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Structural effects on the interaction of sterols with the $(Ca^{2+} + Mg^{2+})$-ATPase

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The fluorescence quenching properties of a brominated derivative of androstenol 5α,6β-dibromoandrostan-3β-ol have been used to study binding to phospholipid bilayers and to the $(Ca^{2+} + Mg^{2+})$-ATPase purified from sarcoplasmic reticulum of rabbit skeletal muscle. It is shown that androstenol is excluded from the phospholipid/protein interface of the ATPase but can bind to other (non-annular sites) on the ATPase. Binding to these sites increases in strength with decreasing chain length for the phospholipids present in the system. Binding is also stronger in the presence of phospholipids in the gel phase than in the liquid crystalline phase. Androstenol increases the ATPase activity of the ATPase reconstituted with phosphatidylcholines of chain lengths less than C_{18}, but has no effect on activity for the ATPase reconstituted with phosphatidylcholines of chain lengths C_{18} or greater. The effects of cholestanols on the activity of the ATPase reconstituted with dimyristoleoylphosphatidylcholine depend on the configuration of the sterol, with 5α-cholestan-3α-ol having little effect but the other isomers causing a marked stimulation.

Introduction

Cholesterol and related sterols are found in many biological membranes, and studies have shown that sterols are essential for growth of many types of cell [1]. The reason(s) for the importance of sterols is (are) still unclear. It is known that sterols modify the fluidity of membranes and it has been suggested that membrane fluidity can be an important controlling factor for the function of membrane proteins and receptors and in endocytosis [2]. Direct interactions with membrane proteins have also been suggested [2–4].

There are obvious advantages in studying the effects of sterols in simplified systems. Studies with simple lipid bilayers have shown that sterols, particularly cholesterol, exert a condensing effect on lipids in the liquid crystalline state, reducing the average molecular surface area and reducing membrane permeability [1]. These effects of sterols are structurally specific, but although a planar ring system, a 3β-hydroxyl group and an isooctyl side-chain have been suggested to give optimal interactions, effects are also observed for sterols without these structural features [1,5–7].

There have been relatively few studies of the interactions of sterols with membrane proteins in reconstituted systems. It has been shown that cholesterol alone cannot support activity for the $(Ca^{2+} + Mg^{2+})$-ATPase purified from skeletal muscle sarcoplasmic reticulum [8]. Effects of cholesterol on the activity of the ATPase in the presence of phospholipids are more complex. It has been shown that cholesterol has relatively little effect on the activity of the ATPase reconstituted with the phospholipid that supports highest activity, dioleoylphosphatidylcholine ((18 : 1)PC) [4]. However, addition of cholesterol to the ATPase reconstituted with the short-chain phospholipid dimyristoleoylphosphatidylcholine ((14 : 1)PC) results in a large increase in ATPase activity [4]. Fluorescence quenching methods have been used to study the interactions of cholesterol with the ATPase believed to be responsible for these effects. It has been shown that reconstitution of the ATPase into bilayers of dibromostearoylphosphatidylcholine ((Br18 : 0)PC) results in quenching of approx.

Abbreviations: (12 : 0)PC, didodecylphosphatidylcholine; (14 : 1)PC, dimyristoleoylphosphatidylcholine; (16 : 0)PC, dipalmitoylphosphatidylcholine; (16 : 1)PC, dipalmitoleoylphosphatidylcholine; (18 : 1)PC, dioleoylphosphatidylcholine; (20 : 1)PC, dieicosenoylphosphatidylcholine; (22 : 1)PC, dieurucoylphosphatidylcholine; (24 : 1)PC, dinervonylphosphatidylcholine; (Br14 : 0)PC, di(9,10-dibromomyristoyl)phosphatidylcholine; (Br18 : 0)PC, di(9,10-dibromostearoyl)phosphatidylcholine; dibromoandrostanol, 5α,6β-dibromoandrostan-3β-ol; DPH, diphenylhexatriene; PDA, pyrene-1-dodecanoic acid.

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65% of the tryptophan fluorescence of the ATPase, by a static quenching mechanism [4]. This quenching can be used to detect binding of non-quenching molecules at the lipid-protein interface (annular sites) on the ATPase, since binding of non-quenching molecules at the interface will result in displacement of (Br18 : 0)PC, detected as an increase in fluorescence intensity. Such studies showed that cholesterol was unable to bind at the lipid/protein interface [4]. It was, however, observed that addition of a brominated analogue of cholesterol (5,6-dibromocholestan-3β-ol) to the reconstituted ATPase did result in fluorescence quenching, indicating binding of cholesterol to sites on the ATPase that could not be at the lipid/protein interface. These sites were designated non-annular sites, and it was suggested that they could occur either within the ATPase, possibly between transmembranous α-helices, or at interfaces between ATPase molecules in dimeric or higher aggregates [4]. Subsequent studies have shown that cholesterol hemisuccinate and a variety of other hydrophobic molecules can also bind to these non-annular sites [9,10]. Similar fluorescence studies have shown that cholesterol is also excluded from sites at the lipid/protein interface for the acetylcholine receptor, but can again bind at other sites, possibly at protein/protein interfaces between subunits of the receptor [11]. Cholesterol has also been shown to affect the activity of the hexose transport protein in reconstituted systems [12].

Here we investigate the structural dependence of the effects of sterols on the activity of the ATPase and investigate the dependence of sterol–ATPase interactions on the structure of the phospholipids present in the system.

