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The inhibitors thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone favour the E2 form of the Ca^{2+},Mg^{2+}-ATPase

Matthew Wictome[a], Francesco Michelangeli[b], Anthony G. Lee[a] and J. Malcolm East[a]

[a]*SERC Centre for Molecular Recognition, Department of Biochemistry, University of Southampton, UK* and [b]*Department of Biochemistry, University of Birmingham, Birmingham, B15 2TT, UK*

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2,5-Di(*tert*-butyl)-1,4-benzohydroquinone has been shown to inhibit the Ca^{2+},Mg^{2+}-ATPase of sarcoplasmic reticulum with an affinity of 0.4 μM. It has been shown to shift the E2–E1 equilibrium for the ATPase towards E2, as shown previously for the inhibitor thapsigargin. The shift towards E2 results in a decrease in affinity for Ca^{2+}, as also observed for thapsigargin. A marked decrease in the rate of the E2-E1 transition is observed for both BHQ and thapsigargin. A decrease in the equilibrium level of phosphorylation by P_i and of the steady-state level of phosphorylation by ATP are consistent with a decrease in the equilibrium constant for phosphorylation by P_i and an increase in the rate of dephosphorylation.

Ca^{2+},Mg^{2+}-ATPase; Thapsigargin; 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone

1. INTRODUCTION

It has recently been shown that the Ca^{2+},Mg^{2+}-ATPase of endoplasmic reticulum in a variety of cell types is inhibited by two apparently unrelated molecules thapsigargin, a sesquiterpene lactone from the plant *Thapsia garganica*, and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (BHQ) [1–5]. It has also been shown that thapsigargin inhibits the Ca^{2+},Mg^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) [6–8] and, since the Ca^{2+},Mg^{2+}-ATPase can be purified from SR in high yield, this allows a detailed study of the mechanism of inhibition. The mechanism of the ATPase is best discussed in terms of the E1/E2 scheme shown in simplified form in Scheme 1. In Scheme 1 the ATPase can exist in one of two conformations E1 and E2 which differ in that in E1 the two binding sites for Ca^{2+} on the ATPase are outward facing and of high affinity whereas in the phosphorylated form E2P they face the lumen of the SR and are of low affinity. Following binding of Ca^{2+}- and Mg^{2+}-ATP, the enzyme is phosphorylated and undergoes a conformation change to the E2P conformation from which Ca^{2+} is lost [9].

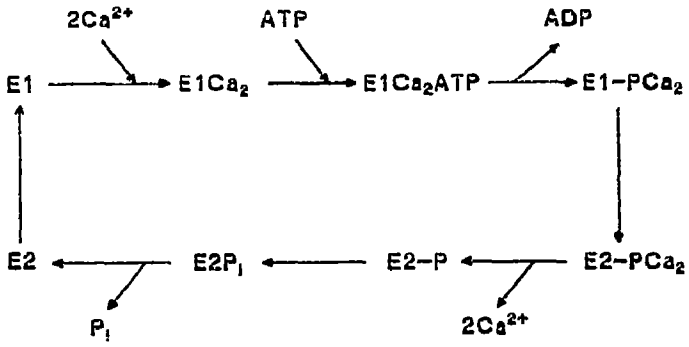
One of several points of controversy about this scheme concerns the existence of the E2 intermediate, since many kinetic experiments are consistent with dephosphorylation of E2P leading directly to E1 [10]. Evidence in favour of E2 as an intermediate has come from studies of the fluorescence of the ATPase labelled with nitrobenzo-2-oxa-1,3-diazole (NBD) which have been shown to be consistent with the E2–E1 model for the ATPase [11]. Here we compare the effects of thapsigargin and BHQ on the fluorescence of NBD-labelled ATPase and on Ca^{2+} binding to the ATPase, and show that both inhibitors shift the E2–E1 equilibrium towards E2.

Abbreviations: BHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; NBD, nitrobenzo-2-oxa-1,3-diazole; SR, sarcoplasmic reticulum; Tg, thapsigargin.

Correspondence address: J.M. East, Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, UK.

