Binding of sesquiterpene lactone inhibitors to the Ca^{2+} -ATPase

Matthew WICTOME, Yamin M. KHAN, J. Malcolm EAST and Anthony G. LEE

Department of Biochemistry and Institute for Biomolecular Sciences, University of Southampton, Southampton, Hants. SO9 3TU, U.K.

The mechanism of inhibition of the Ca^{2+} -ATPase from sarcoplasmic reticulum by the sesquiterpene lactones thapsigargin, trilobolide and thapsivillosin A (TvA) has been determined. A decrease in the affinity of the ATPase for Ca^{2+} is observed in the presence of the inhibitors (I), consistent with a shift in the E1/E2 equilibrium for the ATPase towards E2 forms. Amounts of inhibitor beyond a 1:1 molar ratio with ATPase produce no further decrease in affinity for Ca^{2+} , inconsistent with the formation of a dead-end complex. Measurements of the rate of quenching of the tryptophan fluorescence of the ATPase by TvA are consistent with an association step to give E2I followed by an isomerization to a modified state E2^AI. The kinetics of the

reversal of the effects of TvA by Ca^{2+} at sub-stoichiometric amounts of TvA are bi-exponential, with a fast component whose rate is independent of TvA concentration and equal to the rate observed in the absence of TvA, and a slow component whose rate decreases with increasing TvA concentration. These observations are also consistent with the formation of a modified state E2^AI following the initial binding of I to E2. The equilibrium constant E2^AI/E2I increases in the order TvA < trilobolide < thapsigargin. The results suggest that the effects of the inhibitors on the overall ratio of E2 to E1 forms of the ATPase follow largely from the formation of E2^AI from E2I, and that binding constants are very similar for E1Ca₂, E1 and E2.

INTRODUCTION

Sesquiterpene lactones related to thapsigargin are specific inhibitors of the Ca^{2+} -ATPase of sarcoplasmic reticulum (SR) and of endoplasmic reticulum, with no effects on the Ca^{2+} -ATPase of the plasma membrane or on other P-type ATPases [1–5]. Members of this class of inhibitor include thapsigargin itself and thapsigarginin, both isolated from *Thapsia garganica* [6], thapsivillosin A (TvA) isolated from *Thapsia villosa* [7–9] and trilobolide, extracted from *Laser trilobum* [9,10] (for structures, see insert to Figure 1). The inhibitors are of high affinity, e.g. approx. 0.2 nM for thapsigargin [11]. The stoichiometry of binding to the Ca^{2+} -ATPase is 1:1, although the 1:1 stoichiometry has been reported both with respect to the total ATPase protein present in the preparation [4,12] and with respect to the proportion of the ATPase that is active (typically approx. 30–50%), as defined by the maximum level of phosphorylation of the ATPase by ATP observed under conditions where the rate of dephosphorylation is low [2,3,12].

The effects of thapsigargin on the function of the Ca^{2+} -ATPase have been interpreted in terms of the E2–E1 model for ATPase [13]. It is postulated that, in the absence of any ligands, the ATPase exists in one of two conformations, E1 or E2. In the E1 conformation the ATPase can bind two Ca^{2+} ions on the cytoplasmic side of the SR. Binding of MgATP then leads to phosphorylation of the ATPase and a change of conformation to E2PCa₂, from which Ca^{2+} can dissociate into the lumen of the SR; the ATPase then dephosphorylates and recycles to E1. Binding of Ca^{2+} to E1 is sequential and co-operative, according to Scheme 1 [14].

The E2–E1 equilibrium has been studied using fluorescently labelled ATPase. The fluorescence of the ATPase labelled with 4-

nitrobenzo-2-oxa-1,3-diazole (NBD) is sensitive to the E2–E1 transition, with E2, E1 and E1Ca₂ being states of low, high and intermediate fluorescence intensity respectively [14–17]. The E2–E1 transition can also be detected by observing changes in the fluorescence of the ATPase labelled with fluorescein isothiocyanate (FITC) [18–20], but the intrinsic pH-sensitivity of the fluorescence of the fluorescein group [20] makes this a less convenient probe. From studies of both NBD-labelled [5,9,17] and FITC-labelled [21] ATPase, it has been reported that binding of thapsigargin to the ATPase shifts the E2–E1 equilibrium strongly towards E2. Changes in the fluorescence intensity of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) on binding of Mg²⁺, H⁺ and K⁺ have been shown to parallel changes in the rates of dissociation of Ca^{2+} from the ATPase, and it has been suggested that the fluorescence of Br-DMC-labelled ATPase is sensitive to changes at a 'gating' site controlling access to the Ca^{2+} binding sites on the ATPase [22,23].

Here we use these fluorescence probes in equilibrium and rapid kinetic measurements to characterize conformational changes on the ATPase involved in inhibitor binding.

MATERIALS AND METHODS

ATPase was purified from skeletal muscle SR as described in East and Lee [24]. Concentrations of protein were estimated by using the specific absorption coefficient (1.2 litre · g⁻¹ · cm⁻¹ for a solution in 1% SDS) given by Hardwicke and Green [25]. Thapsigargin was obtained from Calbiochem. Trilobolide was generously provided by M. Holub (Institute of Organic Chemistry, Czechoslovak Academy of Sciences, Prague, Czech Republic).

TvA was extracted from *Thapsia villosa* roots largely as described by Rasmussen et al. [7]. Chopped root material (300 g) was extracted twice with 96% (v/v) ethanol by stirring at room temperature for 24 h. Evaporation of the ethanol yielded a gum which was dissolved in 1:1 (v/v) ethyl acetate/water. The organic layer was concentrated *in vacuo* to produce approx. 30 g of crude extract. A 10 g portion of the crude extract was purified by column chromatography on silica gel 60 (200 g; BDH–Merck;

Scheme 1

0.063–0.2 mM standardized to contain 10 % water) packed with dichloromethane. The column was eluted with dichloromethane containing increasing concentrations of ethyl acetate. Lactone-containing fractions (15–20 % ethyl acetate) were pooled and concentrated *in vacuo* to give approx. 600 mg of product. This was dissolved in ethyl acetate (3 ml) and rechromatographed on silica gel 60 (30 g) packed with petroleum ether (b.p. 60–80 °C). Material was eluted with increasing concentrations of ethyl acetate. The major product was TvA eluting at 25–30 % ethyl acetate, with an R_f value of approx. 0.3 in 1:9 (v/v) methanol/chloroform. It was characterized by IR spectroscopy, fast-ion bombardment mass spectrometry, ^1H NMR and HPLC.

Inhibitors were added to the ATPase from concentrated solutions (7.7 mM) in DMSO.

