Biochimica et Biophysica Acta, 1028 (1990) 58–66
Elsevier

BBAMEM 74989

Fluorescence and kinetic studies of the interactions of pyrethroids with the $(Ca^{2+} + Mg^{2+})$-ATPase

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(Received 23 March 1990)

Key words: Kinetics; Pyrethroid; Fluorescence; ATPase, $(Ca^{2+} + Mg^{2+})$-

The fluorescence quenching properties of a series of brominated and iodinated pyrethroids have been used to study the binding of pyrethroids to the $(Ca^{2+} + Mg^{2+})$-ATPase purified from skeletal muscle sarcoplasmic reticulum. It is suggested that binding at the lipid/protein interface of the ATPase is weak but that binding can occur at other (non-annular sites) on the ATPase. Pyrethroids containing either a brominated fatty acyl or iodinated alcohol moiety quench the tryptophan fluorescence of the ATPase, suggesting that the pyrethroids bound to the ATPase adopt a folded conformation with both the acid and alcohol moieties in contact with hydrophobic regions of the ATPase. Whereas effects of the pyrethroids on the activity of the ATPase in bilayers of dioleoylphosphatidylcholine are small, large increases are observed in the activity of the ATPase reconstituted into bilayers of the short-chain phospholipid, dimyristoleoylphosphatidylcholine (DMPC). The rate of phosphorylation of DMPC-ATPase by ATP is slow, but is increased on addition of pyrethroid. The level of phosphorylation of the ATPase by P_i is reduced on reconstitution into bilayers of DMPC, and this is also increased by addition of pyrethroid.

Introduction

The pyrethroids are a class of hydrophobic esters whose principal mode of action is believed to be on Na-channels in insect nerve membrane [1]. Very high levels of non-specific binding of the pyrethroids to biological membranes have made it difficult to identify directly the proposed binding sites on the Na-channels [2]. Indirect approaches have, however, identified sites on the Na-channel to which the pyrethroids can bind with an affinity of approx. 1–10 μM [2,3]. Effects of pyrethroids are stereospecific, despite the fact that DDT can apparently bind to the same site on the Na-channel as the pyrethroids [2,4]. The extensive non-specific binding of the pyrethroids to biological membranes means that they are likely to affect the activities of membrane proteins other than the Na-channel. Thus both acetylcholine [5,25] and GABA receptors [6] have been shown to be inhibited by pyrethroids. Effects of pyrethroids and other insecticides on the function of ion-transport ATPases have been reported which could be responsible for some of the effects observed on insect physiology [27]. In particular, Clark and Matsumura [7,8] have demonstrated that pyrethroids inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase from synaptosomal preparations of squid optic nerve and of microsomal preparations of cockroach brain. Studies of the effects of pyrethroids on the $(Ca^{2+} + Mg^{2+})$-ATPase purified from skeletal muscle sarcoplasmic reticulum have shown that whereas high concentrations of pyrethroids inhibit the ATPase, low concentrations can increase the activity of the ATPase, the effect depending on the structure of the phospholipids present in the system [9].

The activity of the $(Ca^{2+} + Mg^{2+})$-ATPase has been shown to be very sensitive to the chemical structures of the molecules surrounding it in the membrane. Thus it has been shown that activity varies markedly with changes in the structure of the phospholipids in the membrane, with dioleoylphosphatidylcholine (DOPC) supporting highest activity with phospholipids with other fatty acyl chain lengths or head groups supporting lower activities [11]. Effects on ATPase activity of a wide variety of hydrophobic molecules that can parti-

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Abbreviations: BrPC, bis(9,10)-dibromostearoyl)phosphatidylcholine; DMPC, dimyristoleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PDA, pyrene-1-dodecanoic acid.

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tion into the membrane have been shown to be dependent both on the structure of the additive and on the structures of the phospholipids present in the system. It has been suggested that charged molecules (long chain amines and acids) can bind extensively at the lipid/protein interface of the ATPase, such binding resulting in inhibition of ATPase activity [28]. Uncharged molecules (long chain alcohols and esters, sterols) appear to bind weakly at the lipid/protein interface but can bind to other sites on the ATPase, such binding having relatively little effect on the activity of the ATPase reconstituted into bilayers of DOPC but resulting in a large increase in activity when the ATPase is reconstituted with the short-chain phospholipid dimyristoleoylphosphatidylcholine (DMPC) [9,15–17,24,28].

In the previous paper [10], a number of brominated and iodinated pyrethroids were introduced as fluorescence quenchers, and fluorescence quenching was used to study interactions of the pyrethroids with phospholipid bilayers. Here, we study the effects of a variety of pyrethroids of related structure on the activity of the ATPase and use fluorescence quenching to study binding of the pyrethroids to the $(Ca^{2+}+Mg^{2+})$-ATPase.

Materials and Methods

Phospholipids were obtained from Lipid Products or Avanti Polar Lipids and pyrene-1-dodecanoic acid (PDA) from Calbiochem. 1,2-bis(9,10-Dibromostearoyl)phosphatidylcholine (BrPC) was prepared as described in Ref. 11. The pyrethroids used in these studies (Fig. 1) were synthesised as described in Ref. 10.