2. MATERIALS AND METHODS

Thapsigargin and BHQ were obtained from Calbiochem and Aldrich, respectively. Ca^{2+},Mg^{2+}-ATPase was purified from rabbit skeletal muscle SR and ATPase activities were determined using a coupled enzyme assay as described in Michelangeli et al. [12]. The ATPase was labelled with NBD using a slight modification of the method of Wakabayashi et al. [11] as described previously [6]. The fluorescence of NBD-labelled ATPase was recorded using an SLM 8000 spectrofluorimeter with excitation and emission wavelengths of 430 and 510 nm, respectively at 25°C in 50 mM HEPES/KOH, pH 7.2. For measurements of the Ca^{2+} dependence of fluorescence, the buffer also contained 1 mM Ca^{2+}; EGTA was added to give the required free Ca^{2+} concentration. Ammonium vanadate was dissolved in 100 mM



Scheme 1.

KOH to give a 100 mM stock solution and was added to the fluorescence samples to give a final concentration of 1 mM.

Ca^{2+} binding to the ATPase was measured by the dual labelling method of Yamaguchi and Watanabe [13]. ATPase (1 mg) was incubated in 5 ml of buffer (50 mM HEPES/KOH, pH 7.2) containing 20 mM $MgSO_4$, 400 μM $^{45}Ca^{2+}$ (3 Ci/mol), 500 μM [^{3}H]glucose and EGTA to give the required free Ca^{2+} concentration. 1 ml samples were rapidly filtered through Millipore HAWP 0.45 μm filters. After drying, 10 ml of Optiphase HiSafe 3 scintillant was added and the filters counted for both ^{3}H and $^{45}Ca^{2+}$ in a liquid scintillation counter. The amount of [^{3}H]glucose trapped on the filter was used to calculate the filter wetting volume and thus the proportion of the total $^{45}Ca^{2+}$ on the filter that was bound to the ATPase. Free Ca^{2+} concentrations were calculated using the binding constants given in [14].

Phosphorylation of the ATPase by [γ-^{32}P]-ATP was determined as described previously [6,12].

3. RESULTS

Fig. 1 shows the inhibitory effect of BHQ on steady-state ATPase activity. The data fits to a simple inhibition curve with a K_i of 0.4 μM. As shown the effect of BHQ could be reversed by addition of Triton X-100. As reported previously [6], addition of thapsigargin to the ATPase results in a marked reduction in the binding of $^{45}Ca^{2+}$ and, in the absence of Mg^{2+}, it is not possible to determine a Ca^{2+} binding curve for the ATPase in the presence of thapsigargin because of extensive non-specific binding of $^{45}Ca^{2+}$ observed at mM concentrations of Ca^{2+}. However, in the presence of 20 mM Mg^{2+}, non-specific binding is sufficiently reduced to allow the determination of a binding curve as shown in Fig. 2A. In the presence of 2 μM thapsigargin or 30 μM BHQ, the pCa^{2+} value giving half-saturation shifts from 5.8 to 3.8 and 5.5, respectively.

Wakabayashi et al. [15] have shown that the fluorescence intensity of NBD-labelled ATPase is sensitive to the E2–E1 conformation change, with a higher fluorescence intensity in the E1 conformation than in the E2 conformation. The effect of Ca^{2+} on the fluorescence of NBD-labelled ATPase can therefore be used to determine the Ca^{2+} affinity of the ATPase in the absence of Mg^{2+}, since there will be no interference from non-specific binding. As shown in Fig. 2B, under these conditions the half-maximal effect for the control is achieved at a pCa^{2+} of 6.6, shifting to 3.7 and 5.8 in the presence of 1 μM thapsigargin or 30 μM BHQ, respectively. The effect of inhibitors on the time-course of the change in fluorescence intensity of NBD-labelled ATPase following addition of Ca^{2+} is shown in Fig. 3.

Addition of Mg^{2+} to NBD-labelled ATPase at pH 8 shifts the E2–E1 equilibrium towards E1 as shown by the increase in fluorescence intensity (Fig. 4) [6,11]. As

