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PERTURBATIONS OF LIVER PLASMA MEMBRANES INDUCED BY Ca^{2+} ARE DETECTED USING A FATTY ACID SPIN LABEL AND ADENYLATE CYCLASE AS MEMBRANE PROBES

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Ca^{2+} decreased the lipid fluidity of rat liver plasma membranes labeled with 5-nitroxide stearate, I(12,3), as indicated by the order parameter (S). These effects form a reversible, saturable process with an association constant of $1 \cdot 10^3$ M^{-1}. Arrhenius-type plots of S indicated that the lipid phase separation, present in the external leaflet of native membranes between 28 and 19°C, is perturbed by mM Ca^{2+} such that the high temperature onset is elevated to 32–34°C. Fluoride-stimulated adenylate cyclase was similarly inhibited by Ca^{2+} ($ID_{50} = 1$ mM) for the enzyme in membrane-bound or solubilized states. The glucagon-stimulated activity was more sensitive to Ca^{2+} inhibition with an ID_{50} of 0.2 mM. These inhibitory effects are due neither to perturbations of glucagon binding to its receptor nor to fluidity changes, but are instead attributed to direct Ca^{2+}-enzyme interactions. Such binding desensitizes the enzyme to fluidity alterations induced by temperature elevation or benzyl alcohol addition. With Ca^{2+}, Arrhenius plots of glucagon-stimulated activity indicated breaks at 32 and 16°C, whereas those of fluoride-stimulated activity showed one break at 17°C. Without Ca^{2+}, Arrhenius plots exhibited one break at 28°C for glucagon-stimulated activity, whereas fluoride-stimulated plots were linear. We propose that Ca^{2+} achieves these effects through asymmetric perturbations of the membrane lipid structure.

Introduction

Ca^{2+} exerts multiple effects on biological membranes, participating in a wide variety of processes including the regulation of enzyme activities, transduction of hormonal information, stimulus-secretion coupling, membrane fusion, functioning of transport systems, neuronal conduction and muscular contraction. These actions may be achieved by a number of mechanisms, such as the specific flux of Ca^{2+} through protein channels, the binding of Ca^{2+} to regulatory sites on proteins, and the interaction of Ca^{2+} with membrane lipids which can not only decrease bilayer fluidity but also initiate structural re-arrangements in the membrane by clustering acidic phospholipids (for review, see Refs. 1–3).

In rat liver plasma membranes, binding studies have indicated the presence of both high- and low-affinity sites for liganding Ca^{2+}. It would appear that the high-affinity sites are supplied predominantly by proteins, whereas membrane lipids primarily constitute the low-affinity sites [4–8]. One important consequence of the binding of Ca^{2+} to such anionic membrane sites might be the decrease in fluidity that has been observed [9,10], as changes in membrane fluidity can have dramatic

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effects on the functioning of penetrant membrane enzymes (for review, see Ref. 11). This study explores in some detail the effect of Ca^{2+} on the liver plasma membrane bilayer using a fatty acid spin label as a structural probe and the key regulatory enzyme, glucagon-stimulated adenylate cyclase, as a functional probe. Both the activity of this asymmetrically-orientated integral enzyme and the mobility of membrane-incorporated spin labels have been shown to be sensitive to changes in bilayer fluidity and lipid organization [12–14].

Materials and Methods

The *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, I(12,3), was obtained from Syva Co., Palo Alto, CA. Creatine kinase, creatine phosphate, ATP and cyclic AMP were from Boehringer (U.K.) Ltd., East Sussex, U.K. Glucagon was a kind gift from Dr. W.W. Bromer of Lilly Research Laboratories, IN and Lubrol detergents 17A10 and N13 were gifts from ICI Pharmaceuticals, Cheshire, U.K. All other chemicals were of AR grade from BDH Chemicals, Dorset, U.K.

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing between 200 and 300 g as previously described [15]. Adenylate cyclase was assayed as set out in detail earlier [15]. Membranes or solubilized preparations were pretreated for 15 min at 4°C with a given concentration of Ca^{2+} before starting the enzyme assay incubation. The results presented here reflect initial rates calculated from linear time courses under all experimental conditions. Specific binding of ^{125}I-glucagon to liver plasma membranes was assessed as in Houslay et al. [16], as was the protein determination [17]. A fluoride-stabilized, solubilized preparation of adenylate cyclase was achieved using the non-ionic detergents Lubrols 17A10 and N13 as described previously [18,19]. Arrhenius plot data was handled by a computer-fitting process which utilizes least squares minimalization to assign break points and to determine activation energies [17].

Methods for ESR studies were essentially as detailed earlier [9,12]. Samples of liver plasma membranes were added to the I(12,3) spin probe that had initially been deposited on the side of the tube by evaporation from ethanol to yield ratios of μg of I(12,3)/mg membrane protein of 5 to 40. Spectra were recorded on a Varian E-104A ESR spectrometer and the cavity temperature was calibrated as set out by Gordon et al. [9].

The outer ($2T_{\parallel}$) and inner ($2T_{\perp}$) hyperfine splittings were determined from 'unexpanded spectra with magnified wings' [9]. The following order parameter expressions [20] may be used to evalulate the flexibility of the membrane-incorporated fatty acid spin probe:

$$S(T_{\parallel}) = \tfrac{1}{2}\left[\frac{3(T_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right] \quad (1)$$

$$S(T_{\perp}) = \tfrac{1}{2}\left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1\right] \quad (2)$$

$$S = \frac{(T_{\parallel} - T_{\perp})}{(T_{zz} - T_{xx})} \frac{(a_N)}{(a_{N'})} \quad (3)$$

Here, T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor ($\boldsymbol{T}$) parallel to the static Hamiltonian ($\boldsymbol{H}$) principal nuclear hyperfine axes x and z, respectively. The x axis is parallel to the N-O bond direction, and the z axis is parallel to the nitrogen $2p\pi$ orbital. The elements of T used in this study were earlier determined by incorporating nitroxide derivatives into host crystals as substitutional impurities: (T_{xx}, T_{zz}) − (6.1, 32.4) G [21]. $a_{N'}$ and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal states, respectively (i.e., $a_{N'} = 1/3(T_{\parallel} + 2T_{\perp})$ and $a_N = 1/3(T_{zz} + 2T_{xx})$).

