Mechanism of inhibition of Ca^{2+} -ATPase by myotoxin a

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The peptide DCRQKWCKKKGSG [myotoxin-(29–42)], corresponding to residues 29–42 of myotoxin a, inhibits the activity of the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum, with a K_d value of $19.4 \mu\text{M}$ at pH 7.5, in 100 mM KCl. The peptide YKQCHKKGHCFPKEK, corresponding to residues 1–16 of myotoxin a, is a less potent inhibitor. Inhibition by myotoxin-(29–42) is reduced at low pH and at high ionic strength, suggesting that charge interactions are important in binding to the ATPase. Inhibition of the ATPase has been shown to follow from a decrease in the rate of dephosphorylation, with no effect on the rate of phosphorylation of the ATPase or on the rate of the Ca^{2+} transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$). Binding of myotoxin-

(29–42) decreased the affinity of the ATPase for Ca^{2+} and Mg^{2+} , and increased the rate of dissociation of the outer Ca^{2+} ion from the ATPase. Unlike the amphipathic peptide melittin, it is suggested that myotoxin-(29–42) does not bind significantly to the lipid bilayer portion of the sarcoplasmic reticulum. Fluorescence quenching studies suggest that it could bind to the ATPase in the vicinity of Cys-344 in the phosphorylation domain and Lys-515 in the nucleotide binding domain. Inhibition of the ATPase is observed when the ATPase is reconstituted in monomeric form in sealed vesicles, suggesting that aggregation of the ATPase is not involved in inhibition.

INTRODUCTION

Myotoxin a, a polypeptide of 43 amino acids from the prairie rattlesnake *Crotalus viridis viridis*, has been shown to cause muscle degeneration [1]. Binding to the sarcoplasmic reticulum (SR) leads to vacuolization of the SR followed by degeneration of the myofibrils [1]. Myotoxin a has been shown to bind to the major protein in the SR membrane, the Ca^{2+} -ATPase, and to calsequestrin, the Ca^{2+} -binding protein located in the lumen of the SR [2–6]. It has been reported that binding of myotoxin a to the Ca^{2+} -ATPase leads to uncoupling of Ca^{2+} transport from ATP hydrolysis [3].

The regions of myotoxin a that are important for binding to the ATPase have been established by studying the effects of peptide fragments on the ATPase. It was found that a peptide corresponding to the C-terminus of myotoxin a competed better against myotoxin a binding than a peptide corresponding to the N-terminus [4], although both were able to reduce the level of accumulation of Ca^{2+} by SR vesicles [5]. Peptides corresponding to the middle regions of myotoxin a had little effect on Ca^{2+} accumulation [5]. Both the N- and C-terminal peptides were highly basic, the former containing five lysine residues and the latter, four [5]. Specificity in the interaction between myotoxin a and the Ca^{2+} -ATPase is suggested by the report that another basic toxin, cobra cardiotoxin, did not bind to the Ca^{2+} -ATPase [4] although it has been reported that cardiotoxin inhibits Ca^{2+} accumulation by the SR [7].

Other basic peptides have been shown to bind to the Ca^{2+} -ATPase and inhibit ATPase activity. Binding of the hydrophilic domain of phospholamban has been shown to reduce the maximal rate of ATP hydrolysis by the ATPase [8,9] by slowing the rate of dissociation of Ca^{2+} from the phosphorylated ATPase ($\text{E1PCa}_2 \rightarrow \text{E2P}$) [9]. Melittin from bee venom is also a potent

inhibitor of the Ca^{2+} -ATPase [10–12]; inhibition has been suggested to involve basic residues on melittin, and has been shown to follow from a decrease in the rate of the dephosphorylation step ($\text{E2P} \rightarrow \text{E2}$) [12]. Melittin is an amphipathic peptide with strong haemolytic activity [13] whereas myotoxin a, which is not obviously amphipathic, has been shown to have very low haemolytic potency [14].

Here we study the effects on the Ca^{2+} -ATPase of the peptides YKQCHKKGHCFPKEK [myotoxin-(1–16)] and DCRQKWCKKKGSG [myotoxin-(29–42)], corresponding to the N-terminus (residues 1–16) and C-terminus (residues 29–42) respectively of myotoxin a. We show that myotoxin-(29–42), like melittin, inhibits dephosphorylation of the phosphorylated ATPase.

MATERIALS AND METHODS

The peptides YKQCHKKGHCFPKEK and DCRQKWCKKKGSG were synthesized by the method of Merrifield [15] and checked for purity by HPLC on a reversed-phase C_8 column.

SR from rabbit skeletal muscle and the purified Ca^{2+} -ATPase were prepared as described in Michelangeli et al. [16]. ATPase activity was determined at 25 °C by using a coupled enzyme assay in a medium containing, unless otherwise specified, 40 mM Hepes/KOH (pH 7.4), 5 mM MgSO_4 , 100 mM KCl, 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 i.u.), lactate dehydrogenase (18 i.u.) and a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration of $10 \mu\text{M}$) in a total volume of 2.5 ml. Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+} , Mg^{2+} and H^+ to EGTA given by Godt [17].

Ca^{2+} uptake by sealed SR vesicles (0.17 mg of protein/ml) was monitored spectrophotometrically by using the dye murexide in

Abbreviations used: myotoxin-(1–16), YKQCHKKGHCFPKEK; myotoxin-(29–42), DCRQKWCKKKGSG; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; BrF, 5-(bromomethyl)fluorescein; di(C_{18})PC, dioleoyl phosphatidylcholine; FITC, fluorescein isothiocyanate; IAEDANS, 5-((2-iodoacetyl)amino)ethylamino)naphthalene-1-sulphonic acid; NBD, nitro-1,2,3-benzo-oxadiazole; C_{12}E_8 , octa(ethylene glycol) dodecyl monoether; SR, sarcoplasmic reticulum.

40 mM Hepes/KOH, pH 7.4, containing 5 mM Mg^{2+} , 100 mM KCl and 50 μM Ca^{2+} . Spectra were run on an Aminco DW2000 dual-wavelength spectrometer with a wavelength pair of 507 and 542 nm, as described in McWhirter et al. [18].

