Interactions of Dihydroxybenzenes with the Ca^{2+}-ATPase: Separate Binding Sites for Dihydroxybenzenes and Sesquiterpene Lactones†

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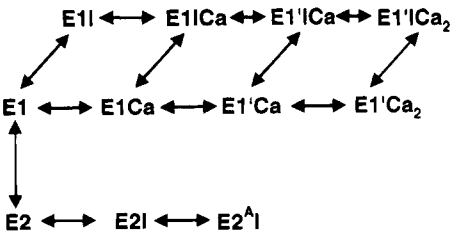
ABSTRACT: The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (BHQ) and other hydrophobic 1,4-dihydroxybenzenes. Inhibitory potency increases on increasing substituent chain length from 2,5-dipropyl-1,4-dihydroxybenzene to 2,5-di-*tert*-amyl-1,4-dihydroxybenzene, the most potent inhibitor, but then decreases for 2,5-bis(7-methylheptyl)-1,4-dihydroxybenzene. Kinetic measurements are consistent with isomerization following the initial binding of BHQ to the ATPase to give a modified E2 conformation, $E2^AI$, as for the binding of sesquiterpene lactones, such as thapsigargin. Binding of BHQ to the ATPase shifts the E1−E2 equilibrium toward E2 because of the formation of $E2^AI$. Measurements of Ca^{2+} binding as a function of BHQ concentration suggest that BHQ can bind to the E1 conformation of the ATPase (but without the subsequent conformational change observed on binding to E2) and that the binding constants of E1 for Ca^{2+} are unaffected by binding of BHQ. Binding of BHQ to the ATPase in the presence of substoichiometric amounts of thapsivillosin A and effects of mixtures of BHQ and thapsivillosin A show that these two inhibitors have separate binding sites on the ATPase.

The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR)[1] is inhibited by two classes of hydrophobic molecule, the sesquiterpene lactones including thapsigargin, thapsivillosin A (TvA), and trilobolide (Thastrup et al., 1990; Sagara & Inesi, 1991; Kijima et al., 1991; Lytton et al., 1991; Wictome et al., 1992a, 1995), and the dihydroxybenzenes (or hydroquinones) including 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (BHQ) (Llopis et al., 1991; Wictome et al., 1992b, 1994; Nakamura et al., 1992). Both the sesquiterpene lactones and BHQ have been shown to shift the E1/E2 equilibrium of the ATPase toward E2 with a decrease in the rate of the E2 → E1 transition, explaining the inhibition of ATPase activity by these compounds (Wictome et al., 1992b). Distinct effects of thapsigargin on the conformational preferences of the Ca^{2+}-ATPase are also suggested by studies of the effects of thapsigargin on the pattern of crystallization (Stokes & Lacapere, 1994).

Measurements of the rate of quenching of the tryptophan fluorescence of the ATPase by TvA have been shown to be consistent with second-order association of the inhibitor with the ATPase to give E2I followed by a first-order isomerization to $E2^AI$ as in Scheme 1, with Trp fluorescence intensity being sensitive to the E2I ⇄ $E2^AI$ change (Wictome et al., 1995). The kinetic and equilibrium data are consistent with binding of TvA to E1 and E2 with equal affinities, the net shift toward E2 following from a favorable equilibrium constant $E2^AI$/E2I (Wictome et al., 1995). In this scheme, the slow step on binding Ca^{2+} to the ATPase in the presence

Scheme 1



of TvA is the E2 → E1 transition, whose effective rate is slow because the fraction of unbound ATPase able to undergo the transition is small. It has also been shown that effects of TvA on the affinity of the ATPase for Ca^{2+} saturate at a 1:1 molar ratio of TvA to ATPase (Wictome et al., 1995). This is not consistent with direct competition between TvA and Ca^{2+} for binding to the ATPase since this would predict a linear decrease in Ca^{2+} affinity with increasing TvA concentration. The results are, however, consistent with Scheme 1 in which TvA can bind to the Ca^{2+}-bound E1 conformations of the ATPase with an affinity equal to that for the Ca^{2+}-free E1 conformation (Wictome et al., 1995). Formation of $E2^AI$ will result in a reduced affinity for Ca^{2+} in the presence of inhibitor since only the E1 conformation has high-affinity binding sites for Ca^{2+} (Wictome et al., 1995). The rate of binding of Ca^{2+} to the ATPase becomes slow in the presence of TvA because only the unbound form of the ATPase can undergo the E2 → E1 transition (Wictome et al., 1995).

It has been suggested that the fluorescence of the ATPase labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) is sensitive to conformational changes at a "gating" site controlling the rate of access of Ca^{2+} to its binding sites (Henderson et al., 1994a). The fluorescence of DMC-labeled ATPase is also sensitive to phosphorylation of the ATPase, suggesting that phosphorylation of the ATPase also affects the gating site (Stefanova et al., 1992). Binding of sesqui-

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[1] Abbreviations: AMPPNP, 5′-adenylylimidodiphosphate; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; FITC, fluorescein isothiocyanate; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; SR, sarcoplasmic reticulum; AHQ, 2,5-di-*tert*-amyl-1,4-dihydroxybenzene; BHQ, 2,5-di-*tert*-butyl-1,4-dihydroxybenzene; PHQ, 2,5-dipropyl-1,4-dihydroxybenzene; TvA, thapsivillosin A.

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terpene lactones to the Ca^{2+}-ATPase also affect DMC fluorescence, suggesting changes at the gating site on the E2I $\rightleftharpoons$ $E2^AI$ transition (Wictome et al., 1995).

We show here that binding of BHQ to the ATPase is also consistent with Scheme 1 but that BHQ and TvA bind to separate sites on the ATPase.

MATERIALS AND METHODS

Sarcoplasmic reticulum (SR) vesicles and purified Ca^{2+}-ATPase were prepared from rabbit skeletal muscle as described in East and Lee (1982). Concentrations of ATPase were estimated from the absorbance at 280 nm in 1% SDS and KOH (5 mM) using the extinction coefficient (1.2 l g^{-1} cm^{-1}) given by Hardwicke and Green (1974), based on a molecular weight of 109 000. Thapsigargin was obtained from Calbiochem, and thapsivillosin A was extracted from *Thapsia villosa* roots as described in Wictome et al. (1995). 2,5-Di-*tert*-amyl-1,4-dihydroxybenzene was obtained from ChemService. 2,5-Dipropyl-, dibutyl-, bis(7-methylheptyl)-, di-2-propenyl-, bis(carboxymethyl)-, bis(ethylamino)-, and disulfonyl-1,4-dihydroxybenzene and 2,3,5,6-tetramethyl-1,4-dihydroxybenzene were gifts from Zeneca. Other compounds were obtained from Aldrich. Inhibitors were added to the ATPase from concentrated solutions in dimethyl sulfoxide.

