The inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase by macrocyclic lactones and cyclosporin A

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The pharmacology of macrocyclic lactones is varied, with many beneficial effects in treating disease processes. FK-506, rapamycin and ascomycin have been utilized as immunosuppressant agents. Ivermectin is typically used to treat parasitic worm infections in mammals. Another immunosuppressant, cyclosporin A, is a cyclic oligotide that has similar immunosuppressant properties to those exerted by macrocyclic lactones. Here we report on the inhibition by these compounds of sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pumps. Ivermectin, cyclosporin A and rapamycin all inhibited the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1). In addition, although ivermectin inhibited brain microsomal endoplasmic reticulum (type 2b) Ca^{2+}-ATPase, cyclosporin A and rapamycin did not. As cyclosporin A also did not inhibit cardiac Ca^{2+}-ATPase activity, this would suggest that it could be an isoform-specific inhibitor. Ivermectin was shown to be the most potent Ca^{2+}-ATPase inhibitor of the macrocyclic lactones ($IC_{50} = 7\ \mu M$). It appears to show a 'competitive' inhibition with respect to high concentrations of ATP by increasing the regulatory binding site K_m but without affecting the catalytic site K_m. In addition, ivermectin stabilizes the ATPase in an E1 conformational state, and inhibits Ca^{2+} release from the enzyme during turnover. This would suggest that ivermectin inhibits Ca^{2+} release from the luminal binding sites of the phosphoenzyme intermediate, a step that is known to be accelerated by high [ATP].

Key words: ATP regulation, immunosuppressant, phosphorylation, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

INTRODUCTION

The pharmacology of macrocyclic lactones has been of considerable interest in recent years, due to their diverse effects on a range of cellular processes [1–5]. FK-506, rapamycin and ascomycin have all been shown to have immunosuppressant actions [1,4]. Furthermore, FK-506 and rapamycin have been shown to have a very high affinity for the FK-506-binding protein (FKBP12), an immunophilin [6] known to interact with the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor [7]. Ivermectin, another macrocyclic lactone, is an anti-helminthic agent [8], which has also been reported to activate neuronal nicotinic acetylcholine receptors [9], glutamate receptors [10] and γ-aminobutyric acid (GABA) receptors [11].

Recently, the macrocyclic lactones have been shown to inhibit ATP-dependent Ca^{2+} uptake in cell extracts from SH-SY5Y cells, a human neuroblastoma cell line [12]. In addition, it was shown that FK-506 inhibited Ca^{2+}-dependent ATPase activity in cardiac sarcoplasmic reticulum (SR) [12]. Furthermore it has been reported that ivermectin inhibits Ca^{2+} uptake into skeletal muscle SR, and enhances Ca^{2+} release via the ryanodine receptor, but not via the inositol 1,4,5-trisphosphate receptors [13].

Another immunosuppressant drug, cyclosporin A (CsA), has been shown to inhibit NO synthesis [14], and, when combined with cyclophilin, to inhibit calcineurin [15,16]. Interestingly, calcineurins are also inhibited by FK-506 [15,16]. CsA has also been reported to increase basal intracellular Ca^{2+} levels in human coronary myocytes [17].

Ca^{2+} re-uptake in SR of muscle cells and the endoplasmic reticulum of non-muscle cells is performed by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), a family of Ca^{2+} pumps that possess three main subtypes. The fast-twitch skeletal muscle SR Ca^{2+}-ATPase is the type 1 isoform of the SERCA family of Ca^{2+} pumps. Isoforms 2a, 2b and 3 are found predominantly in cardiac muscle, smooth muscle and non-muscle cells, respectively [18–20]. Inhibition of SERCA pumps may lead to inhibition of Ca^{2+} uptake, causing a lack of Ca^{2+} release from the cell stores in response to stimulation, and a prolonged elevation in cytosolic [Ca^{2+}]. Such changes in Ca^{2+} homoeostasis can lead to detrimental effects within the cell.

The enzyme activity of the Ca^{2+}-ATPase has been described in terms of an E1–E2 transport cycle, as proposed by de Meis and Vianna [21]. In the E1 form, the ATPase binds Ca^{2+} on the cytosolic side of the sarcoplasmic/endoplasmic reticulum membrane, while in the E2 form, the ATPase binds Ca^{2+} on the luminal side. The original model proposed that there were two Ca^{2+}-bound phosphoenzyme intermediates ($E1 \sim P \cdot Ca_2$ and $E2 \sim P \cdot Ca_2$). In recent years, however, evidence has been shown for the existence of just one phosphoenzyme intermediate ($E \sim P \cdot Ca_2$) [22]. Indeed, if there are two phosphoenzyme intermediates, these cannot be distinguished kinetically.

Here we report on the mechanism of inhibition of the macrocyclic lactones and CsA, and try to rationalize this in terms of its mechanism.

MATERIALS AND METHODS

Ivermectin (primarily ivermectin B1a) and FITC were purchased from Sigma. Rapamycin, ascomycin and CsA (the structures of which are given in Figure 1) were purchased from Calbiochem. [γ-^{32}P]ATP and [^{32}P]P_i were obtained from Amersham Bioscience. All other reagents were of analytical grade.

Abbreviations used: CsA, cyclosporin A; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TNP-ADP, trinitrophenol-ADP.