The time-dependence of phosphorylation of the ATPase by [γ - ^{32}P]ATP at 25 °C was determined using a Biologic QFM-5 system as described in Starling et al. [19]. The time-dependence of dephosphorylation of the ATPase phosphorylated with [γ - ^{32}P]ATP was determined using the triple-mixing capability of the Biologic QFM-5 system [19]. The ATPase (0.2 mg/ml) was incubated in 150 mM Mops/Tris, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl and 100 μM $CaCl_2$. This was mixed with an equal volume of the same buffer containing 100 μM [γ - ^{32}P]ATP. After 200 ms, the mixture was mixed with an equal volume of 5.0 mM unlabelled ATP in the same buffer and then quenched with 12% trichloroacetic acid and 0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed three times with quenching solution and then radioactivity was counted.

The time-dependence of dephosphorylation of the ATPase phosphorylated with [^{32}P]P_i was determined as described in Baker et al. [12]. The ATPase (4 mg/ml) was incubated in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM $MgSO_4$ and 14% (v/v) dimethyl sulphoxide. One volume of this suspension was mixed with 16 volumes of 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4.1 mM $MgSO_4$ and 5.3 mM ATP, followed by quenching as described above. For experiments with peptides, the peptides were present at the required concentrations in all buffers.

The time-dependence of Ca^{2+} dissociation from the ATPase was determined using a Biologic rapid filtration system, at room temperature [21]. ATPase (200 μg) incubated in buffer (150 mM Mops/Tris, pH 7.2) containing 100 mM KCl, 20 mM $MgSO_4$, 100 μM $^{45}Ca^{2+}$ and 500 μM [3H]sucrose was loaded on to a Millipore HAWP filter and then rapidly perfused with the same buffer containing either EGTA (2 mM) or $^{40}Ca^{2+}$ (1 mM) and 20 μM myotoxin-(29–42). The radioactivity on the filter was then counted, and the $^{45}Ca^{2+}$ bound to the ATPase determined [21]. The time-dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined in the same way, perfusing with the same buffer containing 100 μM $^{40}Ca^{2+}$ and 2 mM ATP, as described in Starling et al. [19].

Equilibrium levels of phosphorylation of the ATPase by [^{32}P]P_i were determined in 130 mM Mes/Tris, pH 6.0, containing 5 mM EGTA, 10 mM Mg^{2+} and the required concentration of P_i at 25 °C and a protein concentration of 0.2 mg/ml, as described in Baker et al. [12]. Maximal levels of phosphorylation of the ATPase by [γ - ^{32}P]ATP as a function of free Ca^{2+} concentration were determined by incubation of the ATPase (0.4 mg/ml) in 150 mM Mops/Tris, pH 7.5, containing 100 mM KCl, 1 mM Mg^{2+} , 0.3 mM EGTA and the required concentration of Ca^{2+} , at 25 °C. Samples were quenched after 5 s with 12% trichloroacetic acid and 0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed three times with quenching solution and then the radioactivity was counted.

The ATPase was labelled with nitro-1,2,3-benzo-oxadiazole (NBD), fluorescein isothiocyanate (FITC), 5-(bromomethyl)-fluorescein (BrF), 5-((2-[(iodoacetyl)amino]ethyl)amino)naphthalene-1-sulphonic acid (IAEDANS) or 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) as described [22–25]. Fluorescence measurements were performed at 25 °C using an SLM Amino 8000C fluorimeter. Tryptophan fluorescence was observed with an excitation wavelength of 290 nm and with emission wavelengths of 315 and 340 nm for titrations with Mg^{2+} and Ca^{2+} respectively [26]. Double labelling of the ATPase with IAEDANS

and either BrF or FITC were performed as described in Baker et al. [25].

Reconstitution of the ATPase into sealed vesicles in a monomeric form was performed as described by Heegaard et al. [27]. SR (0.3 mg of protein) was solubilized with octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$, 0.6 mg) in buffer (75 μl ; 30 mM Tris/HCl, pH 7.1) containing 0.4 M KCl, 0.4 M sucrose, 4 mM $MgCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol and 1 mM sodium azide. Dioleoyl phosphatidylcholine [$di(C_{18:1})PC$; 21 mg] was suspended in the same buffer (300 μl) containing 11 mg of cholate and sonicated to clarity in a bath sonicator. The phospholipid and ATPase samples were then mixed and dialysed for 60 h at 5 °C against 0.1 M Na_2HPO_4 (pH 7.1) 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol and 1 mM sodium azide; the dialysis buffer was changed every 10 h.

The effects of myotoxin-(29–42) on the permeability of the reconstituted ATPase to glucose were determined as described by Gould et al. [28]. The ATPase (0.3 mg) was reconstituted with $di(C_{18:1})PC$ at a molar ratio of lipid/ATPase of 3000:1. Phospholipid (6 mg) was dispersed in buffer [350 μl ; 40 mM Hepes/KOH, pH 8.0, containing 15% (w/v) sucrose, 5 mM ATP, 5 mM Mg^{2+} and 4 mg of potassium cholate] by sonication in a bath sonicator. ATPase (0.3 mg) was added and the mixture left on ice for 1 h. It was then dialysed overnight at 4 °C against 40 mM Hepes/KOH, pH 7.4, containing 100 mM KCl and 12.5 mM glucose to form sealed vesicles. External, untrapped glucose was then removed by passage through a Sephadex G-50 column pre-equilibrated with 40 mM Hepes/KOH, pH 7.4, and 100 mM KCl. The vesicle suspension (20 μl , containing 20 μg of protein) was then added to 2.5 ml of 40 mM Hepes/KOH, pH 7.4, containing 100 mM KCl, 5 mM Mg^{2+} , 3 mM ATP, 0.6 mM phosphoenolpyruvate, 0.3 mM NADH, 15 i.u. of pyruvate kinase, 36 i.u. of lactate dehydrogenase and 51 i.u. of hexokinase (yeast, Type III, Sigma) at 30 °C. Leakage of glucose from the vesicles was determined through the rate of oxidation of NADH, monitored at 340 nm. Measurements were continued for about 5 min, and the level of trapped glucose was then established by addition of Triton X-100 to a final concentration of 0.5% (v/v).