$(Ca^{2+}+Mg^{2+})$-ATPase was purified from sarcoplasmic reticulum of skeletal muscle as described in East and Lee [11]. Reconstitutions for fluorescence measurements and for steady-state measurements of ATPase activity were also carried out as described in East and Lee [11]. Phospholipid (1 μmole) 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. 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. For pre-steady state kinetic methods, the reconstitution procedure was modified for a larger scale. Phospholipid (10 μmol) was mixed with buffer (400 μl; 10 mM Hepes-Tris, 15% sucrose, pH 8.0) containing $MgSO_4$ (5 mM) and potassium cholate (13 mg/ml) and sonicated to clarity. ATPase (1.25 mg) in a volume of 60 μl was added and left for 45 min at 5°C to equilibrate. The mixture was then added to 10 ml of cold buffer (10 mM Hepes-Tris, pH 8.0) containing 2 mM dithiothreitol and spun at 200 000 $\times g$ for 1 h. The pellet was rehomogenised in 200–300 μl of 10 mM Hepes-Tris, 15% sucrose, (pH 7.0) to give a final protein concentration of 3–8 mg/ml. In experiments with the pyrethroids, the pyrethroids were added to the phospholipid, prior to solubilization in the original cholate solution.

Steady-state measurements of phosphorylation by $[^{32}P]P_i$ were carried out in 150 mM Mes-Tris (pH 6.2) containing 5 mM EGTA and 10 mM Mg^{2+}, and the required concentration of P_i at 25°C, at a protein concentration of 0.33 mg/ml. After 20s the reaction was quenched by addition of an equal volume of quenching solution (15% trichloroacetic acid, 0.2 M potassium phosphate). The precipitate was collected by

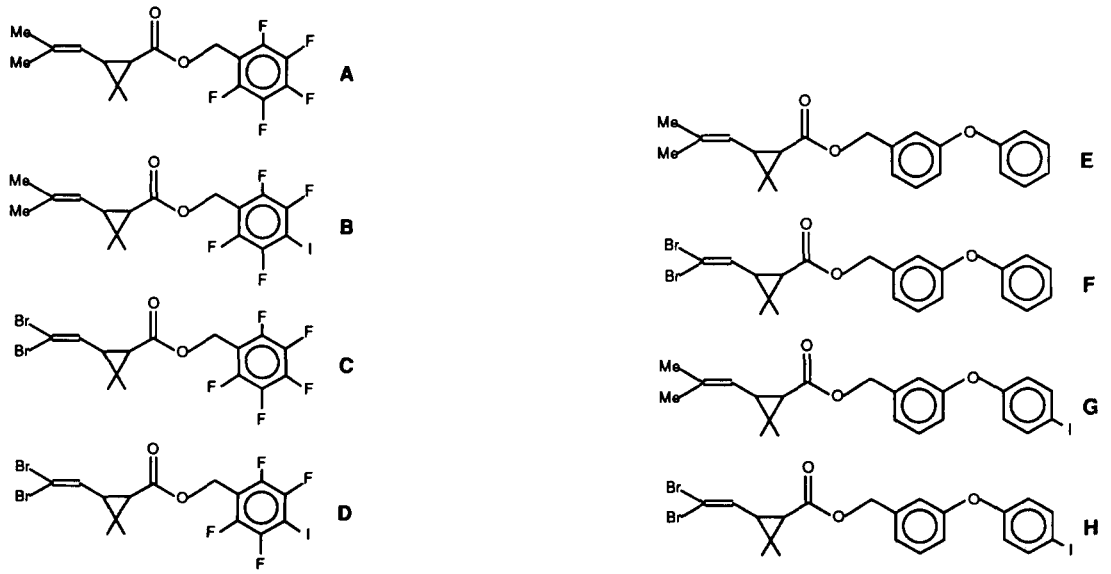

Fig. 1. The structures of the pyrethroids studied: A, *cis*:*trans* 3:7 (*R*,*S*); B, *cis*:*trans* 3:7 (*R*,*S*); C, *cis* (*R*,*S*); D(i), *trans* (*R*,*S*); D(ii), *cis* (*R*,*S*); E, *cis*:*trans* 3:7 (*R*,*S*); F(i), *cis* (*R*,*S*); F(ii), *trans* (*R*,*S*); F(iii), *cis* (1*R*); G, *cis*:*trans* 3:7 (*R*,*S*); H(i), *cis* (*RS*); H(ii), *trans* (*R*,*S*); H(iii), *cis* (1*R*).

filtration through Whatman GF/C glass fibre filters, washed with ice-cold quenching solution and finally counted in Labscint. Controls were performed by first denaturing the ATPase with quench solution followed by addition of [^{32}P]P_i.

Steady-state measurements of phosphorylation by [γ-^{32}P]ATP were performed in a medium containing 40 mM Hepes-Tris, 100 mM KCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$ (pH 7.2) at 25°C. The reaction was started by addition of the required concentration of ATP to the ATPase (0.1 mg/ml), followed by quenching after 10 s with ice-cold 20% trichloroacetic acid, 0.2 M phosphoric acid.

Steady-state measurements of Ca^{2+} release from the ATPase following the addition of ATP were made using Antipyralazo III [12]. Release of Ca^{2+} from the ATPase was followed by measuring the change in dye absorbance at 720–790 nm, using a Shimadzu UV3000 dual-wavelength spectrometer, at 25°C. ATPase (0.4 mg/ml) was added to buffer (150 mM Mes-Tris, pH 6.0, 20 mM $MgSO_4$) containing 100 μM Antipyralazo III. Aliquots of a concentrated solution of $CaCl_2$ were added to a final concentration of 50 μM to calibrate the signal. ATP was added to a final concentration of 40 μM and corrections for dilution were made by measuring the response to the addition of an equal volume of buffer.

ATPase activity was measured using the coupled enzyme assay described in Ref. 11. Samples (12 μl, equivalent to 6 μg of ATPase) were added to a medium 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, the required concentration of ATP, 7.5 IU pyruvate kinase and 18 IU lactate dehydrogenase in a total volume of 2.5 ml, with $CaCl_2$ added to give maximal ATPase activity.

