue. A mitochondrial-lysosomal fraction (15 000 $\times$ *g*, 20 min) was layered on a discontinuous sucrose gradient (30.5, 39.2 and 47.8% (w/w)) and centrifuged at equilibrium, either in a SW27 rotor or a Ti14 zonal rotor. The membranes at the 39.2–47.8% interface were harvested by centrifugation and stored as aliquots in liquid nitrogen at circa 5 mg protein/ml. The resulting plasma membrane fraction was enriched in forskolin-stimulated adenylate cyclase activity 8.9 $\pm$ 2.1-fold in relation to the homogenate, and the yield was 24 $\pm$ 1% (means $\pm$ S.D. of three preparations).

Adenylate cyclase assay. Adenylate cyclase activity was assayed as previously described [21] unless otherwise stated. The incubation medium contained 40 mM Tris-HCl (pH 7.5), 25 mM phospho(*enol*)pyruvate, 40 units of pyruvate kinase/ml, 0.1% bovine serum albumin, 5 μM GTP, 1 mM DTE, 5 mM $MgCl_2$ and 2 mM ATP as final concentrations. Stimulator concentrations were as indicated in the figure legends. cAMP was determined using a protein binding assay [24] with an Amersham assay kit. The linearity of the activity with time and the protein concentration was assessed.

Protein determination. Proteins were determined according to the method of Lowry et al. [25] as modified by Markwell et al. [26] using bovine serum albumin as a standard.

Membrane lipid extraction. Lipids were extracted using chloroform/isopropanol (7 : 11, v/v) essentially according to Rose and Oklander [27] with 2 ml of organic solvent per mg of membrane protein. After evaporation of the solvents, the extract was redissolved in half its volume of chloroform/methanol (2 : 1, v/v), washed and stored as in Yandrasitz et al. [28].

HPLC of the lipid extract. Instrumentation: The chromatographic system consisted of a Beckman (Beckman Inst., Gagny, France) 421 solvent controller connected to two pumps model 110 A (Beckman) equipped with an Altex (Palo Alto, CA, U.S.A.) model 210 injection valve with a 100 μl sample loop. Absorption was measured with a spectromonitor III (LDC, FL, U.S.A.) coupled to an Enica 10 integrator (Delsi Inst., Suresnes, France). The HPLC column (25 cm $\times$ 4.6 mm) was of stainless-steel and was pre-packed with silica gel (Lichrosolv Si-60-10 from Chrompack, Les Ulis, France). A prefilter (30 $\times$ 4.6 mm) packed with the same material was adapted to the column (Brownlee Labs Inc., Santa Clara, U.S.A.).

Choline phospholipids were separated by HPLC in acetonitrile/methanol/water (65 : 21 : 14, by vol.) according to Jungalwala et al. [29] except that the instrumentation was different (see above). For each analysis, an aliquot of lipid extract was evaporated under N_2 and redissolved in ethanol. 100 μl corresponding to 5–7 μg of phosphorus was injected into the column. Absorbance was recorded at 203 nm, with 0.5 or 0.1 absorbance unit full scale.

The other phospholipids and cholesterol were separated according to Yandrasitz et al. [30] using their solvent mixtures, i.e. hexane/isopropanol/water/H_2SO_4 in proportions (by vol.) of 97 : 3 : 0 : 0.025 (A) and 73 : 26 : 0.9 : 0.1 (B), but with the following slight modifications in the elution gradient: (A) for 6 min, then a linear increase to 55% B in 5.5 min with a plateau for 30 min and finally an increase to 100% B in 8.5 min with another plateau for 30 min. An aliquot of lipid extract was evaporated under N_2 and redissolved in solvent A. 100 μl of the resulting solution, containing 12–15 μg of phosphorus was then injected into the column. Absorbance was recorded

at 206 nm, with 0.5 absorbance unit full scale.

For both HPLC procedures, the peaks were collected in the presence of 0.1 ml pyridine and stored at −20°C until use. Collected fractions were then dried and treated as described by Yandrasitz et al. [30]. Lipids were identified by TLC [31] using precoated plates of Silica gel 60 (Merck). The organic phosphorus was determined according to Ames and Dubin [32]. Cholesterol was quantified by comparison with a standard curve drawn by injecting pure cholesterol into the HPLC column.

Fatty acid analysis. Fatty acids were analysed on total lipid extract according to Morrison and Smith [33]. Fatty acid methyl esters were separated by gas chromatography on a Carlo-Erba 4180 analyser with an 'on column' system connected to a grafted Carbowax CPWAX 52B (Chrompack).

Preparation of liposomes from lipid extracts. An aliquot of the lipid extract was evaporated under vacuum overnight. The lipids were resuspended at about 0.02 mg/ml in 60 mM Tris-HCl (pH 7.5) (in the presence of the indicated concentrations of benzyl alcohol or in its absence) by vigorous vortexing and short sonication at room temperature.

Membrane labeling with DPH and TMA-DPH. Stock solutions of 1 mM DPH and TMA-DPH, in tetrahydrofuran and dimethylsulfoxide, respectively, were stored in the dark at room temperature. These solutions were, respectively, diluted in tetrahydrofuran and dimethylsulfoxide, for membrane labeling. Plasma membranes were diluted in 60 mM Tris-HCl buffer (pH 7.5) with or without benzyl alcohol, at 0.04 to 0.08 mg protein/ml. Plasma membrane or liposome samples (1.5 ml) were labeled by rapid injection of 1.5 or 3 μl of diluted probe stock solution and incubation for at least 0.5 h at room temperature before fluorescence measurements. Probe/lipid molar ratios ranged from 1/100 to 1/400. Corrections for blanks were usually negligible.

Fluorescence measurements. Steady-state anisotropy was measured on a SLM 8000 spectrofluorometer (SLM, Urbana, IL) according to Vincent et al. [34]. Nanosecond fluorescence total intensity decays and anisotropy decays were monitored by single photon counting [35,36] with the apparatus already described [34]. The excitation pulses were obtained from the coaxial flash lamp designed by Birch and Imhof [37] (Edinburgh Instr.). Data were accumulated in the memory of an SMT Goupil microcomputer interfaced with an ADC module 8075 from Canberra. The apparatus response function was obtained by the short lifetime standard method [38] with tetraphenylbutadiene ($\tau = 1.76$ ns at 25°C [39]). Data were analysed as previously described [34]. The order parameters of the probes were calculated from r_∞ values [40–42].

Results

Chemical composition of the bovine adrenal cortex plasma membrane fraction

Table I, giving the phospholipid composition, cholesterol and protein content of the plasma membranes tested, indicate that their phospholipid/protein weight ratio was fairly low. Cholesterol content was also very low, whereas the phosphatidylcholine/sphingomyelin ratio was high (9.1 : 1). These two last phospholipid species accounted for more than half the total phospholipids present. The next major lipid after phosphatidylcholine was phosphatidylethanolamine, followed by phosphatidylinositol. The relatively small amount of phosphatidylserine present was comparable to the amount of sphingomyelin. Some cardiolipins ($\approx 5\%$) were also detected and may

TABLE I

COMPOSITION OF PLASMA MEMBRANES FROM BOVINE ADRENAL CORTEX

Data are means ± S.D. for two preparations. For each preparation, the mean value obtained for three lipid extracts has been considered. At least three injections of the same extract have been performed for the HPLC analysis.