Materials and Methods

Lipids were obtained from Avanti polar lipids and pyrene-1-dodecanoic acid (PDA) from Calbiochem. Sterols were from Sigma and Steraloid. 1,2-Bis(9,10-dibromostearoyl)phosphatidylcholine ((Br18 : 0)PC) was prepared from (18 : 1)PC as described in Ref. 13. 1,2-bis(9,10-dibromomyristoyl)phosphatidylcholine ((Br14 : 0)PC) was prepared from (14 : 1)PC by the same method. 5α,6β-Dibromoandrostan-3β-ol (dibromoandrostanol) was prepared from 5-androsten-3β-ol. 5-Androsten-3β-ol (100 mg) was dissolved in diethyl ether (10 ml) at $-20\,^{\circ}$C, to which was added 10 ml of Fiesers' reagent (20 mg anhydrous sodium acetate, 1 ml bromine and 30 ml glacial acetic acid). The mixture was left for 2 h with occasional shaking. A concentrated aqueous solution of sodium dithionite was then added until no bromine colouration was observed in the organic phase. The acetic acid was neutralized by addition of a saturated aqueous solution of sodium bicarbonate. The ether layer was separated and dibromoandrostanol was obtained by rotary evaporation. The product gave a single spot on TLC, and was characterised by IR spectroscopy. The specific rotation, α_D, of the product was determined by using a Perkin Elmer 141C Polarimeter. The value of α_D (-57.5) showed the product to be the planar *trans*-diaxial-5α,6β-dibromoandrostan-3β-ol, since the (5β,6α) isomer would give a positive α_D (see Ref. 14).

Fluorescence spectra were recorded with a Spex Fluorolog fluorimeter. In studies of the effects of sterols on the fluorescence of PDA in mixed liposomes of (18 : 1)PC and (Br18 : 0)PC, PDA, (18 : 1)PC, (Br18 : 0)PC, and sterol were mixed in chloroform solution, dried onto the sides of flasks, and resuspended in buffer (40 mM Hepes, 100 mM NaCl, 1 mM EGTA (pH 7.2)) at 37 °C, to give final concentrations of PDA and phospholipid of 0.4 and 200 μM, respectively, with a molar ratio of (Br18 : 0)PC to (18 : 1)PC of 1 : 2. Fluorescence intensities were measured relative to samples containing the same molar ratios of PDA to phospholipid, but containing (18 : 1)PC as the only phospholipid. Fluorescence was excited at 342 nm and the monomer emission intensity was recorded at 395 nm. Effects of dibromoandrostanol on the fluorescence of PDA in liposomes of (18 : 1)PC were studied in a similar way, at a molar ratio of PDA to phospholipid of 1 : 500, at 25 °C.

Measurements of fluorescence polarisation were made using an Aminco-Bowman fluorimeter fitted with quartz Polacoat filters. Samples were prepared in buffer at a molar ratio of diphenylhexatriene (DPH) to phospholipid of 1 : 100. Fluorescence was excited at 360 nm and emission was detected at 430 nm. Measurements were corrected for light scatter using identical samples but containing no DPH. Fluorescence polarisation was calculated from the equation:

$$P = \frac{I_{0,0} - I_{0,90}(I_{90,0}/I_{90,90})}{I_{0,0} + I_{0,90}(I_{90,0}/I_{90,90})} \quad (1)$$

($Ca^{2+} + Mg^{2+}$)-ATPase was purified from sarcoplasmic reticulum of skeletal muscle as described in East and Lee [13]. Reconstitutions were performed as described in East and Lee [13]. Phospholipid (1 μmol) was mixed with buffer (40 μl; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose (pH 8.0)) containing $MgSO_4$ (5 mM), ATP (6 mM) and potassium cholate (12 mg/ml) and sonicated to clarity in a bath sonicator (Megason). ATPase (0.125 mg) in a volume of 3–10 μl was then added and left for 1 h at 5 °C to equilibrate before being diluted with buffer (200 μl) and stored on ice until use. Fluorescence measurements were made using a Spex Fluorolog fluorimeter. ATPase activities were measured using a coupled enzyme assay. Samples (typically 12 μl, equivalent to 6 μg of ATPase) were added to the medium described in Ref. 13, containing 40 mM Hepes-KOH (pH 7.2), 5 mM $MgSO_4$, 1.01 mM EGTA,

0.42 mM phospho*enol*pyruvate, 0.15 mM NADH, 7.5 IU pyruvate kinase and 18 IU lactate dehydrogenase in a total volume of 2.5 ml, with $CaCl_2$ added to give a maximally stimulating concentration of Ca^{2+}, and the required concentration of ATP. Concentrations of protein were estimated by using the absorption coefficient given by Hardwicke and Green [15].

Analysis of quenching data

Quenching of fluorescence of PDA in a phospholipid bilayer can be described by the Stern-Volmer equation:

$$F_0/F = 1 + K_{sv}[Q_m] \tag{2}$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively; $[Q_m]$ is the concentration of quencher in the membrane (units of moles per litre); and K_{sv} is the quenching constant. For unlimited binding of the quencher to the membrane (simple partition) the distribution of quencher between membrane and aqueous phases is given by a partition coefficient K_p where:

$$K_p = [Q_m]/[Q_a] \tag{3}$$

where $[Q_a]$ is the concentration of quencher in the aqueous phase. As shown elsewhere (Michelangeli, East and Lee, submitted), the Stern-Volmer equation can then be expressed as:

$$F_0/F = 1.0 + K_{app}[Q_t] \tag{4}$$

where

$$K_{app} = K_p K_{sv}/[K_p \alpha_m + (1 - \alpha_m)] \tag{5}$$

where α_m is the volume fraction of the membrane:

$$\alpha_m = V_m/V_t \tag{6}$$

where V_m and V_t are the volumes of the membrane phase and the total volume, respectively, the former being calculated assuming a vesicle density of 1 g/ml. A graph of F_0/F against $[Q_t]$ will be a straight line with slope K_{app}, and a plot of $1/K_{app}$ against α_m will be a straight line with slope given by:

$$\text{slope} = (1/K_{sv}) - (1/K_p K_{sv}) \tag{7}$$

and an intercept given by:

$$\text{intercept} = 1/K_p K_{sv} \tag{8}$$

Alternatively, binding can be described by a Langmuir adsorption isotherm, with the maximum extent of binding to the bilayer being limited by the maximum number of molecules, N, adsorbed per phospholipid molecule, equivalent to a site concentration given by $N[E_t]$, where $[E_t]$ is the total phospholipid concentration. The concentration of bound quencher $[Q_b]$ is then given by:

$$[Q_b] = [Q_a][E_f]/K_d \tag{9}$$

where $[E_f]$ is the free-site concentration, $[Q_a]$ the aqueous quencher concentration, and K_d the dissociation constant for binding. In terms of total concentrations:

$$[Q_b] = \left\{ A - (A^2 - 4N[E_t][Q_t])^{1/2} \right\}/2.0 \tag{10}$$

where

$$A = K_d + N[E_t] + [Q_t] \tag{11}$$

The Stern-Volmer equation then becomes:

$$F_0/F = 1.0 + K'_{sv}[Q_b]/N[E_t] \tag{12}$$

where the different Stern-Volmer quenching constant K'_{sv} arises because of the different concentration units.

