Evidence for a Global Inhibitor-Induced Conformation Change on the Ca^{2+}-ATPase of Sarcoplasmic Reticulum from Paired Inhibitor Studies†

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ABSTRACT: The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by a variety of hydrophobic, hydroxy-containing molecules. A kinetic method has been used to study competition between binding of pairs of inhibitors to the ATPase. The presence of 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (BHQ) decreases the affinity of the ATPase for 2,5-dipropyl-1,4-dihydroxybenzene (PHQ), suggesting that PHQ and BHQ bind to the same site on the ATPase. In contrast, the presence of BHQ increases the affinity of the ATPase for curcumin and vice versa. This suggests that BHQ and curcumin bind to separate sites on the ATPase and that binding of the first inhibitor to the ATPase results in a change to a conformation with higher affinity for the second inhibitor. This is consistent with previous experiments with BHQ and thapsigargin suggesting a conformation change on inhibitor binding, $E2 + I \rightleftharpoons E2I \rightleftharpoons E2^AI$, with $E2^AI$ having a higher affinity for the second inhibitor than E2. The affinity for BHQ is also increased by binding of diethylstilbesterol, ellagic acid, or nonylphenol, and the affinity for curcumin is also increased by ellagic acid. These results showing that binding of a variety of inhibitors of very different structures all result in a general increase in inhibitor affinity point to a global conformational change on the Ca^{2+}-ATPase caused by inhibitor binding, as well as any local, inhibitor-specific changes in conformation.

The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by a variety of hydrophobic, hydroxy-containing compounds. The inhibitors of highest affinity are the sesquiterpene lactones related in structure to thapsigargin, including thapsigargin itself and thapsigargicin, both isolated from *Thapsia garganica*, thapsivillosin A, isolated from *Thapsia villosa*, and trilobolide, extracted from *Laser trilobum* (*1*−*5*). These inhibitors bind with a 1:1 stoichiometry to the Ca^{2+}-ATPase, with an affinity of the order of 0.2 nM (*6*−*8*). The interaction is structurally specific; thapsigargin does not inhibit the plasma membrane Ca^{2+}-ATPase or other P-type ATPases (*1*, *2*). The Ca^{2+}-ATPase is also inhibited by other hydroxy-containing compounds including nonylphenol (*9*−*11*), 2,5-di-*tert*-butyl-1,4-benzohydroquinone (BHQ),[1] and related 1,4-dihydroxybenzenes (*12*−*14*), bisphenol (*15*), ellagic acid (*16*), curcumin (*17*, *18*), and diethylstilbesterol (*19*). These molecules have relatively little in common except that they are all hydrophobic and contain −OH groups (Figure 1).

The kinetics of the Ca^{2+}-ATPase are usually interpreted in terms of the E1−E2 scheme for the ATPase (*20*, *21*). This scheme proposes that, in the absence of any ligand, the ATPase exists as a mixture of two conformations, E1 and E2. In the E1 conformation the ATPase can bind two Ca^{2+} ions from the cytoplasmic side of the membrane whereas in the E2 conformation these binding sites are closed (*21*).

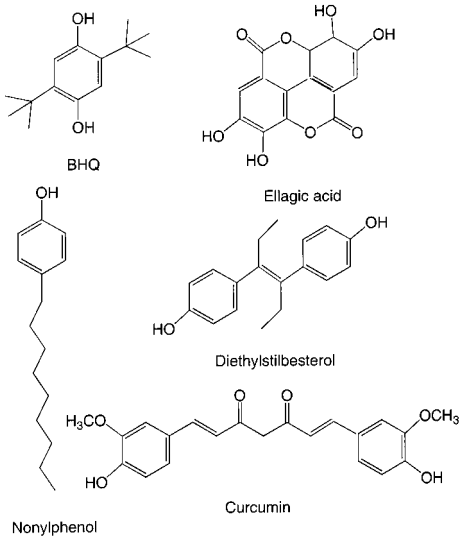

FIGURE 1: Structures of some inhibitors of the Ca^{2+}-ATPase. BHQ is 2,5-di-*tert*-butyl-1,4-dihydroxybenzene.