The order parameters $S(T_{\parallel})$, $S(T_{\perp})$ and S reflect the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). $S(T_{\parallel})$, $S(T_{\perp})$ and S may each assume values between 0 and 1; these extreme order parameters indicate that the probe samples fluid and immobilized environments, respectively. The order parameter S, which requires both hyperfine splittings, corrects for small polarity differences between the membrane and reference crystal. Although $S(T_{\parallel})$ and $S(T_{\perp})$ do not include corrections for polarity contributions, these expressions have been found to be useful approximate measures of the fluidity in those cases where only one

of the hyperfine splittings is available [20,22].

If Ca^{2+} affects liver plasma membrane fluidity by binding to specific membrane sites, then an association constant (K_a) may be calculated if the following assumptions are valid: (i) there are a finite number of Ca^{2+} sites; (ii) Ca^{2+} binding is reversible; (iii) ΔS, the percentage change in fluidity induced by Ca^{2+} is proportional to the density of membrane sites occupied by Ca^{2+}; and (iv) one class of membrane sites is present. Thus,

$$Ca^{2+} + B \overset{K_a}{\rightleftharpoons} Ca^{2+}\text{-}B \tag{4}$$

hence,

$$K_a = \frac{[Ca^{2+}\text{-}B]}{[Ca^{2+}][B]} \tag{5}$$

here, $[Ca^{2+}]$ is the concentration of free Ca^{2+}, [B] is the concentration of unliganded Ca^{2+}-membrane binding sites, and $[Ca^{2+}\text{-}B]$ is the concentration of bound Ca^{2+}-membrane binding sites.

Assumption (iii) presupposes that,

$$\Delta S = (\Delta S)_{max} \cdot V \tag{6}$$

where, ΔS is the percentage change in the order parameter S induced by Ca^{2+}, $(\Delta S)_{max}$ is the maximum change in S induced by Ca^{2+} at 'saturating' concentrations and V is the fraction of membrane sites occupied by Ca^{2+}. Thus,

$$V = \frac{[Ca^{2+}\text{-}B]}{[B] + [Ca^{2+}\text{-}B]} \tag{7}$$

Substituting Eqn. 5 into Eqn. 7 yields

$$V = \frac{K_a[Ca^{2+}]}{1 + K_a[Ca^{2+}]} \tag{8}$$

Eqn. 8 may now be substituted into Eqn. 6 and rearranged to yield:

$$\frac{(\Delta S)_{max}}{\Delta S} = 1 + \frac{1}{K_a[Ca^{2+}]} \tag{9}$$

If the above binding model accurately describes the actions of Ca^{2+} on the order parameters of I(12,3)-labeled membranes, then plots of $(\Delta S_{max}/\Delta S)$ vs. $1/[Ca^{2+}]$ should yield linear regression lines with high coefficients of determination and y intercepts close to 1.0.

Results

ESR studies of the effect of Ca^{2+} on rat liver plasma membranes

We have previously shown that the order parameters calculated from I(12,3)-labeled rat liver plasma membranes were not necessarily independent of the probe concentration employed [9,12]. Although $S(T_{\parallel})$ was relatively insensitive to the probe concentration over a wide range at 37°C, S and $S(T_{\perp})$ were each found to decrease substantially at high loading. As the amount of I(12,3) per mg of protein was increased from 9 to 45 μg, S and $S(T_{\perp})$ decreases by 6 and 10%, respectively, while $S(T_{\parallel})$ was unaffected [12]. These effects were attributed to the clustering of all or a portion of I(12,3) at high probe concentrations [9]. Earlier titration experiments at 37°C indicated that 'intrinsic' order parameters of rat liver plasma membranes could be measured only for probe concentrations less than 10 μg of I(12,3) per mg of protein where nitroxide radical interactions were negligible [12]. At these experimentally-determined low probe concentrations, the ESR spectra may be quantitatively interpreted in terms of a membrane-incorporated probe undergoing rapid, anisotropic motion about its long axis (see Ref. 12).

At 37°C, $CaCl_2$ (6 mM) increased the values of the order parameters S, $S(T_{\parallel})$ and $S(T_{\perp})$ of liver membranes labeled with a low probe concentration (e.g., 5 μg of I(12,3) per mg of protein in Fig. 1). To test further whether spectral perturbations were due to changes in the flexibility and/or polarity of the local environment of the probe, or were instead the result of alterations in probe-probe interactions, Ca^{2+} was added to membranes containing a wide range of probe concentrations. The small slopes and low coefficients of determination (r^2) for the regression lines obtained from plots of the ΔS, $\Delta S(T_{\parallel})$ and $\Delta S(T_{\perp})$ versus μg of probe/mg of protein show that the increase in each other parameter induced by Ca^{2+} was independent of radical interaction effects (Fig. 1). Furthermore, the mean increase in S, $S(T_{\parallel})$ and $S(T_{\perp})$ (i.e., $\Delta S = +5.5 \pm 0.8\%$, $\Delta S(T_{\parallel}) = +7.6 \pm 1.8\%$,