It has been shown that quenching of the tryptophan fluorescence of the ATPase reconstituted into mixtures of phospholipid containing brominated phospholipids can be described by Eqn. 13:

$$F/F_0 = (F_0 - F_{min})(1 - f_a)^{1.6} + F_{min} \tag{13}$$

where F_0 is the fluorescence intensity in the absence of the brominated phospholipid and F is the fluorescence intensity when the fraction of annular sites on the ATPase occupied by brominated phospholipid is f_a. F_{min} is the fluorescence intensity observed when the ATPase is reconstituted in pure brominated phospholipid. For mixtures of phosphatidylcholines it has been shown that f_a is equal to the mole fraction of brominated phospholipid present in the phospholipid mixture [13]. It has been shown [4,9,10] that a variety of brominated hydrophobic molecules can bind to the ATPase at sites other than the annular sites, and in such cases it has been shown that fluorescence quenching can be described by the equation:

$$F/F_0 = 0.15 + A(1 - f_{na})^{1.6} + B(1 - f_a)^{1.6}(1 - f_{na})^{1.6} \tag{14}$$

where f_a and f_{na} are the fraction of annular and non-annular sites respectively occupied by the quencher. The constants A and B represent, respectively, the fractions of total fluorescence that can be quenched by binding to non-annular sites alone or by binding to both annular and non-annular sites. The constant 0.15 represents the fraction of the total fluorescence intensity that is not quenchable by hydrophobic quenchers, consistent with the proposed location of two tryptophan residues (15% of the total) in hydrophilic regions of the ATPase

away from the phospholipid phase [10]. For the experiments reported here, concentrations of quencher and lipid are high with respect to the concentration of ATPase so that the fraction f of annular or non-annular binding sites occupied by sterol is:

$$f = x_L/(K_d + x_L) \quad (15)$$

where K_d is the dissociation constant (in mole ratio units) and x_L is the ratio of sterol to phospholipid in the membrane. Concentrations of sterol bound to annular and non-annular sites on the ATPase and bound to the phospholipid component of the membrane were calculated using the Bisection method, as described [10].

Results

Binding to phospholipid bilayers

Fluorescence quenching methods can be used to study binding of hydrophobic molecules to phospholipid bilayers. As a probe for the bilayer we used pyrene dodecanoic acid (PDA), since this has been shown to bind strongly to phospholipid bilayers with the pyrene group located in the fatty acyl chain region of the bilayer [16]. As shown in Fig. 1, dibromoandrostanol is an efficient quencher of PDA fluorescence. The extent of quenching at any given concentration of dibromoandrostanol decreases with increasing phospholipid concentration, as expected for partitioning or binding to the phospholipid bilayer. The straight line plots shown in Fig. 1 show that, over this concentration range, there is no significant saturation of sites in the bilayer, so that a determination of the number of sites, N, per phospholipid molecules is not possible. However, other studies [17] have suggested a value of 3, and, as shown in Fig. 1A the data fits well with this number of sites with a value for K_d of 70 μM. As shown in Fig. 1B, the data also fits well to the partition model, and a plot of $1/K_{app}$ derived from the initial gradients of the lines shown in Fig. 1A against the volume fraction α_m gives a value for the Stern-Volmer constant of 6.17 M^{-1} and a partition coefficient of 35 000. Identical quenching plots are obtained in bilayers of (14 : 1)PC or dinervonylphosphatidylcholine ((24 : 1)PC), showing that phospholipid acyl chain length has no significant effect on partitioning (data not shown).

Preparation of quenching derivatives of sterols is difficult for sterols which do not contain double bonds across which bromine can be added. An alternative procedure was therefore adopted to study the binding of such sterols to phospholipid bilayers. If PDA is incorporated into bilayers containing mixtures of (18 : 1)PC and (Br18 : 0)PC, then the (Br18 : 0)PC will quench PDA fluorescence, the extent of quenching depending on the concentration of (Br18 : 0)PC in the bilayer. Addition of a hydrophobic molecule like a sterol to the bilayer will dilute the (Br18 : 0)PC in the bilayer, thus reducing fluorescence quenching. As shown in Fig. 2, the fluorescence of PDA in phospholipid bilayers is reduced by 40% by (Br18 : 0)PC at a phospholipid mol fraction of 0.33. Addition of a variety of sterols to the bilayer results in an increase in fluorescence intensity, demonstrating binding of the sterols to the bilayer. The effect of 5β-cholestan-3β-ol appears to saturate at about a molar ratio of sterol to phospholipid of 1 : 1, suggesting that, as for cholesterol, binding cannot exceed a 1 : 1 molar ratio. The other sterols can apparently bind to higher stoichiometries. From the areas occupied by the sterols in the phospholipid bilayer surface, it is possible to estimate binding constants for the sterols from these experiments [17] but such an analysis will not be attempted here.