When in the Ca^{2+}-bound $E1Ca_2$ state, binding of ATP leads to hydrolysis of ATP and transport of Ca^{2+} across the membrane.

One possible explanation for the effect of thapsigargin (Tg) is that thapsigargin binds only to the E2 conformation of the ATPase forming a "dead-end complex" E2Tg with the ATPase that is unable to bind to Ca^{2+}. However, an alternative proposal is that thapsigargin binds to both the E1 and E2 conformations of the ATPase and that inhibition of ATPase activity follows from a conformational change

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[1] Abbreviations: PHQ, 2,5-dipropyl-1,4-dihydroxybenzene; BHQ, 2,5-di-*tert*-butyl-1,4-dihydroxybenzene; Tg, thapsigargin.

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of the initially formed E2Tg complex to a modified E2 state, $E2^{A}Tg$ (*5*). A very similar mechanism with formation of a modified inhibitor-bound E2 state was suggested for inhibition of the ATPase by BHQ, despite the fact that BHQ and thapsigargin bind to different sites on the ATPase (*12*).

The observation that two inhibitors (I) binding at different sites on the ATPase both cause formation of a modified E2 state suggests that the $E2I \rightleftharpoons E2^{A}I$ conformation change on the ATPase reflects a global change in ATPase conformation rather than a change just in the immediate vicinity of the inhibitor binding site. A recent study by Young et al. (*22*) has located the binding site for thapsigargin between two loops connecting the transmembrane α-helices of the Ca^{2+}-ATPase on the lumenal side of the membrane. This proposed location contrasts with the results of site-directed mutagenesis, which showed that residues in the S3 stalk helix on the cytoplasmic side of the membrane, close to the membrane surface, were important in binding (*23*, *24*). These results also suggest that binding of thapsigargin results in global changes in structure of the ATPase (*23*, *25*) so that binding to a site on the lumenal side of the membrane is linked to changes on the cytoplasmic side of the membrane (*22*).

A global change in structure of the ATPase on binding an inhibitor could cause a change in affinity for another inhibitor binding to a second inhibitor binding site on the ATPase. We show here that kinetic experiments do, indeed, provide evidence for such linkage of inhibitor binding sites, with multiple binding sites on the ATPase at which binding results in increased affinity for other inhibitors.

MATERIALS AND METHODS

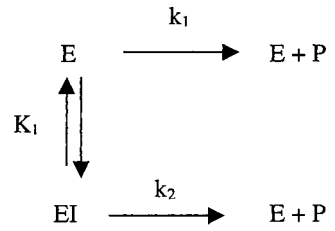
BHQ, nonylphenol, and ellagic acid were obtained from Aldrich, curcumin was obtained from ICN, and 2,5-dipropyl-1,4-dihydroxybenzene (PHQ) was obtained from ChemService.

Sarcoplasmic reticulum was purified from rabbit skeletal muscle as described (*26*). ATPase activities were determined at 25 °C 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, 0.52 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), lactate dehydrogenase (18 IU), and 5 μg of A23187 in a total volume of 2.5 mL. The protein concentration was 0.25 μg/mL. The reaction was initiated by the addition of an aliquot of a 50 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 was ca. 10 μM). Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+}, and H^{+} to EGTA given by Godt (*27*). Experimental data were fitted using the nonlinear least-squares fitting routine in SigmaPlot.

RESULTS

PHQ and BHQ Compete for Binding to the ATPase. Although the kinetics of the Ca^{2+}-ATPase are complex (*20*), it has been shown (*12*) that inhibition of ATPase activity by PHQ and BHQ at fixed concentrations of ATP and Ca^{2+} fits to a single inhibitor binding site model (see Scheme 1). In this scheme K_1 is the apparent dissociation constant for binding of inhibitor I. The measured rate of ATP hydrolysis ($k_{effective}$) depends on the fraction of ATPase bound and

Scheme 1



unbound by inhibitor and is given by

$$k_{effective} = [k_1K_1/(K_1 + [I])] + [k_2[I]/(K_1 + [I])] \quad (1)$$

For ease of comparison, ATPase activities are expressed as a fraction of the activity measured in the absence of inhibitor (typically 3.2 IU/mg of protein). Inhibition by PHQ fits to eq 1 with a dissociation constant of 11.3 ± 0.5 μM and a residual fractional activity (k_2) of 0.14 ± 0.03 (Figure 2, Table 1). Similarly, inhibition by BHQ fits to eq 1 with a dissociation constant of 0.19 ± 0.03 μM and a residual fractional activity (k_2) of 0.14 ± 0.03 (Table 1). Figure 2