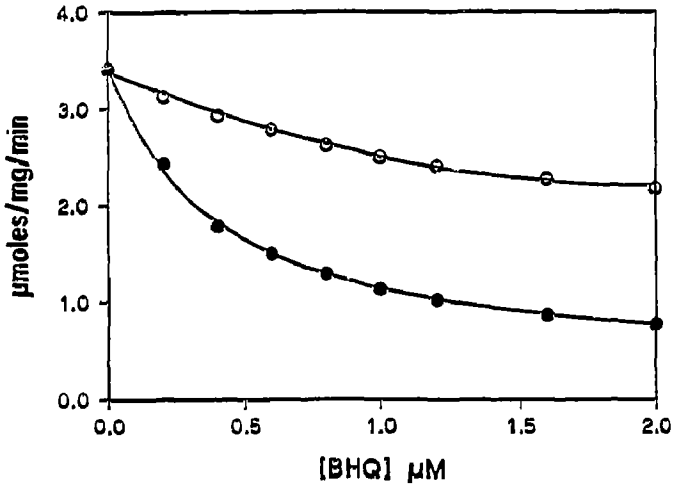


Fig. 1. The effect of BHQ on steady state ATPase activity. (●) ATPase (70 nM) was incubated with the given concentration of BHQ for 30 s and then assayed for activity at 25°C, pH 7.2, ATP = 2.1 mM and free Ca^{2+} = 10 μM. For the points (○), Triton X-100 (0.005%) was added following the incubation with BHQ and incubated for 2 min before ATPase assay.

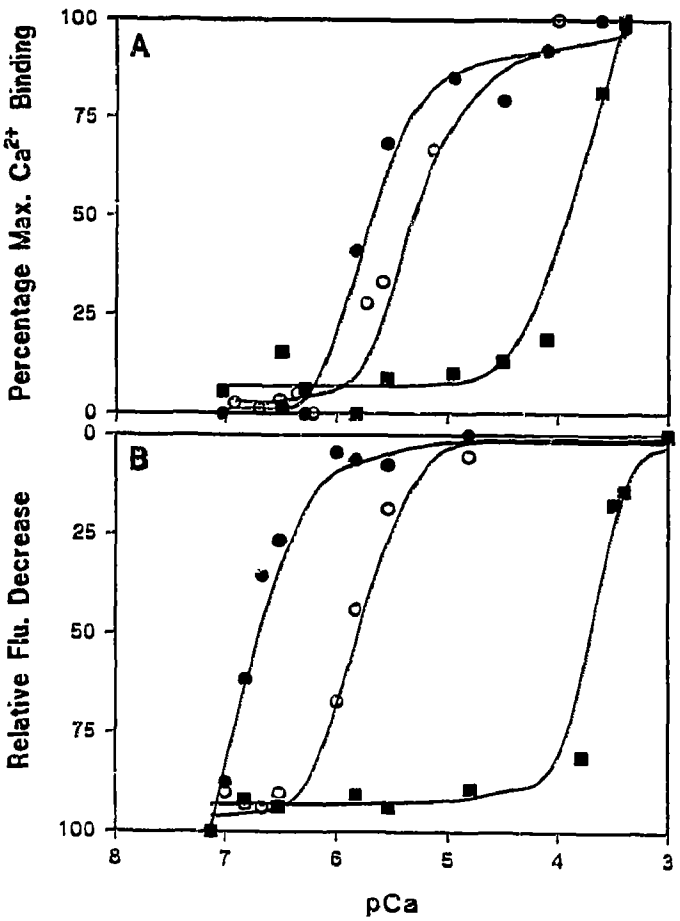


Fig. 2. Effects of inhibitors on binding of Ca^{2+} to the ATPase. (A) Ca^{2+} binding to the ATPase (2 μM) was measured as a function of the free Ca^{2+} concentration in 50 mM HEPES/KOH, pH 7.2, 20 mM $MgSO_4$ in the absence of inhibitors (■) or in the presence of 2 μM thapsigargin (■) or 30 μM BHQ (○). Ca^{2+} binding is expressed as % of the maximal binding observed in the absence of inhibitor (typically 12 nmoles Ca^{2+} bound/mg protein). (B) The % decrease in fluorescence intensity for NBD-labelled ATPase (1 μM) was measured as a function of the free Ca^{2+} concentration in 50 mM HEPES/KOH, pH 7.2, in the absence of inhibitor (●) or in the presence of 1 μM thapsigargin (■) or 30 μM BHQ (○).