RESULTS

Effects of peptides on steady-state ATPase activity and accumulation of Ca^{2+}

Addition of myotoxin-(29–42) to SR vesicles in the presence of the Ca^{2+} ionophore A23187 led to an immediate decrease in ATPase activity, followed by a slow further decrease, continuing over a period of hours (Figure 1a). In the absence of A23187, addition of myotoxin-(29–42) to SR vesicles led to a slow increase in ATPase activity, over a time period matching the slow decrease in activity observed in the presence of A23187 (Figure 1a). Addition of myotoxin-(1–16) had no significant effect on the ATPase activity of SR vesicles in the absence of A23187 (Figure 1a). Addition of myotoxin-(29–42) to SR vesicles led to a slow decrease in the level of accumulation of Ca^{2+} by the vesicles, whereas again myotoxin-(1–16) had little effect (Figure 1b).

The level of inhibition seen immediately following addition of myotoxin-(29–42) to the purified ATPase, present as unsealed membrane fragments, decreased with decreasing pH (Figure 2). The concentration-dependence of inhibition fitted to a single binding site model with a maximum inhibition of 100% and K_d values of 15.4 ± 0.8 , 19.4 ± 0.6 , 36.0 ± 0.9 and 131.1 ± 2.8 μM at pH values of 8.0, 7.5, 7.0 and 6.5 respectively, in the presence of 100 mM KCl. Inhibition of ATPase activity by myotoxin-(1–16) at pH 7.5 also fitted to a maximum inhibition of 100%, with a K_d value of 214 ± 10 μM (Figure 2).

Figure 1 Effects of myotoxin-(1-16) and myotoxin-(29-42) on ATPase activity and Ca^{2+} accumulation by SR vesicles

(a) The ATPase activities of SR vesicles ($8 \mu\text{g}$ of protein/ml) were measured in the absence (○, □) or presence (△) of $0.4 \mu\text{g}/\text{ml}$ A23187, following incubation with $6 \mu\text{M}$ myotoxin-(1-16) (□) or $6 \mu\text{M}$ myotoxin-(29-42) (○, △) for the given times. ATPase activities were measured in 40 mM Hepes/KOH, pH 7.4, containing 2.1 mM ATP, 5 mM Mg^{2+} and $25 \mu\text{M}$ free Ca^{2+} , at 25°C . (b) The maximal level of accumulation of Ca^{2+} by SR vesicles ($170 \mu\text{g}$ of protein/ml) was measured following incubation with $6 \mu\text{M}$ myotoxin-(1-16) (□) or $6 \mu\text{M}$ myotoxin-(29-42) (○) for the given times. Accumulation of Ca^{2+} was measured spectrophotometrically by using the dye murexide in 40 mM Hepes/KOH, pH 7.4, containing 5 mM Mg^{2+} , 100 mM KCl and $50 \mu\text{M}$ Ca^{2+} following the addition of $50 \mu\text{M}$ ATP. Data are expressed as percentage inhibition of the Ca^{2+} accumulation observed in the absence of peptides.

Increasing the ionic strength of the medium decreased the effect of myotoxin-(29-42) on the ATPase activity of the uncoupled ATPase. Thus the inhibition of activity observed with $30 \mu\text{M}$ myotoxin-(29-42) at pH 7.4 decreased to 8% on addition of 310 mM choline chloride (results not shown). The level of inhibition of ATPase activity by $20 \mu\text{M}$ myotoxin-(29-42) was found to be independent of ATP concentration between $0.3 \mu\text{M}$ and 3 mM ATP, implying no effect on the affinity of the ATPase for ATP (results not shown).

Effects of myotoxin-(29-42) on pre-steady-state kinetics

Myotoxin-(29-42) had no effect on the rate of phosphoenzyme formation when the ATPase incubated in the presence of Ca^{2+} was mixed with $25 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at pH 7.2; rates of phosphoenzyme formation were $121 \pm 11 \text{ s}^{-1}$ and $124 \pm 11 \text{ s}^{-1}$ in the absence and presence respectively of $20 \mu\text{M}$ myotoxin-(29-42) (results not shown). Myotoxin-(29-42) did, however, affect the rate of dephosphorylation (Figure 3). The ATPase was first phosphorylated by incubation with $[\text{P}^{32}]\text{P}_i$ at pH 6.0 in the absence of Ca^{2+} and the presence of 14% dimethyl sulphoxide to give a level of phosphorylation of 1.8 mmol of $[\text{EP}]/\text{mg}$ of

Figure 2 pH-dependence of the effect of myotoxin-(29-42) on ATPase activity

Shown is the percentage inhibition of the ATPase activity of the purified ATPase ($8 \mu\text{g}/\text{ml}$) immediately following addition of myotoxin-(29-42) (○, ▽, □, △) or myotoxin-(1-16) (●) in 40 mM Hepes/KOH, 2.1 mM ATP, 5 mM Mg^{2+} , 100 mM KCl, $25 \mu\text{M}$ Ca^{2+} , at 25°C and pH values of 8.0 (○), 7.5 (▽, ●), 7.0 (□) and 6.5 (△). The solid lines show fits to a single-binding-site model, with a maximum inhibition of ATPase activity of 100% under all conditions, with K_d values given in the text.

protein. Dephosphorylation was then induced by mixing with an excess of a pH 7.5 medium containing KCl and ATP. Dephosphorylation under these conditions fitted to a single exponential process with rate constants of $17.3 \pm 1.3 \text{ s}^{-1}$ and $11.3 \pm 1.5 \text{ s}^{-1}$ in the absence and presence respectively of $20 \mu\text{M}$ myotoxin-(29-42) (Figure 3a).

The rate of dephosphorylation in the presence of Ca^{2+} was determined in a triple mixing experiment in which the ATPase was first incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Ca^{2+} to induce formation of radiolabelled ATPase and was then mixed with excess (5.0 mM) unlabelled ATP to induce loss of radiolabel. Under these conditions, dephosphorylation at pH 7.2 again fitted to a single exponential process, with rate constants of $23.2 \pm 1.20 \text{ s}^{-1}$ and $16.6 \pm 2.7 \text{ s}^{-1}$ in the absence and presence respectively of $20 \mu\text{M}$ myotoxin-(29-42) (Figure 3b). At pH 6.0, the presence of $20 \mu\text{M}$ myotoxin-(29-42) had no significant effect on the rate of dephosphorylation, which was $7.5 \pm 0.7 \text{ s}^{-1}$ and $7.8 \pm 1.3 \text{ s}^{-1}$ in the absence and presence respectively of myotoxin-(29-42) (Figure 3b).