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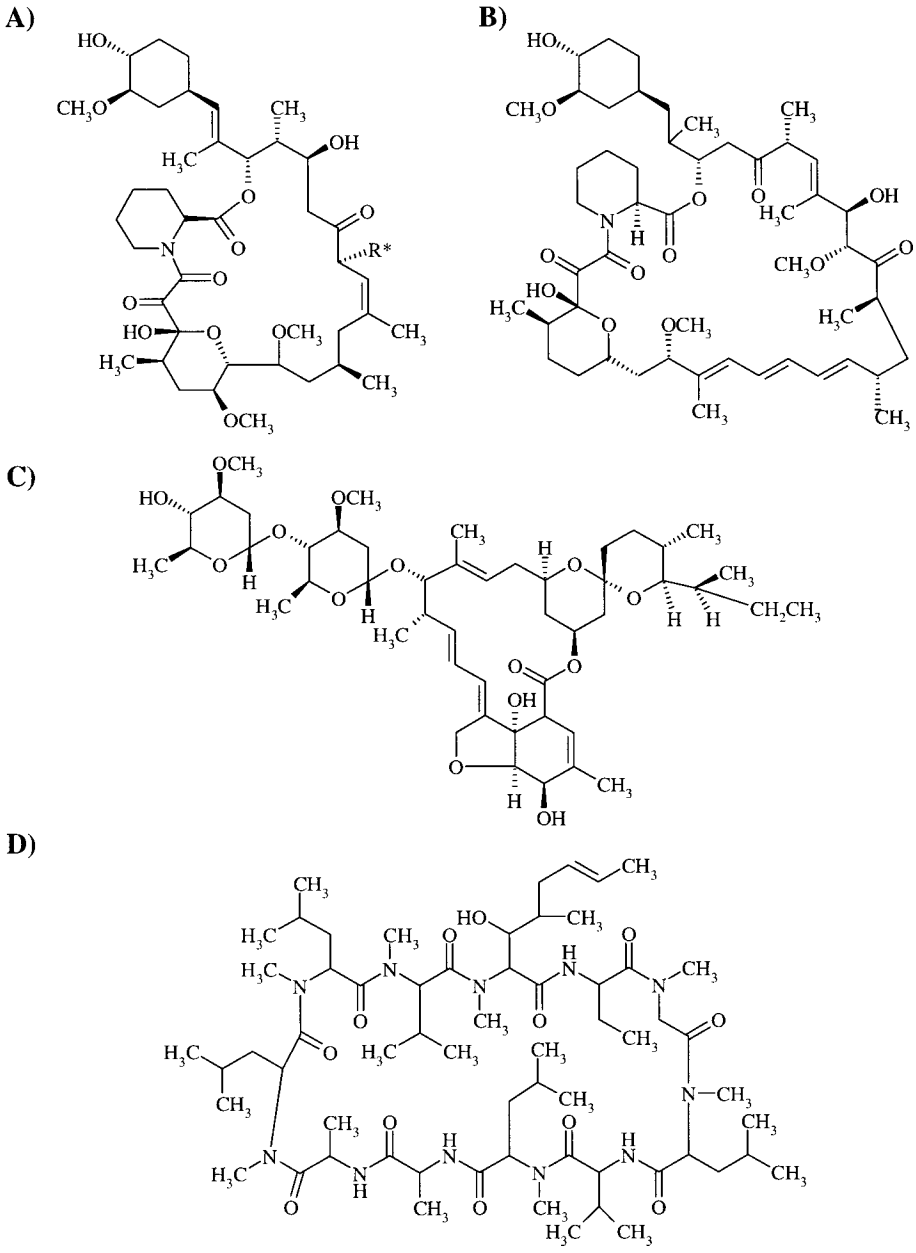

Figure 1 Structures of FK-506, ascomycin, rapamycin, ivermectin and CsA

(**A**) Structure of ascomycin (where R is CH_2—CH_3) and FK-506 (where R is CH_2—CH_2=CH_2). (**B**) Structure of rapamycin. (**C**) Structure of ivermectin B1a. (**D**) Structure of CsA.

Tissue preparation

Fast-twitch skeletal muscle SR and the purified Ca^{2+}-ATPase were prepared from rabbit as described by Michelangeli and Munkonge [23]. The rabbit skeletal muscle SR was determined to contain $\geqslant 90\%$ SERCA1 compared with SERCA2. This was determined using a SERCA2-specific antibody obtained from Santa Cruz Biotechnology and Y1F4 (a gift from Dr J. M. East, Southampton University, Southampton, U.K.), an antibody which recognizes all SERCA isoforms [24]. This was used to determine the relative proportions of all SERCA isoforms in SR and the microsomal membranes. Porcine brain microsomes were prepared as described by Bilmen et al. [25] and porcine cardiac SR was prepared as described by Tovey et al. [26].

Ca^{2+}-dependent ATPase activities

Ca^{2+}-dependent ATPase activities in skeletal muscle SR were performed using the phosphate-liberation assay as described by Longland et al. [27]. Briefly, rabbit skeletal muscle SR (2 μg/ml) or porcine brain microsomes (40 μg/ml) were resuspended in 1 ml of buffer containing 45 mM Hepes/KOH (pH 7.0), 6 mM $MgCl_2$, 2 mM NaN_3, 0.25 M sucrose, 12.5 μg/ml A23187 ionophore and EGTA with $CaCl_2$ added to give a free Ca^{2+} concentration of 10 μM. Assays were pre-incubated at 37 °C for 10 min prior to initiation with ATP (final concentration 6 mM). The reaction was then stopped by addition of 0.25 ml of 6.5 % (w/v) trichloroacetic acid. The assays were placed on ice for 10 min prior to centrifugation for 10 min at 20000 ***g***. Then,

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0.5 ml of the supernatant was added to 1.5 ml of buffer containing 11.25% (v/v) acetic acid, 0.25% (w/v) copper sulphate and 0.2 M sodium acetate. Ammonium molybdate [0.25 ml of 5% (w/v)] was then added and mixed thoroughly. ELAN (0.25 ml; *p*-methylaminophenol sulphate and 5% sodium sulphite) solution was then added. The colour intensity was measured after 10 min at 870 nm absorbance and related to amounts of inorganic phosphate released by the construction of calibration curves from known phosphate standards.

The effects of ivermectin on Ca^{2+}-ATPase activity as a function of [Ca^{2+}], [ATP], [Mg^{2+}] and pH were studied on purified skeletal muscle SR Ca^{2+}-ATPase using the coupled enzyme assay as described in [23]. Typically, 2–6 μg of purified ATPase was assayed in a buffer containing 40 mM Hepes/KOH, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 7.5 units of pyruvate kinase, 18 units lactate dehydrogenase and 1.01 mM EGTA, pH 7.2. For experiments undertaken at pH 6.0, the experiments were performed in 50 mM Mes/KOH, 5 mM $MgSO_4$, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 22.5 units of pyruvate kinase, 54 units of lactate dehydrogenase and 1.01 mM EGTA. ATP (2.1 mM) was present during Ca^{2+}-dependent experiments. Free Ca^{2+} concentrations were calculated based on the method and binding affinities described by Gould et al. [28].

Effects of drugs on the E2 (low-affinity Ca^{2+} binding)–E1 (high-affinity Ca^{2+} binding) transition

Purified ATPase was labelled with FITC according to the method described by Michelangeli et al. [29] to monitor the E2–E1 transition. Briefly, purified ATPase was added in an equal volume to the starting buffer (containing 1 mM KCl, 0.25 M sucrose and 50 mM dipotassium monohydrogen phosphate, pH 8.0). FITC in dimethylformamide was then added to give a molar ratio of FITC/ATPase of 0.5:1. The reaction was then incubated for 1 h at 25 °C. Subsequently, 0.25 ml of stopping buffer (containing 0.2 M sucrose/50 mM Tris/HCl, pH 7.0) was added and left to incubate for 30 min at 30 °C, prior to being placed on ice until required. Measurements were undertaken in a buffer containing 50 mM Tris, 50 mM maleate, 5 mM $MgSO_4$, 100 mM KCl and 100 μM EGTA at either pH 6.0 or 7.0. Free Ca^{2+} concentrations were calculated as 5 μM for pH 6.0 and 0.1 μM at pH 7.0, after addition of 100 μM EGTA, based on the constants given in [28]. Fluorescence was measured on a PerkinElmer LS50B fluorescence spectrophotometer at 25 °C (excitation 495 nm, emission 525 nm); 400 μM Ca^{2+} or 400 μM orthovanadate was added to measure change in fluorescence. Fluorescence changes were recorded as a change relative to the initial FITC fluorescence. Graphs were plotted as a relative change in FITC fluorescence when compared with that of control data.