The ATPase was labeled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) following the protocol of Wakabayashi et al. (1990a) with some modifications. SR (20 mg of protein) was suspended in buffer (10 mL; 13 mM MOPS/KOH, pH 7.0, 0.2 M KCl, 1 mM $CaCl_2$) containing 0.6 mg of 5′-adenylylimidodiphosphate (AMPPNP)/mL. A 120 μL amount of a stock solution of 7-chloro-4-nitrobenz-2-oxa-1,3-diazole in ethanol (5 mg/mL) was added with mixing, and the mixture was incubated in the dark for 1 h at 25 °C. The reaction was then stopped by diluting into 4 vol of ice cold buffer (10 mM histidine/HCl, pH 6.8, 0.1 M KCl, 0.3 M sucrose) and centrifuged at 37 000*g* for 30 min at 4 °C. The pellet was resuspended in buffer (4.5 mL; 20 mM MOPS/NaOH, pH 7.0, 0.1 M NaCl, 0.3 M sucrose) containing 0.3 mg of AMPPNP/mL. A 0.5 mL amount of 100 mM DTT was then added followed by 10 min incubation in the dark at 25 °C. The reaction was stopped by dilution into 8 vol of ice cold buffer (20 mM MOPS/NaOH, pH 7.0, 0.1 M NaCl, 0.3 M sucrose) and centrifuged for 30 min at 37 000*g* at 4 °C. The pellet was resuspended in buffer and frozen until use.

SR was labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) by incubation of Br-DMC and SR at a molar ratio of label to protein of 10:1 at room temperature for 1 h in the dark, in 50 mM Tris/HCl (pH 7.0) containing 200 mM sucrose. Unbound label was removed by centrifugation through two columns of Sephadex G-50 (Stefanova et al., 1992). ATPase was labeled with fluorescein isothiocyanate (FITC) by incubation with a 1:1 molar ratio of label to protein in 50 mM potassium phosphate (pH 8.0) containing 100 mM KCl and 200 mM sucrose, for 45 min at room temperature, which was followed by removal of unreacted FITC on a Sephadex column as above.

The tryptophan fluorescence of the ATPase was recorded by using an SLM-Aminco 8000C fluorometer, with excitation and emission wavelengths of 295 and 330 nm, respectively. DMC fluorescence was excited at 350 nm and observed at 425 nm. NBD fluorescence was excited at 430 nm and observed at 520 nm; to reduce the signal due to scattered light, the excitation beam was passed through a 450 nm long wavelength cutoff filter (450FLO T-50, Andover Corporation) and the emission was passed through a Hoya Y50 500 nm short wavelength cutoff filter. Ca^{2+} titrations were performed by addition of aliquots of a stock solution of EGTA (500 mM) to vary the free Ca^{2+} concentrations. Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+}, and H^+ to EGTA given by Godt (1974). The time dependence of the effects of Ca^{2+} on the fluorescence of the ATPase incubated in the presence of inhibitor were determined by injection of a stock solution of Ca^{2+} from a Hamilton syringe into the sample stirred in the fluorescence cuvette with a Cell-Spinbar magnet (Bel-Art products).

Rapid kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorometer with BioKine V3.20a software for data acquisition and analysis (Henderson et al., 1994b). Tryptophan fluorescence was excited through a monochromator set at 290 nm and emitted light was collected at 90° and filtered by a high-pass cutoff filter with 50% transmission at 320 nm (UV 32, Andover Corporation).

ATPase activities were determined at 25 °C by using a coupled enzyme assay in a medium containing 40 mM Hepes/KOH (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 mL. The reaction was initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents, to give a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration of ca. 10 μM).

Kinetic simulations were performed using FACSIMILE v 3.0 (AEA Technology, Harwell) running on an IBM-compatible PC using the parameters for Ca^{2+} binding given in Lee et al. (1995). Simulations were fitted to single or double exponentials using the same package (BioKine V3.20a) that was used for fitting the experimental data.

For simulations of the equilibrium binding experiments, a set of equations were written describing the individual equilibrium binding steps which were then solved using the method of bisection (Press et al., 1986).

RESULTS

Inhibition of ATPase Activity. Addition of BHQ to the ATPase resulted in inhibition of ATPase activity, the inhibition curve being consistent with binding of BHQ at a single site with an apparent K_d value of 0.4 μM with a maximal inhibition of 100% (Figure 1). Inhibition was also observed with a variety of other hydrophobic 1,4-dihydroxybenzene derivatives (Figure 1, Table 1). 2,6-Di-*tert*-butyl-1,4-dihydroxybenzene, 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol, and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde had no effect on ATPase activity at concentrations up to 60 μM.

Stoichiometry of Binding and Effect of BHQ. Binding of BHQ to DMC-labeled SR resulted in a decrease in fluorescence intensity (Figure 2A), the magnitude of the effect being comparable to that reported on binding TvA (Wictome et al., 1995). The concentration at which the effect of BHQ saturates varied between preparations of SR, as was found previously for TvA (Wictome et al., 1995). The fluorescence

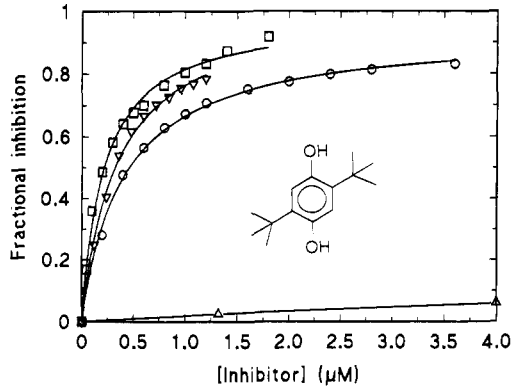

FIGURE 1: Effects of 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (○), 2,5-di-*tert*-amyl-1,4-dihydroxybenzene (□), 2,5-dipropyl-1,4-dihydroxybenzene (△), and 2,5-dibutyl-1,4-dihydroxybenzene (▽) on the ATPase activity of uncoupled SR vesicles (0.04 μM ATPase) measured at pH 7.2 and 25 °C in 100 mM KCl, 2.1 mM ATP, and 50 μM Ca^{2+} in the presence of 4.0 μg of A23187/mL. Results are expressed relative to the activity measured in the absence of inhibitor (3.4 IU/mg of protein). The insert shows the structure of 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (BHQ).