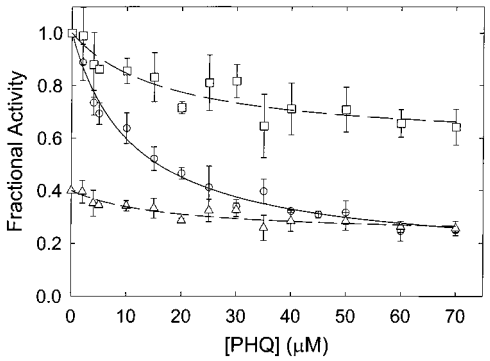


FIGURE 2: Effect of PHQ on Ca^{2+}-ATPase activity in the presence or absence of BHQ. Activities were measured in the presence of the given concentrations of PHQ in the absence (○) or presence (△) of 0.4 μM BHQ. Activities are expressed as a fraction of the activity measured in the absence of inhibitor, typically 3.2 IU/mg of protein. Activities measured as a function of PHQ concentration in the presence of 0.4 μM BHQ are also plotted as a fraction of the activity measured in the absence of PHQ (□). The lines show fits to a single binding site model, giving the K_d values and residual rates given in Table 1.

also shows the effect of PHQ in the presence of 0.4 μM BHQ. Addition of 0.4 μM BHQ results in a 60% decrease in ATPase activity; subsequent addition of PHQ results in further reduction in activity, but with a lower apparent affinity for PHQ than in the absence of BHQ. The change in apparent affinity for PHQ is most clearly shown by plotting the activity in the presence of 0.4 μM BHQ as a fraction of the activity in the absence of PHQ (Figure 2). The effect of PHQ in the presence of 0.4 μM BHQ fits to a dissociation constant of 46.4 ± 5 μM for PHQ (Figure 2, Table 1).

Given the very similar structures of PHQ and BHQ, the two inhibitors would be expected to bind competitively to the same site on the ATPase, as shown in Scheme 2. If the concentrations of the two inhibitors I and J are [I] and [J], respectively, then the fraction F_I of the enzyme in the I-bound form EI is

Table 1: Analysis of Inhibitor Binding to the Ca^{2+}-ATPase

inhibitor	second inhibitor	dissociation constant for stimulatory site (μM)	dissociation constant for inhibitory site (μM)	stimulated fractional rate (k_4)[a]	residual fractional rate (k_2)[b]
PHQ			11.3 ± 0.5		0.14 ± 0.03
	BHQ (0.4 μM)		46.4 ± 5.0		0.14 (fixed)
BHQ			0.19 ± 0.03		0.14 ± 0.04
	curcumin (6 μM)		0.07 ± 0.01		0.15 ± 0.01
curcumin		0.01 (fixed)	3.0 ± 0. 5	1.17 ± 0.03	0
	BHQ (0.4 μM)		0.81 ± 0.06		0.10 ± 0.02
ellagic acid		0.01 (fixed)	25.5 ± 7.5	1.17 ± 0.02	0
	BHQ (0.4 μM)	0.01 (fixed)	9.78 ± 2.6	1.17 ± 0.05	0
	curcumin (3 μM)	0.01 (fixed)	6.6 ± 2.7	1.22 ± 0.07	0
diethylstilbesterol		0.01 (fixed)	5.24 ± 1.19	1.16 ± 0.07	0.09
	BHQ (0.4 μM)		1.28 ± 0.20		0.31 ± 0.03
	curcumin (3 μM)		5.08 ± 1.26		0.18 ± 0.06
nonylphenol[c]		1 (fixed)	11.1 ± 1.9	2.51 ± 1.8	0
	BHQ (0.4 μM)	1 (fixed)	6.76 ± 1.55	2.18 ± 3.0	0

[a] See Scheme 3. [b] See Scheme 1. [c] n fixed at 3.