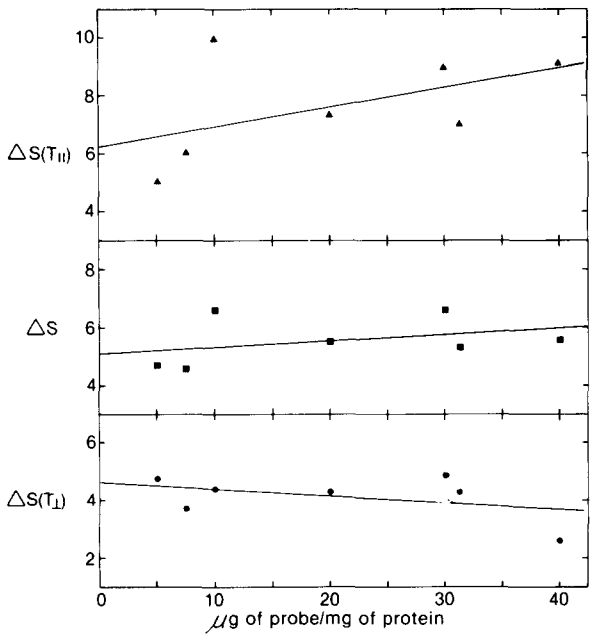


Fig. 1. The effects of increasing I(12,3) probe concentration on the percentage of changes in order parameters induced by 6 mM $CaCl_2$ at 37°C. ΔS, $\Delta S(T_{\parallel})$, and $\Delta S(T_{\perp})$, the percentage of changes in the liver plasma membrane order parameters induced by 6 mM $CaCl_2$, are plotted as a function of I(12,3) probe concentration; S, $S(T_{\parallel})$ and $S(T_{\perp})$ were calculated as indicated under Materials and Methods. Linear regression lines are drawn through the ΔS, $\Delta S(T_{\parallel})$ or $\Delta S(T_{\perp})$ versus probe concentration plots with slopes, y intercepts and coefficients of determination (r^2) of 0.2, +5.1, 0.12, and 0.07, +6.3, 0.26 and 0.2, +4.6, 0.17, respectively. Low-range order parameters were calculated from native liver membranes suspended in 50 mM triethanolamine, 8% sucrose, pH 7.6, and labeled with less than 10 μg of I(12,3)/mg of protein, whereas high range order parameters were measured from native liver membranes with 10 to 40 μg of probe/mg of membrane protein [12]. The presence of probe-probe interactions in native membranes at high loading characteristically alters the respective order parameters, as discussed in Results.

$\Delta S(T_{\perp}) = +4.1 \pm 0.8\%$ (errors given are S.D.)) mediated by Ca^{2+} for the seven membrane samples used in Fig. 1 agreed well with the respective extrapolated y intercepts (i.e., $\Delta S = +5.1\%$, $\Delta S(T_{\parallel}) = +6.3\%$ and $\Delta S(T_{\perp}) = +4.6\%$). The y intercepts reflect the magnitude of the Ca^{2+} effect at 'zero' probe concentrations. These data suggest that, although Ca^{2+} influences the order parameters of the membrane-incorporated probe, this cation does not affect the ability of the spin probe to cluster in the membrane (see Refs. 9 and 22).

Ca^{2+} clearly increases the value of the polarity-corrected order parameter S for membranes labeled with low probe concentrations (Fig. 1), indicating

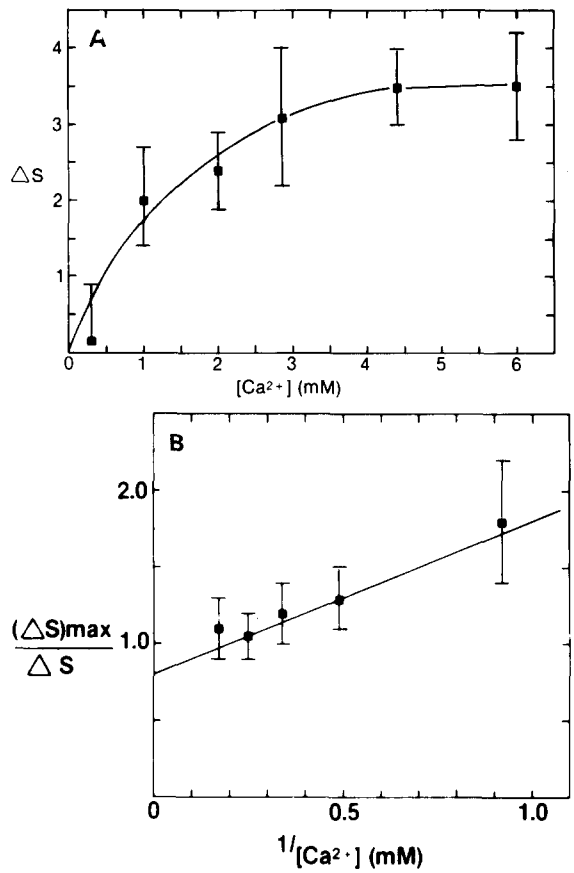
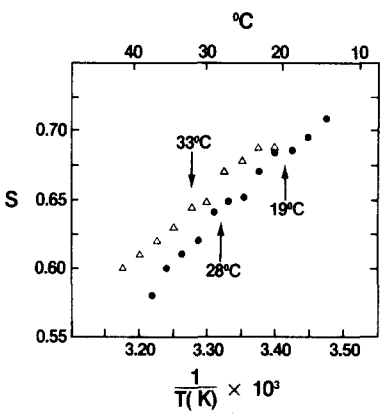