Table 1: Inhibition of the ATPase by 1,4-Dihydroxybenzene Derivatives[a]

1,4-dihydroxybenzene 2,5-substituent	apparent K_d (μM)
$CH_2CH_2CH_3$	58
$CH_2CH{=}CH_2$	122
$C(CH_3)_3$	0.4
$CH_2CH_2CH_2CH_3$	0.3
$CH_2C(CH_3)_3$	0.2
$CH_2CH_2CH_2CH_3CH_2CH(CH_3)_2$	39
CH_2COOH	nd[b]
$CH_2CH_2NH_2$	nd
SO_3	nd
other compounds	
2,3,5,6-tetramethyl-1,4-dihydroxybenzene	nd
2,6-di-*tert*-butyl-1,4-dihydroxybenzene	nd
3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol	nd
3,5-di-*tert*-butyl-4-hydroxybenzaldehyde	nd

[a] ATPase activities were measured at pH 7.2, 2.1 mM ATP, 50 μM Ca^{2+}, 40 nM ATPase, 25 °C. [b] nd, no inhibition detected at concentrations up to 60 μM.

data cannot be fitted using a simple Scatchard plot since the concentration range over which BHQ has its effect is comparable to the concentration of ATPase in the sample (1 μM for the experiment shown in Figure 2) so that binding significantly depletes the concentration of free, unbound BHQ. For other hydrophobic molecules, binding has been demonstrated to both the protein and the lipid components of the membrane (Froud et al., 1986). Addition of vesicles of dioleoylphosphatidylcholine at concentrations up to 500 μM to DMC-labeled SR had no significant effect on the response to BHQ (Figure 2A). We conclude that BHQ does not bind to the lipid component of the SR membrane at the concentration of lipid present in these experiments (90 μM, calculated for a molar ratio of lipid:ATPase of 90:1; Warren et al., 1974).

For a single binding site on the ATPase with no binding to lipid, the fluorescence curve can be fitted to the equation

$$F = F_{max}(A - (A^2 - 4nEI)^{1/2})/2$$

where F is the decrease in fluorescence intensity observed

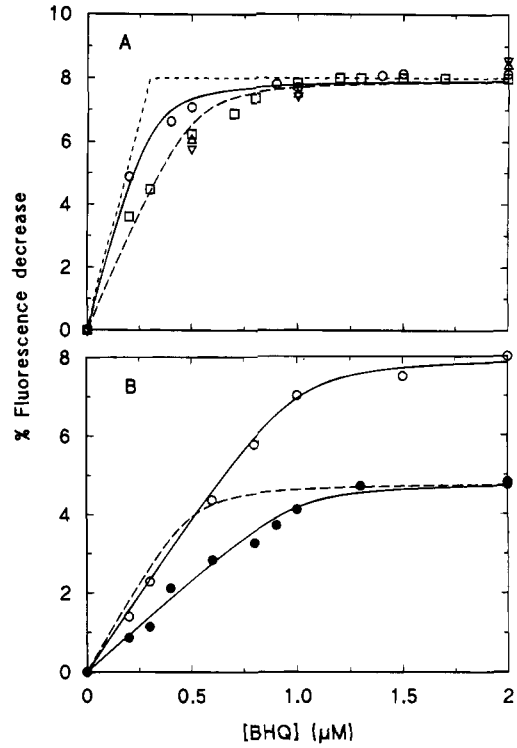

FIGURE 2: Effect of BHQ on the fluorescence intensity of DMC-labeled SR. (A) (○, □) two different preparations of DMC-labeled SR (1 μM); (△, ▽), the preparation of DMC-labeled SR used to obtain the data points (□) but in the presence of 100 (△) or 500 μM (▽) dioleoylphosphatidylcholine added as sonicated vesicles. The solid and broken lines represent simulations for a K_d value of 0.02 μM and 0.3 and 0.5 site per ATPase molecule, respectively. The dotted line represents a simulation with K_d = 1 nM and 0.3 site per ATPase molecule. (B) Effect of BHQ on the fluorescence of DMC-labeled SR (2 μM) in the absence (○) or presence (●) of 0.5 μM TvA. The buffer was 40 mM Tris/HCl, pH 6.0, containing 1 mM EGTA. The solid lines represent simulations with a K_d value of 0.02 μM and 0.5 site per ATPase molecule, and the broken line represents a simulation with K_d = 0.02 μM and 0.25 site per ATPase molecule.

at concentrations of BHQ and ATPase of I and E, respectively, n is the number of binding sites per ATPase molecule, and A is given by

$$A = K_d + I + nE$$

where K_d is the dissociation constant for the inhibitor. A free fit to this equation gave a value for F_{max} of 8% with n = 0.45 ± 0.04 and 0.25 ± 0.04 for the two sets of binding data and K_d values of 0.03 ± 0.01 and 0.05 ± 0.01 μM, respectively. The maximum level of phosphoenzyme formation observed for our preparations of the Ca^{2+}-ATPase varies between 2.5 and 4 nmol of [EP]/mg of protein [see Starling et al. (1993)], corresponding to between 29% and 46% of that expected for a pure protein of molecular weight 109 000. The reason for the lower than expected level of phosphorylation is unclear [see Coll and Murphy (1984) and Barrabin et al. (1984)]. The preparation of ATPase is essentially pure by SDS-PAGE (Gould et al., 1987). The stoichiometry of Ca^{2+} binding to the ATPase is 2:1 based on the maximal level of phosphorylation rather than on the total amount of protein present (Starling et al., 1993). However, the stoichiometry of labeling with reagents such as FITC (Froud & Lee, 1986), Br-DMC (Stefanova et al., 1992), and 5-(bromomethyl)fluorescein (Stefanova et al., 1993) is 1:1 with

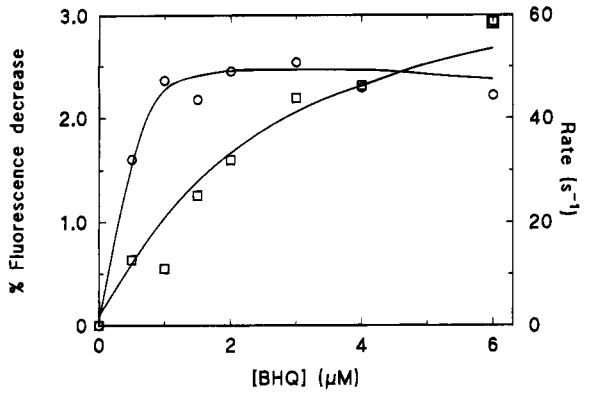

FIGURE 3: Rate of quenching of Trp fluorescence of the ATPase by BHQ. Decreases in the Trp fluorescence of the ATPase were determined as a function of time following mixing of the ATPase in buffer containing 1 mM EGTA at pH 7.2 with an equal volume of the same buffer containing 1 mM EGTA and BHQ in a stop-flow fluorimeter, to give 2 μM ATPase and the given concentrations of BHQ. Fluorescence changes were fitted to single exponentials; rate constants (□) and amplitudes (○) are plotted as a function of BHQ concentration. The solid lines show simulations of the rates and amplitudes, calculated as described in the text assuming 0.3 binding site per ATPase molecule and scaling the amplitude change to a maximum quenching of 2.4%.

respect to total protein. It is therefore assumed that the preparation of ATPase contains a variable proportion of "inactive" ATPase, unable to bind Ca^{2+} and be phosphorylated by ATP but still with a sufficiently native structure to ensure specific labeling with a variety of fluorescence labels. The variability in the stoichiometry of inhibitor binding is then presumed to depend on the extent of inhibitor binding to the "inactive" ATPase present in the preparation.