Changes in intrinsic tryptophan fluorescence to follow Ca^{2+}-induced conformational changes

The conformational change induced by the addition of Ca^{2+} to purified ATPase was monitored by observing a change in intrinsic tryptophan fluorescence [29]. These experiments were performed in the presence of 0.05 mg/ml purified ATPase in a buffer containing 20 mM Hepes/Tris, 100 mM KCl and 5 mM $MgSO_4$, pH 7.2. The conformational change was measured as a percentage change in total tryptophan fluorescence, over a range of free Ca^{2+} concentrations (0.01–100 μM), as previously defined by calculations based on constants given in [28]. Fluorescence was measured on a PerkinElmer LS50B fluorescence spectrophotometer at 25 °C (excitation 295 nm, emission 330 nm).

Measurement of Ca^{2+} release from the purified ATPase during enzyme turnover

The Ca^{2+}-ATPase binds Ca^{2+} in the absence of ATP. Upon addition of ATP, Ca^{2+} is released from the ATPase as the enzyme passes through Ca^{2+}-free steps in the Ca^{2+}-transport cycle. The amount of Ca^{2+} released from purified Ca^{2+}-ATPase during enzyme turnover was measured in 2.5 ml of buffer containing 150 mM Mes/Tris (pH 6.0), 20 mM $MgSO_4$ and 0.1 mM Antipyralazo III at 25 °C. Purified Ca^{2+}-ATPase (0.4 mg/ml) was added and incubated in buffer 10 min prior to the start of the experiment [30]. Measurements were taken on a Shimadzu UV-3000 dual-wavelength spectrophotometer, measuring the absorbance difference between 720 and 790 nm. Calibrations were performed with each experiment by addition of known amounts of Ca^{2+}. Addition of 5 μl of a 10 mg/ml ATP solution was used to start the reaction.

Phosphorylation studies

Maximum levels of phosphorylation of the ATPase by [γ-^{32}P]-ATP were measured at 25 °C as described by Bilmen et al. [25]. Purified ATPase was diluted to 75 μg/ml in 20 mM Hepes/Tris (pH 7.2) containing 100 mM KCl, 5 mM $MgSO_4$ and 1 mM $CaCl_2$ in a total volume of 1 ml. Stocks of 0.5 and 5 mM ATP were made to cover the range between 0 and 100 μM (specific radioactivities 100 and 10 Ci/mol, respectively). The reaction was initiated by addition of [γ-^{32}P]ATP and stopped 15 s later by addition of ice-cold 40% (w/v) trichloroacetic acid. The assay was then placed on ice for 30 min subsequent to the addition of BSA (final concentration, 1 mg/ml). The precipitate was then collected by filtration through Whatman GF/C filters. The filters were washed with 12% (w/v) trichloroacetic acid/0.2 M H_3PO_4, and left to dry. The filters were then placed in scintillant and counted for radioactivity.

Phosphorylation by [^{32}P]P_i was carried out as described in [31]. Purified ATPase was added to a buffer containing 150 mM Mes/Tris (pH 6.2), 5 mM EGTA and 10 mM $MgSO_4$, to give a final concentration of 0.6 mg/ml protein in 300 μl. The experiment was initiated by addition of 2 mM P_i (specific radioactivity, 17 Ci/mol) and stopped 20 s later by addition of 250 μl of 40% (w/v) trichloroacetic acid/0.2 M H_3PO_4. The precipitate was collected by rapid filtration through Whatman GF/C filters and washed with 30 ml of 12% (w/v) trichloroacetic acid/0.2 M H_3PO_4. The filters were left to dry, then placed in scintillant and counted for radioactivity.

Trinitrophenol-ADP (TNP-ADP) binding to the Ca^{2+}-ATPase

Binding of TNP-ADP, a spectroscopic analogue of ATP, to the purified Ca^{2+}-ATPase was measured in the absence and presence of drugs as described in [32]. Briefly, purified ATPase was diluted to 0.8 mg/ml in a buffer containing 50 mM Mops/KOH (pH 7.0)/1 mM $CaCl_2$. This was titrated with TNP-ADP (0–14 μM) in a Shimadzu UV-3000 dual-wavelength spectrophotometer, and the absorbance difference was measured at 422 and 390 nm.

RESULTS

Figure 2 shows the effects of the various drugs on Ca^{2+}-dependent ATPase activity in skeletal muscle SR and porcine brain microsomal extracts. All experiments were fully uncoupled due to the presence of Ca^{2+} ionophore A23187. In Figure 2(A), the

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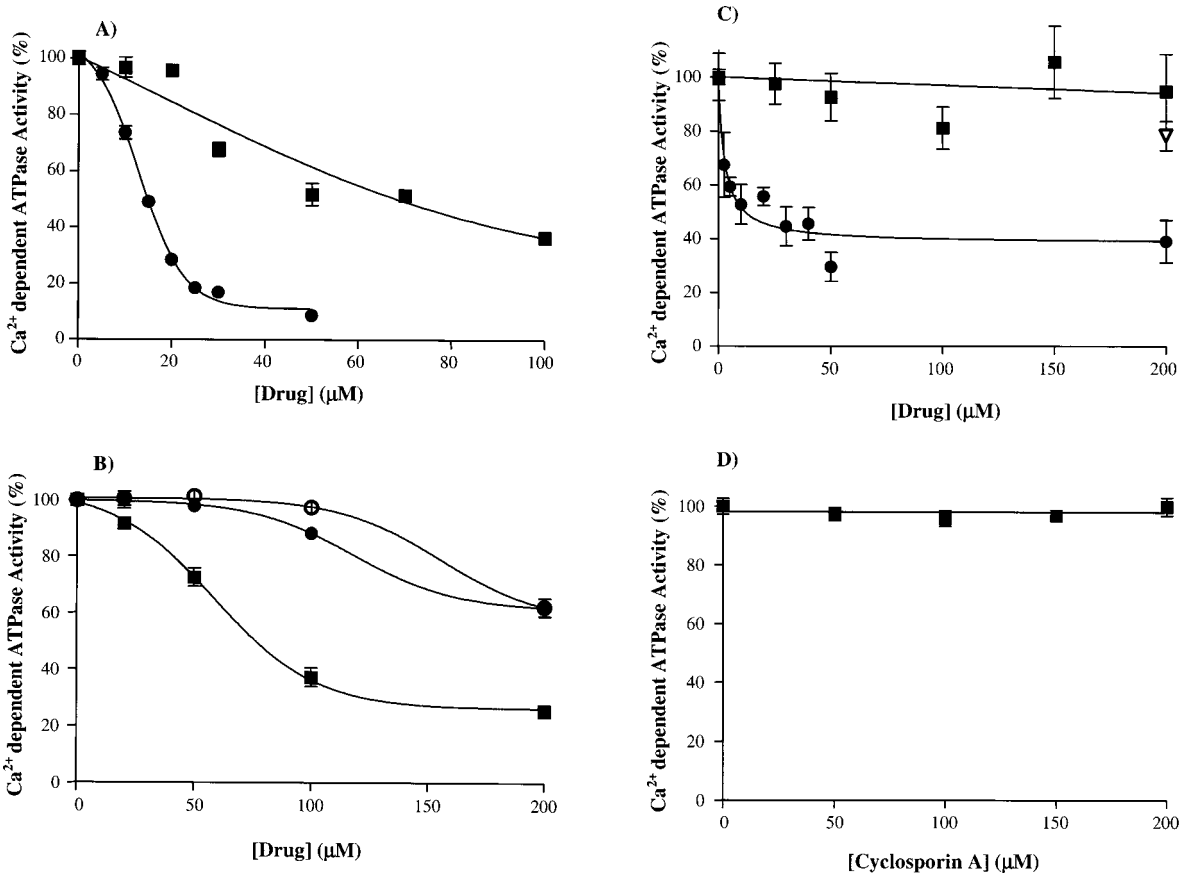

