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Quinidine and melittin both decrease the fluidity of liver plasma membranes and both inhibit hormone-stimulated adenylate cyclase activity

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Increasing concentrations of either quinidine or melittin gave a dose-dependent inhibition of both the glucagon- and fluoride-stimulated activities of adenylate cyclase in the liver plasma membranes. At similar concentrations these agents increased the order of liver plasma membranes as detected by a fatty acid ESR probe, doxyl stearic acid. This increase in bilayer order (decrease in 'fluidity') is suggested to explain the inhibitory action of quinidine on adenylate cyclase activity but only in part contributes to the inhibitory action of melittin on adenylate cyclase. Arrhenius plots of fluoride-stimulated activity became non-linear in the presence of either quinidine or melittin, with a single well-defined break occurring at around 12°C in each instance. Arrhenius plots of the glucagon-stimulated activity also exhibited such a novel break at around 12°C when either quinidine or melittin were present as well as exhibiting a break at around 28°C, as was seen in the absence of these ligands. The fatty acid spin probe inserted into liver plasma membranes detected a novel lipid phase separation occurring at around 12°C when either quinidine or melittin was present and showed that the lipid phase separation occurring at around 28°C in native membranes was apparently unaffected by these ligands.

Introduction

Adenylate cyclase is a key regulatory enzyme whose activity can be modulated by a variety of hormones and neurotransmitters [1,2]. These ligands bind to specific receptors on the cell surface and transduce their stimulatory action on the catalytic unit of adenylate cyclase, via the stimulatory guanine nucleotide regulatory protein, G_s [3–5]. In the liver plasma membrane these entities are orientated asymmetrically with the binding site of the receptor facing the cell surface and with the G_s and adenylate cyclase located at the cytosol surface of the bilayer [6].

The lipid bilayer of rat liver plasma membranes is structurally asymmetric with the acidic phospholipids found almost exclusively in the cytosol half of the bilayer [7]. It exhibits a lipid phase separation occurring at around 28°C being due to the lipids of the external half of the bilayer only (for reviews, see Refs. 8 and 9). We have demonstrated that agents which increase bilayer fluidity [10–13] stimulate adenylate cyclase activity whereas those that decrease fluidity cause a reduction in activity [14–16]. The physical properties of

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the two halves of the bilayer can be perturbed selectively by charged local anaesthetics [8,11,12], by Ca^{2+} [16] and by cholesterol manipulation [14–17]. We have shown in rat [6,8] and hamster liver plasma membranes [18] and in rat brain plasma membranes [19] that the activity of adenylate cyclase, when stimulated through G_s, by either NaF or guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG), is influenced only by the lipid environment of the inner half of the bilayer as the functional, globular domains of both G_s and the catalytic unit of adenylate cyclase reside there [8,9]. However, the presence of glucagon causes its receptor to couple structurally and functionally to G_s and adenylate cyclase forming a transmembrane complex [6,20] whose activity is regulated by the lipid environment of both halves of the bilayer [8,9].

Melittin is a basic amphipathic peptide which has been shown to interact strongly with phospholipid vesicles and biological membranes [21,22]. Indeed, like myelin basic protein [23,24], melittin can interact preferentially with acidic phospholipids [22] in membranes. The anti-arrhythmic drug, quinidine has also been shown to interact with acidic phospholipids, causing them to cluster, forming relatively rigid domains [25].

We [26] and others [27] have recently demonstrated that acidic phospholipids can modulate adenylate cyclase activity by affecting regulation by guanine nucleotide regulatory proteins. In this study we show that both melittin and quinidine inhibit adenylate cyclase activity in a dose-dependent manner. Part of this inhibitory effect can be attributed to a decrease in bilayer fluidity achieved by these ligands.