Figure 2B shows the effect of 1 μM TvA on a titration with BHQ in the presence of 2 μM DMC-labeled ATPase. In the absence of TvA, the data fits to $F_{max} = 8\%$, with $n = 0.50 \pm 0.01$ and $K_d = 0.02 \pm 0.01$ μM, and, in the presence of TvA, to $F_{max} = 4.8\%$, with $n = 0.53 \pm 0.03$ and $K_d = 0.02 \pm 0.01$ μM. If BHQ and TvA competed for binding at the same site on the ATPase, then, if dissociation of TvA was fast on the time scale of the experiment, the presence of TvA would have resulted in an increased K_d for BHQ. Alternatively, if dissociation of TvA was very slow, then if TvA and BHQ bound to the same site on the ATPase, the presence of TvA would have resulted in an apparent decrease in the stoichiometry of BHQ binding to the ATPase with no change in K_d (see the broken line in Figure 2B). The observation of no change in either n or K_d with a reduced value for F_{max} shows that BHQ and TvA bind at separate sites on the ATPase, with binding at either site resulting in the same conformational change on the ATPase as detected by DMC fluorescence.

Conformational Change Occurs on Binding BHQ. Binding of BHQ to the ATPase results in a decrease in Trp fluorescence intensity as observed previously for TvA (Wictome et al., 1995). The time course of the change in Trp fluorescence observed on mixing the ATPase with BHQ fits to a single exponential with the rate constants shown as a function of BHQ concentration in Figure 3. The dependence of the rate on the concentration of BHQ is nonlinear, although the nonlinearity is less marked than with TvA (Wictome et al., 1995). The simplest model consistent with these results is a second-order association step ($E \rightleftarrows EI$) followed by a first-order isomerization ($EI \rightleftarrows E^AI$), with

Table 2: Rate Parameters Describing Binding of BHQ to the ATPase and the Effects of Ca^{2+} According to Scheme 1[a]

step	rate constant
E1 → E1I	1.0×10^7 M^{-1} s^{-1}
E1I → E1	6 s^{-1}
E2 → E2I	7.0×10^7 M^{-1} s^{-1}
E2I → E2	42 s^{-1}
E2I → $E2^AI$	72 s^{-1}
$E2^AI$ → E2I	2.5 s^{-1}

[a] Rate constants for the steps $E2 \rightleftarrows E1$ and $E1Ca \rightleftarrows E1'Ca \rightleftarrows E1'Ca_2$ are as given in Lee et al. (1995); rate constants for the Ca^{2+} binding steps are unaltered by binding BHQ.

fluorescence quenching corresponding to the isomerization, as previously suggested for the binding of TvA (Wictome et al., 1995). In terms of Scheme 1, these steps correspond to $E2 \rightleftarrows E2I$ and $E2I \rightleftarrows E2^AI$, respectively. The time dependence of binding of BHQ to the ATPase was simulated in terms of Scheme 1 and fitted to a single expontial using the same fitting program used to fit the experimental data. Satisfactory agreement with the experimental data (Figure 3) can be obtained with rate constants of 7×10^7 M^{-1} s^{-1} and 72 s^{-1} for $E2 \rightleftarrows E2I$ and $E2I \rightleftarrows E2^AI$, respectively (Table 2). The simulations were almost independent of the rate of BHQ binding to E1; a rate constant of 1.0×10^7 M^{-1} cm^{-1} was therefore assigned to this step, the value assumed for binding of TvA (Table 2). The exact value of this rate constant had no effect on any of the simulations reported here.

Effects on E2−E1 Equilibrium. Reaction of the ATPase with NBD-Cl labels a number of Cys residues on the Ca^{2+}-ATPase, but subsequent treatment with dithiothreitol removes the majority of the label except that on Cys-344 (Wakabayashi et al., 1990a). The intensity of the fluorescence of the NBD group at Cys-344 has been shown to be sensitive to the E2−E1 equilibrium of the ATPase, with E2, E1, and $E1Ca_2$ being states of low-, high-, and intermediate-fluorescence intensity, respectively (Wakabayashi et al., 1990a,b; Wictome et al., 1992b; Henderson et al., 1994a). We find that some fluorescence also derives from NBD-labeled Cys residues in transmembrane regions of the ATPase, since fluorescence can be quenched with 12-doxylstearic acid; the fluorescence from these groups is not sensitive to the E2−E1 conformational change (unpublished observations). Addition of BHQ to NBD-labeled ATPase in the presence of 1 mM vanadate results in a decrease in NBD fluorescence intensity, attributed to a direct quenching of NBD-labeled Cys residues in the transmembrane region of the ATPase (Wictome et al., 1994). The effect of BHQ on the fluorescence of NBD-labeled ATPase at pH 6.0 (Figure 4) is comparable to the effect of BHQ in the presence of vanadate and is thus probably due to quenching. BHQ has a larger effect on fluorescence intensity at more alkaline pH values, attributable to a shift in E1−E2 equilibrium toward E2, the low-fluorescence form. The E2−E1 equilibrium for the ATPase is pH sensitive, low pH favoring the E2 state because of H^+ binding at a site with an affinity for H^+ of 5×10^5 M^{-1} in the E1 state and 3.0×10^8 M^{-1} in the E2 state, the equilibrium constant E1/E2 for the unprotonated forms of the ATPase being 4 (Henderson et al., 1994a). The effect of BHQ can be simulated assuming a 30-fold decrease in the E1/E2 equilibrium constant with no change in the H^+ binding constants (Figure 4) with fluorescence intensities of

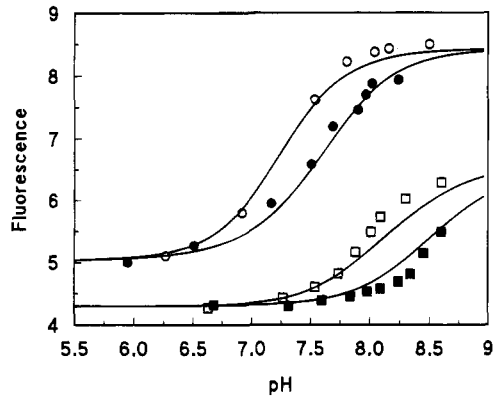

FIGURE 4: Effects of BHQ on the fluorescence intensity of NBD-labeled ATPase as a function of pH. NBD-labeled ATPase (1 μM) was incubated in buffer containing 0.3 mM EGTA and 100 mM KCl in the absence (○, ●) or presence of (□, ■) of 5 μM BHQ in the absence (●, ■) or presence (○, □) of 5 mM Mg^{2+} The 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, in the absence of BHQ and 4.3 and 7.8, respectively, in the presence of BHQ.