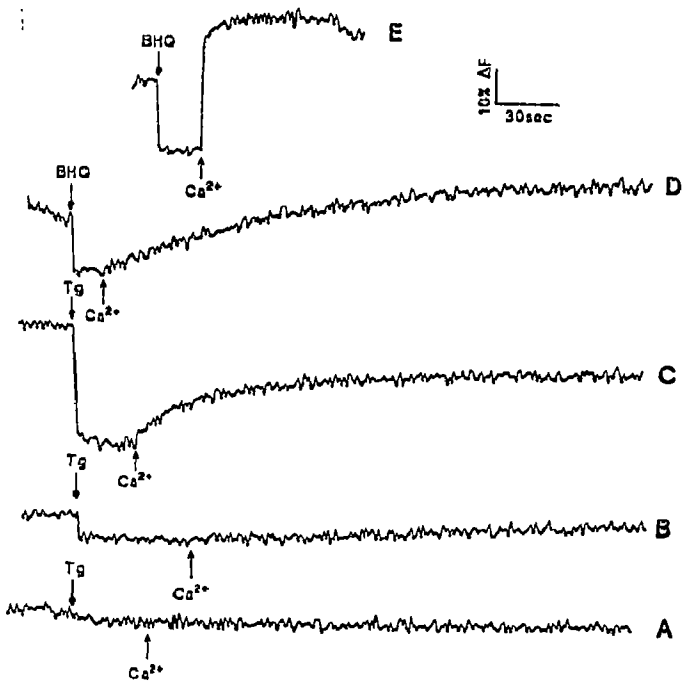


Fig. 3. Effect of inhibitors on the time-course of the fluorescence change of NBD-labelled ATPase following addition of Ca^{2+}. NBD-labelled ATPase (1 μM) was incubated in: (A,D; pH 6.0), 50 mM MES/KOH (B,E; pH 7.0), 50 mM MOPS/KOH, or (C; pH 8.5), 50 mM Tris-HCl, containing 0.3 mM EGTA. At the marked times, 1 μM thapsigargin (Tg; A–C) or 32 μM BHQ (D,E) were added, followed by 0.7 mM Ca^{2+}.

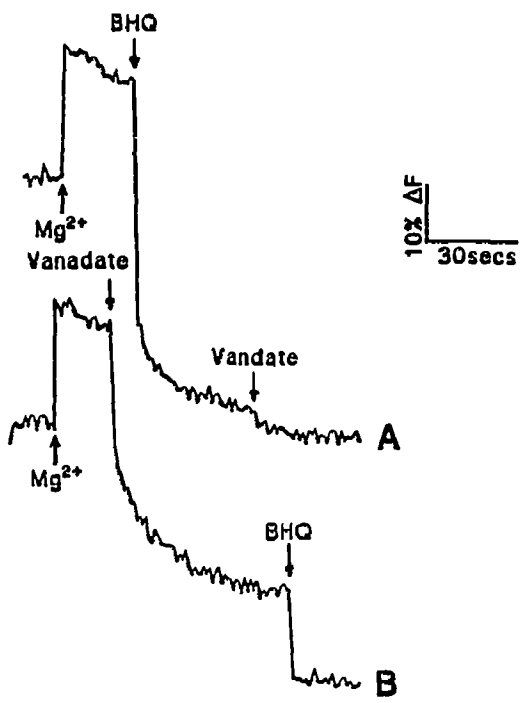


Fig. 4. Effect of BHQ on the fluorescence intensity of NBD-labelled ATPase. NBD-labelled ATPase (1 μM) was incubated in 50 mM Tris/KOH, pH 8.0 containing 0.3 mM EGTA. At the marked times, the following additions were made: (A), 5 mM Mg^{2+}, 32 μM BHQ, 1 mM vanadate; (B), 5 mM Mg^{2+}, 1 mM vanadate, 32 μM BHQ.

shown in Fig. 4, this effect can be reversed by addition of BHQ. Vanadate, an analogue of P_i, is thought to bind to the ATPase shifting the equilibrium towards E2 and forming a complex analogous to E2P in Scheme 1 [16]. Addition of vanadate to NBD-labelled ATPase following addition of BHQ had very little effect on fluorescence intensity (Fig. 4A). Addition of vanadate before BHQ resulted in a large decrease in fluorescence intensity, but now addition of BHQ was found to have little effect (Fig. 4B).

The effects of BHQ and thapsigargin on the steady-state level of phosphorylation of the ATPase by ATP observed in the presence of 1 mM Ca^{2+} are shown in Table I. The effect of BHQ on phosphorylation of the ATPase by P_i is shown in Table II.