Fig. 2. The effects of $CaCl_2$ on the order parameter S of I(12,3)-labeled rat liver membranes and the determination of the association constant (K_a) for binding sites that regulate the membrane fluidity. (A) ΔS, the percentage of change in S, is plotted as a function of the $CaCl_2$ concentration. Liver membranes were labeled with 9 μg of I(12,3)/mg of protein and suspended in 50 mM triethanolamine, 8% sucrose, pH 7.6 and 110 mM NaCl. Additions were performed by incubating a given concentration of $CaCl_2$ with the labeled membranes for 10 min before recording the ESR spectra at 37°C. The error bars for each point represent ±1 S.D. obtained from four separate determinations. (B) $(\Delta S)_{max}/\Delta S$ is plotted versus $1/[Ca^{2+}]$ (mM), where $(\Delta S)_{max}$ is the maximum percentage change in S (i.e., +3.5%) induced by 'saturating' concentrations of $CaCl_2$ (see Materials and Methods). Each point and error bar represent the mean ±1 S.E. from four separate determinations. A linear regression line drawn through this data exhibited a y intercept and coefficient of determination (r^2) of 0.80 and 0.72. The K_a determined from this plot employing Eqn. 9 is $1 \cdot 10^3$ M^{-1} (1 mM^{-1}).

that this cation reduces the mobility of the probe. On the other hand, Ca^{2+} does not exert any significant effect on the polarity of the environment of the probe, which is verified by noting that the isotropic hyperfine coupling constant ($a_{N'}$) in the presence or absence of $CaCl_2$ (6 mM) is 15.35 ± 0.03 G or 15.41 ± 0.03 G, respectively (errors given are S.D. with $n = 3$).

As addition of NaCl (110 mM) had no significant effect on the order parameter S for I(12,3)-labeled membranes ($\Delta S = +0.7 \pm 0.5\%$) at 37°C, the above Ca^{2+}-dependent ordering was not simply due to an increase in the ionic strength of the medium. This was confirmed by noting that the change in S induced by 6 mM $CaCl_2$ was only slightly different whether 110 mM NaCl was absent ($\Delta S = +5.5 \pm 0.8\%$) or initially present ($\Delta S = +4.2 \pm 1.2\%$) in the medium. All increases in the order parameters induced by Ca^{2+} could be completely reversed upon addition of excess EGTA (ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid).

The cationic local anesthetic prilocaine (10 mM) increases the fluidity of I(12,3)-labeled liver membranes at 37°C, as evidenced by a decrease in $S(T_{\parallel})$ ($\Delta S(T_{\parallel}) = -2.0 \pm 0.5\%$). Pre-incubation of the membranes with this drug concentration markedly reduced by the ability of Ca^{2+} to de-



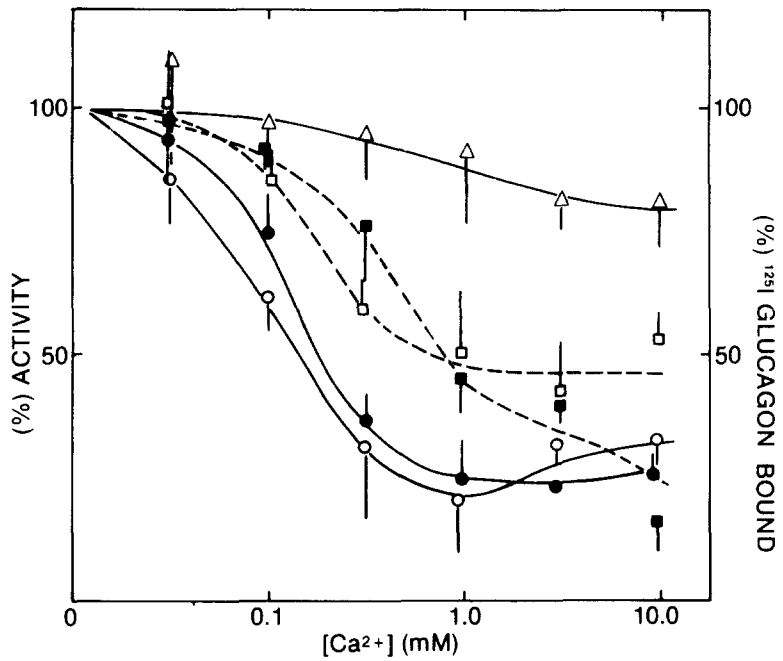


Fig. 3. The temperature dependence of the order parameter S of I(12,3)-labeled liver plasma membranes in the presence (△) and absence (●) of 3 mM $CaCl_2$. Liver plasma membranes (4 mg protein/ml) were labeled with 9 μg probe/mg of protein and suspended in 50 mM triethanolamine, pH 7.6, 8% sucrose. Characteristic temperatures corresponding to the onset and ending temperatures of the lipid phase separation at 19 and 28°C are indicated for native plasma membranes. The high temperature onset of the lipid phase separation for Ca^{2+}-treated membranes at 33°C is also indicated. All data were obtained by progressing from low to high temperatures.

Fig. 4. Effects of $CaCl_2$ on hepatic adenylate cyclase activity and on the specific binding of ^{125}I-glucagon to rat liver plasma membranes. The fluoride-stimulated activity was assessed for either membrane-bound (■-----■) or Lubrol 17A10-solubilized preparations (□-----□). The glucagon-stimulated activity of membrane-bound adenylate cyclase was determined in the presence (○——○) and absence of (●——●) of 0.1 mM GTP. All enzyme assays were carried out at 37°C, taking initial rates from linear time courses. The number of determinations for each of the membrane-bound activities was 6, while that for the solubilized preparation was 4. The actions of $CaCl_2$ on the specific binding of ^{125}I-glucagon (△——△) were evaluated at 37°C for six separate determinations as set out in Materials and Methods. Each point and error bar represent the mean ± 1 S.D.

crease the bilayer fluidity at 37°C. Addition of $CaCl_2$ (3 mM) increased the order parameter to a much smaller degree in the presence of prilocaine ($\Delta S(T_{\parallel}) = +3.6 \pm 0.5\%$) than in its absence ($\Delta S(T_{\parallel}) = +7.6 \pm 1.8\%$).