Phospholipid/protein (w/w) [a]	0.44 ± 0.02
Cholesterol [b]	3.6 ± 0.4
Phospholipid (% (w/w))	
Phosphatidylcholine	51.1 ± 2.1
Phosphatidylethanolamine	17.2 ± 0.4
Phosphatidylinositol	11.7 ± 0.1
Sphingomyelin	5.5 ± 0.6
Phosphatidylserine	5.4 ± 0.4
Diphosphatidylglycerol	5.2 ± 2.1
Unidentified	3.9 ± 0.7

[a] Mean phospholipid molecular weight was taken as 800.
[b] Molar ratio cholesterol/phospholipid × 100.

TABLE II

FATTY ACID COMPOSITION OF PLASMA MEMBRANES FROM BOVINE ADRENAL CORTEX

Data are means ± S.D. for two preparations. Other fatty acids, detected as traces (< 0.1%), include: C18:3 $n-6$, C20:4 $n-3$, C22:1 $n-11$, C22:1 $n-9$, C22:1 $n-7$, C23:0, C24:0.

Fatty acids	% (w/w)	Fatty acids	% (w/w)
C14:0	0.7 ± 0.1	C20:2 $n-6$	0.3 ± 0.1
C15:0	0.5 ± 0.1	C20:3 $n-9$	0.3 ± 0.1
C16:0	14.9 ± 0.8	C20:3 $n-6$	1.5 ± 0.4
C16:1 $n-9$	0.6 ± 0.1	C20:4 $n-6$	20.9 ± 0.9
C16:1 $n-7$	0.9 ± 0.1	C20:5 $n-3$	1.7 ± 0.1
C17:0	1.0 ± 0.1	C22:0	0.2 ± 0.1
C18:0	20.6 ± 2.2	C22:4 $n-6$	1.2 ± 0.1
C18:1 $n-9$	14.5 ± 0.8	C22:5 $n-6$	0.3 ± 0.1
C18:1 $n-7$	3.0 ± 0.3	C22:5 $n-3$	4.1 ± 0.6
C18:2 $n-6$	11.2 ± 1.1	C22:6 $n-3$	0.5 ± 0.3
C18:3 $n-3$	0.7 ± 0.1	C24:1 $n-9$	0.3 ± 0.2
C20:0	0.1 ± 0.1	Saturated [a]	38.0 ± 1.4
C20:1 $n-9$	0.1 ± 0.1	Unsaturated	62.0 ± 1.3

[a] % calculated from whole fatty acid analysis.

partly have come from a mitochondrial contaminant.

As regards the fatty acyl chain profile of the total lipid extract (Table II), five main fatty acids accounted for more than 80% of the total fatty acid content: they were stearic and arachidonic acid, present in equal amounts, followed by palmitic, oleic and linoleic acid. Long chain C_{22} fatty acids were also detected (e.g. C22:5). The ratio of unsaturated over saturated fatty acids was 1.6:1.

Use of fluorescence probes to ascertain membrane fluidity

The experiments in this section were performed in order to evaluate membrane fluidity under different conditions and in particular to quantify the effect of benzyl alcohol as a fluidifying agent.

Total fluorescence intensity decays of DPH and TMA-DPH in plasma membranes and liposomes obtained from the lipid extracts: effects of temperature and benzyl alcohol. Whatever the experimental conditions, the time-resolved total fluorescence intensity decays of the DPH and TMA-DPH probes incorporated into the plasma membranes from bovine adrenal cortex or into liposomes obtained from the lipid extracts of these membranes, were

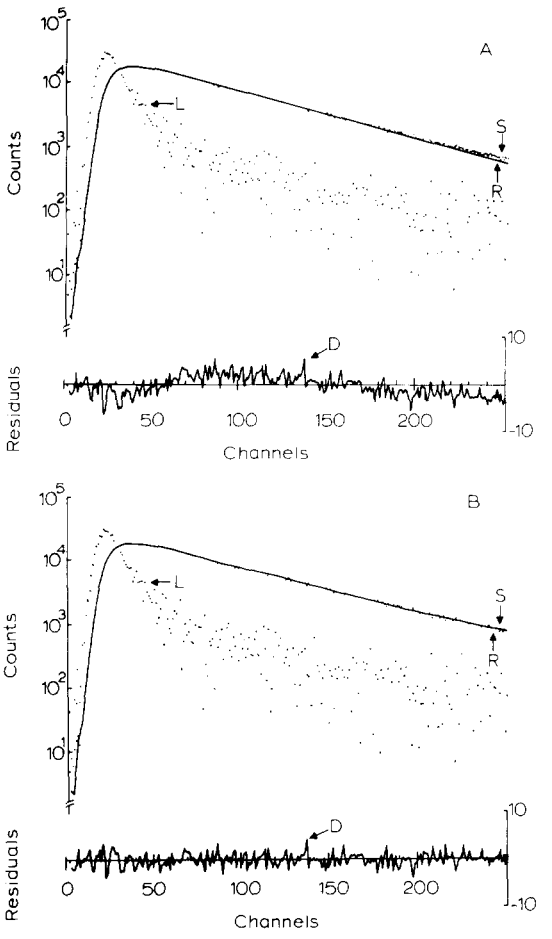

Fig. 1. Total fluorescence intensity decay of DPH in plasma membranes from bovine adrenal cortex. Excitation wavelength: 356 nm (5 nm bandwidth), emission: Schott KV 418 cutoff filter. S (·····) experimental curve $S(t) = I_{VV}(t)G + 2I_{VH}(t)$ where G is a correction factor as previously defined [34]; L (·····) calculated apparatus response function; R (———) reconvoluted curve; D: deviation function. Time resolution: 0.174 ns/channel. Temperature: 25 °C. Panel A: monoexponential model $i(t) = a \exp(-t/\tau)$ with $\tau = 9.01$ ns and $\chi^2 = 4.90$. Panel B: biexponential model $i(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$ with $\tau_1 = 12.2$ ns, $\tau_2 = 7.4$ ns. a_1, α_1 and a_2, α_2 are, respectively, the amplitudes and the fractional amplitudes of the first and second exponential. $\alpha_1 = 0.29$ and $\alpha_2 = 0.71$, $\chi^2 = 1.94$.

not monoexponential. Fig. 1 shows, for DPH, that a biexponential model, which reduces the χ^2 values significantly, is necessary for a proper fit of the curve – compare Fig. 1A (one exponential) with Fig. 1B (two exponentials). This is particularly obvious from the deviation function (D). Consequently, this model was used throughout the

TABLE III

TOTAL FLUORESCENCE INTENSITY DECAY PARAMETERS FOR DPH AND TMA-DPH IN BOVINE ADRENAL CORTEX PLASMA MEMBRANES AND IN LIPOSOMES FROM LIPID EXTRACTS

Excitation and emission conditions were as in Fig. 1. The data were fitted by a biexponential model: $i(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. α_1 and α_2 are the fractional amplitudes. Mean excited-state lifetimes were calculated as $\langle\tau\rangle = \Sigma a_i\tau_i^2/\Sigma a_i\tau_i$ [44]. Data are means ± S.D. of two to four measurements. Temperature: 25 °C.