$$F_I = \frac{K_2[I]}{K_1K_2 + K_2[I] + K_1[J]} \quad (2)$$

This can be written as

$$F_I = \frac{[I]}{(K_{eff} + [I])} \quad (3)$$

where

$$K_{eff} = (K_1K_2 + K_1[J])/K_2 \quad (4)$$

Thus the presence of the inhibitor J simply increases the dissociation constant for the inhibitor I by a factor of $(1 + [J]/K_2)$. With dissociation constants of 0.19 and 11.3 μM for BHQ and PHQ, respectively (Table 1), the affinity for PHQ in the presence of 0.4 μM BHQ can be calculated from eq 4 to be 35.0 μM, in good agreement with the experimental value of 46.4 ± 5 μM.

Effects of Mixtures of Inhibitors of Unlike Structure. Some inhibitors of the Ca^{2+}-ATPase increase ATPase activity at low concentrations, with inhibition only being observed at higher concentrations. For example, curcumin causes a small increase in activity (ca. 10%) at low concentrations (up to ca. 0.8 μM) followed by a decrease in activity at higher concentrations (Figure 3). Since the inhibitors of highest specificity and affinity, the sesquiterpene lactones, show only inhibition with no stimulatory phase, it is likely that stimulation and inhibition follow from binding to different sites on the ATPase. A variety of small hydrophobic molecules are known whose binding stimulates the activity of the ATPase, including diethyl ether (*28*), short-chain alcohols (*29*), jasmone (*30*), and the pyrethroid deltamethrin (*31*). As described previously (*17*), the concentration dependence of the effect of curcumin on ATPase activity fits to a two-site model with separate stimulatory and inhibitory sites. If the dissociation constant for inhibitor binding at the stimulatory site is K_{stim} (see Scheme 3), then the stimulated rate is given as a function of the concentration of I by

$$k_{stim} = \frac{k_1K_{stim}}{K_{stim} + [I]} + \frac{k_4[I]}{K_{stim} + [I]} \quad (5)$$

The observed rate is then calculated as the product $k_{stim}k_{effective}$. As described previously (*17*), K_{stim} is not well defined by the data; any value for K_{stim} less than about 0.1 μM gives an equally good fit. The value for K_{stim} was therefore fixed at 0.01 μM, giving a dissociation constant for curcumin at the inhibitory site of 3.0 ± 0. 5 μM with $k_2 = 0$ (Figure 3, Table 1).

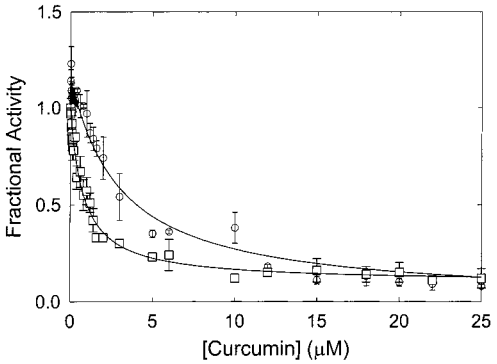
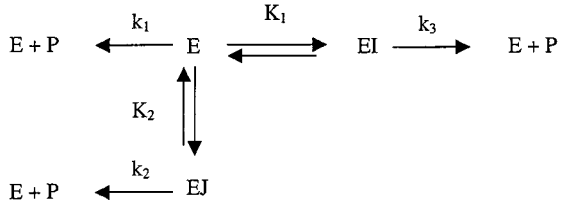
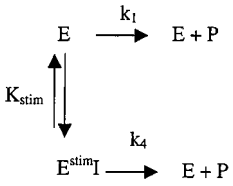


FIGURE 3: Effect of curcumin on Ca^{2+}-ATPase activity. Activities were measured in the presence of the given concentrations of curcumin at an ATP concentration of 2.1 mM in the absence (○) or presence (□) of 0.4 μM BHQ. Activities are expressed as a fraction of the activity measured in the absence of curcumin. The solid lines show fits to the two-site model for stimulation/inhibition (○) or the one-site model for inhibition (□), giving the values listed in Table 1.