The time-dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP was measured at 25°C using a Hi-Tech PQ-43 preparative quench flow system. ATPase (0.2 mg/ml) in buffer (20 mM Hepes-Tris, 100 mM KCl, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.2) was mixed with an equal volume of [γ-^{32}P]ATP in buffer, followed by quenching with 2 ml of 1 M perchloric acid. The quenched samples were mixed with 1 ml of 40% trichloroacetic acid, 0.2 M phosphoric acid and the precipitate was collected by filtration through Whatman GF/C glass fibre filters.

Fluorescence measurements were made using a Spex Fluorolog fluorimeter. The tryptophan fluorescence of the ATPase was excited at 280 nm and recorded at 340 nm, for an ATPase concentration of 1 μM, in 40 mM Hepes-KOH, 100 mM NaCl, 1 mM EGTA (pH 7.2), 25°C.

Concentrations of protein were estimated by using the extinction coefficient (1.2 litre $\cdot$ g^{-1} $\cdot$ cm^{-1} for a solution in 1% SDS) given by Hardwicke and Green [13]. Kinetic simulations were carried out using the FACSIMILE programme [14] running on an IBM 3090 computer.

Analysis of quenching data

It has been shown that the tryptophan fluorescence of the ATPase is quenched if the ATPase is reconstituted into bilayers of BrPC or on addition of brominated hydrophobic molecules to the ATPase which can bind either at the lipid/protein interface (annular sites) or at other sites (non-annular sites) on the ATPase, possibly at protein-protein interfaces either between α-helices within the ATPase or between ATPase molecules in dimeric or higher aggregate structures [15–17]. It has been shown that in such cases quenching can be described by the equation:

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

where f_a and f_{na} are the fractions 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 bilayer [17]. The level of fluorescence quenching observed on reconstitution of the ATPase into bilayers of pure BrPC is given by $(0.15 + A)$, and is typically 0.4 [11,17]. Some variation is observed between experiments, probably reflecting some variation in the degree of bromination of DOPC during the preparation of BrPC, and thus A (and, consequently B) in Eqn. 1 was treated as a variable to match the observed level of quenching in BrPC. For the experiments reported here, concentrations of pyrethroid and phospholipid were high with respect to the concentration of ATPase so that binding to annular and non-annular sites could be described by the equation:

$$f = x_L/(K_d + x_L) \quad (2)$$

where K_d is the dissociation constant for binding to the ATPase (in mole ratio units) and x_L is the mole ratio of quencher to phospholipid in the membrane. Binding constants for pyrethroids to phospholipid bilayers were taken from Ref. 10. Concentrations of pyrethroid 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 [17].

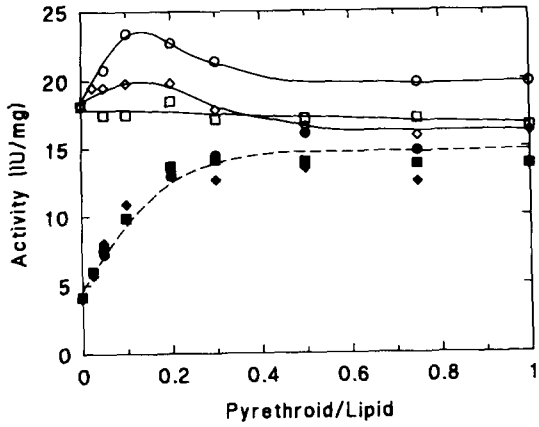

Fig. 2. The effects of pyrethroids on the activity (IU/mg protein) at 37°C and 2.1 mM ATP of DOPC-ATPase (open symbols) and DMPC-ATPase (filled symbols) at the given molar ratios of pyrethroid to phospholipid. Pyrethroids: F(i) (○); E (◇); H(i) (□); F(i) (●); F(ii) (◆); F(iii) (□).

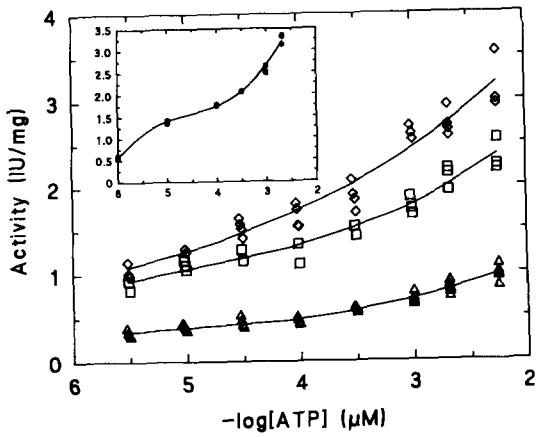

Fig. 4. Effects of pyrethroid D(ii) on the activity (IU/mg protein) of DMPC-ATPase at 25°C and the given concentration of ATP at molar ratios of pyrethroid to DMPC of: 0:1 (△); 0.1:1 (□), 0.3:1 (◇). The insert shows the activity (IU/mg protein) of the native ATPase as a function of ATP concentration, expressed as −log[ATP] (μM).

Results

Effects on the activity of the (Ca^{2+} + Mg^{2+})ATPase

The effects of pyrethroids on the ATPase activity of the reconstituted ATPase measured at 37°C at optimal concentrations of Ca^{2+} and 2.1 mM ATP are shown in Fig. 2. Pyrethroids E and F(i) containing the 3-phenoxybenzyl moiety cause a slight increase in activity for DOPC-ATPase at low concentrations, with inhibition at higher concentrations, but pyrethroid H(i) in which the 3-phenoxybenzyl moiety is iodinated had no effect on the activity of DOPC-ATPase (Fig. 2). In contrast, as shown in Figs. 2 and 3, all the pyrethroids cause a considerable stimulation of activity for DMPC-ATPase. As shown in Fig. 3, the effect of the iodinated pyrethroid H(i) on the activity of DMPC-ATPase is less than that of the other pyrethroids. No significant differences were observed between the isomeric forms of pyrethroid H (Fig. 3) or pyrethroid F (Fig. 2). Effects of pyrethroids containing the pentafluorobenzyl moiety (pyrethroid A, C, D) were rather similar to those containing the 3-phenoxybenzyl moiety, the greatest level of stimulation being seen for pyrethroid A. The effect of pyrethroid D(ii) on the activity of DMPC-ATPase at 25°C as a function of ATP concentration is shown in Fig. 4.