The tryptophan fluorescence of the ATPase was recorded by using an SLM-Aminco 8000C fluorimeter, with excitation and emission wavelengths of 295 and 340 nm respectively. Ca^{2+} titrations were performed by addition of aliquots of a stock solution of EGTA (500 mM) to vary the free Ca^{2+} concentration. Free concentrations of Ca^{2+} were calculated using the binding constants for binding of Ca^{2+} , Mg^{2+} and H^+ to EGTA given by Godt [26]. The ATPase was labelled with NBD as described [5]. Fluorescence measurements were performed with excitation and emission wavelengths of 430 and 520 nm respectively. To reduce the signal caused by scattered light, the excitation beam was passed through a 450 nm long-wavelength cut-off filter (450FLO T-50; Andover Corporation) and the emission was passed through a Hoya Y50 500 nm short-wavelength cut-off filter. The ATPase was labelled with Br-DMC as described by Stefanova et al. [27]. DMC fluorescence was excited at 350 nm and observed at 425 nm. The ATPase was labelled with FITC as described by Froud and Lee [20].

Rapid kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorimeter with BioKine V3.20a software for data acquisition and analysis [22]. Tryptophan fluorescence was excited through a monochromator set at 290 nm and emitted light was collected at 90° and filtered by a high-pass cut-off filter with 50 % transmission at 320 nm (UV 32; Andover Corporation). Fluorescein fluorescence was excited at 490 nm and observed through a cut-off filter with 50 % transmission at 530 nm.

Kinetic simulations were performed using FACSIMILE v. 3.0 (AEA Technology, Harwell, U.K.) running on an IBM-compatible PC using the parameters for Ca^{2+} binding given in Lee et al. [23]. Simulations were fitted to single or double exponentials using the same package (BioKine V3.20a) used for fitting the experimental data.

For simulations of the equilibrium binding experiments, a set of equations was written describing the individual equilibrium binding steps which was then solved using the method of bisection [28].

RESULTS

Stoichiometry of binding

Binding of TvA to NBD-labelled ATPase at pH 8 resulted in a decrease in fluorescence intensity which, for the preparation of NBD-labelled ATPase used in this experiment, saturated at a molar ratio of TvA to ATPase of approx. 1:2 (Figure 1a). Binding of TvA to DMC-labelled ATPase at pH 6 also resulted in a decrease in fluorescence intensity but, for the preparation shown, this was saturated at a molar ratio of inhibitor to ATPase of approx. 1:1 (Figure 1a). The stoichiometry of binding observed for NBD- and DMC-labelled ATPase was found to vary between preparations from approx. 1:4 to 1:1. The maximum level of

Figure 1 Effects of TvA on the fluorescence of the ATPase

Effects of TvA on the fluorescence intensities of: (a) ○, NBD-labelled ATPase in 50 mM Tris/HCl, pH 8.0, containing 0.3 mM EGTA; ●, DMC-labelled ATPase in 40 mM Tris/HCl, pH 6.0, containing 1 mM EGTA; (b) Trp fluorescence of the ATPase in 50 mM Hepes/KOH, pH 7.2, containing 1 mM EGTA (□) or 1 mM Ca^{2+} (○). △ shows the difference between the fluorescence intensities measured in the absence and presence of Ca^{2+} . For all experiments, the ATPase concentration was 1 μM. The lines represent simulations of binding, calculated as described in the text. The inset shows the structures of the sesquiterpene lactones. Thapsigargin: R¹, $\text{CO}(\text{CH}_2)_2\text{CH}_3$; R², $\text{OCO}(\text{CH}_2)_6\text{CH}_3$; trilobolide: R¹, $\text{COCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$; R², H; TvA: R¹, $\text{COCH} = \text{C}(\text{CH}_3)_2$; R², $\text{OCOC}(\text{CH}_3) = \text{CHCH}_3$.

phosphoenzyme formation observed with our preparations of Ca^{2+} -ATPase varies between 2.5 and 4 nmol of [EP]/mg of protein (see [29]), corresponding to between 27 and 44 % of that expected for a pure protein of M_r 109000. The reason for the lower than expected level of phosphorylation is unclear (see [30,31]). The measured stoichiometry of inhibitor binding presumably depends on the extent of inhibitor binding to the 'inactive' ATPase present in the preparation. Since the stoichiometry of labelling of the ATPase with DMC is 1:1 with respect to total ATPase [27], the 'inactive' ATPase must have a structure that is at least partly still native.

The variation in fluorescence intensity with concentration of TvA is almost linear up to the saturation point (Figure 1a), so that it is not possible to derive an affinity for TvA from such plots; simulations show that the affinity must be greater than approx. 10^8 M^{-1} to give linear plots at 1 μM ATPase.

Binding of TvA to the Ca^{2+} -ATPase resulted in a decrease in Trp fluorescence intensity which increased with increasing TvA concentration beyond a 1:1 molar ratio with ATPase (Figure 1b). In the presence of 1 mM Ca^{2+} , a lower level of fluorescence quenching was observed, which increased linearly with con-

Figure 2 Effects of inhibitors on the fluorescence intensity of NBD-labelled ATPase as a function of pH

NBD-labelled ATPase ($1 \mu\text{M}$) was incubated in buffer containing 0.3 mM EGTA in the absence (○) or presence of $1 \mu\text{M}$ TvA (□) or $1 \mu\text{M}$ thapsigargin (△), and fluorescence intensities were determined as a function of pH. The lines are simulations calculated as described in the text, with relative fluorescence intensities of 5.0 and 8.5 for the E2 and E1 conformations respectively.

Scheme 2

centration of TvA above $0.5 \mu\text{M}$ and thus represents non-specific quenching. The difference between the quenching observed in the presence and absence of Ca^{2+} is seen to saturate at a molar ratio of TvA to ATPase of approx. 1:1 (Figure 1b).

Effects on the E2–E1 equilibrium

The decrease in fluorescence intensity of NBD-labelled ATPase on addition of TvA at pH 8.0 is consistent with a shift in the equilibrium towards E2, the form with low fluorescence (Figure 1). The effects of TvA and thapsigargin are shown as a function of pH in Figure 2. The E2–E1 equilibrium has been shown to be pH-sensitive, with low pH favouring the E2 state because of H^+ binding at a site with an affinity for H^+ of $5 \times 10^5 \text{ M}^{-1}$ in the E1 state and $3.0 \times 10^8 \text{ M}^{-1}$ in the E2 state; the equilibrium constant E1/E2 for the unprotonated forms of the ATPase is 4 [14]. The lower fluorescence levels observed in the presence of TvA can be simulated assuming a 35-fold decrease in the E1/E2 equilibrium constant with no change in the H^+ binding constants; in the presence of thapsigargin the decrease in the E1/E2 equilibrium constant is greater, being at least 500-fold (Figure 2).