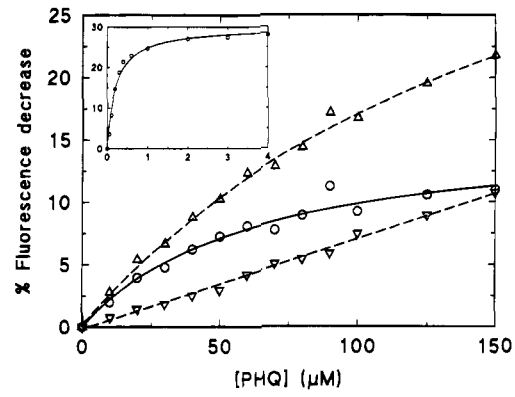

FIGURE 5: Effects of PHQ and AHQ on the fluorescence intensity of NBD-labeled ATPase (1 μM) at pH 8.0 in 50 mM Tris/HCl. Shown are the percent decreases in fluorescence intensity on addition of PHQ in the absence (△) or presence (▽) of 0.7 mM Ca^{2+}, and the difference between the two curves (○). The solid line shows a fit to a single binding site with K_d of 63 μM and a maximum fluorescence decrease of 16%. The insert shows the decrease in fluorescence intensity (%; y-axis) as a function of AHQ concentration (μM; x-axis).

5.0 and 8.5 for the E2 and E1 conformations, respectively, in the absence of BHQ and 4.3 and 7.8 for E2 and E1, respectively, in the presence of BHQ. In terms of Scheme 1, a 30-fold decrease in the equilibrium constant E1/E2 in the presence of BHQ is consistent with an equilibrium constant $E2^AI/E2I$ of 29 with equal inhibitor binding constants K^{E1} and K^{E2} for the E1 and E2 conformations, respectively. A 30-fold shift in the E1/E2 equilibrium constant is consistent with the shift in Ca^{2+} affinity reported below.

Higher fluorescence intensities are observed for NBD-labeled ATPase in the presence of Mg^{2+} than in its absence, attributable to stronger binding of Mg^{2+} to the E1 conformation of the ATPase than to the E2 conformation (Henderson et al., 1994a). Effects of BHQ on the fluorescence of NBD-labeled ATPase in the presence of Mg^{2+} can also be simulated (Figure 4) in terms of a 30-fold decrease in the E1/E2 equilibrium with the Mg^{2+} binding constants given in Henderson et al. (1994a). This implies that BHQ has no effect on Mg^{2+} binding to the ATPase.

Titration of NBD-labeled ATPase with 2,5-dipropyl-1,4-dihydroxybenzene (PHQ) at pH 8.0 in the presence of Ca^{2+} results in a linear decrease in fluorescence intensity with increasing concentration of PHQ (Figure 5), attributable to direct quenching of NBD fluorescence by PHQ. In the absence of Ca^{2+}, quenching is of greater magnitude, and the difference between the two quenching curves shows saturation (Figure 5). The difference curve fits to a K_d value of 63 ± 13 μM and a maximum fluorescence change of −16% ± 1% (Figure 5). Assuming equal fluorescence intensities for PHQ- and BHQ-bound forms, this maximum fluorescence change is consistent with a 5-fold decrease in the E1/E2 equilibrium constant. A similar titration with 2,5-di-*tert*-amyl-1,4-dihydroxybenzene (AHQ) gives a maximum fluorescence change of −28% (Figure 5), which is very similar to that recorded with BHQ in the absence of KCl. Thus effects of BHQ and AHQ on the E1/E2 equilibrium are very similar. Figure 5 also makes clear the much higher affinity of the ATPase for AHQ than for PHQ.

Effect of BHQ on Ca^{2+} affinity. Binding of Ca^{2+} to the ATPase can be followed very conveniently by following the changes in Trp fluorescence for the ATPase that follow from Ca^{2+} binding (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984; Henderson et al., 1994a; Lee et al., 1995) or from the changes in NBD fluorescence for the NBD-labeled ATPase following from the E2 → E1 Ca_2 transition (Wictome et al., 1992b). Addition of 1 μM BHQ to 1 μM 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 half-maximal response is observed at Ca^{2+} concentrations of 1 and 5 μM in the absence and presence of BHQ, respectively (Figure 6A). Figure 6A also shows that increasing the concentration of BHQ beyond a 1:1 molar ratio with ATPase results in no further significant decrease in Ca^{2+} affinity. Comparable Ca^{2+} affinities for 1 μM NBD-labeled ATPase are also observed at 1 and 2 μM BHQ if Ca^{2+} binding is monitored through changes in NBD fluorescence intensity; the Ca^{2+} affinity measured in the presence of 0.5 μM BHQ is, however, slightly higher than that observed at 1 μM BHQ (Figure 6B). In the presence of Mg^{2+}, the affinity of the ATPase for Ca^{2+} is reduced, which is attributable both to binding of Mg^{2+} at the Ca^{2+} binding sites and to binding of Mg^{2+} at a "gating" site controlling the rate of access of Ca^{2+} to the binding sites on the ATPase (Henderson et al., 1994a,b). Shifts in Ca^{2+} affinity caused by BHQ in the presence of Mg^{2+} are very similar to those in the absence of Mg^{2+} (Figure 6C), suggesting that Mg^{2+} binding to the ATPase is unaffected by BHQ.

It has been shown that if Ca^{2+} and inhibitor are in direct competition for binding to the ATPase, then the affinity of the ATPase for Ca^{2+} will decrease linearly with increasing amount of inhibitor beyond a 1:1 molar ratio with ATPase (Wictome et al., 1995). This is not observed experimentally (Figure 6). Saturation of the effect of inhibitor at a 1:1 molar ratio with ATPase follows if inhibitor can bind to the Ca^{2+}-bound ATPase as in Scheme 1 (Wictome et al., 1995). Binding of Ca^{2+} to the ATPase can be described using the binding parameters given in Henderson et al. (1994a) and Lee et al. (1995). With the same Ca^{2+} binding constants for E1 and E1I, a 30-fold stronger binding of BHQ to the E2 conformation than to the E1 conformation of the ATPase results in a shift in the Ca^{2+} concentration, giving 50% binding of Ca^{2+} from 1 μM in the absence of BHQ to 4.2