Figure 2 Inhibition of Ca^{2+}-dependent ATPase activity by macrocyclic lactones and CsA

(**A**) The inhibition of skeletal muscle SR Ca^{2+}-dependent ATPase activity (2 μg/ml) by ivermectin (●) and CsA (■). (**B**) SR Ca^{2+}-dependent ATPase activities in the presence of various concentrations of rapamycin (■), ascomycin (○) and FK-506 (●). (**C**) The inhibition of brain microsomal ER Ca^{2+}-dependent ATPase activities by ivermectin (●), CsA (■) and rapamycin (▽). (**D**) The inhibition of cardiac SR by CsA (■). All experiments were performed at 37 °C, pH 7.2. Each data point represents the mean ± S.D. of three determinations.

compounds ivermectin and CsA can be seen to inhibit the Ca^{2+}-dependent ATPase activity in skeletal muscle SR (SERCA1), with IC_{50} values of 14.9 ± 0.35 μM and 62 ± 12 μM, respectively. At the highest concentration of ivermectin used (50 μM) greater than 90 % of the activity was inhibited. In Figure 2(B), the effects of ascomycin, FK-506 and rapamycin were tested. Only rapamycin appeared to inhibit Ca^{2+}-dependent ATPase activity substantially ($IC_{50} = 77 \pm 2$ μM), whereas ascomycin and FK-506 required considerably high concentrations to cause any effect.

Figure 2(C) illustrates the effects of ivermectin and CsA on the Ca^{2+}-dependent ATPase activity in brain microsomes (SERCA2b). In the presence of ivermectin, the Ca^{2+}-dependent ATPase activity appears to be maximally inhibited by about 60 %. This maximal inhibition is achieved at a concentration of about 30 μM ivermectin, with higher concentrations showing little extra effect on activity. Interestingly, CsA and rapamycin (at 200 μM) had no significant effects on the Ca^{2+}-dependent ATPase activity of brain microsomes that could be detected. In addition, Figure 2(D) shows that no Ca^{2+}-ATPase inhibition was observed in cardiac SR (SERCA2a). This possibly implies that CsA could be an isoform-specific inhibitor.

We have previously shown that around 70 % of the Ca^{2+}-pump activity in brain microsomes is thapsigargin-sensitive [33]. With the preparation used in this study, it was found that the inhibition induced by either 200 μM ivermectin alone or in conjunction with 3 μM thapsigargin gave similar levels of inhibition (approx. 60 %). Thus both ivermectin and thapsigargin affect the same Ca^{2+}-ATPase pool.

Figure 3 illustrates the inhibition of the purified Ca^{2+}-ATPase from skeletal muscle using the coupled enzyme assay. Differing concentrations of ATPase were used to determine the inhibitory effects of ivermectin. When a concentration of 2.8 μg/ml purified Ca^{2+}-ATPase was assayed, ivermectin exhibited a strong inhibition, with an IC_{50} of 6.4 ± 1.0 μM. However, when 5.6 μg/ml ATPase was used, the inhibition of activity by ivermectin decreased, with an IC_{50} of 19.2 ± 2.0 μM. We also investigated its effect on the inhibition of the Ca^{2+}-ATPase at pH 6.0 (Figure 3, inset). The inhibitor appears to be more effective at pH 6.0, with an IC_{50} of 2 ± 0.5 μM, although the level of inhibition appeared to plateau after about 4 μM, with about 65 % of the activity inhibited.

The inhibition of ivermectin on purified ATPase activity (2.8 μg/ml) can be reversed by addition of phosphatidylcholine liposomes. Purified ATPase activity in the presence of 5 μM ivermectin was 10.1 ± 0.3 i.u./mg. However, after addition of 100 μM phosphatidylcholine, this inhibition was reversed (Ca^{2+}-ATPase activity of 16.0 ± 0.7 i.u./mg). This shows ivermectin to be a reversible inhibitor that may equilibrate in the lipid phase, and therefore any excess lipid present may act as a 'sink' for the drug. This effect may explain the different IC_{50} values observed

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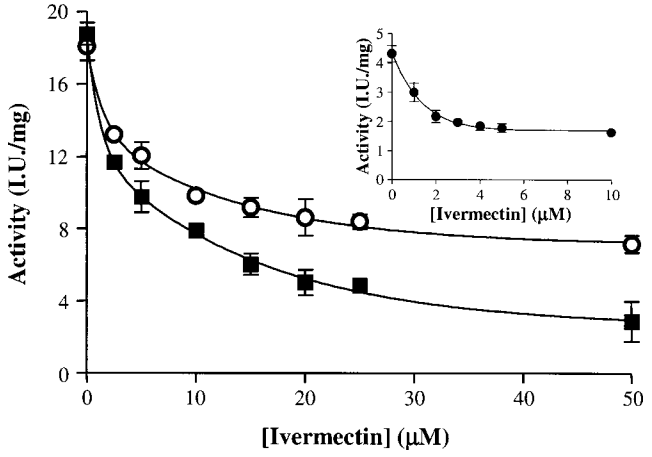

Figure 3 Inhibition of purified Ca^{2+}-ATPase activity by ivermectin

Ca^{2+}-ATPase activity was measured at 2.8 μg/ml (○) and 5.6 μg/ml (■) ATPase, pH 7.2. Inset: ATPase activity measured at 2.8 μg/ml ATPase, pH 6.0. All experiments were performed at 37 °C. Each data point represents the mean ± S.D. of three determinations.

at various ATPase concentrations and with various membrane preparations.