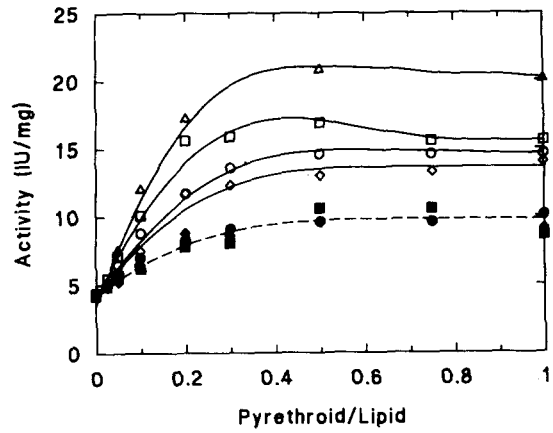

Fig. 3. The effects of pyrethroids on the activity (IU/mg protein) of DMPC-ATPase at 37°C and 2.1 mM ATP at the given molar ratios of pyrethroid to phospholipid. Pyrethroids: A (△); D(i) (□); D(ii) (○); C (◇); H(i) (●); H(ii) (◆); H(iii) (■).

Further insight into the mechanism of the effects of pyrethroid on the activity of the ATPase comes from pre-steady state measurements. In any analysis of such kinetic data, an important parameter is the proportion of the ATPase in the preparation in active form. This has been equated to the maximum possible level of phosphorylation observed with [γ-^{32}P]ATP under conditions (high Ca^{2+}) where the rate of dephosphorylation is low [21]. For preparations of the ATPase which appear > 95% pure on the basis of sodium dodecylsulphate-polyacrylamide gel electrophoresis, maximal observed levels of phosphorylation are typically between 2.3 and 5 nmol/mg protein, corresponding to preparations containing a fraction of active ATPase between 0.26 and 0.58, respectively [20]. Table I shows levels of maximal phosphorylation for the reconstituted ATPase after incubation for 10 s with the given concentrations of [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}. The maximal observed level of phosphorylation corresponds to a fraction of active ATPase of 0.5, and is unaltered by reconstitution with either DMPC alone or DMPC in the presence of pyrethroid D(ii). Lower levels of phosphorylation are observed at low concentrations of ATP, both as a result of significant hydrolysis of the ATP during the 10 s incubation period and as a result of changes in the balance between phosphorylated and non-phosphorylated forms of the ATPase with changing ATP concentration (Michelangeli, East and Lee, unpublished data (1990)). Levels of phosphorylation seen for

TABLE I

Phosphorylation of the (Ca^{2+} + Mg^{2+})ATPase by [γ^{32}P]ATP

The ATPase (0.1 mg/ml) was incubated with the given concentration of [γ-^{32}P]ATP for 10 s in the medium given in Materials and Methods containing 1 mM Ca^{2+} at pH 7.2, 25°C before quenching the reaction.

Concn. of ATP (μM)	Level of phosphorylation (nmol/mg protein)		
	native ATPase [a]	DMPC-ATPase [a]	DMPC-ATPase + pyrethroid D(ii) [b]
100	4.4	4.4	4.4
20	3.5	3.5	3.8
5	2.3	3.3	2.0
2.5	1.1	2.7	1.1
1	0.5	1.7	0.5

[a] Michelangeli, East and Lee, unpublished data (1990).
[b] Molar ratio pyrethroid/DMPC 0.3:1.

DMPC-ATPase plus pyrethroid D(ii) at low concentrations of ATP are more similar to those observed for native ATPase than for DMPC-ATPase in the absence of pyrethroid (Table I).

We have shown that phosphorylation of the ATPase on addition of [γ-^{32}P]ATP is considerably slower for DMPC-ATPase than for DOPC-ATPase or native ATPase, and that addition of androstenol, which increases the ATPase activity of DMPC-ATPase, increases the rate of phosphorylation of the ATPase to a value comparable to that seen for the native ATPase (Michelangeli, East and Lee, unpublished data (1990)). Fig. 5 illustrates the effect of pyrethroid D(ii) on the rate of phosphoenzyme formation for DMPC-ATPase on addition of 5 μM [γ-^{32}P]ATP, for a preparation for which the maximal observed level of phosphorylation

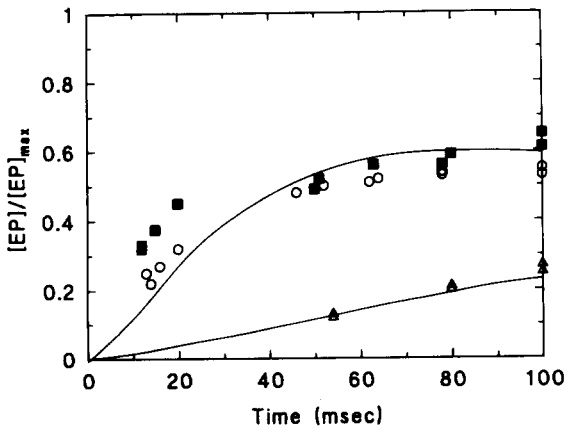

Fig. 5. Rate of phosphorylation of native ATPase (○), DMPC-ATPase (△) and DMPC-ATPase plus pyrethroid D(ii) at a molar ratio of pyrethroid to DMPC of 0.3:1 (■), on addition of 5 μM [γ^{32}P]ATP at pH 7.2, Mg^{2+} = 5 mM, K^+ = 100 mM, Ca^{2+} = 0.1 mM, 25°C. The final protein concentration was 0.1 mg/ml and EP_{max} was put equal to 2.8 nmol/mg protein (corresponding to an ATPase purity of 0.33). Curves are simulations (see text).