Addition of androstenol to bilayers of (16 : 1)PC at 25 °C (when it will be in the liquid crystalline phase) results in an increase in the fluorescence polarisation of DPH in the bilayers, whereas addition of androstenol to

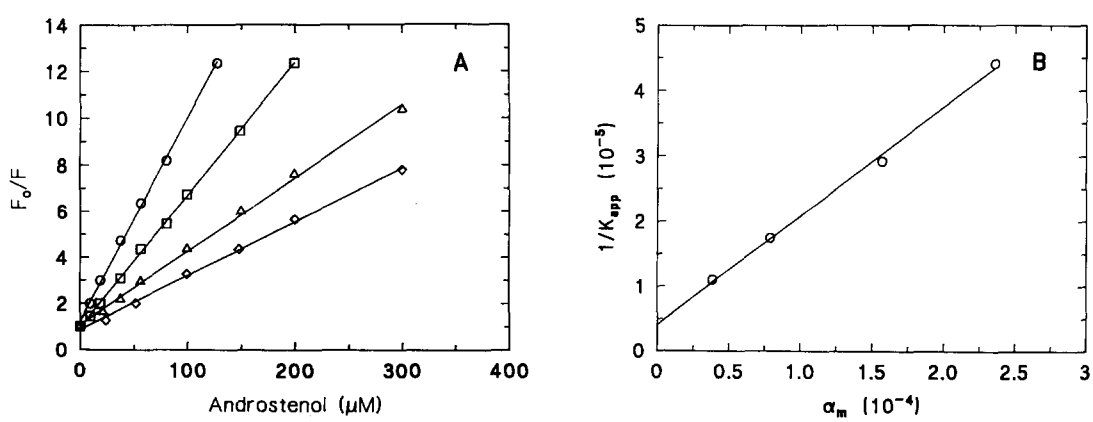

Fig. 1. (A). Fluorescence quenching (F_0/F) of PDA in liposomes of (18 : 1)PC at 25 °C on addition of dibromoandrostanol, at phospholipid concentrations of: 50 μM (○); 100 μM (□), 200 μM (△), 300μM (◇). The molar ratio of PDA to phospholipid was 1 : 500. The lines are simulations in terms of the binding model described in the text. (B) Plot of $1/K_{app}$ calculated from the initial slopes of the lines in Fig. 1A against the membrane volume fraction α_m.

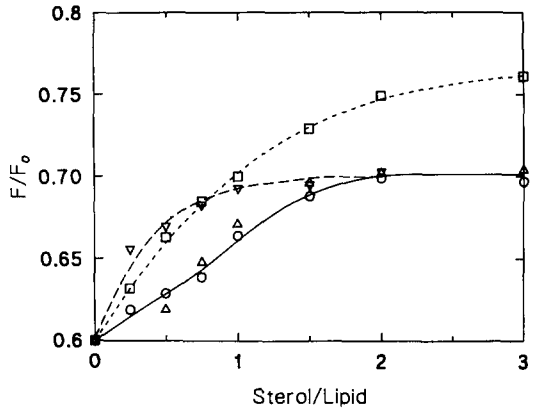

Fig. 2. Effects of addition of sterols on the fluorescence of PDA (0.4 μM) in liposomes containing (18:1)PC (133 μM) and (Br18:0)PC (67 μM) at 37°C. Fluorescence intensity is expressed as a fraction of that for the same system but in the presence of (18:1)PC (200 μM) as the only phospholipid. 5β-Cholestan-3β-ol (▽), 5α-cholestan-3β-ol (□), 5β-cholestan-3α-ol (○), 5α-cholestan-3α-ol (△).

bilayers of (16:0)PC at 25°C (when it will be in the gel phase) has no significant effect on fluorescence polarisation (data not shown).

Interaction with (Ca^{2+} + Mg^{2+})-ATPase

Interaction of cholesterol with the ATPase has been studied by measurement of the changes in tryptophan fluorescence of the ATPase reconstituted into bilayers of (18:1)PC or (Br18:0)PC on addition of cholesterol or dibromocholestanol [4]. Quenching of fluorescence by bromine occurs by a process referred to as heavy atom quenching and involves an intimate collision between the fluorophore and the quencher [26]. Since the exchange time for phospholipids between the bulk phase and annular sites on the ATPase is approx. 0.1 μs [4] and the hopping frequency of sterols in lipid bilayers is approx. $3 \cdot 10^6$ [27] compared to a tryptophan fluorescence lifetime of approx. 4 ns [4], quenching would be expected to be by a static mechanism: this has been confirmed for spin-labelled fatty acids binding to the ATPase [4]. Fig. 3 shows that, as reported previously, the fluorescence intensity for the ATPase in (Br18:0)PC is approx. 35% of that in (18:1)PC. Addition of dibromoandrostanol to the ATPase reconstituted with (18:1)PC results in marked fluorescence quenching, and addition of dibromoandrostanol to the ATPase reconstituted with (Br18:0)PC, results in further quenching; addition of androstenol to the ATPase reconstituted with (Br18:O)PC, however, has no significant effect on fluorescence intensity (Fig. 3A). Fluorescence quenching of the ATPase reconstituted in (Br14:0)PC is more marked than in (Br18:0)PC, although the pattern of effects observed on addition of dibromoandrostanol are the same (Fig. 3B). It has been observed in other systems that fluorescence quenching for the (Br18:0)PC system can be described by Eqn. 14 with values for A and B of 0.195 and 0.655, respectively