Myotoxin-(29-42) at $20 \mu\text{M}$ had no effect on the level of phosphoenzyme formation observed with $[\text{P}^{32}]\text{P}_i$ at pH 6.0 in the absence of Ca^{2+} , at a Mg^{2+} concentration of 10 mM and P_i concentrations between 1 and 10 mM (results not shown).

The time-dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined by incubating SR vesicles with $100 \mu\text{M}$ $^{45}\text{Ca}^{2+}$ in buffer containing 20 mM Mg^{2+} and 100 mM KCl at pH 7.2 in the presence of A23187 and then perfusing with the same medium containing $100 \mu\text{M}$ $^{40}\text{Ca}^{2+}$ and 2 mM ATP. The rate of dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase under these conditions gives the rate of dissociation of Ca^{2+} from the phosphorylated ATPase [29,30]. The data fit to single-exponential processes, with rate constants of $9.7 \pm 1.4 \text{ s}^{-1}$ and $10.2 \pm 1.8 \text{ s}^{-1}$ in the absence and presence respectively of $10 \mu\text{M}$ myotoxin-(29-42) (results not shown).

Information about the relative proportions of Ca^{2+} -bound and

Figure 3 Effect of myotoxin-(29-42) on the rate of dephosphorylation of the Ca^{2+} -ATPase phosphorylated with P_i or ATP

(a) The enzyme syringe contained ATPase (4.0 mg/ml) in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [^{32}P]P $_i$, 20 mM Mg^{2+} and 14% (v/v) dimethyl sulphoxide. The second syringe contained 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM Mg^{2+} and 5.3 mM ATP. Syringes also contained either no peptide (○) or 20 μ M myotoxin-(29-42) (□). The contents of the enzyme syringe were mixed in a 1:16 volume ratio with the dephosphorylation mixture (the pH after mixing was 7.3) and the reaction quenched at the given times. The solid lines represent fits to single exponentials with the parameters given in the text. (b) The enzyme syringe contained ATPase (0.2 mg/ml) in 150 mM Mops/Tris, pH 7.2, 5 mM Mg^{2+} , 100 mM KCl and 100 μ M Ca^{2+} , at pH 7.2 (○, □) or pH 6.0 (●, ■). This was mixed in a 1:1 ratio with a solution containing 100 μ M [γ - ^{32}P]ATP in the same buffer. The mixture was incubated for 200 ms, and then mixed in a 1:1 ratio with the same buffer containing 5.0 mM unlabelled ATP, and quenched at the given times. The syringes contained either no peptide (○, ●) or 20 μ M myotoxin-(29-42) (□, ■). The solid lines represent fits to single exponentials with the parameters given in the text.

Ca^{2+} -free forms of the ATPase at steady state in the presence of ATP can be obtained by measurement of the Ca^{2+} released on addition of ATP to the Ca^{2+} -bound ATPase. Addition of 2 mM ATP to the ATPase at pH 7.2 led to a decrease in the amount of bound Ca^{2+} from 10.6 to 8.6 nmol/mg of protein in the absence of myotoxin-(29-42), and from 10.8 to 9.0 nmol/mg of protein in the presence of 20 μ M myotoxin-(29-42). The release of only a small amount of Ca^{2+} indicates that, under steady-state conditions, the ATPase was predominantly present in a Ca^{2+} -bound form (E1PCa_2), with relatively little Ca^{2+} -free forms (E2P) being present in either the absence or presence of myotoxin-(29-42).

Effect of myotoxin-(29-42) on Ca^{2+} binding to the ATPase

The dependence of ATPase activity on Ca^{2+} concentration is complex (Figure 4a) with low concentrations of Ca^{2+} activating the ATPase, attributable to binding of Ca^{2+} to the Ca^{2+} -binding sites on the unphosphorylated ATPase (E1), and high con-

Figure 4 Effect of myotoxin-(29-42) on the Ca^{2+} -dependence of ATPase activity and phosphorylation by ATP

(a) ATPase activity (i.u./mg of protein) for the purified ATPase (0.07 μ M) as a function of Ca^{2+} concentration in the absence (○) or presence (□) of 20 μ M myotoxin-(29-42) measured at pH 7.4, 2.1 mM ATP, 100 mM KCl and 5 mM Mg^{2+} at 25 °C. (b) Maximal levels of phosphorylation (nmol of [EP]/mg of protein) observed after incubation of the purified ATPase in the absence (○) or presence (□) of 20 μ M myotoxin-(29-42) with 100 μ M [γ - ^{32}P]ATP for 5 s in 150 mM Mops/Tris, pH 7.5, containing 100 mM KCl and 1 mM Mg^{2+} at the given free concentrations of Ca^{2+} , at 25 °C.

centrations of Ca^{2+} inhibiting the ATPase, attributable both to binding of Ca^{2+} to the phosphorylated ATPase (E2P) with a subsequent decrease in the rate of dephosphorylation, and to the formation of CaATP , which is hydrolysed more slowly by the ATPase than MgATP [31–33]. The concentration of Ca^{2+} giving 50% of maximal activity at pH 7.4 shifted from a pCa value of 6.3 to 5.9 on addition of 20 μ M myotoxin-(29-42), whereas myotoxin-(29-42) had little effect on the inhibition observed at high Ca^{2+} concentrations, with 50% inhibition being observed at pCa values of 3.7 and 3.6 in the absence and presence respectively of myotoxin-(29-42) (Figure 4a). A decrease in affinity for Ca^{2+} of the high-affinity Ca^{2+} -binding sites in the presence of myotoxin-(29-42) is also suggested by measurements of the Ca^{2+} -dependence of phosphorylation by ATP (Figure 4b). As shown, the Ca^{2+} concentration required for half-maximal phosphorylation shifts from 0.5 μ M in the absence of myotoxin-(29-42) to 4 μ M in the presence of 20 μ M myotoxin-(29-42).