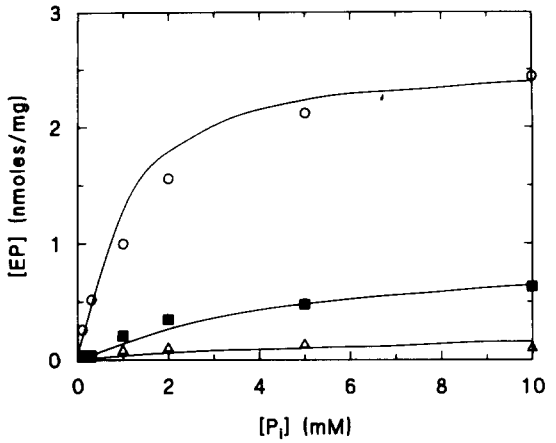

Fig. 6. Equilibrium level of phosphorylation of the ATPase (nmol/mg protein) by P_i at pH 6.2, Mg^{2+} = 10 mM, 25°C in the absence of K^+ and Ca^{2+}; DOPC-ATPase, (○); DMPC-ATPase, (△); DMPC-ATPase plus pyrethroid D(ii) at a molar ratio of pyrethroid to DMPC of 0.3:1 (■). The solid lines represent simulations with the parameters given in Froud and Lee [19] for DOPC-ATPase and with the equilibrium constant for phosphorylation reduced by a factor of 30 (for DMPC-ATPase) and 15 (for DMPC-ATPase plus pyrethroid).

was 2.8 nmol/mg protein, corresponding to a fraction of active ATPase of 0.33. It is clear that the rate of phosphoenzyme formation for DMPC-ATPase is increased considerably on addition of pyrethroid, and becomes equal to that observed for the native ATPase.

The ATPase can be phosphorylated by P_i in the presence of Mg^{2+} and absence of external Ca^{2+} [21]. Fig. 6 illustrates that, as shown previously (Michelangeli, East and Lee, unpublished data (1990)), the equilibrium level of phosphorylation observed for DMPC-ATPase is considerably less than for DOPC-ATPase, and Fig. 6 also shows that addition of pyrethroid D(ii) at a molar ratio of pyrethroid : DMPC of 0.3 : 1 results in a significant increase in the level of phosphorylation, although not to that observed for DOPC-ATPase.

Information about the steady-state distribution between the various phosphorylated and non-phosphorylated forms of the ATPase can be obtained from measurements of the release of Ca^{2+} from the ATPase when ATP is added to the ATPase previously equilibrated with Ca^{2+} [12]. As shown in Table II, on addition of ATP to the native ATPase incubated with 50 μM Ca^{2+}, an amount of Ca^{2+} is released into the medium, corresponding to 5.5 nmol Ca^{2+} per mg protein. For DMPC-ATPase, Ca^{2+} release on addition of ATP is immeasurable. For DMPC-ATPase plus pyrethroid D(ii) at a molar ratio of pyrethroid : DMPC of 0.15 : 1, a Ca^{2+} release of 1.7 nmol/mg protein is measured.

Fluorescence studies of interactions with the ATPase

Reconstitution of the ATPase with BrPC results in quenching of approx. 60% of the tryptophan fluorescence of the ATPase, by a static mechanism [11]. Ad-

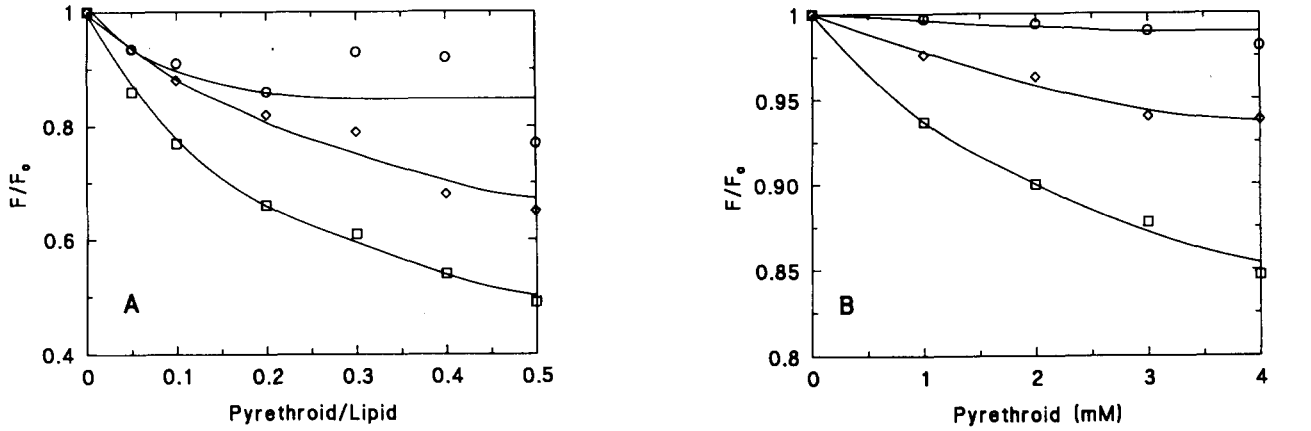

Fig. 7. (A) Quenching of the fluorescence (F/F_0) of DOPC-ATPase by pyrethroid at the given molar ratio of pyrethroid to DOPC and (B) of pyrene-1-dodecanoic acid (1 μM) in ethanol at the given concentration of pyrethroid (mM). Pyrethroid F(i) (○); H(i) (◇); G (□).