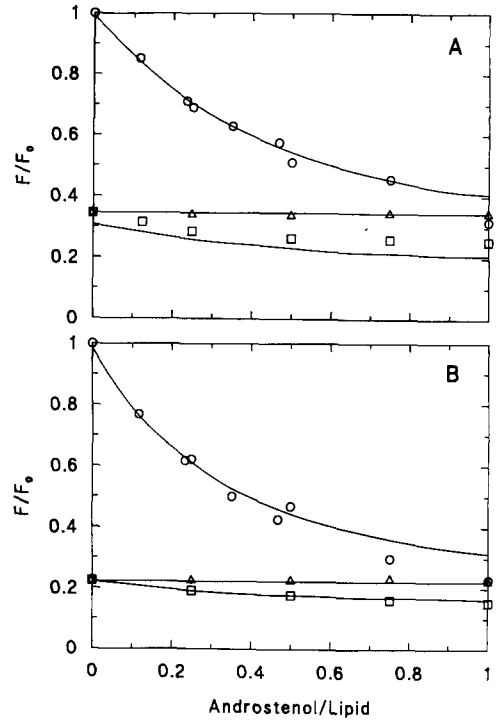

Fig. 3. Effects of androstenol at the given molar ratios of androstenol to phospholipid on the tryptophan fluorescence intensity of the (Ca^{2+} + Mg^{2+})-ATPase reconstituted with: (A) (18:1)PC and (Br18:0)PC and (B) (14:1)PC and (Br14:0)PC at 37°C. (18:1)PC or (14:1)PC plus dibromoandrostanol (○); (Br18:0)PC or (Br14:0)PC plus dibromoandrostanol (□); (Br18:0)PC or (Br14:0)PC plus androstenol (△). The lines are simulations with the binding parameters in Table I.

[4,9,10] and, as shown in Fig. 3A, quenching by dibromoandrostanol for the (18:1)PC-ATPase system can be fitted well with these values, with the dissociation constants for binding given in Table I. The greater extent of quenching seen with (Br14:0)PC (Fig. 3B) can be simulated assuming a change in the parameters A and B in Eqn. 14 to 0.075 and 0.775, respectively, to

TABLE I

Dissociation constants for binding of dibromoandrostanol to non-annular sites on the (Ca^{2+} + Mg^{2+})-ATPase

Constants were determined from fluorescence quenching plots as described in the text. Binding at annular sites was too weak to be detectable.

Phosphatidylcholine fatty acyl chain	Dissociation constant K_d	
	mole ratio units	μM [a]
12:0	0.33	23
14:1	0.33	23
16:1	0.40	28
18:1	0.50	35
20:1	0.50	35
24:1	0.50	35
16:0 at 17°C	0.17	12
16:0 at 45°C	0.35	24

[a] Calculated from K_d in mole ratio units and the binding constant for dibromoandrostanol to phospholipid.

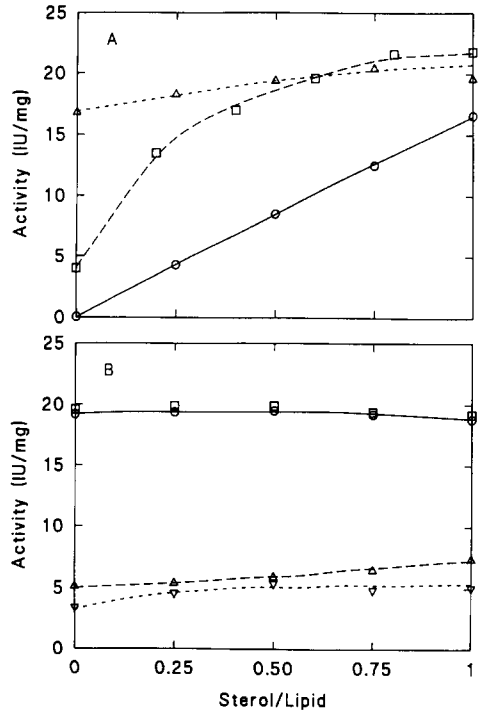

Fig. 4. Effects of dibromoandrostanol at the given molar ratio of dibromoandrostanol to phospholipid on the activity (IU/mg protein) of the $(Ca^{2+} + Mg^{2+})$-ATPase at 37 °C (pH 7.2), 2.1 mM ATP. (A) (12:0)PC (○), (14:1)PC (□), (16:1)PC (△). (B) (18:1)PC (○), (20:1)PC (□), (22:1)PC (△), (24:1)PC, (▽).

give the binding parameters given in Table I. As shown in Table I, for both the (18 : 1)PC-ATPase and (14 : 1)PC-ATPase systems, binding of dibromoandrostanol to annular sites is negligible. Under these conditions, Eqn. 14 can be simplified to:

$$F/F_0 = 0.15 + 0.85(1 - f_{na})^{1.6}$$

Binding constants of dibromoandrostanol can be obtained from fluorescence quenching profiles of dibromoandrostanol added to bilayers of non-brominated phospholipids. Table I shows binding parameters obtained in this way for the ATPase reconstituted with phosphatidylcholines of chain lengths between 12 and 24. Binding constants derived from similar quenching plots for the ATPase reconstituted with (16 : 0)PC at 17 and 45 °C (data not shown) are also given in Table I.

The effects of dibromoandrostanol on the activity of the reconstituted ATPase measured at 37 °C at optimal concentrations of Ca^{2+} and 2.1 mM ATP are shown in Fig. 4. As shown in Fig. 4A, addition of dibromoandrostanol to the ATPase reconstituted with the short-chain phosphatidylcholines (12 : 0)PC and (14 : 1)PC results in a large increase in activity, whereas addition of dibromoandrostanol to the ATPase reconstituted with (16 : 1)PC results in a small increase in activity and addition of dibromoandrostanol to the ATPase reconstituted with longer chain phosphatidylcholines had no significant effect on activity (Fig. 4B). As shown in Fig. 5, addition of androstenol to the ATPase reconstituted with (16 : 0)PC resulted in an increase in ATPase activity both at 45 °C, when the phospholipid will be in the liquid crystalline phase, and at 25 °C, when the phospholipid will be in the gel phase. Effects of androstenol on the activity of the ATPase reconstituted with (14 : 1)PC at 25 °C as a function of ATP concentration are shown in Fig. 6. Fig. 7 contrasts the effects of androstenol on the activity of the ATPase reconstituted with (14 : 1)PC with those of cholesterol, epicholesterol and the four isomers of cholestanol: all except 5α-cholestan-3α-ol produced a large increase in activity. Addition of pregnenolone or cortisone up to a 2 : 1 molar ratio with phospholipid had no effect on activity (data not shown).