4. DISCUSSION

BHQ has been reported to inhibit the Ca^{2+},Mg^{2+}-ATPase of endoplasmic reticulum [1,3,5]. We show here that it also inhibits the Ca^{2+},Mg^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (Fig. 1). The dissociation constant for BHQ is 0.4 μM, indicating considerably weaker binding than for the inhibitor thapsigargin which has a dissociation constant in the nM range [6–8]. Nevertheless, the mechanisms of inhibition by BHQ and thapsigargin appear very similar. We have shown elsewhere that thapsigargin favours the E2 conformation of the ATPase [6] and we show here that BHQ has the same effect.

It has been suggested that the E2–E1 equilibrium for the ATPase can be monitored by changes in the fluorescence intensity of the ATPase labelled with NBD-Cl, since the fluorescence intensity of NBD-labelled ATPase is relatively low in the E2 conformation and higher in the E1 conformation [15]. Addition of 32 μM BHQ to NBD-labelled ATPase in the presence of Ca^{2+} (data not shown) or vanadate (Fig. 4) results in a 12% decrease in fluorescence intensity and a 30% decrease is also seen in the intensity of tryptophan fluorescence of the ATPase (data not shown); these changes presumably follow from direct effects of BHQ on fluorescence intensity. At pH 8, addition of Mg^{2+} to NBD-labelled ATPase results in an increase in fluorescence intensity, attributable to a shift to E1 and subsequent addition of BHQ results in a 60% decrease in fluorescence intensity, indicating a shift in the equilibrium towards E2 (Fig. 4). Since subsequent addition of vanadate, which binds preferentially to the E2 conformation of the ATPase [16], has little further effect on fluorescence we conclude that in the presence of BHQ the ATPase is predominantly in the E2 conformation. If vanadate is added to the NBD-labelled ATPase before BHQ, a 45% decrease in fluorescence is observed, representing the shift to E2.

Subsequent addition of BHQ results in a smaller change in fluorescence intensity, of the same magnitude as observed on addition of BHQ to the NBD-labelled ATPase in the presence of Ca^{2+} (data not shown).

The dependence of fluorescence intensity on Ca^{2+} concentration is illustrated in Fig. 2B. In the presence of a 1:1 molar ratio of thapsigargin to NBD-labelled ATPase, the pCa^{2+} value giving the half-maximal effect is 3.7 compared to 6.6 in the absence of thapsigargin. BHQ (at a molar ratio of 32:1 with ATPase) also decreases the pCa^{2+} value required for half-maximal effect, but only by 0.8. Corresponding shifts are seen in Ca^{2+}-binding curves measured directly using $^{45}Ca^{2+}$ (Fig. 2A). In the presence of thapsigargin, a binding curve could only be established in the presence of 20 mM Mg^{2+} to reduce non-specific binding to the membrane. Under these conditions thapsigargin at a 1:1 molar ratio with ATPase results in a decrease in the pCa^{2+} value giving half-saturation of sites of ca. 2 units. Previously we suggested that in the presence of thapsigargin only a single Ca^{2+} could bind to the ATPase instead of the usual 2 [6], but the data shown in Fig. 2A suggests that our previous observation followed from the considerable decrease in affinity of the ATPase for Ca^{2+}. Addition of BHQ at a 15:1 molar ratio with ATPase also results in a decrease in affinity for Ca^{2+}, but only by 0.4 units.

The observed decreases in affinity for Ca^{2+} are consistent with the suggested shift of the E2–E1 equilibrium towards E2. In terms of the E2–E1 model presented in Froud and Lee [17], the results shown in Fig. 2 suggest decreases in the E1/E2 equilibrium constant by factors of 10 and 10^4 in the presence of BHQ and thapsigargin, respectively. If the inhibitors bind only to the E2 form of the ATPase, inhibitor binding would produce a dead-end complex as suggested by Sagara and Inesi [7]. The slow E2–E1 change seen on addition of Ca^{2+} in the presence of inhibitor (Fig. 3) would then correspond to a slow dissociation of inhibitor, [E2 inhibitor] → [E2] → [E1] → [E1Ca_2]. An alternative explanation of the results would be that inhibitor can bind to both E2 and E1, but with stronger binding to E2 to explain the shift of the equilibrium towards E2. If the rate of dissociation of the inhibitor from E2 is slower than the rate of the transition [E2 inhibitor] → [E1 inhibitor], then the pathway followed on addition of Ca^{2+} would be [E2 inhibitor] → [E1 inhibitor] → [E1] → [E1Ca_2]. Such a model would be consistent with the pH dependence of the transition shown in Fig. 3. The rate of the E2–E1 transition increases with increasing pH but in the absence of inhibitor is too fast to follow in a conventional fluorimeter [17]. In the presence of thapsigargin the rate of the transition becomes immeasurably slow at pH 6, but significant at pH 8.5. Effects of BHQ are, as expected, less marked with the rate at pH 6 being slow, although that at pH 7 is too fast to follow.