Materials and Methods

The I(12.3) spin label (*N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-oxostearic acid) was obtained from Syva Co, Palo Alto, CA, U.S.A. Creatine kinase, phosphocreatine, ATP, cyclic AMP, and triethanolamine hydrochloride were from Boehringer (U.K.) Ltd. Melittin and quinidine were from Sigma (U.K.) Ltd. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g as described previously [28,29]. Fluoride- and glucagon-stimulated adenylate cyclase (EC 4.6.1.1) were assayed over linear time-courses at constant pH as previously described [28]. Adenylate cyclase assays were performed in a medium containing final concentrations of 25 mM triethanolamine-HCl, 1 mM EDTA, 5 mM $MgSO_4$, 10 mM theophylline and an ATP-regenerating system of 7.4 mg/ml creatinine phosphate and 1 mg/ml creatine kinase at a final pH 7.6. Enzyme activity was initiated by the addition of ATP to a final concentration of 1.5 mM. Quinidine and melittin were shown not to affect the method of determination of cyclic AMP. The determination of break points in Arrhenius plots was performed using a least-squares fitting procedure as described previously by us [18]. Protein was determined using a modified microbiuret method [18].

Electron spin resonance measurements were made using a Varian E-9 X band spectrometer equipped with variable temperature accessory (Varian E-257). Measurements were made at a 1.0 G modulation amplitude, 0.30 s time constant, 4 min time scan, 10 mW microwave power on a scan range of 100 G. Sample spectra were compared with a standard manganese sample in a microwave cavity operating in the H014 mode. The spectrometer was used in conjunction with a Nicolet 1170 signal averager and the inner and outer hyperfine splittings, $2T_\perp$ and $2T_\parallel$ were measured in terms of channels on the averager, calibrated by comparison with the separation of the manganese marker peaks. The order parameter S of rat liver plasma membranes was calculated as previously detailed [16]. The value of S reflects the motion, both rotational and translational, of the membrane incorporated spin probe and, by inference, the 'fluidity' of the membrane. S may assume a value between 0 and 1, representing a free fluid or immobilised environment, respectively. Spin-labelling of rat liver plasma membranes was done using an experimentally determined 'low probe' concentration of 9 μg of probe per mg of protein as described before by us [30]. The same medium was used as that employed for the assays of adenylate cyclase activity.

Results

Increasing concentrations of quinidine (Fig. 1) and melittin (Fig. 2) caused a dose-dependent inhibitory effect on both fluoride- and glucagon-stimulated adenylate cyclase activity. Over the same range of concentrations at which inhibition ensured, both of these ligands caused an increase in the value of the order parameter, *S*, for the incorporated fatty acid spin probe (Fig. 2, compare Figs. 1A and 2A), thus indicating a decrease in bilayer order. We did note, however, that at much lower concentrations of quinidine, there appeared to be a small decrease in bilayer order. There was no evidence to suggest that either quinidine or melittin enhanced 'probe–probe' interactions at the concentrations of spin probe and drugs used in this study.

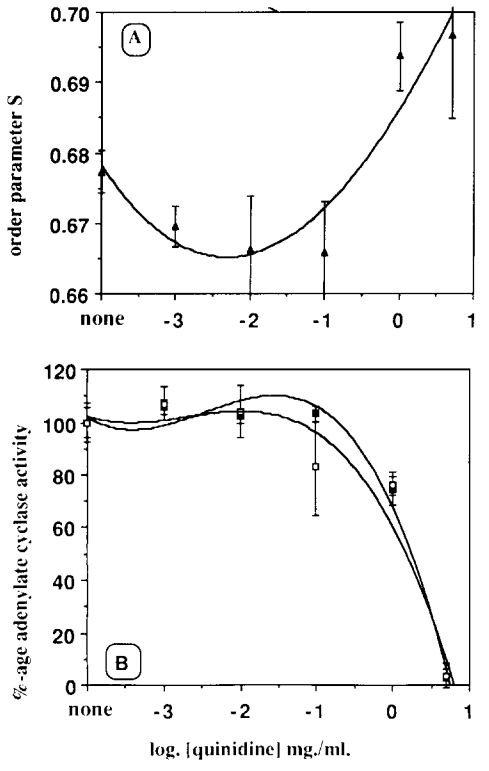

Fig. 1. Action of quinidine on adenylate cyclase activity and on membrane order. (A) Order parameter, *S* for ESR spin probe, doxyl stearic acid. (B) Fluoride (□)- and glucagon (■)-stimulated adenylate cyclase activities. Results presented are means ± S.D. for $n = 3$ experiments. Experiments were performed at 30 °C.