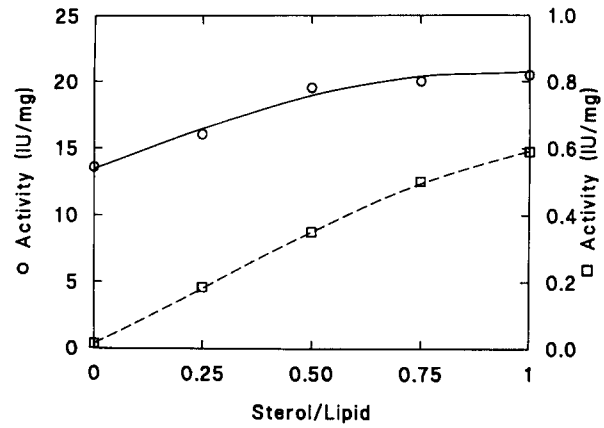

Fig. 5. Effects of androstenol at the given molar ratio of androstenol to phospholipid on the activity (IU/mg protein) of the $(Ca^{2+} + Mg^{2+})$-ATPase at pH 7.2, 2.1 mM ATP reconstituted with (16 : 0)PC at 25 °C (□) and 45 °C (○).

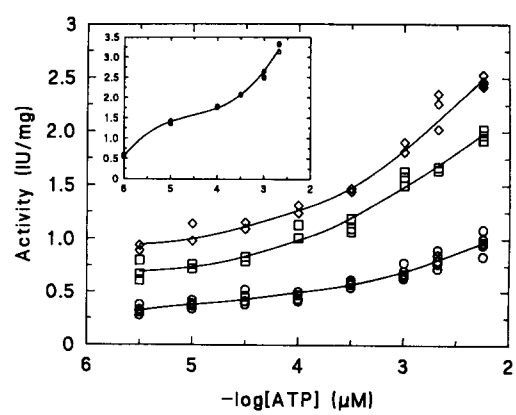

Fig. 6. Effects of androstenol on the activity (IU/mg protein) of the $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted with (14 : 1)PC, at pH 7.2, 25 °C and the given concentration of ATP at molar ratios of androstenol to phospholipid of: 0 : 1 (○), 0.45 : 1 (□) and 1.2 : 1 (◇). The inset shows the activity (IU/mg protein) of the native ATPase as a function of ATP concentration, expressed as −log[ATP] (μM).

The results presented in Table I suggest stronger binding of dibromoandrostanol at non-annular sites on the ATPase when the ATPase is reconstituted with short-chain phospholipids than when reconstituted with long-chain phospholipids. In previous studies we have suggested that pyrene-1-methanol binds to non-annular sites on the ATPase whereas PDA binds to both annular and non-annular sites [16]. The overlap between the emission spectrum of tryptophan and the absorption spectrum of pyrene means that pyrene fluorescence can be excited by Forster energy transfer from tryptophan residues on the ATPase when fluorescence is excited at 285 nm; the efficiency of transfer from tryptophan to pyrene is distance dependent, so that fluorescence will only be observed from molecules bound to the membrane, with a greater transfer efficiency for those molecules bound directly to the ATPase than for those bound to the phospholipid bilayer component of the membrane. Fig. 8A shows pyrene fluorescence intensity for pyrene-1-methanol added to the reconstituted ATPase as a function of protein concentration. Fluorescence intensity excited through the tryptophans increases with increasing protein concentration, and is higher for (14 : 1)PC-ATPase than for (18 : 1)PC-ATPase, suggesting stronger binding to non-annular sites for (14 : 1)PC-ATPase than for (18 : 1)PC-ATPase.

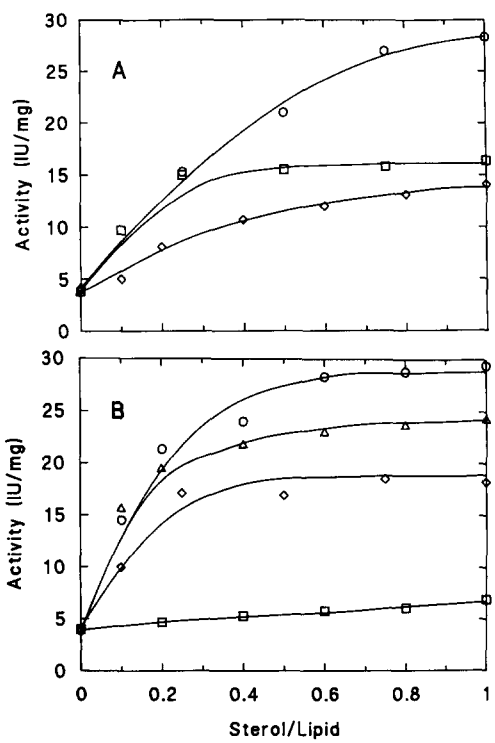

Fig. 7. Effects of sterols at the given molar ratios of sterol to phospholipid on the activity (IU/mg protein) of the $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted with (14:1)PC, at pH 7.2, 2.1 mM ATP, 37 °C. (A) Androstenol (○), cholesterol (□); epicholesterol (◇). (B) 5β-Cholestan-3β-ol (○), 5β-cholestan-3α-ol (△), 5α-cholestan-3β-ol (◇), 5α-cholestan-3α-ol (□).