The effects of the inhibitors on the ATPase can be described in terms of Scheme 2. A 35-fold decrease in the equilibrium constant E1/E2 in the presence of a saturating concentration of TvA implies weaker binding of TvA to E1 than to E2 ($K^{E2} = 35K^{E1}$, where K^{E1} and K^{E2} are inhibitor binding constants for the E1 and E2 conformations respectively).

Figure 3 Effects of inhibitors on the Ca^{2+} affinity of the ATPase as determined by changes in Trp or NBD fluorescence

Shown are the changes in (a) Trp fluorescence intensity for unlabelled ATPase ($1 \mu\text{M}$) and (b) NBD fluorescence for NBD-labelled ATPase ($1 \mu\text{M}$) in buffer containing 0.3 mM EGTA and 1.3 mM Ca^{2+} , on addition of EGTA to produce the given free Ca^{2+} concentration. Fluorescence changes are expressed as a fraction of the change observed between pCa values of 3.0 and 7.5. (a) In 50 mM Hepes/KOH, pH 7.2, in the absence of inhibitor (○), or in the presence of 1 (■) or 2 (▼) μM TvA, or 1 (□), 2 (○) or 4 (◇) μM thapsigargin. (b) In 50 mM Hepes/KOH, pH 7.2, in the absence of inhibitor (○), or in the presence of 0.5 (△), 1 (□) or 3 (◇) μM TvA, or $1 \mu\text{M}$ trilobolide (■); or in 50 mM Mes/KOH, pH 6.0, in the absence of inhibitor (●) or in the presence of $1 \mu\text{M}$ TvA (▲). The lines are simulations calculated as described in the text assuming a concentration of functional ATPase of $1 \mu\text{M}$.

Binding of Ca^{2+} to the ATPase in the presence of inhibitor

Studies using $^{45}\text{Ca}^{2+}$ have shown that the affinity of the ATPase for Ca^{2+} is reduced in the presence of thapsigargin [17]. A more convenient experimental technique to monitor Ca^{2+} binding is by observation of changes in NBD fluorescence for the NBD-labelled ATPase (following from the $\text{E2} \rightarrow \text{E1Ca}_2$ transition) [17], or by monitoring the changes in Trp fluorescence for the ATPase that follow from Ca^{2+} binding [14,23,32,33]. Addition of $1 \mu\text{M}$ TvA or $1 \mu\text{M}$ thapsigargin to $1 \mu\text{M}$ ATPase at pH 7.2 results in a decrease in the affinity of the ATPase for Ca^{2+} as monitored by changes in Trp fluorescence, the effect being larger for thapsigargin than for TvA (Figure 3a). Figure 3(a) also shows that increasing the concentration of TvA or thapsigargin beyond a 1:1 molar ratio with ATPase results in no further decrease in Ca^{2+} affinity for the ATPase. A similar result is shown in Figure

Scheme 3

3(b), monitoring changes in the fluorescence of NBD-labelled ATPase as a function of Ca^{2+} concentration. In the presence of $0.5 \mu\text{M}$ TvA and $1 \mu\text{M}$ ATPase, the Ca^{2+} binding curve appears biphasic. Ca^{2+} binding curves obtained at concentrations of TvA of 1 and $3 \mu\text{M}$ are identical to each other, and identical to those determined by monitoring changes in Trp fluorescence (Figure 3a). The effect of $1 \mu\text{M}$ trilobolide is greater than that of $1 \mu\text{M}$ TvA (Figure 3b) and comparison with the results for thapsigargin in Figure 3(a) shows that the effect of the inhibitors increases in the order $\text{TvA} < \text{trilobolide} < \text{thapsigargin}$. Shifts in Ca^{2+} affinity as a result of TvA binding are very similar at pH 7.2 and pH 6.0 (Figure 3b).

It is shown in the Appendix that if Ca^{2+} and inhibitor are in direct competition for binding to the ATPase according to Scheme A1 or A2, then the Ca^{2+} affinity would be expected to decrease when the amount of inhibitor is increased beyond a 1:1 molar ratio with the ATPase. This is not observed experimentally (Figure 3). The effects of inhibitor binding are not complicated by any significant binding of the inhibitor to the lipid component of the membrane, since addition of vesicles of dioleoyl-phosphatidylcholine at concentrations up to $500 \mu\text{M}$ had no effect on the observed responses to TvA. Saturation of the effect of the inhibitor at a 1:1 molar ratio with ATPase will occur (see the Appendix) if inhibitor can bind to the Ca^{2+} -bound ATPase as in Scheme 3. Stronger binding of inhibitor to the E2 than to the E1 conformation will result in a reduced affinity for Ca^{2+} in the presence of inhibitor. Binding of Ca^{2+} to the ATPase can be described using the binding parameters given in Henderson et al. [14] and Lee et al. [23]. Assuming that the parameters describing binding of Ca^{2+} to E1 and E1I are identical, 35-fold stronger binding of TvA to the E2 conformation than to the E1 conformation of the ATPase (Figure 2) results in a shift in the pCa value for 50% binding of Ca^{2+} of 0.7 in the presence of $1 \mu\text{M}$ TvA at pH 7.2, in agreement with the observed shift (Figures 3a and 3b). The observed shifts seen on binding of trilobolide and thapsigargin correspond respectively to 150- and 5000-fold stronger binding to E2 than to E1 (Figures 3a and 3b).

A conformation change occurs on binding of inhibitor

The difference between the extent of quenching of Trp fluorescence by TvA in the presence and absence of Ca^{2+} is greater when total fluorescence is observed through a 320 nm cut-off filter in a stop-flow fluorimeter (Figure 4) than when fluorescence intensity is observed at 340 nm in a conventional fluorimeter (Figure 1b). The rates of change in Trp fluorescence intensity on mixing the ATPase with TvA in the absence of Ca^{2+} fit to single-exponential processes with the rates and amplitudes shown in Figure 4. Rates observed at pH 6.0 and pH 7.2 are very similar. As shown, the rate reaches a limiting value of approx. 34 s^{-1} at concentrations of TvA above approx. $4 \mu\text{M}$ when the concentration of ATPase is $1 \mu\text{M}$. This behaviour is consistent with an association step followed by an isomerization, with