Binding of Ca^{2+} to the high-affinity binding sites on the ATPase can also be monitored by observation of the resulting changes in tryptophan fluorescence intensity [26,34–36]. In the presence of 20 μ M myotoxin-(29-42), the pCa value giving a half-maximal fluorescence change decreased by approx. 1.1 at pH 7.2 and pH 8.0, but myotoxin-(29-42) had no effect at pH 6.0

Figure 5 Ca^{2+} -dependence of tryptophan fluorescence intensity in the presence and absence of myotoxin-(29-42)

The figure shows the percentage decrease in tryptophan fluorescence intensity of the ATPase ($0.7 \mu\text{M}$) on addition of EGTA to the given pCa value in 150 mM Mops/Tris, 100 mM choline chloride, at a total Ca^{2+} concentration of 1 mM at pH 6.0 (a), pH 7.2 (b) and pH 8.0 (c), in the absence (○) or presence (□) of $20 \mu\text{M}$ myotoxin-(29-42). The lines are simulations of Ca^{2+} occupancy of the ATPase calculated using the parameters in Henderson et al. [26] in the absence of toxin and with modified binding constants for Ca^{2+} in the presence of toxin, as described in the text, and scaled to match the observed change in fluorescence intensity. Fluorescence was excited at 290 nm and observed at 340 nm.

(Figure 5). In the presence of 300 mM choline chloride, the effect of myotoxin-(29-42) was reduced, the shift in pCa value for a half-maximal fluorescence change at pH 7.2 being 0.3 for $20 \mu\text{M}$ myotoxin-(29-42) (results not shown).

Addition of Mg^{2+} to the ATPase results in a small increase in tryptophan fluorescence intensity when fluorescence is excited at 290 nm and observed at 315 nm [26,37,38]; it has been suggested

Figure 6 Effect of myotoxin-(29-42) on the rate of dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase

Dissociation of $^{45}\text{Ca}^{2+}$ when the ATPase incubated in buffer (150 mM Mops/Tris, pH 7.2) containing 100 mM KCl, 20 mM Mg^{2+} and $100 \mu\text{M}$ $^{45}\text{Ca}^{2+}$ was perfused with the same buffer containing either 2 mM EGTA (○, □) or 1 mM $^{40}\text{Ca}^{2+}$ (●, ■) in the absence (○, ●) or presence (□, ■) of $10 \mu\text{M}$ myotoxin-(29-42). The lines show fits to single or double exponential decays, as described in the text.

that this change results from binding of Mg^{2+} at the outer of the two Ca^{2+} -binding sites on the ATPase [26]. The concentration of Mg^{2+} causing a half-maximal fluorescence change shifted from 3.1 mM in the absence of myotoxin-(29-42) to 6.6 and 12.5 mM in the presence of 10 and $20 \mu\text{M}$ myotoxin-(29-42) respectively. In the presence of 300 mM choline chloride, addition of $20 \mu\text{M}$ myotoxin-(29-42) had no significant effect on the Mg^{2+} response.

An effect of myotoxin-(29-42) on the Ca^{2+} -binding sites on the ATPase is also shown by measurements of the rate of dissociation of Ca^{2+} from the ATPase. On washing $^{45}\text{Ca}^{2+}$ -bound ATPase with EGTA essentially all the bound $^{45}\text{Ca}^{2+}$ was lost from the ATPase, whereas on washing with 1 mM $^{40}\text{Ca}^{2+}$ only half the $^{45}\text{Ca}^{2+}$ was lost (Figure 6). In the presence of $10 \mu\text{M}$ myotoxin-(29-42), an identical result was obtained, except that the rate of dissociation of $^{45}\text{Ca}^{2+}$ was faster. On washing with $^{40}\text{Ca}^{2+}$, dissociation of Ca^{2+} fitted to a single-exponential process with rates of $14.6 \pm 2.3 \text{ s}^{-1}$ and $28.0 \pm 2.0 \text{ s}^{-1}$ in the absence and presence respectively of $10 \mu\text{M}$ myotoxin-(29-42) (Figure 6). As described in Starling et al. [21], dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase according to the sequential process in Scheme 1:

Scheme 1

can be represented as the sum of two exponentials of equal amplitude, with the rate of dissociation of the first Ca^{2+} being equal to the rate of dissociation of $^{45}\text{Ca}^{2+}$ observed in the presence of $^{40}\text{Ca}^{2+}$. As shown in Figure 6, dissociation of $^{45}\text{Ca}^{2+}$ on washing with EGTA can be fitted to this scheme, with rates for dissociation of the second Ca^{2+} of $8.5 \pm 1.9 \text{ s}^{-1}$ and $8.4 \pm 2.7 \text{ s}^{-1}$ in the absence and presence respectively of myotoxin-(29-42).

Figure 7 Effect of myotoxin-(29-42) on the fluorescence intensity of NBD-, FITC- or DMC-labelled ATPase

The graph shows the effects of myotoxin-(29-42) on the fluorescence intensity (a) of NBD-labelled ATPase or FITC-labelled ATPase and (b) DMC-labelled ATPase. (a) ○, NBD-labelled ATPase in 150 mM Mops/Tris, pH 7.2; □, FITC-labelled ATPase in 150 mM Mops/Tris, pH 7.2, 200 mM choline chloride. (b) DMC-labelled ATPase in 150 mM Mops/Tris, pH 7.2, 0.3 mM EGTA in the absence (○) or presence (□) of 200 mM choline chloride; or in the presence of buffer, 200 mM choline chloride and 0.4 mM Ca²⁺ (free Ca²⁺ concentration of 100 μM) (△), 5 mM Mg²⁺ (▽) or 20 mM Mg²⁺ (◇). The lines show fits to single binding sites with the K_d values given in the text.

At high pH values, Mg²⁺ has been shown to inhibit ATPase activity, probably by binding to the Ca²⁺ sites on the phosphorylated ATPase [39,40]. At pH 8.0, the level of inhibition of ATPase activity caused by 20 μM myotoxin-(29-42) (42%) was found to be independent of Mg²⁺ concentration between 0.5 and 20 mM (results not shown).