Ca^{2+} can be demonstrated to achieve a decrease in the fluidity of liver plasma membranes by a concentration-dependent, saturable process reaching a plateau at about 3.5 mM (Fig. 2A). The binding model presented in Materials and Methods assumes that Ca^{2+} binds reversibly to a finite number of sites on the membrane, and indeed a plot of $(\Delta S)_{max}/\Delta S$ versus $1/[Ca^{2+}]$ is linear, with a regression line yielding a high coefficient of determination (r^2) (Fig. 2B). Thus, the decrease in fluidity is apparently achieved by Ca^{2+} binding to a single class of binding sites. These are of low-affinity, yielding a K_a of 1 mM^{-1}, as determined from the reciprocal to the slope of the line (Eqn. 9 and Fig. 2B).

The temperature dependence of the S of I(12,3)-labeled plasma membranes in the absence and presence of $CaCl_2$ (3 mM) is shown in Fig. 3. The S versus $1/T$(K) plot obtained in the absence of $CaCl_2$ exhibits significant curvature and may be interpreted as having 'breaks' or 'discontinuities' at 28 and 19°C. This data was previously attributed to a thermotropic lipid phase separation between 19 and 28°C (see Discussion and Refs. 9 and 22]. Addition of 3 mM Ca^{2+} acts to displace the Arrhenius-type plot of S to higher temperatures, and appears to elevate the high temperature onset from 28 to 33°C (Fig. 3). The low temperature onset of the phase separation of Ca^{2+}-treated membranes is, however, difficult to define because the probe enters a rigid environment at reduced temperatures and no longer executes rapid anisotropic motion about its long molecular axis. Hence, the order parameter formalism is no longer applicable and S cannot be meaningfully calculated.

The actions of Ca^{2+} on adenylate cyclase activity

Increasing concentrations of Ca^{2+} progressively inhibited the fluoride-stimulated activity up to the highest concentration tested (10 mM); the Ca^{2+} concentration yielding 50% inhibition of the original activity (ID_{50}) was 1 mM (Fig. 4). Addition of Ca^{2+} also inhibited the solubilized, fluoride-pre-

TABLE I

EFFECTS OF $CaCl_2$ OR PRILOCAINE ON THE BREAK POINTS AND ACTIVATION ENERGIES DERIVED FROM ARRHENIUS PLOTS OF THE ACTIVITY OF HEPATIC ADENYLATE CYCLASE IN THE MEMBRANE-BOUND OR SOLUBILIZED STATES

Values represent means ± 1 S.D., employing at least three separate preparations.

Preparation	Ligand	Break points (°C)		Activation energy ($kJ \cdot mol^{-1}$)		
		Upper	Lower	Above upper	Above lower	Below lower
Native [a]	Glucagon	27.8 ± 0.5		58.6 ± 12.6	127.3 ± 2.9	
Native [a]	Fluoride	Linear		84.1 ± 3.8		
+ 1 mM $CaCl_2$	Glucagon	32.3 ± 1.4	15.5 ± 2.0	16.8 ± 7.0	51.0 ± 6.4	84.8 ± 15.0
+ 1 mM $CaCl_2$	Fluoride		17.1 ± 1.1		55.6 ± 10.0	76.0 ± 4.8
+ 10 mM prilocaine [b]	Glucagon	28.3 ± 0.4	10.8 ± 1.2	25.2 ± 7.1	52.5 ± 5.5	102.5 ± 35.3
+ 10 mM prilocaine [b]	Fluoride		11.0 ± 1.7		55.9 ± 7.6	101.2 ± 20.6
Lubrol 17A10 solubilized [c]	Fluoride	22.8 ± 2.5		37.0 ± 7.5	75.0 ± 9.4	
Lubrol N13 solubilized [c]	Fluoride	20.4 ± 1.0		11.4 ± 1.7	51.2 ± 7.3	
Lubrol 17A10 solubilized + 1 mM $CaCl_2$	Fluoride	27.3 ± 0.8		76.3 ± 4.3	34.8 ± 0.2	
Lubrol N13 solubilized + 1 mM $CaCl_2$	Fluoride	26.6 ± 1.2		78.5 ± 9.5	39.5 ± 6.5	

[a] Data adapted from Dipple and Houslay [24].
[b] Data adapted from Houslay et al. [36].
[c] Data adapted from Dipple and Houslay [18].

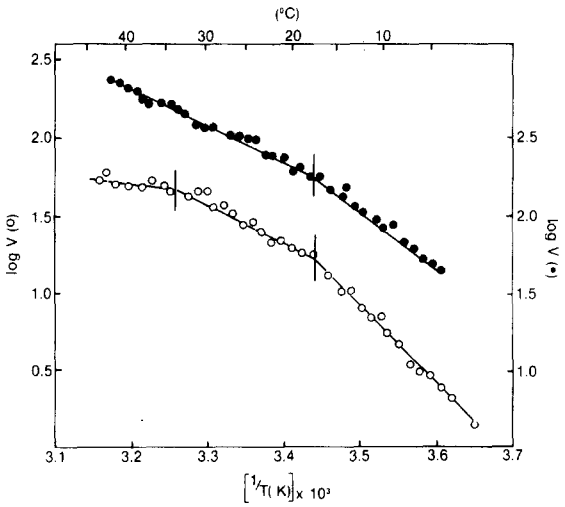


Fig. 5. Arrhenius plots of the fluoride-stimulated (uncoupled) adenylate cyclase activity (●) and the glucagon-stimulated (coupled) adenylate cyclase activity (○) of rat liver plasma membranes in the presence of 1 mM $CaCl_2$. All enzyme assays were performed by taking initial rates from linear time courses. The activity (v) is expressed as pmol cyclic AMP per mg protein per min. Vertical hash marks in the Arrhenius plot of the glucagon-stimulated activity indicate high and low temperature breaks at around 33 and around 16°C, respectively. The dash mark in the Arrhenius plot of the fluoride-stimulated activity indicates a break at 17°C (see Table I).