Figure 4 Rate of quenching of the Trp fluorescence of the ATPase by TvA

Decreases in the Trp fluorescence of the ATPase were determined as a function of time following mixing of the ATPase in buffer containing 5 mM EGTA at pH 6.0 (Δ , $\circ$) and pH 7.2 (∇) with an equal volume of the same buffer containing 5 mM EGTA and TvA in a stop-flow fluorimeter, to give $1 \mu\text{M}$ ATPase and the given concentrations of TvA. Fluorescence changes were fitted to single exponentials; rate constants obtained at pH 6.0 (Δ) and pH 7.2 (∇) and amplitudes obtained at pH 6.0 ($\circ$) are plotted as a function of TvA concentration. Also shown ($\square$) are the amplitudes of the fluorescence changes obtained on mixing TvA with the ATPase at pH 6.0 in the presence of 1 mM Ca^{2+} ; the rates in the presence of Ca^{2+} were approx. 32 s^{-1} at pH 6.0 or pH 7.2, at TvA concentrations between 0.5 and $16 \mu\text{M}$. The solid line shows a simulation of the rates at pH 6.0, calculated as described in the text assuming a concentration of functional ATPase of $0.5 \mu\text{M}$; calculated rates at pH 7.2 were identical to those at pH 6.0.

Scheme 4

fluorescence quenching corresponding to the isomerization shown in Scheme 4. The maximum observed rate gives the rate of the conformation change $\text{EI} \rightarrow \text{E}^{\text{A}}$ as 34 s^{-1} , and simulations of Scheme 4 at low concentrations of TvA give the rate of the $\text{E} \rightarrow \text{EI}$ change as $1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figure 4).

Reversal of the effects of inhibitors by addition of Ca^{2+}

The effects of the sesquiterpene lactones on the fluorescence of the NBD-labelled ATPase [17] or the FITC-labelled ATPase [21] can be reversed by addition of high concentrations of Ca^{2+} . The effects of the addition of 0.4 mM Ca^{2+} to $1 \mu\text{M}$ NBD-labelled ATPase or DMC-labelled ATPase incubated in the presence of $1 \mu\text{M}$ TvA at pH 6.0 are shown in Figure 5 as a function of time. Also shown are the effects on the Trp fluorescence of the unlabelled ATPase and of the NBD-labelled ATPase. All curves fit to single exponentials, with rates between 0.02 and 0.03 s^{-1} . The observed equal rates for all of these fluorescence responses suggest that, under these conditions, all respond to the same conformation change on the ATPase. The observed equal rates for the Trp fluorescence response for the unlabelled ATPase and for the NBD-labelled ATPase show that labelling does not affect the rate of the conformation change.

A different result is obtained in the presence of sub-stoichiometric amounts of TvA. Two kinetically resolvable processes are observed when Ca^{2+} is added to $1 \mu\text{M}$ NBD-labelled ATPase incubated in the presence of $0.33 \mu\text{M}$ TvA, with rates of 0.027 ± 0.002 and $0.22 \pm 0.02 \text{ s}^{-1}$ and fractional intensities of 0.52

Figure 5 Kinetics of the reversal of the effects of TvA by Ca^{2+}

Shown are the increases in NBD fluorescence for NBD-labelled ATPase (a), DMC fluorescence for DMC-labelled ATPase (c) and Trp fluorescence for unlabelled ATPase (b) or NBD-labelled ATPase (d) on addition of Ca^{2+} to the ATPase ($1 \mu\text{M}$) at pH 6.0 in the presence of $1 \mu\text{M}$ TvA and 0.3 mM EGTA, to give a final free Ca^{2+} concentration of 0.4 mM .

Figure 6 Kinetics of the reversal of the effects of TvA by Ca^{2+} at sub-stoichiometric concentrations of TvA

Shown are the changes in NBD fluorescence for NBD-labelled ATPase (a) and in DMC fluorescence for DMC-labelled ATPase (b) on addition of Ca^{2+} to the ATPase ($1 \mu\text{M}$) at pH 6.0 in the presence of $0.33 \mu\text{M}$ (a) or $0.5 \mu\text{M}$ (b) TvA and 0.3 mM EGTA, to give a final free Ca^{2+} concentration of 0.4 mM (a) or 0.1 mM (b). (a) Dotted line, experimental data; solid line, best single-exponential fit; broken line, best double-exponential fit. (b) Dotted line, experimental data; solid line, best single-exponential fit.

Figure 7 Effect of TvA on the fast component of the fluorescence response of FITC-labelled ATPase to Ca^{2+}

FITC-labelled ATPase was incubated with TvA in buffer at pH 7.2 (●, ○) or pH 6.0 (□, ■) containing 0.3 mM EGTA and mixed in a stop-flow fluorimeter with an equal volume of buffer containing Ca^{2+} to give final concentrations of $2 \mu\text{M}$ ATPase, 0.6 mM free Ca^{2+} and the given concentrations of TvA. The resulting change in fluorescence intensity was fitted to the sum of a single exponential and a sloping baseline (representing the slow component of the fluorescence change), and the rates (○, □) and relative intensities of the fast component (●, ■) are plotted. The lines represent the results of simulations as described in the text, assuming a concentration of functional ATPase of $1 \mu\text{M}$.

and 0.48 respectively (Figure 6a). These experiments were performed by addition of Ca^{2+} to the sample in a stirred cuvette in a conventional fluorimeter, so that the rate of the fast component will underestimate the true rate; the slow component is, however, sufficiently slow to be measured accurately in this way. A similar experiment recording changes in DMC fluorescence of $1 \mu\text{M}$ DMC-labelled ATPase incubated with $0.5 \mu\text{M}$ TvA fits well to a single exponential with a rate ($0.034 \pm 0.01 \text{ s}^{-1}$) comparable with that of the slow component seen with NBD-labelled ATPase (Figure 6b).

Changes observed on addition of Ca^{2+} to FITC-labelled ATPase in the presence of sub-stoichiometric quantities of TvA are two-component (results not shown), as described above for NBD-labelled ATPase. The rate of the fast component has been determined by stop-flow fluorescence measurements. We have found that FITC-labelled ATPase is more convenient than NBD-labelled ATPase for such measurements, because of reduced interference from light scatter. The fast component fits to a single exponential. The rate is independent of TvA concentration and is equal to that observed in the absence of TvA (Figure 7). The rate is faster at pH 7.2 than at pH 6.0, and the magnitude of the fast component decreases in intensity with increasing TvA concentration at both pH 6.0 and 7.2 (Figure 7). In the absence of TvA, the change in FITC fluorescence observed on addition of Ca^{2+} has been shown to be due to the E2-E1 conformation change (see [22]); the rates of the changes observed here at both pH 7.2 and 6.0 are comparable with those calculated using the parameters given in Henderson et al. [22] (Figure 7).