Probe	Sample	τ_1 (ns)	α_1	τ_2 (ns)	α_2	$\langle\tau\rangle$ (ns)
DPH	Plasma membranes	10.3 ± 1.0	0.46 ± 0.17	6.0 ± 1.2	0.54 ± 0.17	9.16 ± 0.16
	Liposomes	11.5 ± 0.2	0.27 ± 0.03	7.7 ± 0.4	0.73 ± 0.03	9.12 ± 0.01
TMA-DPH	Plasma membranes	6.3 ± 0.1	0.44 ± 0.01	1.5 ± 0.2	0.56 ± 0.01	5.23 ± 0.04
	Liposomes	5.5 ± 0.5	0.44 ± 0.06	1.0 ± 0.4	0.56 ± 0.06	4.70 ± 0.14

present work. The mean excited-state lifetimes, which are characteristic of the probe molecules but also depend on the microenvironment of the probe (see Discussion) are given in Table III, where decay intensity parameters for DPH and TMA-DPH are compared in membranes and liposomes, and in Table IV, in which the effect of benzyl alcohol on the plasma membranes is evaluated. Both show that the mean excited-state lifetimes of TMA-DPH are shorter than those of DPH. This is mainly because both lifetime components (τ_1 and τ_2) are shorter for TMA-DPH than for DPH. The presence of membrane proteins had no significant effect on the mean excited-state

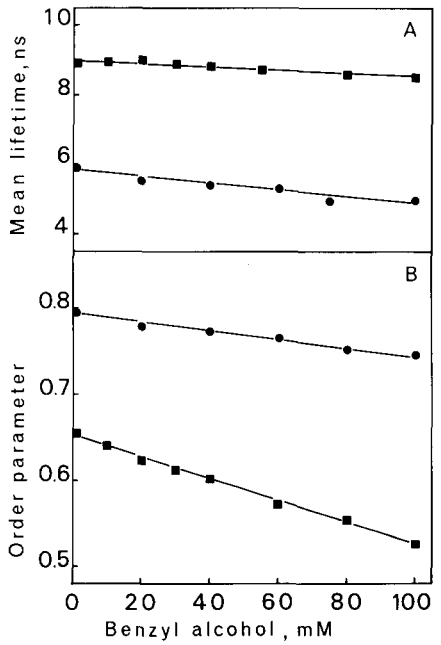

Fig. 2. Benzyl alcohol concentration dependence of the mean excited-state lifetime $\langle\tau\rangle$ (Panel A) and the order parameter S (Panel B) of DPH and TMA-DPH in bovine adrenal cortex plasma membranes. DPH (■) and TMA-DPH (●) were embedded in the plasma membranes at a probe/lipid molar ratio of approx. 1/200 and approx. 1/100, respectively. Each data point is the mean of two determinations. Temperature: 20 °C.

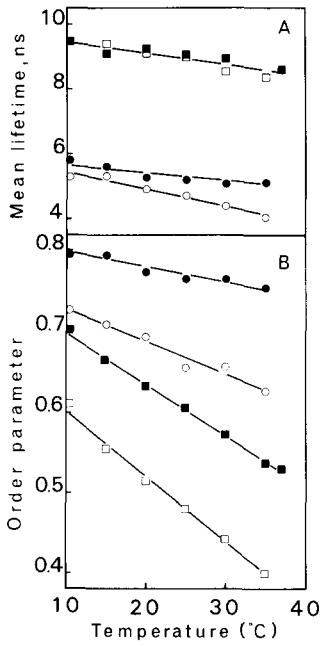

Fig. 3. Temperature dependence of the mean excited-state lifetime $\langle\tau\rangle$ (Panel A) and the order parameter S (Panel B) of the fluorescent probes DPH and TMA-DPH in bovine adrenal cortex plasma membranes and the liposomes from their lipid extracts. DPH (■, □) and TMA – DPH (●, ○) were embedded in plasma membranes (closed symbols) and liposomes obtained from their lipid extracts (open symbols). Probe/lipid molar ratio was approx. 1/400 for DPH and approx. 1/100 for TMA-DPH. Each data point is the mean of two determinations.

TABLE IV

EFFECT OF BENZYL ALCOHOL ON TOTAL FLUORESCENCE INTENSITY DECAY PARAMETERS FOR DPH AND TMA-DPH IN BOVINE ADRENAL CORTEX PLASMA MEMBRANES

Parameters are as described in Table III. Data are means ± S.D. of two measurements. Temperature: 20 °C.

Probe	Sample	τ_1 (ns)	α_1	τ_2 (ns)	α_2	$\langle\tau\rangle$ (ns)
DPH	Plasma membranes	10.0 ± 0.6	0.62 ± 0.02	3.6 ± 1.6	0.38 ± 0.02	8.87 ± 0.30
	Plasma membranes + 100 mM benzyl alcohol	9.4 ± 0.1	0.65 ± 0.01	2.9 ± 0.1	0.35 ± 0.01	8.52 ± 0.08
TMA-DPH	Plasma membranes	7.3 ± 0.1	0.48 ± 0.01	3.0 ± 0.2	0.52 ± 0.01	5.96 ± 0.08
	Plasma membranes + 100 mM benzyl alcohol	6.4 ± 0.1	0.39 ± 0.01	2.1 ± 0.1	0.61 ± 0.01	4.98 ± 0.01

lifetime of DPH, as shown in Table III. As regards TMA-DPH, a slight decrease in this lifetime was observed in the liposomes compared to the total membranes.

The membrane perturbing agent benzyl alcohol, used at concentrations of 10 to 100 mM, shortened the mean excited-state lifetime of both probes (Table IV and Fig. 2, panel A). This effect was more pronounced for the polar probe TMA-DPH than for DPH, since the maximal relative decreases of these probes were 17 and 5%, respectively. For DPH, this effect was entirely due to a decrease in the two lifetime component values τ_1 and τ_2. For TMA-DPH, there was also a slight enhancement of the fractional amplitude of the shorter lifetime component value α_2 (Table IV).

The variations with temperature in the mean excited-state lifetime of the probes (Fig. 3, panel A) shows that temperature had a similarly small effect on the excited-state lifetime of DPH in the plasma membranes and in the liposomes. For TMA-DPH, a slightly larger decrease was observed in the liposomes than in the plasma membranes.