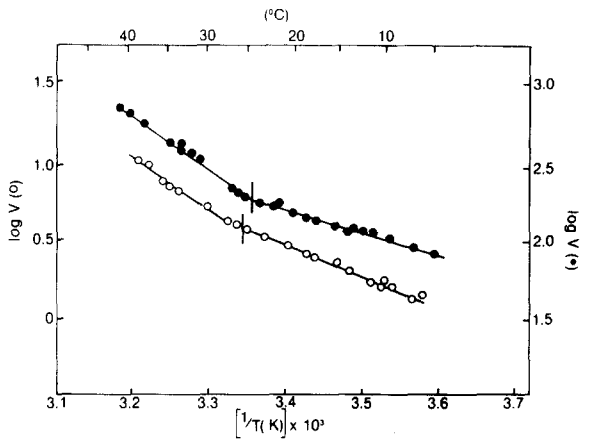


Fig. 6. Arrhenius plots of the fluoride-stimulated activity of adenylate cyclase solubilized by either Lubrol N13 (●) or Lubrol 17A10 (○) in the presence of 1 mM $CaCl_2$. All enzyme assays were performed by taking initial rates from linear time courses. The activity (v) is expressed as pmol cyclic AMP per mg protein per min. The vertical dash marks in the Arrhenius plots of the adenylate cyclase solubilized by Lubrol N13 or Lubrol 17A10 indicate breaks at 25 or 26°C, respectively.

activated enzyme with a similar ID_{50} equal to 1 mM. Only for Ca^{2+} concentrations greater than 2 mM was the membrane-bound activity somewhat more inhibited than the solubilized preparation (Fig. 4).

The glucagon-stimulated activity was found to be more sensitive to inhibition by Ca^{2+} (Fig. 4) with an ID_{50} of 0.2 mM; similar results were obtained whether the glucagon-stimulated activity of the membrane-bound adenylate cyclase was assayed in the presence or absence of 0.1 mM GTP. No attempt was made to assess the effect of Ca^{2+} on detergent-solubilized preparations in the presence of glucagon, since the solubilized enzyme does not respond to hormone [18,19] and our preparation is fluoride-preactivated. Over the full range of Ca^{2+} concentrations tested in Fig. 4, Ca^{2+} did not alter the specific ^{125}I-glucagon binding to its liver plasma membrane receptor. All of these effects were fully reversible upon washing the membranes.

In the presence of Ca^{2+} (1 mM), Arrhenius plots of glucagon-stimulated adenylate cyclase exhibited two well-defined breaks occurring at around 32°C and 16°C, whereas the fluoride-stimulated activity exhibited a single break at around 17°C (Table I; Fig. 5). However, Arrhenius plots of fluoride-preactivated adenylate cyclase activity solubilized either with Lubrol 17A10 or N13 exhibited single breaks at around 25–27°C in the presence of Ca^{2+} (1 mM) (Fig. 6; Table I).

Discussion

Millimolar Ca^{2+} concentrations lower the fluidity of rat liver plasma membranes, as monitored with the 5-nitroxide stearate spin probe, I(12,3). This is in accord with its action on a number of other plasma membrane systems (for review, see Ref. 3). Here, Ca^{2+} appears to influence the fluidity of I(12,3)-labeled rat liver plasma membranes by binding reversibly to low-affinity sites with a K_a of 1 mM^{-1} (Fig. 2). These presumably reflect, at least in part, the low-affinity, protease-insensitive sites detected in binding studies carried out on rat liver plasma membranes using $^{45}Ca^{2+}$ [5–8]. As extensive investigations [17,23] have failed to pro-

vide evidence for significant permeability barriers in our membrane preparation, the decrease in fluidity reported here is due to the binding Ca^{2+} to membrane components in either the outer or inner half to the bilayer, or both.

Our observation that Ca^{2+} reduces the fluidity of spin-labeled rat liver plasma membranes agrees well with results from a recent fluorescent probe study of this system [10]. There it was demonstrated that the addition of mM Ca^{2+} to lipid dispersions extracted from liver plasma membranes lowered the mobility of the incorporated fluorescent label diphenylhexatriene (DPH), and this was attributed to a direct action of Ca^{2+} on anionic sites of the lipid bilayer. The effects of Ca^{2+} on the respective lipid fluidities detected by diphenylhexatriene and I(12,3) are probably related, since in both cases the action of Ca^{2+} was rapid and reversible.