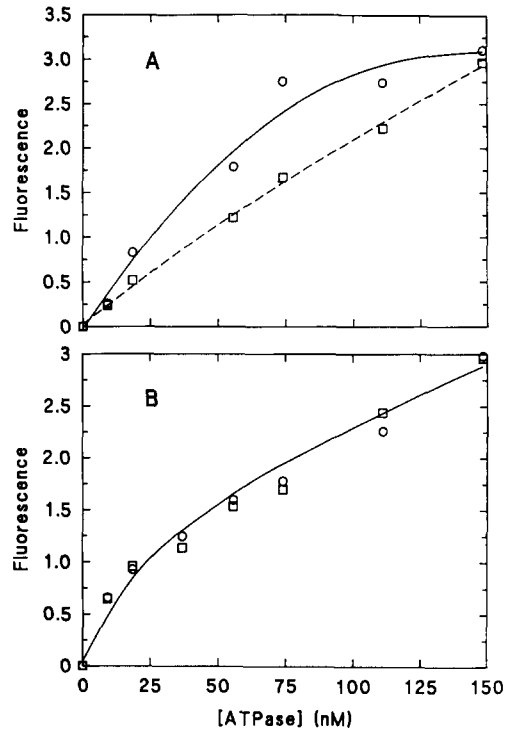

Fig. 8. Fluorescence intensity for (A) pyrene-1-methanol and (B) PDA excited by energy transfer via the $(Ca^{2+} + Mg^{2+})$-ATPase, for concentrations of 60 nM pyrene derivative and the given protein concentration, for the ATPase reconstituted with (14:1)PC (○) or (18:1)PC (□), at a molar ratio of phospholipid/ATPase of 900:1, at 37 °C.

In contrast, Fig. 8B shows that the fluorescence of PDA as a function of ATPase concentration is the same for (14 : 1)PC-ATPase and (18 : 1)PC-ATPase, suggesting that binding at annular sites is little dependent on phospholipid structure.

Discussion

High levels of cholesterol are found in cell outer membranes, where typically the molar ratio of cholesterol to phospholipid is close to 1 : 1, the maximum amount of cholesterol that can be incorporated into a phospholipid bilayer before separation of a pure cholesterol phase [18]. Activities of a number of proteins found in outer cell membranes have been reported to be modified by the presence of cholesterol. Perhaps the clearest example is that of the acetylcholine receptor for which a variety of functions are thought to require the presence of cholesterol [11]. The function of the hexose transporter from human erythrocyte membranes has also been reported to be very sensitive to levels of cholesterol in the membrane [12]. It has also been reported that high levels of cholesterol inhibit the activity of the $(Na^+ + K^+)$-ATPase of human erythrocyte membranes, although the effects are rather small [19].

The cholesterol content of inner cell membranes are generally very low. Studies of the effects of cholesterol and other sterols on the function of proteins from such inner cell membranes will therefore serve to define the general features that can be expected for sterol–membrane protein interactions and can thus help to define effects (if any) that must arise from specific interactions with particular proteins in outer cell membranes where cholesterol does occur. It has been shown that cholesterol is essentially absent (< 10%) from sarcoplasmic reticulum [20,21]. Previous studies using fluorescence quenching techniques have shown that cholesterol is excluded from the lipid-protein interface of the $(Ca^{2+} + Mg^{2+})$-ATPase (annular sites) but can bind at other sites (non-annular sites) on the ATPase either at protein/protein interfaces in dimers or higher aggregates of the ATPase or at sites within the ATPase, possibly between α-helices [4,9]. Studies using spin-labelled sterols have been interpreted in terms of relatively strong binding at lipid/protein interfaces [22], but we have shown that these studies are also consistent with exclusion of sterols from the lipid/protein interface with binding at non-annular sites [10,23]. In this paper we explore effects of sterol structure on the interaction with the $(Ca^{2+} + Mg^{2+})$-ATPase and effects of the structure of the membrane phospholipids on the sterol–ATPase interaction.

Based on the results obtained previously with cholesterol [4] we would expect sterols to bind to both the phospholipid and protein components of the membrane. Characterisation of sterol binding to the ATPase therefore requires prior determination of binding to phospholipid, since binding to phospholipid will be in competition with binding to the ATPase. Binding to phospholipid bilayers of hydrophobic molecules that are quenchers of fluorescence can be quantitated from an analysis of their quenching of the fluorescence of a suitable probe incorporated into the membrane. We have shown that pyrenedodecanoic acid (PDA) is suitable for such studies [16]. Bromination of 5-androsten-3β-ol gives 5α,6β-dibromoandrostan-3β-ol (dibromoandrostanol) whose planar *trans*-diaxial structure will be comparable to that of the parent molecule. As shown in Fig. 1, binding of dibromoandrostanol to bilayers of (18 : 1)PC can be quantitated in this way, either in terms of a partition model with a partition coefficient K_p of 35 000 or in terms of a binding model with three binding sites per phospholipid molecule and a K_d of 70 μM; the binding model is, however, insensitive to the value of N, the number of binding sites per phospholipid molecule, over the experimental concentration range as long as the ratio K_d/N is kept constant. Binding is identical in phosphatidylcholines with chain lengths C_{14}–C_{20} (data not shown). The relatively high K_d value implies that, under the conditions used in our experiments, not all the dibromoandrostanol will be bound to the membrane. This is consistent with the data of Demel et al. [24] who showed that under conditions where cholesterol was bound to phospholipid bilayers to give a 1 : 1 molar ratio of bound sterol to phospholipid, androstanol was bound only to a molar ratio of 0.7 : 1.

Binding of non-quenching molecules to phospholipid bilayers can also be quantitated by observation of the reduction in quenching of PDA fluorescence in mixed bilayers of (Br18 : 0)PC and (18 : 1)PC that follows from binding. As shown in Fig. 2, binding of 5β-cholestan-3β-ol appears to saturate at a molar ratio of sterol to phospholipid close to 1 : 1 as was observed for cholesterol (data not shown), whereas the other sterols can apparently bind to higher stoichiometries. If it is assumed that all the sterols occupy similar areas in the bilayer surface, then the greater effect of 5β-cholestan-3β-ol and 5α-cholestan-3β-ol suggests stronger binding of these isomers than of the corresponding cholestan-3α-ol isomers, consistent with the results of Demel et al. [24]. NMR studies of the binding of epicholesterol to phospholipid bilayers suggested that sterols with 3α-hydroxyl groups bind with rather similar orientations of the sterol ring to that adopted by cholesterol, but with a less polar environment for the -OH group [6]. The data reported here suggests that, as expected, the 3β orientation of the -OH group is the more favourable for interaction with phospholipid bilayers. The conformation at the 5 position has relatively little effect on binding to phospholipid bilayers. The importance of an alkyl chain at the C-17 position, as in cholesterol, is shown by the stronger binding of cholesterol than androstenol.