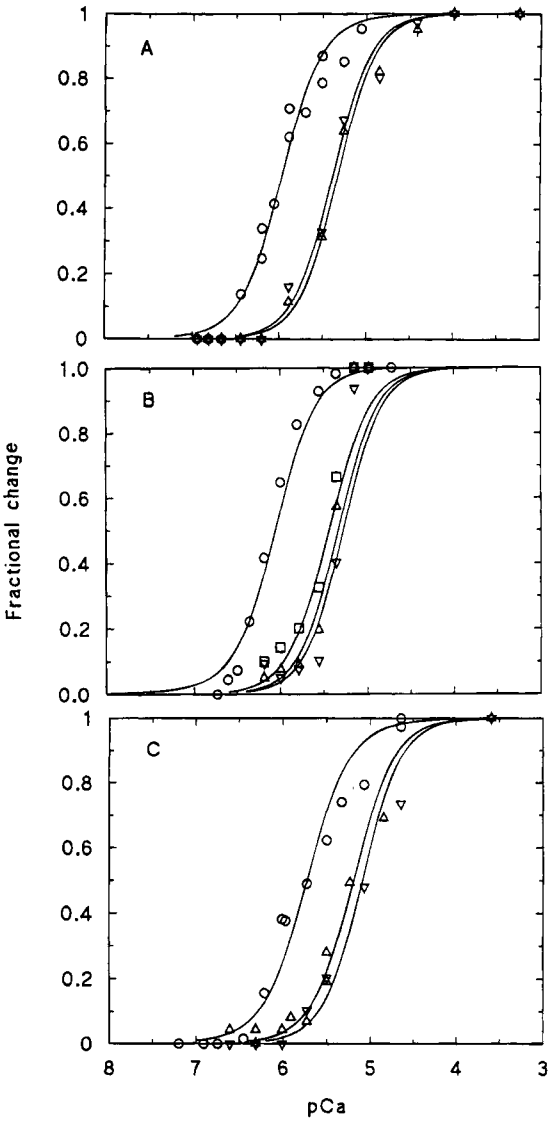

FIGURE 6: Effects of BHQ on the Ca^{2+} affinity of the ATPase as determined by changes in Trp or NBD fluorescence. Shown are the changes (A, C) in Trp fluorescence intensity for unlabeled ATPase (1 μM) or (B) NBD fluorescence for NBD-labeled ATPase (1 μM) in buffer containing 0.3 mM EGTA and 1.3 mM 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 and 7.5. (A, C) in 50 mM Hepes/KOH, pH 7.2, in the absence of BHQ (○), or in the presence of 1 (△) or 2 μM (▽) BHQ, in the absence (A) or presence (C) of 5 mM Mg^{2+}. (B) in 50 mM Hepes/KOH, pH 7.2, in the absence of inhibitor (○), or in the presence of 0.5 (□), 1 (△) or 2 μM (▽) BHQ. The lines are simulations calculated as described in the text assuming 0.3 binding site per ATPase molecule.

and 5.2 μM in the presence of 1 and 2 μM BHQ, respectively, which is in good agreement with the experimental data (4.1 ± 1.0 and 4.4 ± 1.0 μM, respectively; Figure 6).

The shifts in Ca^{2+} affinity caused by binding of 1 μM TvA and 1 μM BHQ are additive; the observed total shift can be simulated as the sum of the shifts caused by TvA and BHQ alone (Figure 7). In conjunction with the results shown in Figure 6 this implies that BHQ and TvA bind to independent sites on the ATPase.

Reversal of the Effects of Inhibitors by Addition of Ca^{2+}. Effects of BHQ on the fluorescence intensity of the labeled ATPase can be reversed by addition of high concentrations

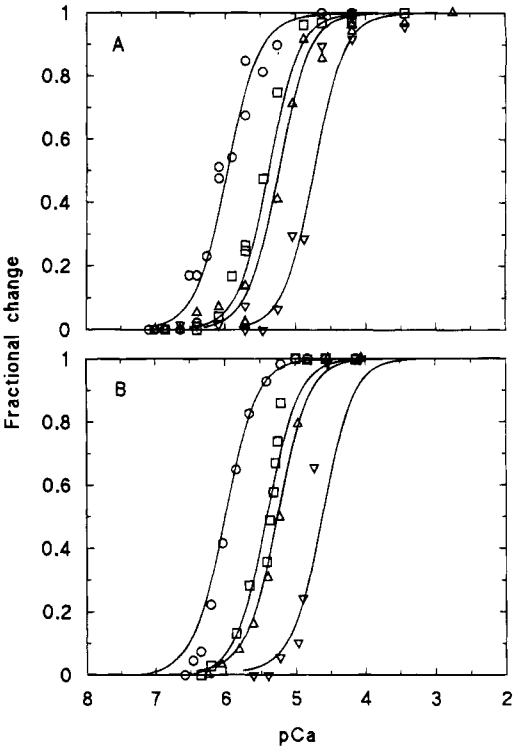

FIGURE 7: Effects of BHQ and TvA on the Ca^{2+} affinity of the ATPase as determined by changes in Trp or NBD fluorescence. Shown are the changes (A) in Trp fluorescence intensity for unlabeled ATPase (1 μM) or (B) NBD fluorescence for NBD-labeled ATPase (1 μM) in buffer determined as in the legend to Figure 6 in the absence of inhibitor (○), or in the presence of 1 μM BHQ (□), of 1 μM TvA (△), or a mixture of 1 μM BHQ plus 1 μM TvA (▽). The lines are simulations calculated as described in the text assuming 0.3 binding site for BHQ per ATPase molecule and 0.5 binding site for TvA per ATPase molecule.

of Ca^{2+}. The fluorescence of the ATPase labeled with fluorescein isothiocyanate (FITC) is sensitive to the E2−E1 conformational change (Pick & Karlish, 1982; Pick, 1982; Froud & Lee, 1986). The time dependences of the changes in DMC and FITC fluorescence on addition of 0.7 mM Ca^{2+} to 1 μM DMC- or FITC-labeled ATPase, respectively, incubated in the presence of 1 μM BHQ and 0.3 mM EGTA at pH 7.0 are shown in Figure 8A. Both fit to single exponentials with a rate constant of 0.1 s^{-1}. The time courses of the changes in Trp fluorescence for the unlabeled ATPase, for NBD-labeled ATPase (Figure 8) or for FITC- or DMC-labeled ATPase (data not shown) also fit to a single exponential, with a rate constant of 0.1 s^{-1}. In contrast, the rate of change of NBD fluorescence for NBD-labeled ATPase is much slower, with a rate constant of 0.02 s^{-1} (Figure 8A). These results suggest that Trp, FITC, and DMC fluorescence all respond to the same conformational change in the system but that NBD fluorescence is affected by a separate process.

The rates of the Trp, DMC, and FITC responses decrease with increasing concentration of BHQ, and the Trp and FITC responses are slower at pH 6.0 than at pH 7.0 (Figure 9A,B). According to Scheme 1, the rate of the fluorescence response will depend on the rate of the E2−E1 transition and on the binding constant for BHQ. The rate of the E2−E1 transition was calculated from the parameters in Henderson et al. (1994a) to be 1.4 and 5.3 s^{-1} at pH 6.0 and 7.0, respectively. Simulations of the rates of Ca^{2+} binding in terms of Scheme 1 are in agreement with the experimental data for a value of