Arrhenius plots of adenylate cyclase activity in native liver plasma membranes gave, as reported by us previously [8–10,28], a linear result for the fluoride-stimulated activity and a biphasic result for glucagon-stimulated activity with a single well-defined break occurring at 28 °C. In contrast, quinidine and melittin both altered the form of these Arrhenius plots dramatically. Both agents caused Arrhenius plots of the fluoride-stimulated activity to be markedly biphasic with a break occurring at around 12 °C (Figs. 3 and 4; Table I). Their presence in assays of glucagon-stimulated activity resulted in Arrhenius plots, whilst still exhibiting a high temperature break at around

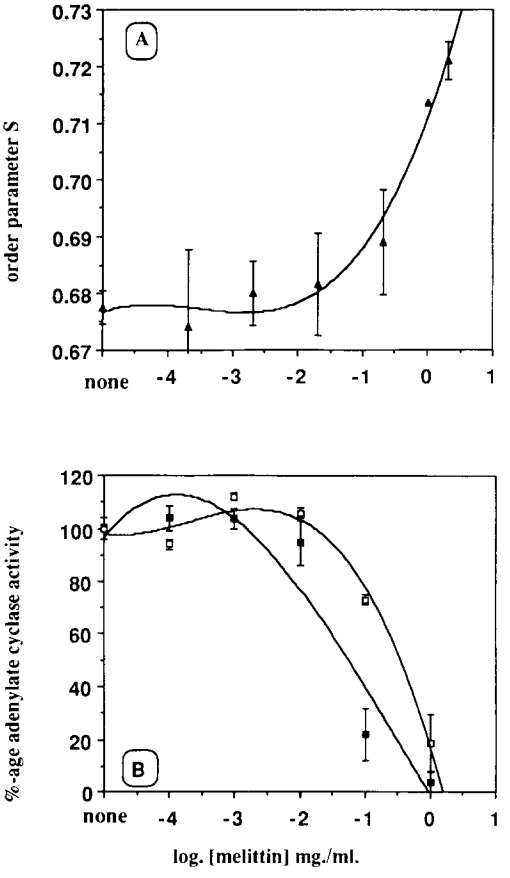

Fig. 2. Action of melittin on adenylate cyclase activity and on membrane order. (A) Order parameter, *S* for ESR spin probe, doxyl stearic acid. (B) Fluoride (□)- and glucagon (■)-stimulated adenylate cyclase activities. Results presented are means ± S.D. for $n = 3$ experiments. Experiments were performed at 30 °C.