Scheme 2



Scheme 3



Unexpectedly, inhibition by curcumin in the presence of BHQ occurs over a lower curcumin concentration range than in the absence of BHQ (Figure 3), showing that, rather than

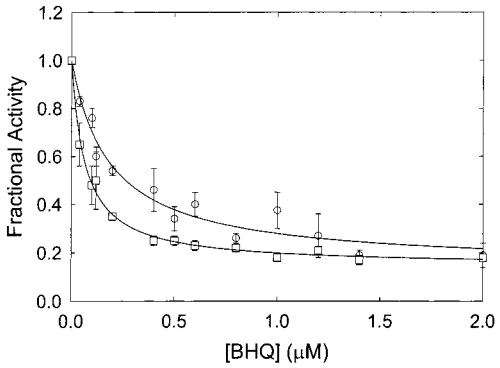
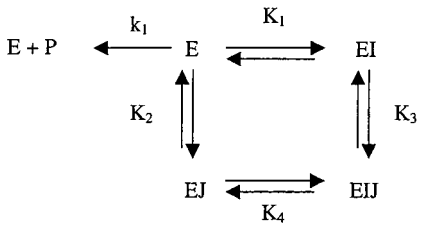


FIGURE 4: Effect of BHQ on the activity of the ATPase in the presence or absence of curcumin. Activities were measured in the presence of the given concentrations of BHQ in the absence (○) or presence (□) of 6 μM curcumin. Activities are expressed as a fraction of the activity measured in the absence of BHQ. The lines show fits to single binding site models, with the parameters given in Table 1.

Scheme 4



BHQ and curcumin competing for binding to the ATPase, the presence of BHQ *increases* the affinity of the ATPase for curcumin. Inhibition by curcumin in the presence of 0.4 μM BHQ fits to a simple single site model for inhibition with no evidence for an initial stimulatory phase (Figure 3). The data fit to a dissociation constant for curcumin of 0.81 $\pm$ 0.06 μM compared to an inhibitory dissociation constant of 3.0 $\pm$ 0.5 μM in the absence of BHQ (Table 1). A corresponding effect is seen in studies of the effect of curcumin on inhibition of the ATPase by BHQ where the presence of curcumin leads to an increase in affinity for BHQ (Figure 4, Table 1).

These results show that curcumin and BHQ must bind to different sites on the ATPase and, further, that binding of one inhibitor to the Ca^{2+}-ATPase results in a conformational change to a state with a higher affinity for the other (see Scheme 4). In Scheme 4 if $K_4 < K_1$ (and $K_3 < K_2$), then binding of I will increase the affinity for J, and binding of J will increase the affinity for I.

The fraction F of enzyme that is unbound by inhibitor is given by

$$F = \frac{K_1K_2K_3}{K_1K_2K_3 + K_2K_3[\mathrm{I}] + K_1K_3[\mathrm{J}] + K_2[\mathrm{I}][\mathrm{J}]} \quad (6)$$

In the presence of J alone the fraction of enzyme free of inhibitor is

$$F_{\mathrm{J\ alone}} = \frac{K_2}{K_2 + [\mathrm{J}]} \quad (7)$$

If the activity of the inhibitor-bound ATPase is zero, then the fractional activity in the presence of J, relative to that in the absence of J, is given by the ratio $F/F_{\mathrm{J\ alone}}$:

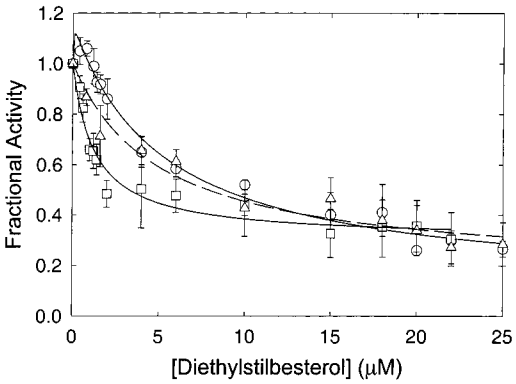


FIGURE 5: Effect of diethylstilbesterol on the activity of the ATPase in the presence or absence of other inhibitors. Activities were measured in the presence of the given concentrations of diethylstilbesterol in the absence (○) or presence of 0.4 μM BHQ (□) or 3 μM curcumin (△). Activities are expressed as a fraction of the activity measured in the absence of diethylstilbesterol. The data for diethylstilbesterol alone were fitted to the two-site model for stimulation/inhibition, giving the parameters listed in Table 1. The data in the presence of BHQ or curcumin were fitted to a single site model, giving the parameters again listed in Table 1.