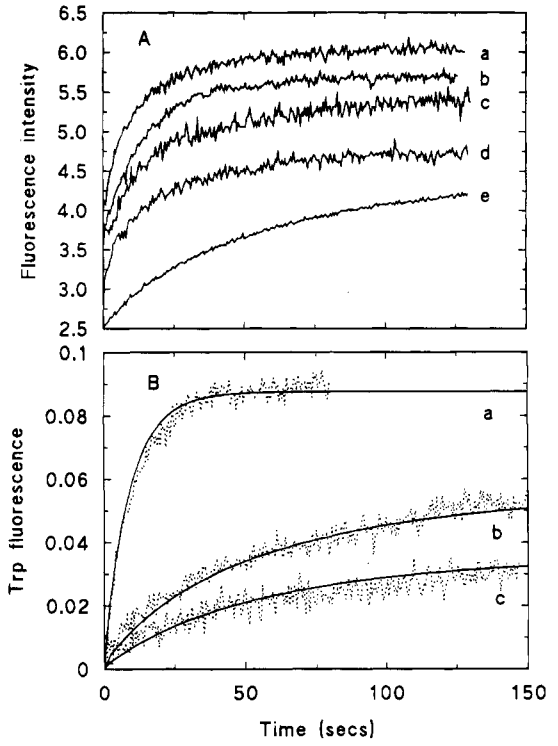

FIGURE 8: Kinetics of the reversal of the effects of BHQ by Ca^{2+}. (A) Shown are the increases in Trp fluorescence for unlabeled ATPase (a), FITC fluorescence for FITC-labeled ATPase (b), DMC fluorescence for DMC-labeled ATPase (c), and Trp (d) and NBD (e) fluorescence for NBD-labeled ATPase on addition of Ca^{2+} to the ATPase (1 μM) at pH 7.0 in the presence of 1 μM BHQ and 0.3 mM EGTA, to give a final free Ca^{2+} concentration of 0.4 mM. Traces have been offset vertically for clarity. (B) Increase in Trp fluorescence intensity for unlabeled ATPase (1 μM) incubated in the presence of 1 μM BHQ (a), 1 μM TvA (b) or a mixture of 1 μM BHQ plus 1 μM TvA (c), on addition of Ca^{2+} at pH 7.0. Dotted lines, experimental; solid lines, simulations as described in the text.

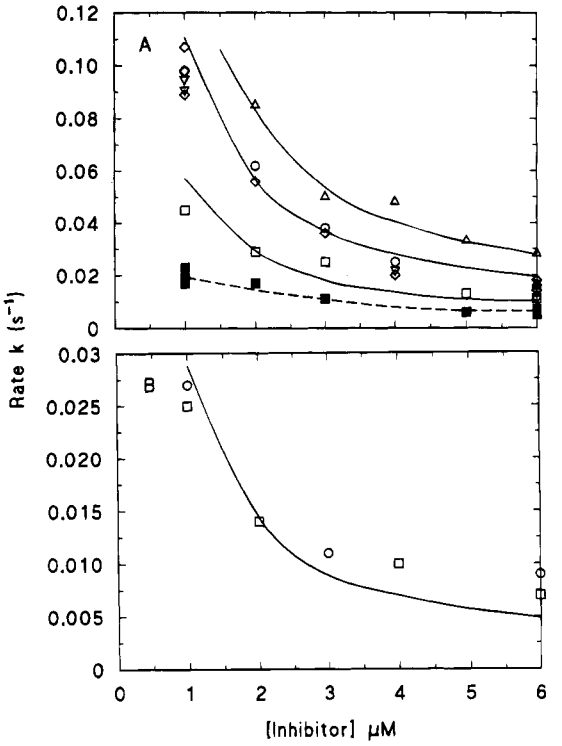

FIGURE 9: Effect of inhibitor concentration on the rate of fluorescence responses of the Ca^{2+}-ATPase to Ca^{2+}. (A) 1 μM unlabeled ATPase (◇), FITC-labeled ATPase (○), DMC-labeled ATPase (▽) or NBD-labeled ATPase (■) was incubated in buffer containing 0.3 mM EGTA at pH 7.0 with the given concentrations of BHQ and Ca^{2+} added to a final free concentration of 0.4 mM. Trp (◇), FITC (○), DMC (▽), and NBD (■) fluorescence responses were fitted to single exponentials, and the rates plotted as a function of BHQ concentration. The rates of responses of FITC-labeled ATPase to the addition of Ca^{2+} were also determined in the presence of 2,5-di-*tert*-amyl-1,4-dihydroxybenzene (□) and 2,5-dibutyl-1,4-dihydroxybenzene (△). The solid lines show the results of simulations calculated as described in the text, assuming 0.2 inhibitor binding site per ATPase molecule. (B) The rates of the fluorescence responses for FITC-labeled ATPase were determined as a function of BHQ concentration at pH 6.0, as described above: FITC fluorescence (□), Trp fluorescence (○). The solid line is a simulation calculated as described in the text.

the overall dissociation constant for BHQ ($E2 \rightleftharpoons E2^AI$) of 0.02 μM (Figure 9). This is consistent with the range of values (0.02−0.05 μM) obtained from titrations of the effect of BHQ on the fluorescence of DMC-labeled SR (Figure 2). A slightly faster fluorescence response for FITC-labeled ATPase is observed in the presence of 2,5-dibutyl-1,4-dihydroxybenzene whereas in 2,5-di-*tert*-amyl-1,4-dihydroxybenzene responses are slower than for BHQ (Figure 9). These data can also be simulated in terms of Scheme 1 with the binding constants given in Table 3. Effects of addition of Ca^{2+} on the Trp fluorescence of the ATPase in the presence of 2,5-bis(7-methylheptyl)-1,4-dihydroxybenzene, 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol, or 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde were too fast to be measured on a conventional fluorimeter (data not shown).

The effect of addition of Ca^{2+} on the Trp fluorescence intensity of the ATPase incubated in the presence of a mixture of BHQ and TvA is shown in Figure 8B. The response is slower than in the presence of either TvA or BHQ alone and can be simulated assuming separate binding sites for TvA and BHQ on the ATPase and an equilibrium constant for the conformational change of the doubly-bound species ($E2^A$·TvA·BHQ/E2·TvA·BHQ) equal to the product (986) of the equilibrium constants for TvA-bound (34; Wictome et al., 1995) and BHQ-bound (29) species alone (Figure 8B).

DISCUSSION

2,5-Di-*tert*-butyl-1,4-dihydroxybenzene (BHQ) has been shown to be an inhibitor of the ER/SR class of Ca^{2+}-ATPases but to have little effect on the plasma membrane Ca^{2+}-ATPase (Moore et al., 1987; Llopis et al., 1991; Wictome et al., 1992b, 1994; Nakamura et al., 1992). BHQ has been shown to reduce the affinity of the ATPase for Ca^{2+}, due to a shift in the E1−E2 equilibrium toward the E2 conformation (Wictome et al., 1992b). These effects of BHQ are very similar to those observed for thapsigargin and other sesquiterpene lactone inhibitors of the Ca^{2+}-ATPase (Thastrup et al., 1990; Sagara & Inesi, 1991; Kijima et al., 1991; Lytton et al., 1991; Wictome et al., 1992a, 1995). As also observed with thapsigargin, BHQ reduces phosphorylation of the ATPase by P_i (Wictome et al., 1992b), which is unexpected since in the E1−E2 model for the ATPase it is the E2 conformation which is phosphorylated by P_i (de Meis, 1981). For thapsigargin, effects were interpreted in terms of Scheme 1, with a conformational change following binding of inhibitor to E2, to give $E2^AI$, which could not be phosphorylated by P_i. It was suggested that the conformations E2 (and E2I) and $E2^AI$ could differ in the degree of closure of

a cleft between the phosphorylation and nucleotide binding domains (Wictome et al., 1995).