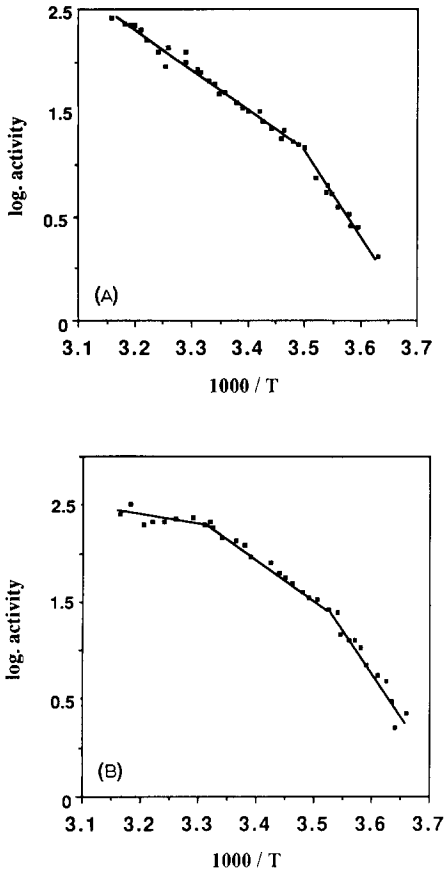

Fig. 3. Action of quinidine on the thermodependence of adenylate cyclase activity. (A) Fluoride-stimulated activity. (B) Glucagon-stimulated activity. Typical data for experiments repeated three times are shown. Quinidine was used at 1 mg/ml. Temperature in K.

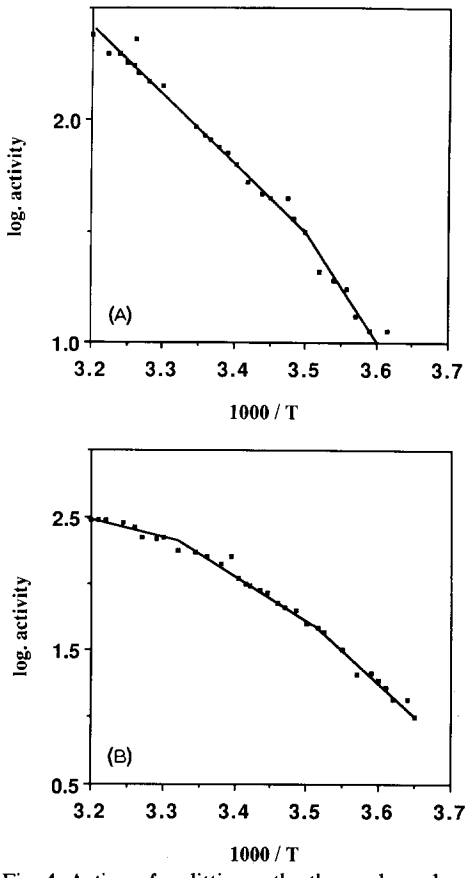

Fig. 4. Action of melittin on the thermodependence of adenylate cyclase activity. (A) Fluoride-stimulated activity. (B) Glucagon-stimulated activity. Typical data for experiments repeated three times are shown. Melittin was used at 0.05 mg/ml. Temperature in K.

TABLE I

ARRHENIUS PLOTS OF ADENYLATE CYCLASE ACTIVITY IN LIVER PLASMA MEMBRANE

Experiments were performed with 1 mg/ml quinidine and 0.05 mg/ml melittin. FSAC, fluoride-stimulated adenylate cyclase activity; GSAC, glucagon-stimulated adenylate cyclase activity.

Additions	Stimulatory ligand	Upper break point (°C)	Lower break point (°C)	E_a above upper break point (kJ/mol)	E_a above lower break point (kJ/mol)	E_a below lower break point (kJ/mol)
None	FSAC	–	–	–	65.4± 3.3	–
	GSAC	29.1±0.5	–	27.2±3.1	73.2±10.5	–
Quinidine	FSAC	–	11.8±1.1	–	62.6±15.0	96.3± 5.4
	GSAC	27.5±0.7	10.6±0.6	27.0±5.4	73.8± 9.0	148.0±38.2
Melittin	FSAC	–	12.8±1.1	–	61.6±10.9	101.1±20.4
	GSAC	27.0±1.4	11.9±1.2	34.7±2.8	79.9± 4.1	107.8±10.9