The rate of the slow component of the NBD response decreases, and its amplitude increases, with increasing concentrations of TvA; the rate is slower at pH 6.0 than at pH 7.0 (Figures 8a and 8b). These observations are inconsistent with Scheme 3. If the dissociation of inhibitor (I) from the complex E2I were slow compared with the rate of the $\text{E2} \rightarrow \text{E1}$ transition, then the fast component would correspond to the $\text{E2} \rightarrow \text{E1}$ transition for

Figure 8 Effect of TvA concentration on the rate of the slow component of the fluorescence response of NBD- or DMC-labelled ATPase to Ca^{2+}

A concentration of $1 \mu\text{M}$ NBD-labelled ATPase (a, b) or $1 \mu\text{M}$ DMC-labelled ATPase (c) was incubated in buffer containing 0.3 mM EGTA at pH 6.0 (a) or pH 7.0 (b, c) with the given concentrations of TvA and with Ca^{2+} added to a final free concentration of 0.4 mM (a, b) or 0.1 mM (c). The fluorescence response was fitted to the sum of two exponentials, and the rates ($\circ$) and relative amplitudes ($\square$) of the slow response are plotted. The lines show the results of simulations as described in the text, assuming concentrations of functional ATPase of (a) $0.35 \mu\text{M}$, (b) $0.5 \mu\text{M}$ and (c) $0.75 \mu\text{M}$.

unbound E2 and the rate of the fast component would be independent of the concentration of I, but the rate of the slow component, corresponding to the rate of dissociation of I from E2I, would also be independent of the concentration of I. The data are also inconsistent with Scheme 3 if it is postulated that the slow step corresponds to a slow $\text{E2I} \rightarrow \text{E1I}$ transition, since again the rate of the slow component would be independent of the concentration of I. Furthermore, the data are inconsistent with Scheme 3 if it is postulated that the rate of dissociation of I from E2I is fast compared with the rate of the $\text{E2} \rightarrow \text{E1}$ transition; under these conditions the response to the addition of

Scheme 5

Figure 9 Effect of Ca^{2+} concentration on the rate of the slow component of the fluorescence response of NBD-labelled ATPase to Ca^{2+}

Fluorescence responses of NBD-labelled ATPase ($1 \mu\text{M}$) incubated at pH 6.0 in the presence of $1 \mu\text{M}$ TvA to the addition of Ca^{2+} were determined as described in the legend to Figure 8: rates ($\circ$) and amplitudes ($\square$) of the slow response are plotted as a function of Ca^{2+} concentration. The solid lines show the results of simulations as described in the text, assuming a concentration of functional ATPase of $0.35 \mu\text{M}$. The broken line shows a simulation of equilibrium Ca^{2+} binding under these conditions, calculated as described in the legend to Figure 3.

Ca^{2+} would be single-exponential, with a rate which would decrease with increasing concentration of inhibitor, as given by the quasi-equilibrium approach [34]. However, the data are consistent with Scheme 3 if it is postulated that a conformation change occurs following binding of the inhibitor to E2 (Scheme 4), as in Scheme 5, as long as the equilibrium $\text{E2I} \rightleftharpoons \text{E2}^{\text{A}}\text{I}$ strongly favours $\text{E2}^{\text{A}}\text{I}$ and the rate of the step $\text{E2}^{\text{A}}\text{I} \rightarrow \text{E2I}$ is slow compared with the rate of the $\text{E2} \rightarrow \text{E1}$ transition (in the absence of inhibitor). With sub-stoichiometric amounts of TvA at pH 6.0 or 7.2 in the absence of Ca^{2+} , the ATPase will then exist predominantly as a mixture of E2 and $\text{E2}^{\text{A}}\text{I}$, with very small amounts of unbound inhibitor. Addition of Ca^{2+} will lead to a rapid transition of E2 to E1Ca_2 at a rate that is unaffected by the presence of TvA. Subsequent conversion of $\text{E2}^{\text{A}}\text{I}$ to E2I will lead to the formation of E2 and a rate of Ca^{2+} binding that is dependent on the rate of the $\text{E2} \rightarrow \text{E1}$ transition and the fraction of unbound E2, as given by the quasi-equilibrium approach [34].

For DMC-labelled ATPase in the presence of sub-stoichiometric amounts of TvA, the rate of the DMC fluorescence response to the addition of Ca^{2+} is single-exponential at all TvA concentrations and the rate measured at pH 7.0 is comparable with that of the slow component seen with NBD-labelled ATPase (Figure 8c).

The rate of the fluorescence change seen on addition of Ca^{2+} to $1 \mu\text{M}$ NBD-labelled ATPase in the presence of $1 \mu\text{M}$ TvA at pH 6.0 increases, and its magnitude decreases, with decreasing

Figure 10 Effect of pH on the rate of the slow component of the fluorescence response of NBD- and DMC-labelled ATPase to Ca^{2+}

Fluorescence responses to the addition of 1 mM Ca^{2+} to 1 μM NBD-labelled ATPase ($\circ$, $\square$, $\triangle$) or 1 μM DMC-labelled ATPase ($\bullet$) incubated in buffer at the given pH containing 0.3 mM EGTA and 1 μM TvA ($\circ$, $\bullet$), 1 μM trilobolide ($\square$) or 1 μM thapsigargin ($\triangle$) were determined as described in the legend to Figure 8. Shown are rates obtained from single-exponential fits to the data. The lines are the results of simulations performed as described in the text, assuming a concentration of functional ATPase of 1 μM .

Ca^{2+} concentrations (Figure 9). The rate of the response increases with increasing pH, and the rate increases in the order thapsigargin < trilobolide < TvA (Figure 10). The rate of the fluorescence change measured for 1 μM DMC-labelled ATPase incubated with 1 μM TvA is very similar to that observed for NBD-labelled ATPase over the pH range studied (Figure 10).

Dependence of the rate of the effect of thapsigargin on the concentration of Ca^{2+}

Addition of thapsigargin to NBD-labelled ATPase incubated in the presence of appropriate concentrations of Ca^{2+} can lead to a reduction in NBD fluorescence, corresponding to the transition from E1Ca_2 to E2 -thapsigargin. The rate of the transition decreases with increasing concentrations of Ca^{2+} (Figure 11), implying that only Ca^{2+} -free forms of the ATPase can undergo the transition to the E2 conformation, as in Scheme 5. Comparable results are obtained at pH 7.2 (not shown).

DISCUSSION

The sesquiterpene lactones thapsigargin [2,3,17,21,35], trilobolide [8,9] and TvA [8] are potent inhibitors of the Ca^{2+} -ATPase with affinities in the nanomolar range [11]. The stoichiometry of binding is 1:1, either with respect to total protein or with respect to 'functional' ATPase (as defined by the maximum level of phosphoenzyme formation obtained with ATP), depending on the preparation of the ATPase [2-4,12].