Effects of myotoxin-(29-42) on fluorescence responses of the ATPase

Addition of myotoxin-(29-42) to NBD-labelled, FITC-labelled or DMC-labelled ATPase results in a large decrease in fluorescence intensity (Figure 7); the fluorescence of FITC-labelled ATPase was found to be unstable at low ionic strength in the presence of myotoxin-(29-42), and so experiments with FITC-labelled ATPase were performed in the presence of 200 mM choline chloride. The fluorescence quenching observed with NBD-labelled ATPase was consistent with binding to a single site with a K_d value of 7.4 ± 1.6 μM, with a maximum fluorescence quenching of 20%. The fluorescence quenching observed with FITC-labelled ATPase in the presence of 200 mM choline chloride was also consistent with binding to a single site, with a K_d value of 22.6 ± 0.7 μM, with a maximum fluorescence quench-

Table 1 Effect of myotoxin-(29-42) and melittin on fluorescence energy transfer

The efficiency of resonance energy transfer was measured by the quenching of IAEDANS fluorescence (F/F_0) for the ATPase doubly labelled with IAEDANS and BrF at molar ratios of IAEDANS/BrF/ATPase of 0.79:0.73:1 and doubly labelled with IAEDANS and FITC at molar ratios of IAEDANS/FITC/ATPase of 0.88:0.70:1. The values of E are calculated for a 1:1 molar ratio of BrF/ATPase and FITC/ATPase from the experimental data (F/F_0). Values of r were calculated using R_0 (Å; 1 Å $\equiv$ 0.1 nm) values of 47.4 [25] and 49 [57] for the IAEDANS/BrF and IAEDANS/FITC pairs respectively. Fluorescence spectra were recorded using 0.036 μM ATPase in 50 mM Hepes/KOH, pH 7.2, 0.3 mM EGTA, and the fluorescence emission intensity for IAEDANS was measured at 465 nm. The myotoxin-(29-42) concentration used was 10 μM, and the melittin concentration was 1.5 μM.

Acceptor	Conditions	F/F_0	E	r (Å)
BrF	Buffer	0.43	0.78	39
	Myotoxin-(29-42)	0.44	0.77	39
	Melittin	0.46	0.74	40
FITC	Buffer	0.71	0.36	54
	Myotoxin-(29-42)	0.72	0.35	53
	Melittin	0.70	0.38	54

ing of 13%. Quenching of the fluorescence of DMC-labelled ATPase fitted to K_d values of 8.3 ± 0.8 and 19.7 ± 3.1 μM in the absence and presence respectively of 200 mM choline chloride with a maximum quenching of 19%. In the presence of 200 mM choline chloride, the K_d values for quenching of DMC fluorescence increased to 39.7 ± 1.4 and 51.8 ± 2.7 μM on addition of 5 and 20 mM Mg²⁺ respectively, and to 30.7 ± 5.3 μM on addition of 100 μM Ca²⁺. Addition of myotoxin-(29-42) to the ATPase labelled with either IAEDANS or BrF had no effect on fluorescence intensity (results not shown).

Resonance energy transfer has been used to measure the distance between Cys-670/674, labelled with IAEDANS, and either Glu-439 labelled with BrF or Lys-515 labelled with FITC [25]. As shown in Table 1, addition of either myotoxin-(29-42) or melittin had no significant effect on the efficiency of energy transfer.

Effects of myotoxin-(29-42) on membrane permeability to glucose

To test for effects of myotoxin-(29-42) on the non-specific permeability of vesicles containing the Ca²⁺-ATPase, the rate of efflux of glucose from reconstituted vesicles was determined as described in the Materials and methods section. The time taken to release half the trapped glucose was greater than 10 min in the absence of any additive, and was unaffected by addition of 20 μM myotoxin-(29-42). In contrast, addition of 3 μM melittin decreased the time taken for release of half the trapped glucose to 3 min, which compared with a half-time of 1 min in the presence of 0.5% (v/v) Triton X-100.

Effect of reconstitution of the ATPase in monomeric form

The possibility that the effects of myotoxin-(29-42) depend on aggregation of the ATPase was studied by reconstituting the ATPase from solution in C₁₂E₈ into phospholipid vesicles at a molar ratio of phospholipid to ATPase of 10000:1, conditions which give sealed vesicles each containing a small number of isolated monomeric ATPase molecules [27,41]. As shown in Table 2, simple addition to the ATPase of vesicles of di(C_{18:1})PC prepared by dialysis from cholate solution had no effect on the level of inhibition observed with 20 μM myotoxin-(29-42). Similarly, addition of vesicles consisting of a 1:1 molar ratio of

Table 2 Effects of myotoxin-(29–42) on the activity of the ATPase reconstituted in monomeric form

ATPase activities were measured at 2.1 mM ATP, 25 μM Ca^{2+} , 25 °C, in 40 mM Hepes/KOH, pH 7.2.

System	ATPase activity (i.u./mg of protein)	
	– Myotoxin-(29–42)	+ 20 μM myotoxin-(29–42)
ATPase	3.0	1.7
ATPase + $\text{di}(\text{C}_{18:1})\text{PC}^*$	3.0	1.8
ATPase + $\text{di}(\text{C}_{18:1})\text{PC}$ + cholesterol†	3.0	1.7
Reconstituted SR/ $\text{di}(\text{C}_{18:1})\text{PC}^\ddagger$	1.3	0.8
Reconstituted SR/ $\text{di}(\text{C}_{18:1})\text{PC}$ + cholesterol§	1.5	0.8

* Reconstituted vesicles of $\text{di}(\text{C}_{18:1})\text{PC}$ added to the purified ATPase at a molar ratio of phospholipid/ATPase of 10000:1.

† Reconstituted vesicles of a 1:1 molar ratio of $\text{di}(\text{C}_{18:1})\text{PC}$ /cholesterol added to the purified ATPase at a molar ratio of phospholipid to ATPase of 10000:1.

‡ ATPase reconstituted in monomeric form with $\text{di}(\text{C}_{18:1})\text{PC}$ at a molar ratio of phospholipid/ATPase of 10000:1.

§ ATPase reconstituted in monomeric form with a 1:1 molar ratio of $\text{di}(\text{C}_{18:1})\text{PC}$ /cholesterol, at a molar ratio of phospholipid/ATPase of 10000:1.