Fluorescence quenching techniques can also be used to study binding of sterols to the ATPase. Androstenol and dibromoandrostanol have been shown to have identical effects on the activity of the ATPase (data not shown), implying very similar interactions with the ATPase, as expected from the similarity of their structures. The fluorescence quenching data shown in Fig. 3 shows that the pattern of binding of androstenol to the ATPase is comparable to that observed for cholesterol [4], with no significant binding at the phospholipid/protein interface (annular sites) but with binding at other, non-annular sites. The dissociation constant for binding at non-annular sites for dibromoandrostanol (0.5 in molar ratio units) is less than that for dibromocholestanol (1.2; ref. 10), indicating stronger binding to the ATPase when binding is expressed in terms of concentration in the bilayer; the stronger binding of dibromocholestanol to the phospholipid bilayer, however, means that binding of dibromocholestanol to the ATPase will be stronger than binding of dibromoandrostanol when expressed in terms of aqueous concentrations.

The binding constant for dibromoandrostanol at the non-annular sites depends on the structure of the phos-

pholipids present in the system. As shown in Table I, the strength of binding of dibromoandrostanol decreases slightly with increasing chain length from C_{12} to C_{24}. As shown in Table I, the physical phase of the phospholipid also affects binding, with stronger binding for phospholipids in the gel phase than for phospholipids in the liquid crystalline phase. Since, as described above, binding of dibromoandrostanol to phospholipid bilayers is independent of phospholipid chain length, the chain length effect on the interaction with the ATPase must reflect a change in the nature of the binding sites on the ATPase. An effect of phospholipid chain length on the affinity of the non-annular sites for hydrophobic molecules is also suggested by the experiments with pyrene derivative sshown in Fig. 8. In previous studies we have suggested that pyrene-1-methanol, like sterols, is largely excluded from annular sites on the ATPase but can bind to non-annular sites [16]. In contrast, PDA can bind extensively to annular sites, as well as to non-annular sites [16]. As shown in Fig. 8, the fluorescence intensity observed for pyrene-1-methanol excited by Forster energy transfer from the tryptophan residues of the ATPase is higher for the ATPase reconstituted with (14 : 1)PC than for that reconstituted with (18 : 1)PC, consistent with stronger binding in the former case. For PDA, there is no change in fluorescence intensity between the (14 : 1)PC and (18 : 1)PC-containing systems, suggesting that binding at annular sites is independent of phospholipid chain length, consistent with our previous observation that binding constants for phospholipids at the phospholipid-protein interface are almost independent of chain length [13].

There has been considerable debate in the literature as to the possible effects of membrane fluidity on the function of membrane proteins (see Refs. 23 and 25). As described in Ref. 25, decreasing membrane fluidity (increasing membrane viscosity) could slow the rate for any particular step in the reaction sequence of a membrane protein by increasing the height of the corresponding energy barrier. Such a decrease in rate would normally be expected to result in a decrease in the overall reaction rate, although it is possible to picture situations where slowing the rate of some particular step (e.g., that leading to a dead-end complex) would lead to an increase in overall reaction rate. Measurements of the fluorescence polarization of DPH show that addition of androstenol to a phospholipid bilayer in the liquid crystalline state leads to a decrease in fluidity, comparable to that seen on addition of cholesterol (data not shown). Several observations, however, suggest that the changes in membrane fluidity are not an important factor in determining effects of sterols on ATPase activity.

The effect of androstenol on the activity of the ATPase depends markedly on the structure of the phospholipid present in the system (Fig. 4). For the ATPase reconstituted with phosphatidylcholines with chain lengths less than C_{18}, addition of dibromoandrostanol increased activity, the magnitude of the effect decreasing with increasing chain length from C_{12} to C_{16}. For chain lengths of C_{18} or longer, addition of dibromoandrostanol has no effect on activity. As shown in Fig. 5, an effect of androstenol on the activity of the ATPase reconstituted with (16 : 0)PC is observed both in the liquid crystalline and gel phases. As shown in Fig. 6, the effect of androstenol on the activity of the ATPase reconstituted with (14 : 1)PC is observed over a wide range of ATP concentrations.

Steroid structure is important in determining effects on ATPase activity, as shown in Fig. 7. Androstenol has a greater effect on the activity of the ATPase reconstituted with (14 : 1)PC than does cholesterol and both have a greater effect than the 3α-isomer, epicholesterol. Also as shown in Fig. 7, the four isomers of cholestanol have different effects on activity, with 5α-androstan-3α-ol having little effect, but marked stimulations of activity being observed for the other isomers. The lack of effect of 5α-androstan-3α-ol and the smaller effect of 5α-androstan-3β-ol than of the 5β-isomers suggest that the conformation at the 5 position (which determines the planarity (5α) or lack of planarity (5β) of the ring system) is much more important in determining interactions with the ATPase than it is for determining binding to phospholipid bilayers. This would be the expected result if the sterols were binding at clefts either within the ATPase molecule itself or at protein/protein interfaces in dimers or higher aggregates of the ATPase.

It is shown elsewhere (Michelangeli, Champeil, East and Lee, unpublished observations) that androstenol has complex effects on the activity of the ATPase reconstituted with (14 : 1)PC. On reconstitution with (14 : 1)PC, the stoichiometry of Ca^{2+} binding changes from the usual two Ca^{2+} per ATPase molecule to one Ca^{2+} per ATPase molecule and there is a reduction in the equilibrium constant for phosphorylation from P_i. All these effects are reversed on addition of androstenol. The observed changes in equilibrium properties of the ATPase show that effects cannot follow from changes in membrane fluidity, which, as a dynamic property, can only change the dynamics of the system, not equilibrium properties [25].

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