When Ca^{2+} is added to assays of adenylate cyclase activity, then the enzyme is reversibly inhibited, with the glucagon-stimulated ($ID_{50} = 0.2$ mM) (Fig. 4) being more sensitive to inhibition than the fluoride-stimulated activity ($ID_{50} = 1$ mM) (Fig. 4). This difference in sensitivity is not due to any effect of Ca^{2+} on the binding of glucagon to its receptor, which remains unaltered over the range of Ca^{2+} concentrations examined (Fig. 4). As the activities of both the glucagon- and fluoride-stimulated enzymes are profoundly augmented by increases in bilayer fluidity achieved using local anesthetics (Table II and Refs. 12–14 and 24), then it would not be untoward to expect these activities to be inhibited by Ca^{2+}-mediated decreases in fluidity. Nevertheless, our present results indicate that the depressed enzyme activities induced by Ca^{2+} are, for the most part, independent of fluidity alterations. For example, both the membrane-bound enzyme and the fluoride-preactivated, solubilized preparation exhibit very similar sensitivity to Ca^{2+} ($ID_{50} = 1$ mM) (Fig. 4), indicating that these inhibitory actions of Ca^{2+} are not transmitted through the native lipid bilayer. Only at high concentrations (approx. 10 mM Ca^{2+} total), where the membrane-bound enzyme is more inhibited than the solubilized preparation, are Ca^{2+}-mediated increases in lipid ordering likely to influence adenylate cyclase activity (Fig. 4). Reductions in fluidity are also not responsible for the inhibition of the glucagon-stimulated activity. At a Ca^{2+} concentration of 0.3 mM, where the hormone-sensitive activity is approx. 34% of its original value (Fig. 4), no significant increase in S was detected (Fig. 2). Nor is it likely that this inhibition is simply a reflection of an acute sensitivity of the enzyme to changes in fluidity, since the glucagon-stimulated activity plateaus for Ca^{2+} concentrations (between 0.3 and 10 mM) over which the bilayer fluidity is significantly decreased (Figs. 2 and 4). A more plausible explanation is that Ca^{2+} exerts its inhibitory effects by directly interacting with protein components of the adeny-

TABLE II

EFFECTS OF $CaCl_2$, TEMPERATURE ALTERATIONS AND BENZYL ALCOHOL ON THE FLUORIDE-, AND GLUCAGON-STIMULATED ACTIVITIES OF ADENYLATE CYCLASE AND THE ORDER PARAMETER $S(T_{\|})$ OF I(12,3)-LABELED RAT LIVER PLASMA MEMBRANES.

The percentage change in $S(T_{\|})$, $\Delta S(T_{\|})$, induced by a given perturbant was determined from baseline values at the initial conditions using membranes labeled with 9μg of probe/mg of protein. Percent activities of fluoride-stimulated adenylate cyclase (%FSAC) and glucagon-stimulated adenylate cyclase (%GSAC) were calculated for each perturbant by setting the respective activities at the initial conditions at 100%. Values represent the mean ± 1 S.D., using at least three separate plasma membrane preparations.

Initial condition	Perturbant	%FSAC	%GSAC	$\Delta S(T_{\|})$
Native membranes at 30°C	Temperature raised to 37°C	222±8	172±8	−9.7±1.6
Native membranes at 30°C + 1 mM $CaCl_2$	Temperature raised to 37°C	135±6	122±8	−8.4±2.0
Native membranes at 30°C	50 mM benzyl alcohol added	155±6	210±6	−5.0±1.0
Native membranes at 30°C + 1 mM $CaCl_2$	50 mM benzyl alcohol added	150±6	135±5	−4.5±0.4
Native membranes at 30°C	1 mM $CaCl_2$ added	50±8	25±6	+2.2±0.5
Native membranes at 30°C	50 mM benzyl alcohol and 1 mM $CaCl_2$	72±6	40±6	−2.4±0.8

late cyclase complex. Indeed, this enzyme has been noted to have divalent cation binding sites that perform regulatory roles [25]. Other factors contributory to our observed inhibition could be due to Ca^{2+} complexing the ATP substrate or to a high-affinity liganding of Ca^{2+} to acidic phospholipids that have been purported to be essential for the functioning of this enzyme [25], and presumably would be associated with protein.

In native rat liver plasma membranes, a thermotropic lipid phase separation occurring between 28 and 19°C can be detected by spin-label techniques (see Fig. 3 and Refs. 9,22,26–28) and a variety of other physical methods, including differential scanning calorimetry (DSC) [29], light scattering [24], electron diffraction [30] and fluorescence probe spectroscopy [29,31]. As the 'breaks' in Fig. 3 are not as dramatic as those seen in the thermal transitions of defined, monocomponent lipids, we suggested that the 28°C 'break' reflects the formation of discrete lipid domains [9,12]. This assignment would be consistent with a recent DSC study which reported a broad transition that was low in both enthalpy and cooperativity [29]. It is of particular interest, then, that transmembrane enzymes, such as glucagon-stimulated adenylate cyclase, and integral enzymes connected with the external half of the bilayer, such as 5'-nucleotidase, exhibit breaks in their Arrhenius plots of activity at around 28°C [24,26,32,33,38]. However, certain integral enzymes associated with the cytosol half of the bilayer, such as fluoride-stimulated adenylate cyclase, do not sense this lipid phase separation [26,33]. Since all of these enzymes are influenced by changes in lipid fluidity [12], we proposed that the phase separation is confined to the outer half of the bilayer [15,26,33]. Such a hypothesis is not unreasonable in view of the known chemical asymmetry of the bilayer, in which negatively charged lipids predominate at the inner leaflet, while neutral and positively-charged lipids are concentrated in the external leaflet [34]. Our model is also consistent with the finding that anionic local anesthetics such as phenobarbital can preferentially fluidize the external half of the liver plasma membrane bilayer by selectively partitioning into the neutral and positively charged lipids residing there; this serves to substantially lower the high temperature onset of the phase separation detected by I(12,3) and the activities of enzymes connected with outer leaflet, such as that of glucagon-stimulated adenylate cyclase [13,35]. Contrarily, cationic local anesthetics such as prilocaine preferentially fluidize the cytosol side of the plasma membrane by virtue of selective charge interactions with acidic phospholipids, where an additional lipid phase separation is induced that is sensed both by I(12,3) and enzymes associated with the inner leaflet, such as fluoride-stimulated adenylate cyclase [35–37].