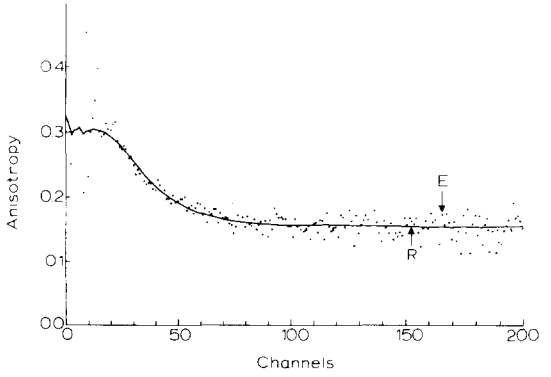

Fig. 4. Fluorescence anisotropy decay of DPH in plasma membranes from bovine adrenal cortex. Experimental conditions as in Fig. 1. The decay fitted the function $r(t) = (r_{t=0} - r_\infty)\exp(-t/\phi) + r_\infty$. Decay parameters; $\phi = 2.4$ ns, $r_{t=0} = 0.323$, $r_\infty = 0.149$. E (· · · · · ·), experimental curve; R (———), reconvoluted curve.

Steady-state and time-resolved fluorescence anisotropy decay measurements, with DPH and TMA-DPH. The fluorescence anisotropy measurements performed on the probes incorporated into either plasma membranes or liposomes reflect the constraints imposed on probe motion by the membrane structure. Fig. 4, showing the nanosecond time-resolved fluorescence anisotropy decays of DPH in plasma membranes clearly indicates that the anisotropy was non-vanishing. Consequently, the data were fitted by the function:

$$r(t) = (r_{t=0} - r_\infty)\exp(-t/\phi) + r_\infty$$

where $r_{t=0}$ is the anisotropy value at time zero, r_∞ is the non-vanishing value of the anisotropy, and ϕ is the apparent correlation time of the wobbling motion. This was the case under all our experimental conditions. The decay parameter values obtained for both probes in plasma membranes and liposomes (Table V) show that in both systems, TMA-DPH exhibited higher r_∞ values than DPH and consequently higher values of the order parameter S. The correlation times ϕ were in the range of 2–3 ns. In most cases, $r_{t=0}$ values were significantly smaller than the fundamental anisotropy r_0 values measured in vitrified medium at low temperatures [14,43]. The r_∞ parameter and

TABLE V

FLUORESCENCE ANISOTROPY DECAY PARAMETERS FOR DPH AND TMA-DPH IN BOVINE ADRENAL CORTEX PLASMA MEMBRANES AND LIPOSOMES FROM THEIR LIPID EXTRACTS

The experimental data fitted the function: $r(t) = (r_{t=0} - r_\infty) \exp(-t/\phi) + r_\infty$. Data are means ± S.D. for four or five determinations. Temperature: 20 °C.

Probe	Sample	ϕ (ns)	$r_{t=0}$	r_∞	S [a]
DPH	Plasma membranes	2.7 ± 0.4	0.328 ± 0.009	0.154 ± 0.003	0.633 ± 0.006
	Liposomes	2.3 ± 0.6	0.330 ± 0.004	0.102 ± 0.005	0.515 ± 0.022
TMA-DPH	Plasma membranes	2.6 ± 0.9	0.320 ± 0.010	0.233 ± 0.010	0.772 ± 0.027
	Liposomes	2.0 ± 0.9	0.390 ± 0.032	0.187 ± 0.012	0.692 ± 0.019

[a] The order parameter S was calculated according to $S = (r_\infty / r_0)^{1/2}$ [40–42] with $r_0 = 0.384$ for DPH [43] and $r_0 = 0.390$ for TMA-DPH [14].

order parameter S were highly sensitive to the presence of proteins (Table V). S values for DPH and TMA-DPH were, respectively, 20% and 13% lower in the liposomes than in the plasma membranes.

The S values for the probes exhibited a linear decrease with increasing temperature from 10 to 35 °C in both plasma membranes and liposomes (Fig. 3B). In the membranes, a relative decrease of 23% in the S value for DPH was observed in this temperature range. The slope of the curve was $4.22 \cdot 10^{-3}$/Cdeg, with a correlation coefficient of $r = 0.971$. TMA-DPH was less sensitive to the effect of temperature, and a relative decrease of only 6% was observed for this probe (curve slope: $1.7 \cdot 10^{-3}$/Cdeg, $r = 0.953$). In the liposomes, the effect of temperature was stronger for both probes than in the membranes (Fig. 3B).

We next examined the effects of benzyl alcohol at different temperatures. Since $\langle \tau \rangle$ was fairly constant (Tables III and IV) steady-state anisotropy measurement readily provided an estimate of membrane fluidity. Fig. 5 shows the effect of 40 mM benzyl alcohol as a function of temperature on the steady-state anisotropy of the DPH probe embedded in plasma membranes or liposomes. Whatever the temperature, this anaesthetic had almost the same fluidifying effect on membranes and liposomes, as shown by the parallel temperature dependence curves of the steady-state anisotropy, obtained in the absence or presence of benzyl alcohol. In fact, at 40 mM, benzyl alcohol had the same fluidifying effect on the region probed by DPH as a 6.5 Cdeg rise in the temperature. When fluorescence anisotropy decay was measured in the presence of 40 mM benzyl alcohol, the S values for DPH exhibited linear

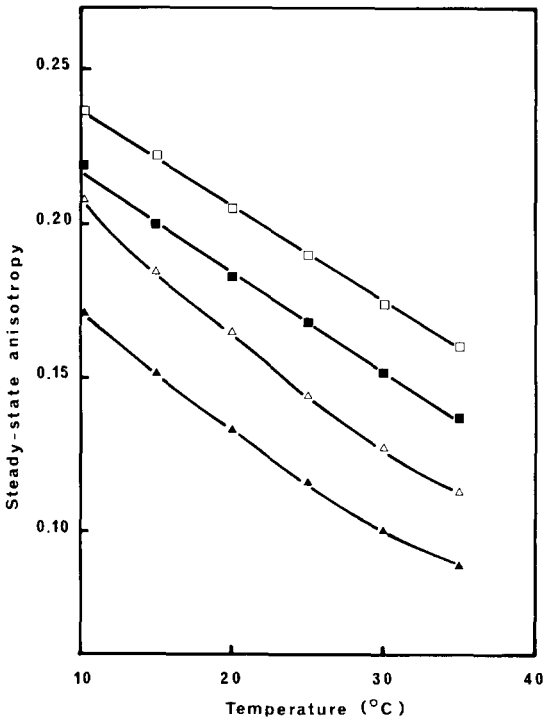

Fig. 5. Effect of benzyl alcohol on the temperature dependence of DPH steady-state anisotropy in bovine adrenal cortex plasma membranes and liposomes from their lipid extracts. DPH was embedded in plasma membranes (■, □) and liposomes (▲, △) in the presence (closed symbols) of 40 mM benzyl alcohol or in its absence (open symbols). Each data point is the mean of five determinations (S.D. = 0.001). Excitation wavelength: 356 nm (2 nm bandwidth). Emission: Schott KV 418 cutoff filter.