Binding of BHQ to the Ca^{2+}-ATPase has been shown here to fit the same Scheme 1 used to describe the binding of thapsigargin. Binding of BHQ to the ATPase involves a second-order association reaction followed by a first-order conformational change (Figure 3). The 30-fold shift in the E2−E1 equilibrium detected from changes in fluorescence intensity for NBD-labeled ATPase (Figure 4) is consistent with the observed decrease in Ca^{2+} affinity (Figure 6). The observation that effects of BHQ on the affinity of the Ca^{2+} ATPase do not increase linearly with increasing BHQ concentration but show saturation at a molar ratio of BHQ: ATPase of ca 1:1 (Figure 6) is consistent with BHQ binding to both Ca^{2+}-bound and Ca^{2+}-free forms of the ATPase.

According to Scheme 1, addition of Ca^{2+} to the ATPase incubated in the presence of BHQ will result in a conformational change from E2^AI to E1'ICa_2, the rate of the change decreasing with increasing BHQ concentration since only the unbound ATPase is able to undergo the E2−E1 transition. The observed rate of responses of Trp, FITC, or DMC fluorescence for the labeled or unlabeled ATPase are equal (Figures 8 and 9), suggesting that the rate-controlling step for all three responses is the same (the E2−E1 step). The concentration dependence of the effect of BHQ at pH 6.0 and 7.0 is consistent with Scheme 1 with the binding constant for BHQ given in Table 3; the binding constant is consistent with that estimated from titrations of the effects of BHQ on the fluorescence of DMC-labeled SR (Figure 2).

An unexpected observation was that the rate of change of NBD fluorescence for NBD-labeled ATPase on addition of Ca^{2+} was much slower than the rate of change of Trp fluorescence for the same sample; the rate of change of Trp fluorescence for NBD-labeled ATPase was identical to that for the unlabeled ATPase (Figure 8A). A similar result was obtained in the presence of AHQ, the NBD response again being slower than the Trp response (data not shown) The nature of the slow process affecting NBD fluorescence is unclear. A direct quenching of NBD fluorescence by BHQ is observed (Wictome et al., 1994; see also Figure 4), possibly due to the presence of NBD-labeled Cys residues in the transmembrane region of the ATPase. Slow dissociation of BHQ from sites in the lipid bilayer from which NBD fluorescence can be quenched could be a possible explanation.

The mechanisms of inhibition of the ATPase by BHQ and by the sesquiterpene lactones therefore appear to be the same. However, BHQ and TvA bind to separate sites on the ATPase. Titrations of DMC-labeled SR with BHQ in the presence of substoichiometric amounts of TvA are inconsistent with binding of BHQ and TvA at the same sites on the ATPase (Figure 2B). Effects of mixtures of TvA and BHQ on the affinity of the ATPase for Ca^{2+} are additive, also inconsistent with binding at the same sites (Figure 7), as is the very slow change in Trp fluorescence intensity observed on binding Ca^{2+} to the ATPase in the presence of a mixture of TvA and BHQ (Figure 8B).

In trilobolide, the trans-vicinal hydroxyl groups at C-7 and C-11 are essential for inhibition of the ATPase as desoxytrilobolide is unable to bind to the ATPase (Wictome et al., 1994). The hydroxyl groups on BHQ are also essential for activity, with neither 2,5-di-*tert*-butyl-1,4-quinone nor 2,5-di-*tert*-butyl-1,4-dihydroxybenzene diacetate inhibiting the ATPase (Wictome et al., 1994). However, the separation between the two essential −OH groups in crystals of trilobolide (3.64 Å; Kutschabsky et al., 1986) is very different to that predicted by modeling for BHQ (5.52 Å). Thus it is unlikely that the same residues on the ATPase could be involved in interaction with the −OH residues of the sesquiterpene lactones and the 1,4-dihydroxybenzenes.

Table 3: Binding Constants for 1,4-Dihydroxybenzenes[a]

inhibitor	binding constant (M^{-1}) E2 + I ⇌ E2I	equilibrium constant E2^AI/E2I	overall binding constant (M^{-1}) E2 + I ⇌ E2^AI
BHQ	1.7×10^6	29	5.0×10^7
AHQ	3.4×10^6	29	1.0×10^8
PHQ	3.2×10^3	4	1.6×10^4
2,5-dibutyl-1,4-dihydroxybenzene			3.3×10^7

[a] Calculated assuming equal binding constants for inhibitors to the E1 and E2 conformations.

The concentration of BHQ giving 50% inhibition of ATPase activity (0.4 μM; Table 1) is considerably greater than the dissociation constant estimated from fluorescence experiments (0.02 μM; Tables 2 and 3). The ATPase activity measurements will underestimate the true binding constant for BHQ because, at the concentration of ATPase used in the assay, the concentration of BHQ will be significantly depleted by binding to the ATPase. For PHQ, where binding is weaker, the binding constant estimated by fluorescence methods (62 μM; Table 3) is equal to that estimated from inhibition of the ATPase (58 μM; Table 2).

Hydrophobicity of the 1,4-dihydroxybenzenes is essential for activity. Thus neither 2,5-bis(carboxymethyl)-, bis-(ethylamino)-, nor disulfonyl-1,4-dihydroxybenzene affects the activity of the ATPase at concentrations up to 60 μM (Table 1). The concentration of inhibitor required for 50% inhibition of ATPase activity decreases with increasing substituent chain length from 2,5-dipropyl-1,4-dihydroxybenzene to 2,5-di-*tert*-amyl-1,4-dihydroxybenzene but is low for 2,5-bis(7-methylheptyl)-1,4-dihydroxybenzene (Table 1). These results are consistent with the results of the fluorescence measurements, giving stronger binding for AHQ than for 2,5-dipropyl-1,4-dihydroxybenzene (Table 3). The fluorescence change observed on addition of Ca^{2+} to the ATPase incubated in the presence of 2,5-bis(7-methylheptyl)-1,4-dihydroxybenzene is too fast to follow in a conventional fluorometer, which is consistent with weak binding to the ATPase. These results show that there is an optimal size for the hydrophobic substituents at the 2 and 5 positions and that binding does not depend solely on hydrophobicity. Since neither 2,3,5,6-tetramethyl-1,4-dihydroxybenzene nor 2,6-di-*tert*-butyl-1,4-dihydroxybenzene affects activity (Table 1), the positions of the hydrophobic groups are also important.

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