Despite the fact that the inhibition of adenylate cyclase activity (Fig. 4) is primarily due to the direct interaction of Ca^{2+} with the enzyme complex, our results indicate that the effect of Ca^{2+} on the membrane lipid structure exert significant 'second-order' perturbations on the temperature dependence of these activities. We note that Ca^{2+} raises the high temperature onset of the lipid phase separation from 28 to around 33°C as detected by the I(12,3) probe (Fig. 3), and this is matched by a similar effect on the temperature at which the break occurs in Arrhenius plots of the glucagon-stimulated adenylate cyclase activity (Fig. 5; Table I). Such an elevation in the onset temperature presumably reflects the interaction of the cation with acidic glycolipids and neutral phospholipids in the external half of the membrane, and would be in accord with the effects of Ca^{2+} on the transition temperatures of model lipid membranes [2,3,39]. However, we also observed that Ca^{2+} mediates a break in the Arrhenius plots of both the fluoride- and glucagon-stimulated activities at around 16–17°C (Fig. 5; Table I). As no such low temperature break was induced by Ca^{2+} in Arrhenius plots of the detergent-solubilized enzyme activities (Fig. 6; Table I), this event is likely to be a lipid-dependent perturbation transmitted through the native membrane.

One explanation of the break at approx. 17°C detected by the membrane-bound adenylate cyclase activities (Fig. 5) is that it is due to the interaction of Ca^{2+} with acidic phospholipids, leading both to cluster formation and to a new lipid phase separation occurring in the inner half of the bilayer. This mechanism would be analogous to that triggered by Ca^{2+} which, when added to mixtures of negatively-charged and isoelectric phospholipids, induces the formation of physically-distinct domains

consisting of phosphatidylcholine and acidic phospholipid-Ca^{2+} complexes [40–42]. It is, unfortunately, not possible to assign the Ca^{2+}-mediated lipid phase separation solely from S versus $1/T$(K) plots, inasmuch as a 'break' occurs at around 19°C in the absence of Ca^{2+} and, in the presence of Ca^{2+}, the order parameter S cannot be measured at lower temperatures for reasons set our earlier. Nevertheless, the interpretation that this event is the result of a new lipid phase separation occurring in the inner leaflet would be consistent with our earlier studies using the cationic drug prilocaine [36]. While prilocaine (10 mM) leaves the high temperature onset at 28°C unaffected, it increases the fluidity of rat liver plasma membranes and induces a new lipid phase separation at around 11°C that is monitored by the I(12,3) probe and the fluoride- and glucagon-stimulated activities (Table I) [36]. Since neutral and anionic fluidizing drugs decrease the high temperature onset of the lipid phase separation [12,13]. We suggested that prilocaine preferentially partitions into the cytosol-facing leaflet and segregates the acidic phospholipids residing there through charge interactions [36]. Our present observation that 10 mM prilocaine blunts the ability of Ca^{2+} to reduce the lipid fluidity indicates that these two agents compete for anionic binding sites on the inner leaflet. Moreover, the fact that Ca^{2+} and prilocaine similarly perturb the thermodependent activities of adenylate cyclase suggests that both act through a common site, namely by selectively interacting with acidic lipids in the inner leaflet such that a lipid phase separation is induced. In addition to both agents mediating low temperature breaks, the activation energies achieved by Ca^{2+} above and below the low temperature are in good agreement with the corresponding values obtained for prilocaine (Table I).

An interesting aspect of the interaction of Ca^{2+} with adenylate cyclase is that it appears to 'desensitize' the glucagon-stimulated activity, and to a lesser extent the fluoride-stimulated activity, to alterations in bilayer fluidity. Table II shows that Ca^{2+} modifies the response of the enzyme activity to increases in fluidity achieved by temperature elevation or benzyl alcohol addition, whereas it does not change the degree of fluidization induced by these agents. Furthermore, joint addition of benzyl alcohol and $CaCl_2$ overrides the Ca^{2+}-dependent lipid ordering such that the bilayer becomes more fluid than untreated membranes, but was unable to return the activities to the original values observed with native membranes (Table II). We suggest that Ca^{2+} interacts with components of the adenylate cyclase system to cause a change in conformation so that the enzyme exists in a relatively 'stable' state whose activity resists changes in membrane fluidity. That Ca^{2+} perturbs the protein conformation due to direct interactions might be construed from its effect on the form of the Arrhenius plots of the solubilized enzyme (Fig. 6; Table I). This may, at least in part, explain our finding that the inhibition of the enzyme activity induced by Ca^{2+} does not result from any cation-medium decrease in fluidity, even though the membrane-bound enzyme still senses Ca^{2+}-dependent perturbations of the lipid structure, as is indicated by alterations in the form of its Arrhenius plots (Fig. 5; Table I).

The extracellular fluid in liver exhibits a Ca^{2+} concentration on the order of 10^{-3} M [3,7]. It is, therefore, probable that, under in vivo conditions, Ca^{2+} will interact with the lipids of the external surface of the plasma membrane, raising the high temperature onset of the lipid phase separation to around 33°C. This would imply that a variety of cell surface phenomena sensitive to the membrane lipid structure will be regulated by the binding of Ca^{2+} to the outer leaflet of the plasma membrane. Indeed, Arrhenius plots of the attachment rate of hepatocytes to protein substrata in the presence of mM Ca^{2+} exhibit a break at around 34°C [43]. Less is known about the free intracellular concentration of Ca^{2+}, but it is probable that these are below $1 \cdot 10^{-6}$ M [3], and such low levels would be unlikely to significantly affect the fluidity of the cytosol-facing leaflet.

It would seem that Ca^{2+} can exert dramatic effects on bilayer fluidity and lipid organization in a biological membrane, in confirmation of earlier predictions using model lipid systems [11,40–42]. Due to the asymmetric nature of biological plasma membranes and the Ca^{2+} gradient across them, this may result in selective perturbations on each half of the bilayer and hence in the activities of asymmetrically-oriented proteins.

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