As described elsewhere [12,18] it is not possible to change the equilibrium constant for just one step alone in the reaction cycle of the ATPase, since the product of the equilibrium constants for all the steps around the cycle is fixed, equal to that for the hydrolysis of ATP. Table II shows that BHQ reduces the level of phosphorylation of the ATPase by P_i, consistent with a reduction in the equilibrium constant for phosphorylation. Table I shows that addition of BHQ also reduces the steady-state level of phosphorylation observed in the presence of 1 mM Ca^{2+} and [γ-^{32}P]ATP. A very similar result was obtained following addition of nonylphenol to the ATPase [18] and attributed to an increase in the rate of dephosphorylation of the ATPase (consistent with a decrease in the equilibrium constant for phosphorylation by P_i).

The studies reported here suggest very similar modes of action for thapsigargin and BHQ, despite their very different structures. Further, nonylphenol has also been

Table I

Effects of thapsigargin and BHQ on phosphorylation of the Ca^{2+}, Mg^{2+}-ATPase by ATP

Additions	nmoles [EP]/mg protein
ATPase + Ca^{2+} + [γ-^{32}P]ATP	2.7
ATPase + thapsigargin + Ca^{2+} + [γ-^{32}P]ATP	0.1
ATPase + Ca^{2+} + thapsigargin + [γ-^{32}P]ATP	0.1
ATPase + BHQ + Ca^{2+} + [γ-^{32}P]ATP	1.5
ATPase + Ca^{2+} + BHQ + [γ-^{32}P]ATP	1.7

Phosphorylation of the ATPase (2 μM) was performed in 0.5 ml of 20 mM HEPES/Tris, 5 mM $MgSO_4$, 100 mM KCl, 100 μM [γ-^{32}P]ATP, pH 7.2, inhibitor and 1 mM Ca^{2+}. Either the ATPase was incubated with inhibitor for 2 min followed by addition of Ca^{2+} and [γ-^{32}P]ATP or the ATPase was incubated with Ca^{2+} followed by addition of inhibitor for 2 min and final addition of [γ-^{32}P]ATP. After 10 s at 25°C the reaction was quenched with 12% trichloroacetic acid, 0.2 M phosphoric acid and the precipitate collected and washed on Whatman GF/B filters with 3 × 5 ml quenching solution. The ^{32}P on the filter was determined by liquid scintillation counting. Inhibitor concentrations were 2 μM thapsigargin or 30 μM BHQ.

Table II

Effect of BHQ on phosphorylation of the Ca^{2+},Mg^{2}-ATPase by P_i

	nmoles [EP]/mg protein	
P_i (mM)	no BHQ	30 μM BHQ
1	1.63	0.16
3	2.84	0.28
10	5.07	0.38

Phosphorylation of the ATPase (3.4 μM) was performed by incubating the ATPase in 0.5 ml of 150 mM MES/Tris, pH 6.2, 20 mM $MgSO_4$, 5 mM EGTA in the presence or absence of BHQ for 2 min followed by addition of 1 ml of the same buffer containing the required concentration of [^{32}P]P_i. After 20 s the reaction was quenched with 12% trichloroacetic acid, 0.2 M phosphoric acid, and the precipitate collected and washed on Whatman GF/C filters. Non-specific binding was assessed by quenching the ATPase before addition of [^{32}P]P_i and typically accounted for 10% of the total counts.

shown to shift the E2/E1 equilibrium towards E2 [18]. It remains to be determined whether one or more binding sites on the ATPase are involved in the effects of these inhibitors.

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