$\text{di}(\text{C}_{18:1})\text{PC}$ and cholesterol had no effect on the level of inhibition. These experiments suggest that, unlike melittin [12], myotoxin-(29–42) does not bind significantly to lipid bilayers. For the reconstituted ATPase, the measured ATPase activity is lower than for the unreconstituted ATPase (Table 2) because of the formation of sealed vesicles in which approx. 50 % of the ATPase molecules are oriented with their ATP-binding sites facing the lumen of the vesicles, and thus are unable to bind ATP [41,42]. However, as shown, addition of 20 μM myotoxin-(29–42) to the reconstituted ATPase resulted in the same level of inhibition of ATPase as observed for the unreconstituted ATPase (Table 2). We conclude that myotoxin-(29–42) is able to inhibit the ATPase when reconstituted in monomeric form in sealed vesicles.

DISCUSSION

The C-terminal region of myotoxin a has been shown to bind to the Ca^{2+} -ATPase of the SR with a higher affinity than the N-terminal region [4,5]. Both the C-terminal and N-terminal regions are highly basic; in this resembling another toxin, melittin, which has been shown to be a potent inhibitor of the Ca^{2+} -ATPase [10–12,43–45]. Melittin, an amphipathic peptide, has been shown to bind strongly to lipid bilayers [13], and it has been suggested that binding of melittin at the lipid/water interface is responsible for the aggregation observed with a variety of membrane proteins [46–48].

Addition of myotoxin-(29–42) to uncoupled SR vesicles led to an immediate decrease in ATPase activity, followed by a slow further decrease in activity which continued over a period of hours (Figure 1). Addition of myotoxin-(29–42) to sealed SR vesicles led to an increase in ATPase activity and a decrease in the level of Ca^{2+} accumulation, with the effect again being slow (Figure 1). These slow effects would be consistent with a slow denaturation of the ATPase in the presence of myotoxin-(29–42), leading to an increased permeability of the SR vesicles to Ca^{2+} , and were not characterized further. The immediate decrease in ATPase activity observed on addition of myotoxin-(29–42) to uncoupled SR vesicles or to the purified ATPase is consistent

with binding to a single site on the ATPase, with a K_d of 19.4 μM at pH 7.5 and 100 mM KCl, this binding leading to complete loss of ATPase activity (Figure 2). The K_d value increases with decreasing pH (Figure 2) and inhibition decreases with increasing ionic strength (results not shown), suggesting that charge interactions are important in the interaction with the ATPase. Myotoxin-(1–16) bound much less strongly than myotoxin-(29–42) to the ATPase (Figure 2).

Myotoxin-(29–42) decreases the rate of dephosphorylation of the phosphorylated ATPase (Figure 3), the 35 % decrease in the rate of dephosphorylation caused by 20 μM myotoxin-(29–42) in the absence of Ca^{2+} at pH 7.3 (Figure 3a) being sufficient to explain the observed decrease in the rate of ATP hydrolysis under these conditions (Figure 2). However, no effect was observed at pH 6.0 (Figure 3b), suggesting weak binding of myotoxin-(29–42) to the ATPase at acid pH values. This is also consistent with the lack of effect of myotoxin-(29–42) on the level of phosphorylation of the ATPase by P_i at pH 6.0 (results not shown) and the weak effect of myotoxin-(29–42) on ATPase activity at pH 6.5 (Figure 2).

Myotoxin-(29–42) had no effect on the rate of phosphorylation of the ATPase by ATP or on the ATP-dependence of the rate of ATP hydrolysis (results not shown). It also had no effect on the rate of the Ca^{2+} transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$) (results not shown) and thus, as expected, had less effect on the rate of dephosphorylation of the ATPase phosphorylated in the presence of Ca^{2+} and ATP ($\text{E1PCa}_2 \rightarrow \text{E2}$) (Figure 3b) than on the rate of dephosphorylation of the ATPase phosphorylated with P_i in the absence of Ca^{2+} ($\text{E2P} \rightarrow \text{E2}$) (Figure 3a). Myotoxin-(29–42) had no effect on the steady-state release of Ca^{2+} from the Ca^{2+} -bound ATPase following the addition of ATP (results not shown), also consistent with the observed lack of effect on the Ca^{2+} transport step.

Myotoxin-(29–42) was found to have significant effects on Ca^{2+} binding to the ATPase, as shown by equilibrium binding studies at pH 7.2 or 8.0 (Figure 5), by studies of the Ca^{2+} -dependence of ATPase activity (Figure 4a) and by studies of the Ca^{2+} -dependence of phosphorylation of the ATPase by ATP (Figure 4b). At pH 6.0, myotoxin-(29–42) had no effect on Ca^{2+} affinity (Figure 5), and the presence of 300 mM choline chloride decreased the shift in pCa value for a half-maximal change in tryptophan fluorescence caused by 20 μM myotoxin-(29–42) at pH 7.2 (results not shown), consistent with the importance of charge interactions in the myotoxin-(29–42)–ATPase interactions, as described above.

The affinity of the outer of the two Ca^{2+} -binding sites on the ATPase for Mg^{2+} measured from changes in tryptophan fluorescence [26] decreased by a factor of 2 on addition of 10 μM myotoxin-(29–42) and by a factor of 4 on addition of 20 μM myotoxin-(29–42) (results not shown). Again, the effect of myotoxin-(29–42) was reversed by addition of 300 mM choline chloride (results not shown). The results shown in Figure 6 would also be consistent with a 2-fold decrease in Ca^{2+} affinity for the outer Ca^{2+} -binding site in the presence of 10 μM myotoxin-(29–42). On washing $^{45}\text{Ca}^{2+}$ -bound-ATPase with $^{40}\text{Ca}^{2+}$, dissociation of $^{45}\text{Ca}^{2+}$ from the outer Ca^{2+} -binding site occurred at a rate of 14.6 s^{-1} , with dissociation of the inner $^{45}\text{Ca}^{2+}$ ion being very slow (Figure 6). On addition of 10 μM myotoxin-(29–42), the rate of dissociation of the outer $^{45}\text{Ca}^{2+}$ ion increased by a factor of 2 (Figure 6).

In Henderson et al. [26] a model was presented for Ca^{2+} binding to the ATPase. The effect of 20 μM myotoxin-(29–42) on the Ca^{2+} affinity of the ATPase at pH 7.2 and 8.0 shown in Figure 5 can be reproduced assuming a decrease in affinity at both Ca^{2+} -binding sites on the ATPase by a factor of 12 (results

not shown), or with a reduction in the affinity of the outer site by 4 and of the inner site by 36 (Figure 5).