Figure 4 illustrates the inhibitory effects of ivermectin on purified Ca^{2+}-ATPase activity at varying concentrations of free [Ca^{2+}], [ATP] and [Mg^{2+}] using the coupled enzyme assay. In Figure 4(A), the effects of varying Ca^{2+} concentrations on purified ATPase (2.8 μg/ml) activity can be seen in the absence and presence of 7 μM ivermectin. In both the absence and presence of drug, the Ca^{2+}-ATPase activity exhibited a classical bell-shaped profile with respect to free [Ca^{2+}] [34]. The stimulatory phase has been associated with the binding of Ca^{2+} to the ATPase in an E1 (high-affinity) form, while the inhibitory phase is, in part, associated with Ca^{2+} binding to the E2 (low-affinity) state. In the absence of drug, the data had a V_{max} of 19.9 ± 0.7 i.u./mg, with an EC_{50} for the stimulatory phase of 0.9 ± 0.01 μM and an IC_{50} value for the inhibitory phase of 0.24 ± 0.02 mM. In the presence of 7 μM ivermectin, the EC_{50} and IC_{50} values did not change significantly (EC_{50} = 0.91 ± 0.03 μM and IC_{50} = 0.24 ± 0.02 mM), while the V_{max} decreased to 10.4 ± 0.6 i.u./mg.

In Figure 4(B) the effects of Mg^{2+} on purified Ca^{2+}-ATPase activity in the absence and presence of 7 μM ivermectin were investigated. Maximal ATPase activity occurs in the presence and absence of ivermectin at 2.5 mM Mg^{2+}. In the control data, half-maximal inhibition of Ca^{2+}-ATPase activity by Mg^{2+} occurred at 13.3 ± 0.8 mM. However, at 7 μM ivermectin, the half-maximal inhibition of Ca^{2+}-ATPase activity by $MgSO_4$ was decreased slightly, to 10.7 ± 0.8 mM.

Figure 4(C) shows the activity of the Ca^{2+}-ATPase at various ATP concentrations. These experiments have previously been shown to fit well to a bi-Michaelis–Menten equation assuming a high-affinity catalytic site and lower-affinity regulatory site [35]. The control data fitted well, assuming high-affinity K_m and V_{max} of 1.43 ± 0.85 μM and 5.19 ± 0.51 i.u./mg, respectively, and a lower-affinity K_m and V_{max} of 0.24 ± 0.07 mM and 16.3 ± 1.4 i.u./mg, respectively. In the presence of ivermectin, good fits of the data were obtained, assuming a high-affinity K_m and V_{max} of 2.29 ± 0.44 μM and 4.87 ± 0.16 i.u./mg, respectively, and a lower-affinity K_m and V_{max} of 1.15 ± 0.39 mM and 8.35 ± 1.22 i.u./mg, respectively. As can be seen, the effects of ivermectin on the ATPase seem to be predominantly on the

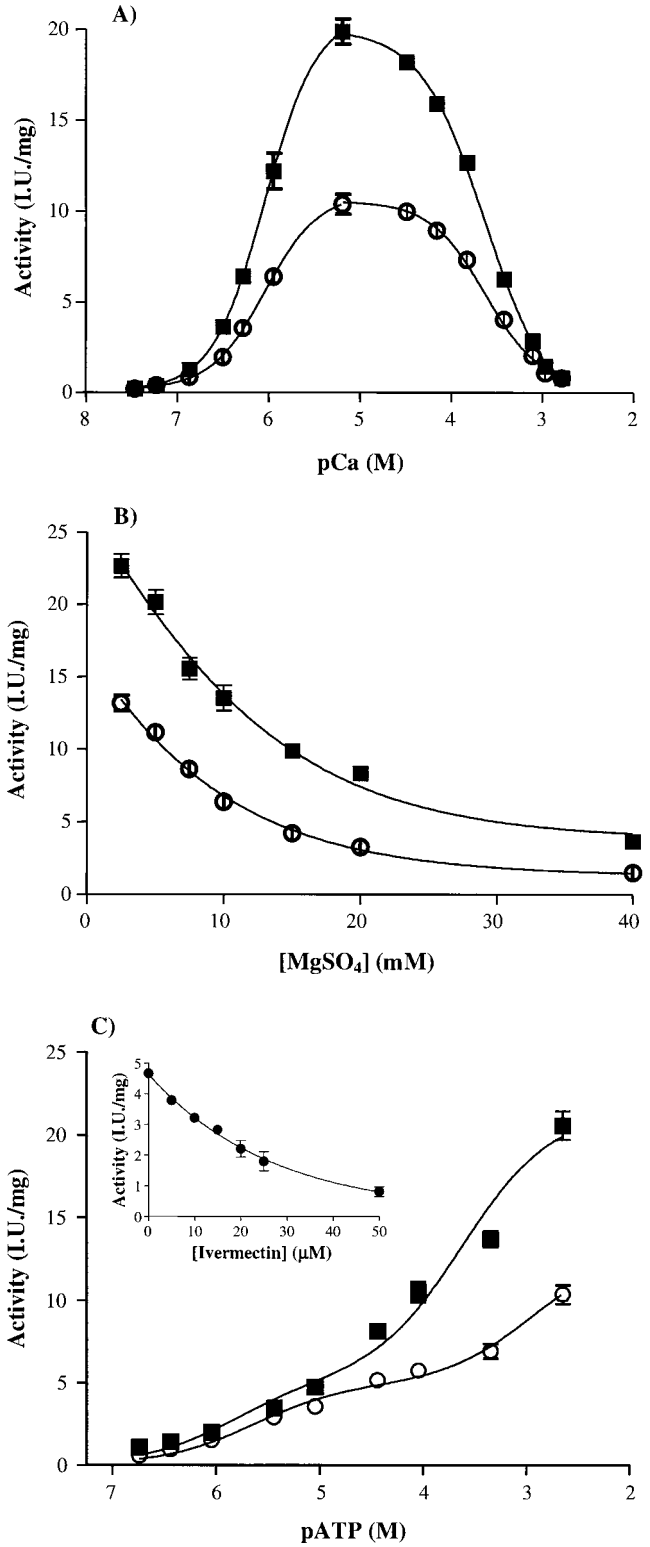

Figure 4 Effects of ivermectin on the purified skeletal muscle Ca^{2+}-ATPase activity as a function of free [Ca^{2+}], [Mg^{2+}] and [ATP]