The inhibitors have been shown to cause a reduction in the affinity of the ATPase for Ca^{2+} [2,17,21] and ATP [3], following from a shift in the E2 - E1 equilibrium of the ATPase towards E2 [3,5,9,17,21]. However, an unexpected observation was a decrease in the level of phosphorylation of the ATPase by P_i [35]; increased phosphorylation by P_i would have been expected, since in the E2 - E1 model for the ATPase it is the E2 conformation that is P_i -reactive [13].

Figure 11 Effect of Ca^{2+} concentration on the rate of the fluorescence response of NBD-labelled ATPase to the addition of thapsigargin

(a) NBD-labelled ATPase (0.9 μM) was incubated in buffer (50 mM Mes/KOH, pH 7.0, 100 mM KCl, 5 mM Mg^{2+}) at the given pCa values. At the time shown thapsigargin (Tg) was added to give a final concentration of 0.9 μM . (b) Simulations of (a), calculated as described in the text.

Further information about the mechanism of inhibition can potentially be obtained from kinetic studies of the competition between inhibitor and Ca^{2+} for binding to the ATPase. Rates of binding of Ca^{2+} to the ATPase in the presence of thapsigargin are found to be extremely low [17,21]. However, we have found that effects of TvA on the ATPase are less marked than those of thapsigargin, and that rates of reversal occur on a more convenient time scale.

Studies of the fluorescence of NBD-labelled ATPase as a function of pH in the presence and absence of TvA are consistent with a 35-fold decrease in the $\text{E1}/\text{E2}$ equilibrium constant for the ATPase; the effects of thapsigargin on the equilibrium are larger (at least a 500-fold decrease) (Figure 2). A shift in the E2 - E1 equilibrium towards E2 is consistent with a reduction in the affinity of the ATPase for Ca^{2+} , as observed (Figure 3). An unexpected observation, however, was that the effects of the inhibitors on the affinity of the ATPase for Ca^{2+} were found to saturate at a 1:1 molar ratio of inhibitor to ATPase (Figure 3). As shown in the Appendix, this is not consistent with the formation of a dead-end complex in which binding of inhibitor and Ca^{2+} to the ATPase are mutually exclusive. Saturation of the effect of the inhibitor at a 1:1 molar ratio with ATPase implies that the inhibitor can bind to the ATPase with high affinity in

Table 1 Rate parameters describing binding of TvA to the ATPase and the effects of Ca^{2+} according to Scheme 5

Rate constants for the steps $\text{E2} \rightleftharpoons \text{E1} \rightleftharpoons \text{E1Ca} \rightleftharpoons \text{E1}'\text{Ca} \rightleftharpoons \text{E1}'\text{Ca}_2$ are as given in Lee et al. [23]; rate constants for the Ca^{2+} binding steps are unaltered by binding of TvA.

Step	Rate constant
$\text{E1} \rightarrow \text{E1I}$	$1.0 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$
$\text{E1I} \rightarrow \text{E1}$	0.9 s^{-1}
$\text{E2} \rightarrow \text{E2I}$	$1.0 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$
$\text{E2I} \rightarrow \text{E2}$	8.8 s^{-1}
$\text{E2I} \rightarrow \text{E2}^{\text{AI}}$	34.0 s^{-1}
$\text{E2}^{\text{AI}} \rightarrow \text{E2I}$	1.0 s^{-1}

both the absence and the presence of Ca^{2+} , so that under the conditions of the experiment the ATPase remains fully bound to inhibitor. A reduction in affinity for Ca^{2+} can then follow if the inhibitor binds more strongly to the E2 conformation of the ATPase than to the E1 conformation (Schemes 3 and 4). The observed reduction in affinity for Ca^{2+} in the presence of TvA (Figure 3) is consistent with the 35-fold stronger binding of TvA to E2 than to E1 estimated above (see Table 2).

Measurements of the rate of reversal of the effects of TvA by addition of Ca^{2+} are also inconsistent with the formation of a dead-end complex, but are consistent with Scheme 5, involving a first-order isomerization to E2^{AI} following the initial second-order association step (Figures 5–7). Measurements of the rate of quenching of Trp fluorescence by TvA are also consistent with such an isomerization (Figure 2). It should be emphasized that, according to Scheme 5, with the rate constants given in Table 1, the slow step on binding of Ca^{2+} in the presence of TvA and the other inhibitors is the $\text{E2} \rightarrow \text{E1}$ transition; it is slow because the fraction of E2 unbound by TvA, and thus able to undergo the transition, is small.

The formation of a modified state E2^{AI} in the presence of inhibitor would explain the reduced phosphorylation of the ATPase by P_i in the presence of inhibitor [35] if phosphorylation of the modified state E2^{AI} by P_i is less favourable than phosphorylation of E2. The existence of two conformational states for the phosphorylated ATPase, E2P and $\text{E2}'\text{P}$, has been suggested on the basis of experiments on the effects of phospholipids on the function of the ATPase [36]. It was suggested that these two states could differ in the degree of closure of a cleft between the phosphorylation and nucleotide binding domains [36]. A cleft closure between these two domains has been suggested to occur following binding of Ca^{2+} and ATP to the ATPase [37]. Thus, as one possible concrete proposal, the states E2I and E2^{AI} could correspond to open and closed forms of the ATPase respectively, with the equilibrium favouring the closed form.

The experimental data reported here have been simulated in terms of Scheme 5 using the FACSIMILE program. For experiments with sub-stoichiometric amounts of TvA, it was necessary to assume a stoichiometry of binding of TvA to the total ATPase protein in the preparations used. As described, this was found to vary between preparations (Figure 1), possibly depending on the extent of binding of TvA to non-functional ATPase. Simulations of the magnitude of the observed fluorescence changes as a function of TvA concentration (Figures 7 and 8) were consistent with TvA binding to between 35 and 100% of the total ATPase protein present (see Figure legends). For our preparations of ATPase, the fraction of active ATPase, as defined by the maximum observable level of phosphorylation by ATP, is generally between 30 and 50%. Changes in the fraction of the ATPase able to bind TvA were found to have only minor effects on the rates of the fluorescence changes in the simulations.