TABLE VI

EFFECT OF BENZYL ALCOHOL ON FLUORESCENCE ANISOTROPY DECAY PARAMETERS FOR DPH AND TMA-DPH IN BOVINE ADRENAL CORTEX PLASMA MEMBRANES

Parameters are as defined in Table V. Data are means ± S.D. of 2 determinations. Temperature: 20 °C.

Probe	Sample	ϕ (ns)	$r_{t=0}$	r_∞	S
DPH	Plasma membranes	2.8 ± 0.6	0.308 ± 0.011	0.165 ± 0.005	0.655 ± 0.010
	Plasma membranes + 100 mM benzyl alcohol	2.2 ± 0.1	0.304 ± 0.004	0.107 ± 0.002	0.528 ± 0.004
TMA-DPH	Plasma membranes	1.8 ± 0.2	0.347 ± 0.014	0.250 ± 0.002	0.800 ± 0.003
	Plasma membranes 100 mM benzyl alcohol	0.9 ± 0.1	0.391 ± 0.018	0.218 ± 0.001	0.746 ± 0.003

temperature dependence in both membranes and liposomes (data not shown), as observed in the absence of benzyl alcohol (Fig. 3B).

Since the effect of benzyl alcohol on membrane fluidity was basically the same at all the temperatures studied, the dependence of fluidity on the benzyl alcohol concentrations was only examined at 20 °C (Table VI and Fig. 2B). The values of the correlation times decreased in the presence of benzyl alcohol, especially for TMA-DPH (Table VI). For the order parameter S, concentration dependence was linear for both probes between 0 and 100 mM benzyl alcohol (Fig. 2B; curve fit: $S = -1.25 \cdot 10^{-3}c + 0.652$ and $S = -0.504 \cdot 10^{-3}c + 0.794$ for DPH and TMA-DPH, respectively). The variations in S induced by benzyl alcohol were larger for DPH than TMA-DPH (Fig. 2B), as observed for the effect of temperature (Fig. 3B) and also for the effect of protein on S (Table V). However, benzyl alcohol seemed more effective than temperature in fluidifying the region of the membrane probed by TMA-DPH compared to the region probed by DPH. Thus, the effect of 40 mM benzyl alcohol was equal to that produced by temperature increases of 12 and 8 Cdeg for TMA-DPH and DPH, respectively, as calculated from the slopes of the S curves given above. It is worth noting that the increase of 8 Cdeg for DPH obtained in time-resolved experiment is about the same as the rise of 6.5 Cdeg obtained in the steady-state experiment.

Adenylate cyclase activity and membrane fluidity

Temperature dependence of adenylate cyclase activity. The temperature dependence of adenylate cyclase activity was studied in the presence of three activators, forskolin (100 μM), NaF (10 mM) and ACTH 1–24 (8 μM). We have previously determined that, at 30 °C, forskolin stimulated

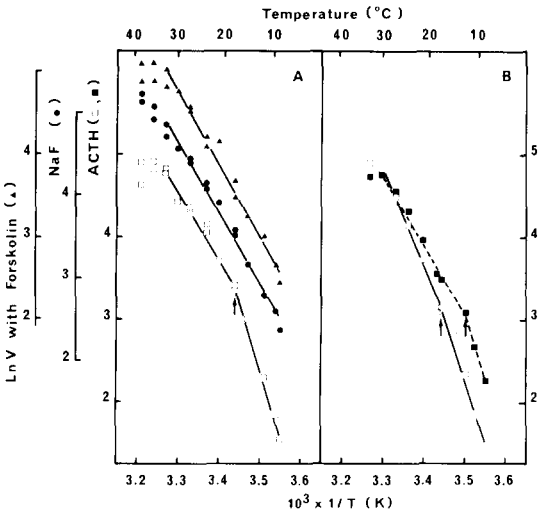

Fig. 6. Arrhenius plots of adenylate cyclase activity in bovine adrenal cortex plasma membranes, with forskolin, NaF or ACTH as stimulators. Effect of benzyl alcohol. Panel A: adenylate cyclase activity was determined in the presence of 100 μM forskolin (▲), 10 mM NaF (●) or 8 μM ACTH 1–24 (□). Membranes were preincubated for 20 min at 4 °C in the assay buffer and for 2 min at the experiment temperature. Incubation times were 10 to 20 min. Each data point corresponds to duplicate incubations at two protein concentrations (S.D. = 5–10%). Velocities are expressed in pmol/min per mg of protein. Panel B: the activity stimulated by 8 μM ACTH 1–24 was determined in the absence (□) or presence (■) of 40 mM benzyl alcohol. Assay conditions were as in Panel A, except for preincubation times at 4 °C, which were reduced to 10 min for the points at 27, 30 and 33 °C. These plots are representative of three separate experiments. The arrows indicate the position of the breaks.

this enzyme's activity 9-fold versus its basal activity, and NaF and ACTH 7-fold [21]. As shown in Fig. 6A, the Arrhenius plots of the forskolin or fluoride-stimulated adenylate cyclase activity fitted linearly between 9 and 33°C (regression coefficients $r = 0.990$ and 0.993, respectively), yielding the same activation energy of 70 kJ · mol^{-1}. However, careful examination of these data revealed a slight inward curvature for the plot obtained in the presence of fluoride. By contrast, in the presence of ACTH 1–24, the Arrhenius plot was best fitted by a biphasic linear curve showing a break at 18°C, as seen in Figs. 6A and 6B which corresponded to two different membrane preparations. In Fig. 6A, the break was more apparent and the data have been obtained simultaneously with the three activators. In that case, above the break, the plot obtained with ACTH was parallel to that obtained with forskolin or fluoride ($E_A = 67$ kJ · mol^{-1}, $r = 0.989$) whereas below, the activation energy doubled ($E_A = 135$ kJ · mol^{-1}, $r = 0.997$). This sudden change in activation energy was found to be reversible: when the membranes were preincubated in the assay buffer for 5 min above the break temperature, at 27°C, and then assayed for adenylate cyclase activity between 10 and 18°C, the same activation energy was obtained within the experimental error margin (data not shown). Above 33°C, the activity reached its maximum with forskolin or with ACTH 1–24, but continued to rise in the presence of fluoride.

Effect of benzyl alcohol on adenylate cyclase activity. The Arrhenius plots of the ACTH-stimulated adenylate cyclase activity were made in the absence or presence of 40 mM benzyl alcohol, which was shown to produce the same fluidifying effect in the membrane region probed by DPH as a rise of 6.5 and 8 Cdeg in the temperature, as assessed by steady-state and time-resolved experiments, respectively (Fig. 6B). At this concentration, benzyl alcohol induced a shift of approx. 6 Cdeg in the break point, from 18 to 12.5°C. Below 12.5°C, both plots were parallel (i.e., activation energy was the same) and were separated by a distance corresponding to a constant 2-fold enhancement by benzyl alcohol of the ACTH-stimulated adenylate cyclase activity. Above the break point temperature, benzyl alcohol reduced the activation energy by 30 ± 2% (mean ± S.D. of three experiments). This decrease was very reproducible, although the values for activity above the break tended to vary from one preparation to another.