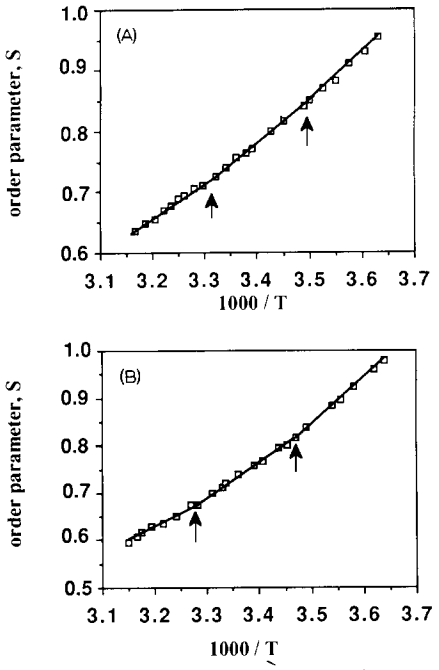

Fig. 5. Action of quinidine and melittin on membrane order. This shows Arrhenius plots of the order parameter S for the nitroide spin labelled liver plasma membranes in the presence of either (A) quinidine (1 mg/ml) or (B) melittin (0.1 mg/ml). A typical experiment of one done at least three times is shown. Temperature in K. Arrows indicate break points.

28°C, now showing a new break around 12°C (Figs. 3 and 4; Table I).

Arrhenius plots of the order parameter, S, have been shown by us and others (for reviews, see Refs. 8 and 9) to identify the lipid phase separation occurring at around 28°C in native liver plasma membranes (28.0 ± 1.5°C in this study). The presence of either quinidine or melittin (Fig. 5) did not appear to affect the occurrence of this lipid phase separation. For, high temperature lipid phase separations were identified in the presence of melittin (0.1 mg/ml) at 29.6 ± 0.8°C and in the presence of quinidine (1 mg/ml) at 30.1 ± 0.7°C. However, the presence of these ligands induced a new lipid phase separation occurring at around 12.3 ± 1.2°C for melittin and at around 13.1 ± 1.5°C for quinidine (errors are S.D., $n = 3$).

Discussion

Glucagon-stimulated adenylate cyclase is a multicomponent integral membrane protein embedded asymmetrically in the cell plasma membrane [1–3,6]. We and others have demonstrated previously that a variety of neutral [10,13,30] or charged [11,12] local anaesthetics, which increase bilayer fluidity, also activate adenylate cyclase activity. Conversely, we have shown that when bilayer fluidity was decreased, using either the neutral molecule dimethylamine [15] or by increasing the cholesterol content of the plasma membrane in vitro [14], the activity of adenylate cyclase was reduced. Quinidine [25] and melittin [22] have been shown to interact preferentially with acidic phospholipid species in model membrane systems where they decrease membrane fluidity (increase their state of order). We see here that both of these ligands decrease the fluidity of a biological membrane in a dose-dependent fashion (Figs. 1 and 2). Over the same concentration range they also inhibit both of the ligand-stimulated activities of adenylate cyclase (Figs. 1 and 2). It is thus tempting to attribute the inhibitory effects of these ligands to the decrease in membrane fluidity that they achieved. Certainly we can calculate for quinidine that at 1 mg/ml it will decrease membrane fluidity by an amount comparable to a temperature fall of around 4 Cdeg assessed using native membranes only. This would be expected to reduce the glucagon- and fluoride-stimulated activities by around 28% and 32%, respectively. This compares well with our observations that quinidine (1 mg/ml) inhibited the glucagon- and fluoride-stimulated activities by 25% and 24%, respectively. For melittin at 1 mg/ml, however, the decrease in fluidity achieved is equivalent to a 6 Cdeg fall in temperature assessed using native membranes. This would give a predicted fall of the glucagon- and fluoride-stimulated activities by 62% and 45%, respectively, of their control values. In the event glucagon- and fluoride-stimulated activities fell by 96% and 78%, respectively, of the control values. On this basis we suggested that the major mechanism whereby quinidine inhibited adenylate cyclase in these experiments was by decreasing membrane fluidity. However, melittin appeared to exert a much more pronounced inhibitory effect on adenylate cyclase than could be attributed to any decrease in membrane fluidity that it caused. Melittin as well as reducing bilayer fluidity, presumably, also exerted either a

direct inhibitory effect on the enzyme or an indirect one by virtue of perturbing interactions with specific lipids essential for enzyme activity.