The interpretation of the measurements of the rate of reversal of the effects of TvA by Ca^{2+} given above requires that the equilibrium $\text{E2I} \rightleftharpoons \text{E2}^{\text{AI}}$ should lie strongly towards E2^{AI} , so that the equilibrium constant $\text{E2}^{\text{AI}}/\text{E2I}$ must be greater than approx. 10. Thus the major factor favouring E2 forms of the ATPase over E1 forms in the presence of TvA is the favourable formation of E2^{AI} . The simplest model for the 35-fold shift in the ratio of E2 to E1 forms of the ATPase in the presence of TvA (Figure 2) is then to propose equal binding of TvA to E1 and E2 with an equilibrium constant $\text{E2}^{\text{AI}}/\text{E2I}$ of 34 (Tables 1 and 2).

In terms of Scheme 5, the rate of the slow step on binding of Ca^{2+} in the presence of inhibitor is determined by the rate of the $\text{E2} \rightarrow \text{E1}$ step, which will be the same in the presence of all the inhibitors, and by the binding constant for binding of inhibitor to E2 and the equilibrium constant $\text{E2}^{\text{AI}}/\text{E2I}$. The results reported here for TvA are consistent with an affinity for E2 of $1.1 \times 10^7 \text{ M}^{-1}$ (Tables 1 and 2). The results for trilobolide and thapsigargin (Figure 10) are consistent with affinities of 6.7×10^6 and 8.0×10^6 respectively (Figure 10; Table 2).

We conclude, therefore, that the inhibitors bind with very similar affinities to E1, E2 and E1Ca_2 but, when bound to E2, they can induce a further, energetically favourable, conformational change. If, as suggested, the differences between E2I and E2^{AI} lie in differences in the phosphorylation and nucleotide binding domains, then it will be the effects of the inhibitors on these domains which will be important. The observed decreases in fluorescence intensity for DMC-labelled ATPase on binding of inhibitor (see Figures 5 and 8c) are consistent with such an observation, since phosphorylation of the ATPase has also been reported to result in a decrease in DMC fluorescence [27]. The hydrophobic nature of the sesquiterpene lactones and related inhibitors suggests a binding site in the *trans*-membrane region of the ATPase [9]. The phosphorylation and nucleotide binding domains are located well above the surface of the membrane [38], so that long-range interactions would be involved in the proposed effects of the inhibitors.

Table 2 Binding constants for the sesquiterpene lactones

Values were calculated assuming equal binding constants for binding of inhibitors to the E1 and E2 conformations.

Inhibitor	Binding constant (M^{-1}) $\text{E2} + \text{I} \rightleftharpoons \text{E2I}$	Equilibrium constant $\text{E2}^{\text{AI}}/\text{E2I}$	Overall binding constant $\text{E2} + \text{I} \rightleftharpoons \text{E2}^{\text{AI}}$
TvA	1.1×10^7	34	4.0×10^8
Trilobolide	6.7×10^6	150	1.0×10^9
Thapsigargin	8.0×10^6	5.0×10^3	4.0×10^{10}

We thank Dr. M. Holub for his gift of the trilobolide, Barbara Griffiths for help with purification of the ATPase, The Wellcome Trust, the BBSRC and the Wessex Medical Trust for financial support, and the BBSRC for a studentship (to Y.M.K.).

REFERENCES

- 1 Thastrup, O., Cullen, P. J., Drbak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- 2 Sagara, Y. and Inesi, G. (1991) *J. Biol. Chem.* **266**, 13503–13506
- 3 Kijima, Y., Ogunbunmi, E. and Fleischer, S. (1991) *J. Biol. Chem.* **266**, 22912–22918
- 4 Lytton, J., Westlin, M. and Hanley, M. R. (1991) *J. Biol. Chem.* **266**, 17067–17071
- 5 Wictome, M., Henderson, I. M. J., Lee, A. G. and East, J. M. (1992) *Biochem. J.* **283**, 525–529
- 6 Christensen, S. B., Larsen, I. K. and Rasmussen, U. (1982) *J. Org. Chem.* **47**, 649–652
- 7 Rasmussen, U., Christensen, S. B. and Sandberg, F. (1981) *Planta Med.* **43**, 336–341
- 8 Wictome, M., Starling, A. P., Sharma, R. P., East, J. M. and Lee, A. G. (1993) *Biochem. Soc. Trans.* **21**, 348S
- 9 Wictome, M., Holub, M., East, J. M. and Lee, A. G. (1994) *Biochem. Biophys. Res. Commun.* **199**, 916–921
- 10 Kutschabsky, L., Kretschmer, R. G. and Ripperger, H. (1986) *Cryst. Res. Technol.* **21**, 627–633
- 11 Christensen, S. B., Andersen, A., Poulsen, J. C. and Treiman, M. (1993) *FEBS Lett.* **335**, 345–348
- 12 Papp, B., Enyedi, A., Kovacs, T., Sarkadi, B., Wuytack, F., Thastrup, O., Gardos, G., Bredoux, R., Levytoledano, S. and Enouf, J. (1991) *J. Biol. Chem.* **266**, 14593–14596
- 13 de Meis, L. (1981) *The Sarcoplasmic Reticulum*, Wiley, New York
- 14 Henderson, I. M. J., Khan, Y. M., East, J. M. and Lee, A. G. (1994) *Biochem. J.* **297**, 615–624
- 15 Wakabayashi, S., Imagawa, T. and Shigekawa, M. (1990) *J. Biochem. (Tokyo)* **107**, 563–571
- 16 Wakabayashi, S., Ogurusu, T. and Shigekawa, M. (1990) *Biochemistry* **29**, 10613–10620
- 17 Wictome, M., Michelangeli, F., Lee, A. G. and East, J. M. (1992) *FEBS Lett.* **304**, 109–113
- 18 Pick, U. and Karlsh, S. J. (1982) *J. Biol. Chem.* **257**, 6120–6126
- 19 Pick, U. (1982) *J. Biol. Chem.* **257**, 6111–6119
- 20 Froud, R. J. and Lee, A. G. (1986) *Biochem. J.* **237**, 197–206
- 21 Sagara, Y., Wade, J. B. and Inesi, G. (1992) *J. Biol. Chem.* **267**, 1286–1292
- 22 Henderson, I. M. J., Starling, A. P., Wictome, M., East, J. M. and Lee, A. G. (1994) *Biochem. J.* **297**, 625–636
- 23 Lee, A. G., Baker, K. J., Khan, Y. M. and East, J. M. (1995) *Biochem. J.* **305**, 225–231
- 24 East, J. M. and Lee, A. G. (1982) *Biochemistry* **21**, 4144–4151
- 25 Hardwicke, P. M. and Green, N. M. (1974) *Eur. J. Biochem.* **42**, 183–193
- 26 Godt, R. E. (1974) *J. Gen. Physiol.* **63**, 722–739
- 27 Stefanova, H. I., East, J. M., Gore, M. G. and Lee, A. G. (1992) *Biochemistry* **31**, 6023–6031
- 28 Press, W. H., Flannery, B. P., Teukolsky, S. A. and Vetterling, W. T. (1986) *Numerical Recipes*, Cambridge University Press, Cambridge
- 29 Starling, A. P., East, J. M. and Lee, A. G. (1993) *Biochemistry* **32**, 1593–1600
- 30 Coll, R. J. and Murphy, A. J. (1984) *J. Biol. Chem.* **259**, 14249–14254
- 31 Barrabin, H., Scofano, H. M. and Inesi, G. (1984) *Biochemistry* **23**, 1542–1548
- 32 Dupont, Y. and Leigh, J. B. (1978) *Nature (London)* **273**, 396–398
- 33 Fernandez-Belda, F., Kurzmack, M. and Inesi, G. (1984) *J. Biol. Chem.* **259**, 9687–9698
- 34 Cha, S. (1968) *J. Biol. Chem.* **243**, 820–825
- 35 Sagara, Y., Fernandezbelda, F., de Meis, L. and Inesi, G. (1992) *J. Biol. Chem.* **267**, 12606–12613
- 36 Michelangeli, F., Grimes, E. A., East, J. M. and Lee, A. G. (1991) *Biochemistry* **30**, 342–351
- 37 Stahl, N. and Jencks, W. P. (1987) *Biochemistry* **26**, 7654–7667
- 38 Baker, K. J., East, J. M. and Lee, A. G. (1994) *Biochim. Biophys. Acta* **1192**, 53–60