Activities of the Ca^{2+}-ATPase were measured at 37 °C using the coupled enzyme assay, at pH 7.2. The activity of purified Ca^{2+}-ATPase was measured as a function of free [Ca^{2+}] (**A**), [Mg^{2+}] (**B**) and [ATP] (**C**), in the absence (■) or presence (○) of 7 μM ivermectin. (**C**) Inset: inhibition of purified Ca^{2+}-ATPase activity by ivermectin at low (10 μM) [ATP]. Each data point is the mean ± S.D. of 3–6 determinations. pCa, −log[Ca]; pATP, −log[ATP]

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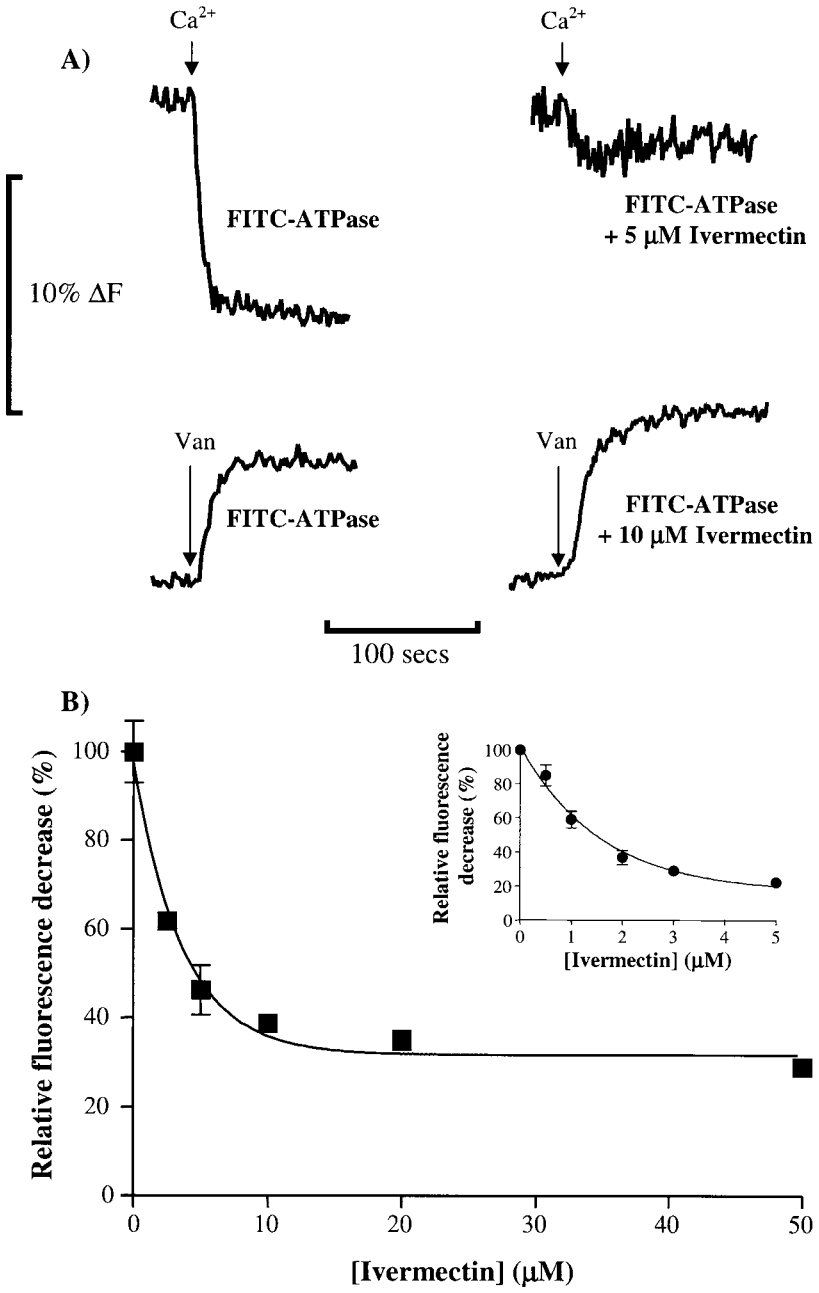

Figure 5 The measurement of E2–E1 conformational change using FITC-labelled Ca^{2+}-ATPase

(**A**) Effects of ivermectin on the fluorescence change in FITC-ATPase induced by either 400 μM Ca^{2+} or 400 μM orthovanadate (Van), in the presence or absence of ivermectin at pH 6.0. (**B**) The effects of ivermectin on the fluorescence changes induced by Ca^{2+} at pH 7.0 (■). Inset: the effects of ivermectin on the fluorescence changes induced by Ca^{2+} at pH 6.0 (●). Experiments were performed at 25 °C and each data point is the mean ± S.D. of three determinations.

lower-affinity site, with little effect on the higher-affinity ATP site. The low-affinity 'regulatory' K_m seems to have increased nearly 5-fold. Ivermectin may therefore preferentially inhibit the lower-affinity ATP-dependent activation of the Ca^{2+}-ATPase. To investigate this further, the inhibition of Ca^{2+}-ATPase activity by ivermectin at low ATP (10 μM) was monitored (Figure 4C, inset). The IC_{50} under these conditions was 19 ± 1 μM, which is nearly a 3-fold decrease in the inhibitory constant for ivermectin (i.e. 6.4 μM at 2.1 mM ATP), clearly demonstrating that ivermectin preferentially exerts its effects at high [ATP], thus affecting the lower-affinity (regulatory) ATP-binding site [30,36].

Figure 5 illustrates the effects of ivermectin on the FITC-labelled Ca^{2+}-ATPase. FITC-ATPase has been shown to change its fluorescence upon transition from the E2 to the E1 state [29]. In Figure 5(A), traces are shown illustrating the change in FITC fluorescence in the absence and presence of ivermectin at pH 6.0. The addition of excess Ca^{2+} to FITC-ATPase at pH 6.0 is known to cause the enzyme to undergo a transition from an E2 state to the E1 state. At pH 6.0 the addition of Ca^{2+} to FITC-ATPase

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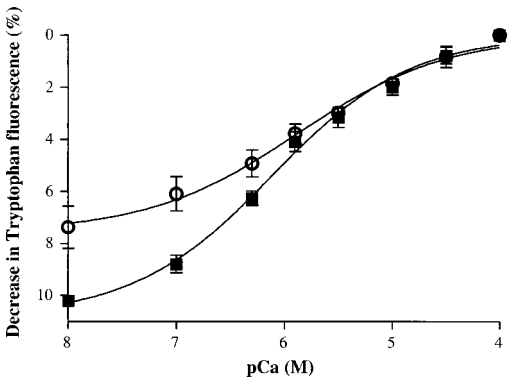

Figure 6 Changes in tryptophan fluorescence of purified Ca^{2+}-ATPase as a function of free [Ca^{2+}] in the absence and presence of 50 μM ivermectin

Purified Ca^{2+}-ATPase (0.05 mg/ml) was incubated in buffer at pH 7.2 and the effects of different free [Ca^{2+}] on the tryptophan fluorescence intensities were observed at 25 °C. The change in tryptophan fluorescence was measured in the absence (■) and presence (○) of 50 μM ivermectin. Each data point represents the mean ± S.D. of 3–4 determinations.

caused an 8.3 ± 0.1 % decrease in fluorescence. In the presence of 5 μM ivermectin, however, the fluorescence change was dramatically reduced to 1.8 ± 0.2 %. Addition of orthovanadate, a drug known to bind the Ca^{2+}-ATPase in an E2 form, caused a fluorescence increase of 5.3 ± 0.3 % in the absence of ivermectin. At 10 μM ivermectin, however, this fluorescence change was increased to 7.7 ± 0.1 %.