dition of hydrophobic, non-quenching molecules that can bind at the lipid/protein interface of the ATPase results in an increase in fluorescence of BrPC-ATPase, as a result of displacement of BrPC from the surface of the ATPase [11,15–17]. Addition of quenching hydrophobic molecules can result in quenching of the tryptophan fluorescence of the ATPase, both from binding at the lipid/protein interface of the ATPase (annular sites) and from binding to other sites to which lipids cannot bind, referred to as non-annular sites [15–17]. Fig. 7A shows that the pyrethroids F(i), G, and H(i) containing the 3-phenoxybenzyl moiety can all quench the fluorescence of DOPC-ATPase, implying that the pyrethroids can bind to the ATPase. Quenching is identical for all three isomers of pyrethroid H (data not shown). The greater level of quenching caused by the iodinated pyrethroid G probably does not reflect stronger binding to the ATPase but, rather, more efficient quenching. Thus Fig. 7B shows that the relative order of quenching of the fluorescence of pyrene-1-dodecanoic acid in solution in ethanol caused by the three pyrethroids is the same as for the tryptophan fluorescence of the ATPase (Fig. 7A). Fig. 8 shows that pyrethroids B, C and D(ii) containing the pentafluorobenzyl moiety also quench the fluorescence of DOPC-ATPase, the greatest quenching again being observed for the iodinated species (pyrethroids B and D(ii)).

TABLE II

Steady-state release of Ca^{2+} from the ATPase in the presence of ATP

The ATPase was incubated at pH 6.0 in 20 mM Mg^{2+}, 50 μM Ca^{2+} and the Ca^{2+} released on addition of 40 μM ATP was measured as described in Materials and Methods.

System	Ca^{2+} released (nmol/mg protein)
Native	5.5
DMPC-ATPase	0
DMPC-ATPase + pyrethroid D(ii) (molar ratio pyrethroid/DMPC 0.15 : 1)	1.7

More detailed information can be obtained from comparative studies of the quenching of the tryptophan fluorescence of BrPC-ATPase and DOPC-ATPase. Addition of pyrethroids not containing bromine or iodine to the ATPase can result in some fluorescence quenching (Figs. 9 and 10), the effect being particularly marked for pyrethroid A containing the pentafluorobenzyl group. The effects of pyrethroids on the fluorescence intensity of the ATPase reconstituted with BrPC was therefore expressed as the ratio of the fluorescence intensity observed for the pyrethroid-BrPC-ATPase system to that observed for the pyrethroid-DOPC-ATPase system (see Ref. 17). Fig. 9 shows that addition of pyrethroid E to BrPC-ATPase up to a molar ratio of pyrethroid/BrPC of 0.5 : 1 results in little increase in fluorescence intensity, indicating weak binding of the pyrethroid at the lipid/protein interface. Some quenching is observed, however, on addition of the bromine-containing analogue F(i) to DOPC-ATPase, and the fluorescence intensity of BrPC-ATPase is further quenched on addition of pyrethroid F(i), indicating

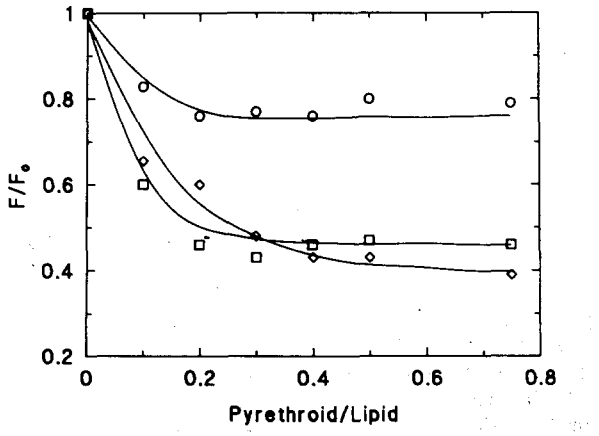

Fig. 8. Quenching of the fluorescence (F/F_0) of DOPC-ATPase by pyrethroid at the given molar ratio of pyrethroid to DOPC. Pyrethroid B (◇); C (○); D(ii) (□).

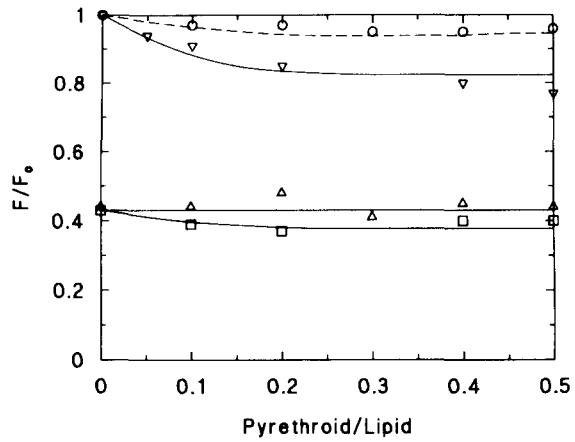

Fig. 9. Effects of pyrethroid on the fluorescence intensity of DOPC-ATPase (○, ▽) and BrPC-ATPase (△, □) at the given molar ratios of pyrethroid to phospholipid. Pyrethroid: E (○, △); F(i) (▽ □). The data for the pyrethroid-BrPC-ATPase system (△) were normalized with respect to the fluorescence intensities for an equal concentration of pyrethroid added to the DOPC-ATPase system (○). The lines are simulations with the binding parameters given in Table III.