APPENDIX

For simplicity, consider binding of an inhibitor I to an enzyme E in competition with binding of a single Ca^{2+} ion (Scheme A1). The dissociation constants for Ca^{2+} and I are K_1 and K_2 respectively and the total concentrations of E and I are $[E^T]$ and $[I^T]$ respectively. If the concentration of Ca^{2+} is buffered and thus constant, then:

$$K_1 = ([E^T] - [\text{ECa}] - [\text{EI}])[\text{Ca}]/[\text{ECa}] \quad (\text{A1})$$

where $[\text{ECa}]$ and $[\text{EI}]$ are the concentrations of the complexes ECa and EI respectively. At the point where E is 50% Ca^{2+} -bound ($[\text{ECa}] = [E^T]/2$), eqn. (A1) gives:

$$[\text{EI}] = ([\text{ECa}] - K_1[E^T])/2[\text{Ca}] \quad (\text{A2})$$

The second equilibrium can be written:

$$K_2 = ([E^T] - [\text{ECa}] - [\text{EI}])([I^T] - [\text{EI}])/[\text{EI}] \quad (\text{A3})$$

When $[\text{ECa}] = [E^T]/2$, eqn. (A2) can be substituted into eqn. (A3) to give:

$$2K_2[\text{Ca}]^2 - 2[\text{Ca}]K_2K_1 - 2[\text{Ca}][I^T]K_1 + [\text{Ca}][E^T]K_1 - [E^T]K_1^2 = 0 \quad (\text{A4})$$

When the term $[E^T]K_1^2$ can be ignored compared with the other terms in eqn. (A4) (with the parameters described below, this is true for $[I^T] \geq 1 \mu\text{M}$), eqn. (A4) simplifies to:

$$[\text{Ca}] = K_1(2[I^T] - [E^T] + 2K_2)/2K_2 \quad (\text{A5})$$

Scheme A1

Thus the concentration of Ca^{2+} giving half-saturation of E increases linearly with increasing concentration of inhibitor. For example, with values of $K_1 = 1 \mu\text{M}$, $K_2 = 1 \text{ nM}$ and $[E^T] = 1 \mu\text{M}$, eqn. (A5) predicts Ca^{2+} concentrations for half-saturation of 0.5 mM at $[I^T] = 1 \mu\text{M}$ and 1.5 mM at $[I^T] = 2 \mu\text{M}$. Figure A1

Figure A1 Dependence of Ca^{2+} binding to E in terms of Schemes A1 and A3

Curve a, binding of Ca^{2+} in the absence of inhibitor, with $[E^T] = 1 \mu\text{M}$ and $K_1 = 1 \mu\text{M}$. Curves b–d, binding of Ca^{2+} according to Scheme A1 with $K_2 = 1 \text{ nM}$ and concentrations of I (μM) of: b, 1; c, 2; and d, 3. The dotted and broken lines show Ca^{2+} binding calculated according to Scheme A3 with $K_2 = 0.1 \text{ mM}$, $K_3 = 0.1 \text{ nM}$ and concentrations of I of 1 μM (broken line) and 3 μM (dotted line)

Scheme A2 **Scheme A3**

shows the full Ca^{2+} binding curves calculated from eqns. (A1) and (A3) for inhibitor concentrations of 1, 2 and 3 μM .

Extension of Scheme A1 to include the two conformations E2 and E1 and the binding of 2 Ca^{2+} ions according to Scheme A2 does not change the result.

The simplest scheme in which increasing the concentration of I beyond a 1:1 molar ratio with E gives no further shift in the Ca^{2+} concentration required to give 50% occupancy of E with Ca^{2+} is one in which Ca^{2+} can bind to the EI complex, but with reduced affinity (Scheme A3). Binding equilibria can be written:

$$[ECa] = [E][Ca]/K_1 \quad (\text{A6})$$

$$[EI] = [E][I]/K_2 \quad (\text{A7})$$

$$[EICa] = [E][I][Ca]/K_2K_3 \quad (\text{A8})$$

These equations can be solved numerically by the method of bisection [1], making use of the conservation relationships:

$$[E^T] = [E] + [ECa] + [EI] + [EICa] \quad (\text{A9})$$

$$[I^T] = [I] + [EI] + [EICa] \quad (\text{A10})$$

The difference between K_1 and K_3 defines the shift in Ca^{2+} affinity on binding I. The requirement that no extra shift in Ca^{2+} affinity is observed at concentrations of I beyond a 1:1 molar ratio with E is that $K_4 (= K_1K_2/K_3)$ is sufficiently small so that, in the presence of Ca^{2+} , E is fully saturated with I. Figure A1 shows a calculation of Ca^{2+} binding using this model, with $[E^T] = 1 \mu\text{M}$, $K_1 = 1 \mu\text{M}$, $K_2 = 0.1 \text{ nM}$, $K_3 = 0.1 \text{ mM}$ and concentrations of $[I^T]$ of 1 and 3 μM .

REFERENCE

- 1 Press, W. H., Flannery, B. P., Teukolsky, S. A. and Vetterling, W. T. (1986) Numerical Recipes, Cambridge University Press, Cambridge