The observations that myotoxin-(29–42) had no effect on the concentration of Ca^{2+} causing inhibition of activity (Figure 4) and no effect on the inhibition of ATPase activity by Mg^{2+} at pH 8.0 (results not shown) suggest that myotoxin-(29–42) had no effect on Ca^{2+} binding to the phosphorylated form of the ATPase (E2P).

It has been reported that myotoxin a has little haemolytic activity [14], consistent with its very polar character. Addition of myotoxin-(29–42) to lipid bilayers resulted in no increase in tryptophan fluorescence intensity (results not shown), suggesting a lack of binding. This is consistent with the observed lack of effect of vesicles of di($\text{C}_{18:1}$)PC on the inhibition of ATPase activity by myotoxin-(29–42) (Table 2). Further, myotoxin-(29–42) had no effect on the permeability of reconstituted vesicles of the ATPase to glucose, showing that myotoxin-(29–42) was unable to disrupt lipid bilayer structure.

Binding of melittin to SR vesicles causes aggregation of the Ca^{2+} -ATPase, and it has been suggested that this aggregation could be responsible for the observed inhibition of ATPase activity [10,11,48]. We have shown that this is unlikely, since inhibition by melittin is also observed for the ATPase reconstituted into lipid vesicles at high molar ratios of phospholipid/ATPase [12], where the lack of cross-linking of the ATPase shows that the ATPase molecules are present in an isolated monomeric form [27,41]. Inhibition by myotoxin-(29–42) is also observed for the ATPase in these reconstituted systems (Table 2), so that aggregation of the ATPase is unlikely to be involved in the mechanism of inhibition.

Evidence for a binding site for myotoxin-(29–42) on the cytoplasmic regions of the ATPase comes from studies of the effect of myotoxin-(29–42) on the fluorescence of the labelled ATPase. Addition of myotoxin-(29–42) to the ATPase labelled with NBD or FITC results in a decrease in fluorescence intensity (Figure 7). Fluorescence intensities of the NBD- and FITC-labelled ATPases have been shown to be sensitive to the E1/E2 equilibrium of the ATPase [22,26,49,50]. However, the reduction in fluorescence intensity observed with myotoxin-(29–42) cannot be attributed to changes in the E1/E2 equilibrium, since quenching of NBD-labelled ATPase occurs in the presence of Ca^{2+} (results not shown) and the decrease in fluorescence intensity for FITC-labelled ATPase is much larger than that attributable to the E1/E2 conformational change [22]. Thus myotoxin-(29–42) appears to have a direct quenching effect on NBD-labelled and FITC-labelled ATPase.

The residue on the ATPase predominantly labelled with NBD under the conditions used here is Cys-344 [51], and the same residue can be labelled with Br-DMC [52]. Myotoxin-(29–42) is also observed to quench the fluorescence of DMC-labelled ATPase (Figure 7). However, myotoxin-(29–42) had no effect on the fluorescence of the ATPase labelled at Glu-439 with BrF [24] or on the ATPase labelled at Cys-670/674 with IAEDANS [25] (results not shown). Quenching of fluorescence could be due to the presence of the three cysteine residues in myotoxin-(29–42); cysteine has been reported to quench the fluorescence of tryptophan by an electron transfer process [53]. The increase in tryptophan fluorescence intensity observed on addition of myotoxin-(29–42) to the ATPase is less than that observed on addition of myotoxin-(29–42) to buffer (results not shown), consistent with quenching of the tryptophan fluorescence of the ATPase by myotoxin-(29–42).

Quenching of NBD-labelled and DMC-labelled ATPase fits to K_d values of 7.4 and 8.3 μM respectively at pH 7.2 in the absence of choline chloride. In the presence of 200 mM choline chloride,

the K_d for quenching of DMC-labelled ATPase increases to 19.7 μM , compared with a value of 22.6 μM for the quenching of FITC-labelled ATPase in 200 mM choline chloride. These values are comparable with the K_d value of 19.4 μM for inhibition of ATPase activity at pH 7.5 in the presence of 100 mM KCl and 5 mM Mg^{2+} (Figure 2), and suggest both that fluorescence quenching of NBD-labelled, DMC-labelled and FITC-labelled ATPase follows from binding at a single site, and that binding at this same site is responsible for inhibition of ATPase activity. Effects of myotoxin-(29–42) on the fluorescence intensity of DMC-labelled ATPase are competitive with those of Mg^{2+} and Ca^{2+} (Figure 7), and effects of Mg^{2+} on the tryptophan fluorescence of the ATPase are competitive with those of myotoxin-(29–42) (results not shown), suggesting that binding at this same site on the ATPase could be responsible for the observed changes in Ca^{2+} binding.

The distance between Cys-344, labelled by NBD-Cl or Br-DMC, and Lys-515, labelled by FITC, has been estimated to be 37 Å [54]. Molecular modelling gives a distance between Cys-30 and Cys-37 on myotoxin-(29–42) of 26 Å in a fully extended conformation, so that simultaneous quenching of NBD and FITC fluorescence by a single bound myotoxin-(29–42) molecule could be possible. This would place the binding site for myotoxin-(29–42) between the phosphorylation domain (containing Cys-344) and the nucleotide-binding domain (containing Lys-515). A photoactivatable analogue of melittin has been shown to label the $(\text{H}^+ + \text{K}^+)\text{-ATPase}$ in the phosphorylation and nucleotide-binding domains [55]. Binding in this region would have to be such as not to affect binding of ATP, since neither the ATP-dependence of ATPase activity nor the rate of phosphoenzyme formation are affected (results not shown). It has also been reported that inhibition of the ATPase by myotoxin a and decavanadate are non-competitive [56], suggesting that myotoxin a does not bind close to the phosphate-binding site on the ATPase.

Fluorescence energy transfer has been used to measure the distances between Cys-670/674 (labelled with IAEDANS), located in the hinge domain, and Glu-439 (labelled with bromo-fluorescein) and Lys-515 (labelled with FITC), in the phosphorylation and nucleotide-binding domains respectively [25]. No significant changes in these distances could be detected on binding of either myotoxin-(29–42) or melittin (Table 1), suggesting little change in overall structure on binding the peptides. Thus the effect of myotoxin-(29–42) could be to prevent conformational changes on the ATPase associated with dephosphorylation rather than itself producing conformational changes on the ATPase.

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