binding of pyrethroid F(i) at sites on the ATPase distinct from those at the lipid/protein interface. As shown in Fig. 9, the fluorescence quenching data fits well to Eqn. 1, with the binding parameters given in Table III. Fig. 10 suggests that pyrethroid A containing the pentafluorobenzyl moiety might be able to bind more strongly to the lipid/protein interface of the ATPase than pyrethroid E, but the large correction that has to be made to the BrPC-ATPase data because of the quenching caused by pyrethroid A in the DOPC-ATPase system makes the conclusion rather uncertain: at most, however, the difference in binding constant between pyrethroids A and E is likely to be no more than a

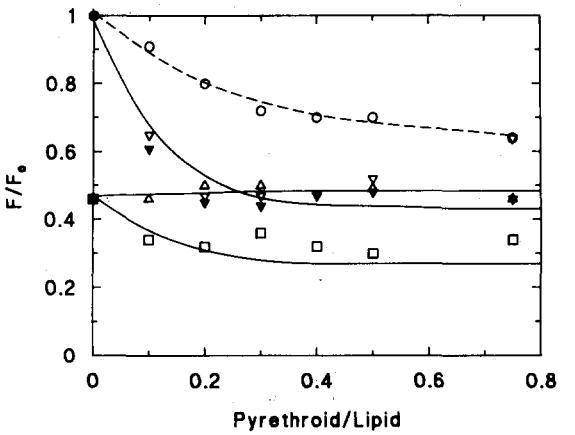

Fig. 10. Effects of pyrethroid on the fluorescence intensity of DOPC-ATPase (○, ▽, ▼) and BrPC-ATPase (△, □) at the given molar ratios of pyrethroid to phospholipid. Pyrethroid: A (○, △); D(i) (▽); D(ii) (▼, □). The data for the pyrethroid-BrPC-ATPase system (△) were normalized with respect to the fluorescence intensities for an equal concentration of pyrethroid added to the DOPC-ATPase system (○). The lines are simulations with the binding parameters given in Table III.

TABLE III

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

Constants were estimated from fluorescence quenching plots as described in the text.

Pyrethroid	Dissociation constant (molar ratio units)	
	annular sites	non-annular sites
E, F	5.0	0.7
A, D	2.0	0.32 [a]

[a] Lower limit (see text).

factor 2.5 (Table III). Quenching caused by the iodinated pyrethroids B(i) and B(ii) is more extensive than that observed for pyrethroid A, with no difference observed between the two isomers. As shown in Fig. 10, the data can be interpreted in terms of Eqn. 1, with the parameters given in Table III. However, it is likely that the iodinated molecules are more efficient quenchers than the corresponding bromine containing molecules and thus the dissociation constant for binding at non-annular sites given in Table III probably represents a lower limit. Nevertheless, it is clear that the binding parameters for the pentafluorobenzyl-containing pyrethroids are very similar to those for the 5-phenoxybenzyl-containing pyrethroids.

Discussion

The pyrethroids have been shown to bind extensively to biological membranes as a result of their marked hydrophobicity [1,2]. Many membrane proteins have been shown to be sensitive to the presence of foreign molecules within the membrane, and thus it might be expected that the pyrethroids would affect the function of membrane proteins other than the Na-channel which is believed to be their primary target [1]. Thus it has been shown that pyrethroids affect the activity of the (Ca^{2+} + Mg^{2+})-ATPase present in synaptosomal membranes, in a microsomal preparation from cockroach brain and in plants [7,8,26]. Unfortunately, the (Ca^{2+} + Mg^{2+})-ATPases in such preparations are not well defined, making detailed studies at the molecular level very difficult. We have therefore studied interactions of pyrethroid with the (Ca^{2+} + Mg^{2+})-ATPase from rabbit skeletal muscle sarcoplasmic reticulum since this can be obtained pure, can be reconstituted into phospholipid bilayer systems of defined composition, and can be obtained in sufficient quantity for detailed kinetic and spectroscopic studies.

The ATPase activity of the (Ca^{2+} + Mg^{2+})-ATPase is dependent on the structure of the phospholipids surrounding it in the membrane, with DOPC supporting highest activity and a phospholipid such as DMPC with shorter fatty acyl chains (C_{14}) supporting lower activity

[11]. In previous studies we have shown that addition of pyrethroids to the ATPase reconstituted with DOPC results in a small increase in activity at low concentrations of pyrethroid and some inhibition at higher concentrations, but that addition of pyrethroids to the ATPase reconstituted with DMPC results in very large increases in activity [9]. We suggested that these effects probably followed from direct interaction with the ATPase rather than indirectly from an effect on the phospholipid component of the membrane such as a change in membrane fluidity [9]. Recently we prepared a series of fluorescence quenching pyrethroids and used their quenching properties to study their interaction with phospholipid bilayers [10]. Here we use these same pyrethroids to study interactions with the ATPase.