To assess the respective effects of changes in temperature and fluidity on adenylate cyclase activity, plasma membranes were treated with different concentrations of benzyl alcohol at 12 and 24°C; both temperatures were 6 Cdeg away from the break point temperature observed when adenylate cyclase activity was stimulated by ACTH. Perturbation of the membranes by benzyl alcohol revealed that this enzyme behaved quite differently with each of the three activators tested (forskolin, NaF and ACTH). With forskolin, a linear decrease in its activity was observed at both 12 and 24°C, for benzyl alcohol concentrations ranging from 0 to 100 mM. When expressed as percentages of the value for control membranes (without benzyl alcohol) the curves for each temperature were fairly similar, and indicated 40–50% inhibition of adenylate cyclase activity at 100 mM benzyl alcohol (Fig. 7A). The effect of benzyl alcohol on the stimulation of this enzyme's activ-

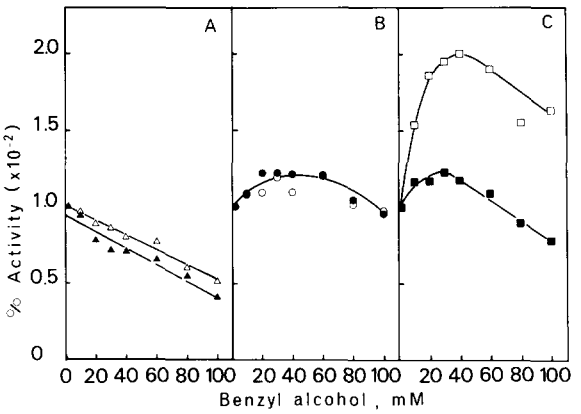

Fig. 7. Effect of benzyl alcohol on ACTH, NaF or forskolin-stimulated adenylate cyclase activity. Adenylate cyclase activity was determined in the presence of (A) 100 μM forskolin (△, ▲); (B) 10 mM NaF (○, ●) or (C) 8 μM ACTH 1–24 (□, ■) at 12°C (open symbols) and at 24°C (closed symbols). Membranes were preincubated for 20 min or 15 min at 4°C in the assay buffer and further incubated for 20 min and 15 min for the assays at 12 and 24°C, respectively. Each data point corresponds to triplicate incubations at one protein concentration (S.D. approx. 5%). Data are expressed as percentage of the control values found in the absence of benzyl alcohol.

ity by fluoride was not much changed by temperature either (Fig. 7B). At both 12 and 24°C, increasing the concentrations of benzyl alcohol in the presence of NaF slightly increased adenylate cyclase activity which was maximal (i.e., enhanced by 20%) between 40 and 60 mM benzyl alcohol. This stimulation disappeared at higher benzyl alcohol concentrations (Fig. 7B). Thus, NaF appeared to stabilize adenylate cyclase activity against benzyl alcohol-induced membrane perturbations.

By contrast, in the presence of ACTH, the effects of benzyl alcohol on this activity differed strikingly at 12 and 24°C (Fig. 7C). At 12°C, benzyl alcohol induced biphasic enhancement of the activity: thus, up to a concentration of 40 mM, it increased the stimulation of this activity by ACTH up to double the control level, and for benzyl alcohol concentrations of 40 to 100 mM, activation decreased. At 24°C, only slight enhancement of adenylate cyclase activity was observed (20% at 30 mM benzyl alcohol) followed by a linear decrease resulting in a final 20% inhibition of activity at 100 mM benzyl alcohol.

Discussion

The present report describes how the membrane structure affects adenylate cyclase activity in the adrenal cortex. Firstly, we have analysed the chemical composition of the plasma membranes. Secondly, we have studied the physical parameters describing membrane fluidity. The values of these parameters were determined by fluorescence anisotropy. Membrane perturbations were induced in order to establish the degree of dependence of adenylate cyclase activity on membrane structure and thus gain insight into the mechanism of activation of the adenylate cyclase complex. The temperature was changed as one kind of perturbation. In this connection, it has long been known that the functions of several membrane-bound proteins exhibit a break in their Arrhenius plots which in some instances might be correlated with lipid phase transition or separation [1,45]. However, since it may be difficult to distinguish between the direct and lipid-mediated effects of temperature on these proteins, the local anaesthetic benzyl alcohol was used as a complementary perturbing agent.

Chemical composition of the plasma membranes

With respect to lipid composition, adrenal cortex plasma membranes were found here to display an unusually low cholesterol/phospholipid molar ratio compared to plasma membranes from other tissues [46]. This result is in agreement with the findings of a previous investigation in which a different adrenal cortex plasma membrane preparation was used [47]. It might be due to the physiological function of the adrenal glands, in which cholesterol acts as a substrate for steroidogenesis and which possess efficient sterol-carrying proteins [48]. The low sphingomyelin/phosphatidylcholine molar ratio observed here is consistent with this low cholesterol/phospholipid ratio, since a correlation between these values has been observed in many biological membranes [49]. Also, as in many systems [49], sphingomyelin and phosphatidylcholine account for about half the total phospholipids. Phosphatidylcholine was by far the largest lipid component of the present membranes. Since naturally occurring phosphatidylcholine is the most fluidifying component of biological membranes – contrarily to cholesterol and sphingomyelin, which are the two main lipid rigidifiers [50] – the lipid composition of these adrenal cortex plasma membranes implied that a more fluid lipid phase could be expected than in plasma membranes from other tissues.

The acyl chain profile was characterized here by the same main fatty acids as those determined in bovine adrenal cortex microsomes and mitochondria [51]. The percentages of unsaturated fatty acids, which have a fluidifying effect contrarily to the corresponding saturated chains, were also similar in the different subcellular fractions (approx. 60%) as well as the percentage of polyunsaturated fatty acids (approx. 40%) [51].

With respect to protein content, the phospholipid/protein ratio corresponded (by weight) to a composition of 70% protein and 30% phospholipid, if the other minor membrane components (e.g. neutral lipids) are not taken into account. This high protein content is consistent with the high buoyant density ($d = 1.17–1.21$ g/ml) at which the membranes were harvested and which was higher than the mean density of mitochondria ($d = 1.15$ g/ml). This protein content was almost as high as the one obtained for mitochondria inner membranes [52,53].