Figure 5(B) illustrates the percentage change in fluorescence decrease of FITC-labelled ATPase by addition of Ca^{2+} at varying ivermectin concentrations. At pH 7.0, the IC_{50} for ivermectin is 4.8 ± 1.0 μM. At pH 6.0, this value is 1.5 ± 0.3 μM. These values are comparable with the activity data (Figure 3). In addition, 20 μM ivermectin induced about a 3 % decrease in fluorescence when added to FITC-ATPase at pH 7.0 in the absence of Ca^{2+}. However, since this change did not occur upon subsequent additions of ivermectin, it is most consistent with it causing an E1 conformational change directly rather than causing non-specific quenching. All these observations taken together would therefore suggest that ivermectin binds to the Ca^{2+}-ATPase and stabilizes it in an E1 conformational state.

To assess whether the conformational change associated with Ca^{2+} binding was affected by ivermectin, the change in tryptophan fluorescence associated with this conformational change was measured (Figure 6). In the absence of drug, the addition of Ca^{2+} gave an ΔF_{max} of 10.2 ± 0.2 % and a K_d value of 1.0 ± 0.2 μM. In the presence of 50 μM ivermectin, however, the ΔF_{max} was slightly decreased to 7.4 ± 0.2 % while the K_d value was unaffected (1.3 ± 0.3 μM). The fact that the K_d was not altered by addition of drug implies that ivermectin has little effect on the Ca^{2+} binding to the ATPase, in agreement with the activity data presented in Figure 4(A).

In order to assess whether ivermectin inhibited Ca^{2+} release from the Ca^{2+}-ATPase during enzyme turnover following the addition of ATP, the effects of ivermectin on Ca^{2+} released from the Ca^{2+}-ATPase at pH 6.0 during cycling were measured. The release of Ca^{2+} from the ATPase is significantly decreased in the presence of 50 μM ivermectin (i.e. Ca^{2+} released during turnover in the absence of inhibitor was 7.79 ± 0.25 nmol/mg of ATPase, while in the presence of 50 μM ivermectin this was reduced to 5.12 ± 0.32 nmol/mg; $P < 0.001$). Thus, in the presence of ivermectin, the ATPase favours the Ca^{2+}-bound state.

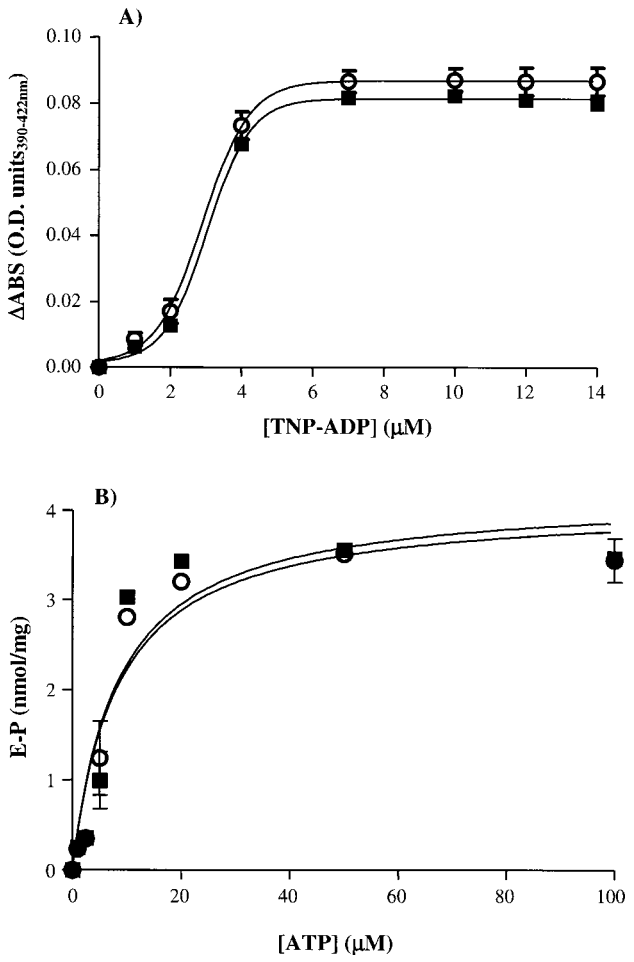

Figure 7 Effects of ivermectin on ATP-dependent phosphorylation and TNP-ADP binding

(**A**) Binding of TNP-ADP (0–14 μM), a spectroscopic analogue of ATP, to the purified Ca^{2+}-ATPase. Binding was observed by measuring the difference in absorbance between 390 and 422 nm, at pH 7.2 and 25 °C in the absence (■) and presence (○) of 50 μM ivermectin. Data points represent the means ± S.D. of three determinations. (**B**) Phosphorylation of purified Ca^{2+}-ATPase by [γ-^{32}P]ATP (0–100 μM) in the absence (■) and presence (○) of 50 μM ivermectin, measured at pH 7.2 and 25 °C. Values represent the mean ± S.D. of 3–5 determinations.

Figure 7(A) shows the effects of ivermectin on the binding of TNP-ADP (an analogue of ATP) to purified Ca^{2+}-ATPase. The control data gave a maximal ΔA of 0.08 ± 0.001 absorbance unit, with a K_d of 3.0 ± 0.1 μM. In the presence of 50 μM ivermectin, these values were not significantly changed (ΔA of 0.086 ± 0.004 absorbance unit and a K_d of 2.9 ± 0.1 μM respectively). Hence it is unlikely that ivermectin inhibits ATP binding to the Ca^{2+}-ATPase at the high-affinity catalytic site.

Figure 7(B) illustrates the ATP-dependent phosphorylation of the purified Ca^{2+}-ATPase, as measured using [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}. Phosphorylation data for the control gave a K_d and $E \sim P_{max}$ (the maximum level of phosphoenzyme that can be formed) of 8.4 ± 3.6 μM and 4.2 ± 0.5 nmol/mg of protein, respectively. These values were little changed in the presence of 50 μM ivermectin (K_d and $E \sim P_{max}$ of 8.3 ± 2.8 μM and 4.1 ± 0.4 nmol/mg of protein, respectively).