We have shown that a variety of hydrophobic molecules can, in principle, bind to at least two classes of site on the ATPase, one set being at the phospholipid/protein interface (annular sites), the other a set of sites on the ATPase from which phospholipids are excluded, possibly at protein/protein interfaces either between α-helices within the ATPase or at the interface between ATPase molecules in dimeric or higher structures in the membrane [15–17]. Binding of non-quenching hydrophobic molecules at the phospholipid/protein interface can readily be studied by observation of the ability of the hydrophobic additive to displace BrPC from the surface of the ATPase reconstituted into bilayers of BrPC; displacement of BrPC is registered as an increase in tryptophan fluorescence intensity. As shown in Figs. 9 and 10, addition of pyrethroids to BrPC-ATPase results in little increase in fluorescence intensity, suggesting very weak binding at the phospholipid/protein interface (Table III). Previous studies have suggested that simple esters of the fatty acids such as methyl oleate are excluded from the phospholipid/protein interface, although the free fatty acids themselves can bind at these sites [17]. Addition of quenching analogues of the pyrethroids does, however, result in fluorescence quenching (Figs. 7–10) indicating that the pyrethroids must be able to bind at other (non-annular) sites on the ATPase. If it is assumed that quenching can be described by the equation developed to explain quenching in other systems (Eqn. 1), then binding constants at these non-annular sites can be derived (Table III). It appears that binding constants for all the pyrethroids are rather similar, and the binding constants are comparable to those estimated previously for fatty acids such as oleic acid, and binding is considerable stronger than for methyl oleate [17].

As shown in Figs. 7 and 8, quenching is observed for pyrethroids containing quenching groups (Br or I) in either the acid or the alcohol moieties. Since quenching by molecules containing bromine or iodine requires an intimate collision between the fluorophore and the quencher [11,18] this implies that the pyrethroids bind to the ATPase in such a conformation that both the alcohol and the acid interact with the hydrophobic regions of the ATPase where the quenchable tryptophan residues are located [17]. We have suggested that on binding to phospholipid bilayers the pyrethroids adopt a horse-shoe conformation, with the ester group located at the lipid-water interface and the acid and alcohol moieties folded back with both penetrating into the phospholipid bilayer [9,10]. The data reported here would be consistent with a similar conformation for the pyrethroids bound to the ATPase.

It has been shown that binding of a variety of hydrophobic molecules, including a series of sterols, to the ATPase reconstituted with DOPC has relatively little effect on ATPase activity whereas large effects follow from binding of the same molecules to the ATPase reconstituted with DMPC [9,15–17,24,28]. It has been suggested that the slow rate of hydrolysis of ATP by the ATPase reconstituted with DMPC follows from a decreased rate of phosphorylation of the ATPase by MgATP, and that the rate of this step can be increased by binding of androstenol (Michelangeli, East and Lee, unpublished data (1990)). A similar pattern of effects has been observed here for the pyrethroids, with binding of the pyrethroids having relatively little effect on the activity of the ATPase reconstituted with DOPC, but resulting in a large stimulation in activity for the ATPase reconstituted with DMPC (Figs. 2–4).

As shown in Fig. 4, comparable effects of pyrethroid D(ii) on the activity of DMPC-ATPase are observed over a range of ATP concentrations, suggesting that the pyrethroid does not have any marked effect on the affinity of the ATPase for ATP. The lower ATPase activity for DMPC-ATPase than for DOPC-ATPase follows, in part, from a slower phosphorylation of the ATPase by ATP (see Fig. 5 and Michelangeli, East and Lee, unpublished data (1990)). It has been suggested by Stahl and Jencks [22] that following binding of ATP to the Ca^{2+}-bound form of the ATPase there is a conformation change before phosphorylation is possible, possibly corresponding to a rearrangement in which the γ-phosphate of ATP bound to one domain on the ATPase is brought close to the residue to be phosphorylated on a second domain. We have suggested that it is this conformation change which becomes slow in DMPC-ATPase (Michelangeli, East and Lee, unpublished data (1990)). As shown in Fig. 5, addition of pyrethroid D(ii) to DMPC-ATPase results in a very large increase in rate for the formation of phosphoenzyme, to a value comparable to that seen for the native ATPase. It has also been shown that reconstitution of the ATPase with DMPC results in a marked decrease in phosphorylation of the ATPase by P_i, attributable to a decrease in the equilibrium constant for formation of phosphoenzyme from P_i-bound ATPase (Michelangeli, East and Lee, unpublished data (1990)). As shown in

Fig. 6, addition of pyrethroid D(ii) to DMPC-ATPase results in a significant increase in phosphoenzyme formation from P_i.

We have shown that measurement of the release of Ca^{2+} from the ATPase at steady state following addition of ATP to the ATPase equilibrated with Ca^{2+} can provide useful information about the relative concentrations of the various phosphorylated and non-phosphorylated forms of the ATPase present at steady state (Michelangeli, East and Lee, unpublished data (1990)). For the native ATPase or DOPC-ATPase, a release of 5.5 nmol Ca^{2+}/mg protein was observed Table II), which for a preparation containing approx. 50% active protein, would indicate that approx. 50% of the protein was in a form with low affinity for Ca^{2+} at steady state in the presence of Ca^{2+} (E_2P in the nomenclature of Ref. 23). For DMPC-ATPase, no Ca^{2+} release was observed on addition of Ca^{2+}, indicating a major change in the distribution between forms of the ATPase on reconstitution with the short-chain phospholipid. On addition of pyrethroid D(ii), significant Ca^{2+} release was observed, although not as great as that observed for the native ATPase.

The kinetic observations reported above would suggest partial reversal of the effects of the short chain phospholipid DMPC on addition of the pyrethroids. The effects do not appear to be very structurally specific, and although differences are observed between the various pyrethroids no differences are observed between the various isomeric forms (Figs. 2 and 3). Very similar effects on the activity of DMPC-ATPase have been observed on addition of androstenol (Michelangeli, East and Lee, unpublished data (1990)), which has also been suggested to bind predominantly at non-annular sites on the ATPase [24], again consistent with relatively non-specific interactions at predominantly hydrophobic sites.

Acknowledgements

F.M. was supported by an SERC CASE studentship with ICI Agrochemicals, Jealott's Hill Research Station, Bracknell. We thank the SERC for financial support, John Williams for technical advice on pyrethroid synthesis and Dr. M.K. Battersby for many helpful discussions.

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