Membrane structure as probed by DPH and TMA-DPH

As in many of the biological and model membranes studied so far, the total fluorescence intensity decays found with DPH and TMA-DPH both in the plasma membranes and liposomes were not monoexponential [14,43,54–58]. Although the origin of this behaviour is not fully understood, it probably arises from the heterogeneity of the probes' microenvironment, since intensity decays are well described by a monoexponential function in organic solvents [55,59]. For the apolar probe DPH, which was shown to be preferentially located near the centre of the bilayer [60], this heterogeneity may result from the location of the probe in membrane regions with different lipid packing. A correlation between the mean excited-state lifetime values of the probes and the acyl chain packing was in fact observed in model systems [14,56,61–63]. Lateral and/or transverse heterogeneity of membrane lipid packing might result from the existence of different lipid domains, as discussed in the literature [64,65]. For the cationic probe TMA-DPH, which is anchored at the lipid/water interface [14,15], another fairly likely hypothesis accounting for lifetime heterogeneity might be the presence of quencher groups such as water or polar phospholipid head groups, in the area near some probe molecules. The presence of membrane proteins is unlikely a major source of probe lifetime heterogeneity, at least for DPH, since in the present work these proteins did not seem to have any significant effect on the intensity decay parameters of this probe. For TMA-DPH, the higher lifetime values observed in the plasma membranes than in the liposomes can be tentatively ascribed to the average ordering effect of the proteins on the bilayer. Such an effect is in fact important in these membranes as shown by the increase in the order parameter values for both probes. This ordering effect of proteins on the membranes was stronger in the hydrophobic region probed by DPH than in the polar region probed by TMA-DPH. This ordering effect is consistent with previous observations in both model and biological membranes with DPH, TMA-DPH or the *n*-anthroyloxy fatty acid probes [43,52,56,66–69]. The reason for this ordering effect is that the fluorescence moiety of all these probes, which is stiff to some extent, senses a so-called 'rigid body orientational order' of the phospholipid acyl chains [69], and this order, unlike the ^{2}H-NMR segmental order [70], is greatly enhanced by proteins.

The effect of temperature on membrane fluidity was also more pronounced in the hydrophobic region of the bilayer than in the polar region. This is in agreement with previous reports showing the increase with temperature of the difference between the anisotropy parameters for DPH and TMA-DPH (either steady-state anisotropy or r_∞) in kidney brush-border membranes [71] and placental microvillus membranes [72]. Here, the absence of any sudden change in the slope of the S variations of both probes in the temperature range studied suggests that neither phase transition nor lateral phase separation of lipids occurs in adrenal cortex membranes. This observation, which is at variance with previously detected thermotropic phase transitions in different kinds of plasma membranes using the same technique [65,72], may indeed be due to the particular lipid composition of the adrenal plasma membrane fraction (i.e., its low cholesterol and sphingomyelin content) and the relatively high level of fatty acid unsaturation, as described earlier. As a consequence, the plasma membranes were fairly fluid. The steady-state anisotropies of DPH at 20 and 25 °C were, respectively, equal to 0.203 ± 0.002 and 0.188 ± 0.002 (mean ± S.D. for two preparations), i.e. 20 to 40% lower than the corresponding one obtained for plasma membranes from other tissues (Refs. 71, 72 and references therein, and Ref. 73).

The effect of benzyl alcohol, when used as a membrane fluidizer at a constant temperature, was examined in detail on our bovine adrenal cortex plasma membranes. Since this perturbing agent is a neutral amphiphile, it was expected to experience a very rapid flip-flop motion in the bilayer, as recently shown for the non-ionic detergent octa(ethyleneglycol) dodecyl monoether when used at non-solubilizing concentrations [74], and thus to induce symmetrical perturbations in the plasma membranes. Benzyl alcohol has been shown to be located in the interfacial region of model systems comprising either surfactant micelles [75] or lipid bilayers [76]. In the latter system, this

location had a rigidifying effect on the glycerol bridge region and top of the alkyl chain, as probed by ^{2}H-NMR [76] whereas a fluidifying effect was observed in the more hydrophobic region of the bilayer, due to the spacing effect of benzyl alcohol on the phospholipid molecules [76,77]. The present measurements of excited-state lifetime and anisotropy made with DPH and TMA-DPH are consistent with the location of benzyl alcohol in the interfacial region, in adrenal cortex plasma membranes. This compound was shown with both DPH and TMA-DPH to have a disorganizing effect on the bilayer but it was greater with DPH than with TAM-DPH, as also observed by steady-state anisotropy measurements in kidney brush-border membranes [71]. By contrast, the decrease in excited-state lifetime caused by benzyl alcohol was more pronounced for TMA-DPH than for DPH, and suggests that with TMA-DPH, benzyl alcohol has a dynamic quenching effect on the fluorescence. The regular decreases in fluorescence intensity and anisotropy decay parameter values with rising benzyl alcohol concentrations ranging from 0 to 100 mM exclude the possibility that benzyl alcohol had dramatically damaging effects on the structure of these plasma membranes, unlike what was observed in other systems at similar concentrations [78]. The linear variation with the benzyl alcohol concentration in the order parameters for both probes makes this agent a suitable fluidifying effector in these membranes.

Adenylate cyclase activation

The temperature dependence of the adenylate cyclase activity did not allow discrimination between its forskolin and the fluoride-activated states, although forskolin is thought to act directly on the catalytic unit, whereas the effect of fluoride is mediated by the stimulatory GTP-binding protein. The two Arrhenius plots for adenylate cyclase activation by these agents were fitted by straight lines of similar slopes. However, a break appeared in the plot when stimulation was triggered by the binding of ACTH to its receptors. Similarly, Houslay et al. [79] showed a straight line for the stimulation by fluoride of adenylate cyclase activity from rat liver plasma membrane, whereas a break appeared when it was stimulated by glucagon. The break therefore seems to be related to the stimulation step involving interaction between the receptor and the stimulatory GTP-binding protein.

In the present case, the break observed when the stimulator was ACTH did not appear to be correlated to any detectable bulk lipid phase transition or phase separation, as shown by the fluorescence data. This does not exclude the possibility that lipid fluidity plays an important part in modulating adenylate cyclase activity, a point we investigated by studying the effect of benzyl alcohol. The importance of lipid fluidity was indeed demonstrated by comparison of the Arrhenius plots for the ACTH-stimulated activity in the presence or absence of 40 mM benzyl alcohol. Benzyl alcohol in fact caused a decrease of 6 Cdeg in the break temperature which counterbalanced rather well the fluidity increase that it induced in the hydrophobic part of the membrane probed by DPH, as assessed by steady-state anisotropy and order parameter values. The break in activity thus appeared to occur at a specific value of membrane fluidity and not at a specific temperature. As long as this fluidity remained below that specific value, the enzyme was in a state which was highly sensitive to an increase in fluidity, as shown by the 2-fold stimulation of its activity produced by 40 mM benzyl alcohol corresponding to only a 10% variation in the steady-state anisotropy value of DPH. Above this fluidity value, the enzyme was in a state insensitive to further increases in fluidity, as shown by the disappearance of stimulation by benzyl alcohol. We define the term 'state' as the distribution and coupling of the three protein components of the cyclase, the receptors, the stimulatory GTP-binding proteins and the catalytic units, which we assume to be the more important parameters in the adenylate cyclase activation, in our conditions.