Ivermectin (50 μM) significantly inhibited phosphorylation of the Ca^{2+}-ATPase by [^{32}P]P_i at pH 6.0 and 10 mM Mg^{2+} (i.e. in

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the absence of inhibitor the amount of E2-P formed was 1.51 ± 0.03 nmol/mg of ATPase, while in the presence of 50 μM ivermectin this was reduced to 0.6 ± 0.04 nmol/mg; $P < 0.001$). This decrease in phosphorylation may be explained by the drug binding the Ca^{2+}-ATPase in an E1 form, thereby reducing the amount of E2 enzyme available to be phosphorylated with P_i.

DISCUSSION

The uses of the macrocyclic lactones in medicine are varied. Ivermectin, an anti-helminthic agent [8], has previously been reported to be an inhibitor of ATP-dependent Ca^{2+} uptake [13]. The immunosuppressive drugs FK-506 and rapamycin are believed to exert their effects, in part, by binding to the immunophilin FK-506-binding protein (FKBP12) [6]. Here we report that ivermectin, CsA and rapamycin are inhibitors of the skeletal muscle SR Ca^{2+}-ATPase (SERCA1), but, of the macrocyclic lactones tested, only ivermectin proved to be an inhibitor of the non-muscle SERCA 2b Ca^{2+} pump, which may indicate an isoform-specific effect. The fact that these drugs are lipophilic in nature, coupled with the fact that different membrane preparations might have different lipid contents, may suggest that the differences seen with CsA on Ca^{2+}-ATPase activity in SR and brain microsomes might be due to their different lipid content. However, in a previous study where another hydrophobic drug, curcumin, was investigated on the Ca^{2+}-ATPase from skeletal muscle SR and brain microsomes using identical procedures as in this study, only about a 2-fold difference in potency was observed [25]. Since in this study we showed that CsA has no effect on brain microsomes at concentrations up to 200 μM, while in SR it has an IC_{50} of 62 μM, the lack of CsA inhibition cannot solely be attributed to excess lipid and therefore there must be some degree of isoform specificity in its inhibition.

The mechanism of inhibition of the SERCA1 Ca^{2+} pump by ivermectin was studied in detail. From the experiments carried out here, we are able to determine that ivermectin seems to inhibit the Ca^{2+}-ATPase by stabilizing it in an E1 conformation, without effects on Ca^{2+} binding. In addition, we have shown that ivermectin does not affect ATP binding to the high-affinity (catalytic) site. Interestingly, the equilibrium phosphorylation by $[^{32}P]P_i$ is significantly reduced in the presence of ivermectin but in the absence of Ca^{2+}. This may be explained by the fact that this compound has been shown to bind the ATPase in an E1 conformation, and hence reduces the amount of E2 form available to bind with P_i.

The enzyme activity at varying [ATP] has previously been shown to fit well to a bi-Michaelis–Menten equation, implying two ATP-binding sites [35]. There have been two explanations proposed for this behaviour: either there are two ATP-binding sites of high (catalytic) and low (regulatory) affinity, or there is a single binding site which changes its affinity for ATP upon phosphorylation, which in turn promotes faster enzyme turnover [28]. The resolved structure of the Ca^{2+}-ATPase to 2.6 Å has only identified a single ATP-binding site, suggesting that the latter explanation is most likely [37].

The fact that ivermectin still binds and affects the Ca^{2+}-ATPase in the presence of FITC, and does not affect TNP-ADP binding, would imply that the compound binds to a site distant from that of the ATP-binding site. From the activity data, it is suggested that ivermectin inhibits the stimulatory effects of ATP observed at high concentrations, as this can be partially reversed by further elevating the ATP concentration. The stimulatory effect of high [ATP] on Ca^{2+}-ATPase activity is pH-dependent, since at pH 6.0 this stimulation is markedly reduced [38]. Ivermectin inhibits the ATPase activity at pH 6.0 with an IC_{50} of 1–2 μM, but can only reduce the overall activity by about 60–65 %, after which no further inhibition is observed (up to 10 μM). This observation can well be explained by the fact that at low concentrations ivermectin specifically reverses the steps stimulated by high [ATP], without affecting the catalytic turnover, which accounts for some 30–40 % of the total activity (Figure 4C).

In the original SR Ca^{2+}-ATPase transport cycle model as proposed by de Meis and Vianna in 1979 [21], it was proposed that there were two phosphoenzyme intermediates with Ca^{2+} bound ($E1 \sim P \cdot Ca_2$ and $E2 \sim P \cdot Ca_2$). Recent work, however, has proposed that there may be only one intermediate [22]. Indeed, if there were two intermediates, then these cannot be kinetically distinguished, since the transition between these steps would be very fast (rate constant $> 100\ s^{-1}$). In addition, the rate of ATP-dependent phosphorylation of the Ca^{2+}-ATPase ($E1 \cdot ATP \cdot Ca_2 \rightarrow E \sim P \cdot Ca_2$) is very fast [22]. Hence these steps are unlikely to be rate-limiting and thus accelerated by high [ATP]. Therefore, it is unlikely that ivermectin is affecting this step.

The fact that ivermectin binds to the Ca^{2+}-ATPase in an E1 form, inhibits the Ca^{2+}-ATPase in a Ca^{2+}-bound form, but does not affect ATP binding to the catalytic site, would imply that the enzyme is inhibited in either an $E1 \cdot ATP \cdot Ca_2$ or an $E \sim P \cdot Ca_2$ form. It has been previously shown that high [ATP] accelerates the Ca^{2+} release from the ATPase into the lumen (i.e. $E \sim P \cdot Ca_2 \rightarrow E2 \sim P$) [30], dephosphorylation of the enzyme (i.e. $E2 \sim P \rightarrow E2$) [36] and transition from the low-affinity Ca^{2+}-binding conformation to the high-affinity Ca^{2+}-binding conformation (i.e. $E2 \rightarrow E1$) [39]. From the data presented here, it would seem most likely that ivermectin inhibits the Ca^{2+}ATPase by slowing Ca^{2+} release from the enzyme into the lumen (i.e. $E \sim P \cdot Ca_2 \rightarrow E2 \sim P$), and thereby reversing the acceleration of this step caused by high concentrations of ATP. At higher concentrations of ivermectin, however, this step is likely to be inhibited further, even at low ATP concentrations. Interestingly, a similar type of inhibition has been proposed for spermine on the Ca^{2+}-ATPase [40].

In summary, of the compounds tested here ivermectin appears to be the most effective inhibitor of the SERCA family of Ca^{2+}-ATPases, and particularly the SERCA1 isoform. The inhibition of the Ca^{2+}-ATPase may play a contributory factor in the side effects of some of these drugs, as ivermectin has been reported to affect muscular activity in animals when given at high doses to treat parasite infestations [41].

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