Liver plasma membranes exhibit a well-defined lipid phase separation occurring at around 28°C that has been detected by a number of investigators using a variety of physical techniques (for reviews, see Refs. 8 and 9). This lipid phase separation has been attributed solely to the lipid in the external leaflet of the bilayer as it is only sensed by proteins which have functional globular regions inserted in this domain; it can be selectively perturbed by cationic local anaesthetics [12] and not anionic species [11] and is lost upon extraction of lipid species from the membrane [9,31]. Indeed, chemical analysis of the phospholipids of these membranes has shown that they are asymmetric with the acidic phospholipids found exclusively at their cytoplasmic surface [7]. We show here that both quinidine and melittin, which decrease membrane fluidity, have no effect upon the lipid phase separation occurring at 28°C in these membranes (Fig. 5). This is in contrast with the action of the neutral compounds dimethylamine [15] or cholesterol-elevation in vitro [14] which both decrease bilayer fluidity and increase the temperature at which this lipid phase separation occurred. However, in contrast to the actions of dimethylamine and of cholesterol elevation, both quinidine and melittin appeared to induce a new lipid pahse separation in these membranes, which occurred at around 12–13°C (Fig. 5). We suggest that this novel lipid phase separation is due to their selective interaction with the lipids of the cytosol-facing (internal) half of the bilayer since we have presented considerable evidence elsewhere (for reviews, see Refs. 8 and 9) that Arrhenius plots of fluoride-stimulated adenylate cyclase activity monitor the lipid environment of the internal half of the bilayer exclusively. Thus in native membranes the plots are linear (Refs. 10, 28; Table I). However, we see here that in the presence of either quinidine or melittin (Figs. 3, 4) they became non-linear with a well-defined break occurring at around 12°C (Table I). When glucagon-stimulated adenylate cyclase activity is followed, then the transmembrane complex which is formed monitors changes occurring in both halves of the bilayer [6,8,9]. However, instead of a single break occurring at around 28°C (Refs. 6, 8, 9; Table I), plots done in the presence of either quinidine or melittin showed two breaks. The upper one, occurring at around 28°C (Figs. 3, 4; Table I), supporting the ESR data which showed no effect of quinidine and melittin on this lipid phase separation and a novel lower break occurring at around 12°C (Fig. 2). We therefore suggest that quinidine and melittin may perturb the inner half of the liver plasma membrane bilayer selectively, decreasing its fluidity and inducing a new lipid phase separation there at around 12°C. This could occur by virtue of their established ability to interact selectively and potently with acidic phospholipids [22,25] that, in the case of liver plasma membranes are known [17] to be found exclusively in the internal leaflet of liver plasma membranes.

The liver plasma membrane thus appears to be a complex membrane with multiple lipid environments. These may be preferentially occupied with various probes and integral membrane proteins. Thus the spin-probe used in this study may present us with an order parameter which reflects the average of that displayed by multiple environments. However, alterations in the form of the Arrhenius plots of glucagon-stimulated adenylate cyclase activity elicited by a variety of drugs, as per this study and others [8,10–13,15,16], and membrane alterations [14,17,26] have been clearly demonstrated to be reflected in comparable changes in Arrhenius plots of the order parameter of this spin probe. This indicates that the doxyl derivative of stearic acid is, at a minimum, a good monitor of changes in the lipid environment of glucagon-stimulated adenylate cyclase activity in liver plasma membranes. We have shown elsewhere that cholesterol depletion [17] and anionic local anaesthetics [12], which increase bilayer fluidity, both appear to induce novel lipid phase separations in the internal half of the bilayer as does the incorporation of small amounts of exogenous acidic phospholipids [26]. Thus, it is tempting to speculate that these acidic phospholipids, by interacting with exogenous species, may play an important role in modulating the properties of the bilayer and hence the functioning of specific integral membrane proteins.

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