The most likely interpretation of our results is that they indicate a transition in the distribution of the receptors. Indeed, since the adenylate cyclase activity mediated by the stimulatory GTP-binding protein in the presence of NaF does not present any break as a function of temperature, an abrupt change in the distribution of the GTP-binding proteins may be excluded. We suggest that the ACTH receptors form clusters when fluidity drops below the value corresponding to the break in the

Arrhenius plot. Dissociation of these clusters by temperature or benzyl alcohol-induced fluidification of the membrane might increase the probability of interaction between the receptor and the stimulatory GTP-binding protein, thus increasing the overall number of activated adenylate cyclase units. Since the receptors generally constitute a minor fraction of the membrane proteins, changes in their distribution to form randomly distributed units instead of clusters would not be detectable in the lipid phase as probed by the fluorescent probes. It is worth noting that a similar shift in the break temperature was also produced by benzyl alcohol for the glucagon-stimulated adenylate cyclase. In that case, this shift correlated well with a depression of the onset of a lipid phase separation [1].

However, since, as explained by Silvius and McElhaney [80], the interpretation of break points in Arrhenius plots is subject to many limitations, once the role of lipid fluidity had been assessed, it was investigated here in more detail by studying the benzyl alcohol concentration dependence curves for adenylate cyclase activity. These experiments revealed striking differences between the mechanisms of this enzyme's activation by forskolin, fluoride and ACTH. With respect to forskolin, its direct interaction with the catalytic unit was demonstrated [17,81,82] but evidence has also been given of the existence of effects dependent on the stimulatory GTP-binding protein [83–85]. The differences between the benzyl alcohol concentration dependence curves in the presence of forskolin on the one hand and NaF or ACTH on the other argues against the existence of effects mediated by the stimulatory GTP-binding protein for forskolin in the adrenal cortex plasma membranes, at 100 μM forskolin. This conclusion is strengthened by the results of previous experiments, in which we demonstrated that stimulation by ACTH and forskolin was additive throughout a wide concentration range of both effectors [21]. These results argue in favour of separate and independent mechanisms of action for ACTH and forskolin. The benzyl alcohol concentration dependence curves obtained here in the presence of forskolin may therefore be interpreted as reflecting gradual inhibition of the catalytic unit. The linear dependence of this inhibition, which was independent of temperature and concomitant with the linear fluidification of the membrane lipid phase, as probed with DPH and TMA-DPH, also implied that it was a lipid-mediated effect. There is no evidence for the existence of specific interactions between adenylate cyclase sites and benzyl alcohol. Similar inhibition of the activity induced by short-chain linear alcohols was also observed for forskolin-stimulated cardiac adenylate cyclase [86]. The correlation between the inhibitory effect and the partition coefficient of the alcohols in the membrane was in agreement with lipid-mediated inhibition. Such inhibition may be attributable to conformational changes in the catalytic unit. These changes may result from modified insertion of the protein into the membrane, the nature of which depends on membrane fluidity [50], or from the relief of the constraints imposed by the surrounding lipids.

With respect to fluoride, the active species has in fact been shown to be the ion AlF_4^- [87], which was suggested to interact with GDP in the nucleotide site of the α-subunit of the GTP-binding regulatory proteins or transducin [88]. In the presence of NaF, the stabilization of the adenylate cyclase activity that we observed following the benzyl alcohol-induced perturbations is consistent with a model in which the active unit of the enzyme is the catalytic unit coupled to one or more subunits of the regulatory GTP-binding proteins. These subunits would prevent the inhibition of the catalytic unit by displacing part of its surrounding lipids, thus impeding their influence. It should be noted that NaF also stabilized adenylate cyclase at temperatures above 33°C. Although the Arrhenius plots of its activity in the presence of ACTH or forskolin flattened in this region, the NaF-stimulated activity continued to increase.

The benzyl alcohol concentration dependence of ACTH-stimulated adenylate cyclase activity confirmed the existence of two states in the adenylate cyclase enzyme complex, as clearly shown by the difference between the normalized curves obtained in the presence of ACTH at 12 and at 24°C. It should be recalled that those obtained in the presence of NaF or forskolin were almost superimposable. These results are consistent with the interpretation formulated earlier

regarding the transition in receptor distribution from clusters to individual units. If this transition indeed occurs, the shape of the curves would also show that it is not a sharp one, but extends over a definite fluidity or temperature range.

The bell shape of the normalized benzyl alcohol concentration dependence curve obtained with ACTH at 12°C illustrates very well the 'optimal fluidity hypothesis' developed by Shinitzky [50], according to which activity curves possessing a peak value at a specific lipid viscosity are expected when two factors related to that activity are induced by the membrane lipid viscosity to shift in opposite directions. If this hypothesis is applied to the present case, the coupling of the receptor with the stimulatory GTP-binding protein would have been enhanced by an increase in fluidity under the conditions in which receptor phase separation occurred, whereas, once formed, the active units would have been inhibited by a fluidity increase, as shown by the decreasing part of the curves observed both at 12 and 24°C. The latter inhibition might also have resulted from an effect on the conformation of the active unit.

The fluidity dependence of the adenylate cyclase activity in the presence of NaF or the hormone differs from that described in pigeon erythrocyte membranes perturbed by octanol, where both fluoride and hormone-stimulated activities paralleled the variations in membrane order [2,89], a situation we only observe in the presence of forskolin. Due to the differences in the membrane systems and experimental conditions, the adenylate cyclase activity in erythrocyte membranes may rather depend upon matching of the interacting sites between its components [89] than on the diffusion of these components which is assumed to be an important parameter in our system.

The fact that the active unit of adenylate cyclase formed in the presence of ACTH appeared to be inhibited by an increase in fluidity (as is clear from the right hand part of Fig. 7C, at 24°C) whereas the unit formed in the presence of NaF was always slightly enhanced (Fig. 7B) argues in favour of the different composition of these two active units of adenylate cyclase. This strengthens previous observations of the same system which showed a divergence between the combined effects of ACTH and forskolin and of NaF and forskolin. Unlike ACTH, NaF was shown to block more than 50% of the stimulation induced by forskolin alone [21]. Both the present and previous experiments indicate that NaF protects the catalytic unit against the influence of external effectors, whether they are lipids or forskolin. These data are in line with recent observations by Verkman et al. [90] who, by target size analysis, determined a higher molecular weight of adenylate cyclase active unit in the presence of NaF than in the presence of hormone. In the former case, the catalytic unit would be activated by coupling with the $\alpha\beta$-subunits of the stimulatory GTP-binding protein, whereas in the latter case, this unit would be activated by the α-subunit alone, as suggested by Gilman et al. [10–12] for the effects mediated by the stimulatory GTP-binding protein. The present results are compatible with such a model for bovine adrenal cortex adenylate cyclase. Alternatively, the increased protection of the catalytic unit that we observed in the presence of NaF and the higher molecular weight of the active unit seen by Verkman et al. might also result from an additional effect of the inhibitory GTP-binding protein on the catalytic unit, according to the fact that NaF is also active on this inhibitory protein [91].

Acknowledgements

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