$$\text{fractional activity} = \frac{K_1K_2K_3 + K_1K_3[\mathrm{J}]}{K_1K_2K_3 + K_2[\mathrm{I}](K_3 + [\mathrm{J}]) + K_1K_3[\mathrm{J}]} \quad (8)$$

Rearranging

$$\text{fractional activity} = \frac{K_1}{K_1 + [\mathrm{I}]\left(\dfrac{1 + [\mathrm{J}]/K_3}{1 + [\mathrm{J}]/K_2}\right)} \quad (9)$$

This is the normal equation for inhibition by I except that K_1 is replaced by $K_1(1 + [\mathrm{J}]K_2)/(1 + [\mathrm{J}]K_3)$. Thus knowing the dissociation constants for I and J alone (K_1 and K_2, respectively), the dissociation constant K_3 for binding of J to I-bound ATPase can be calculated from the affinity of I in the presence of a fixed concentration of J. Substituting the measured dissociation constant for curcumin in the presence of 0.4 μM BHQ (Table 1) into eq 9 gives a dissociation constant for binding BHQ to curcumin-bound ATPase of 0.04 μM, representing a 5-fold increase in affinity for BHQ. Similarly, the measured dissociation constant for BHQ in the presence of 6 μM curcumin (Table 1) gives a dissociation constant for binding curcumin to BHQ-bound ATPase of 0.85 μM, a 3.5-fold increase in affinity for curcumin. The close agreement between these two estimates for the change in affinity (which theoretically should be equal since $K_4/K_1 = K_3/K_2$) gives confidence in the analysis.

Inhibition of the ATPase by diethylstilbesterol is similar to that by curcumin with an initial stimulation of activity followed by inhibition; the data again fit to the two-site stimulation/inhibition model with a dissociation constant for the inhibitory site of 5.24 $\pm$ 1.19 μM (Figure 5, Table 1). In the presence of 0.4 μM BHQ the dissociation constant for diethylstilbesterol decreases to 1.28 $\pm$ 1.3 μM (Figure 5, Table 1), corresponding, from eq 9, to a 5-fold higher affinity for BHQ for diethylstilbesterol-bound ATPase than for unbound ATPase (Table 2). In contrast to the effect of BHQ, the presence of 3 μM curcumin did not lead to any increase in affinity for diethylstilbesterol, and the data fitted to an

Table 2: Dissociation Constants for BHQ and Curcumin Binding to Inhibitor-Bound Ca^{2+}-ATPase

inhibitor	inhibitor bound to the Ca^{2+}-ATPase	dissociation constant (μM)[a]
BHQ	curcumin	0.04
	diethylstilbesterol	0.034
	ellagic acid	0.056
	nonylphenol	0.10
curcumin	BHQ	0.85
	diethylstilbesterol	3.0
	ellagic acid	0.44

[a] Calculated from the data in Table 1 using eq 9.

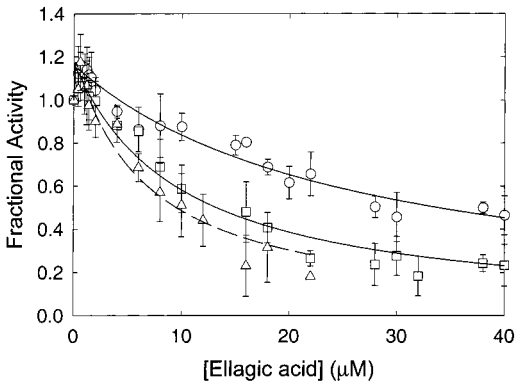


FIGURE 6: Effect of ellagic acid on the activity of the ATPase in the presence or absence of other inhibitors. Activities were measured in the presence of the given concentrations of ellagic acid in the absence (○) or presence of 0.4 μM BHQ (□) or 3 μM curcumin (△). Activities are expressed as a fraction of the activity measured in the absence of ellagic acid. The data were fitted to the two-site model for stimulation/inhibition, giving the parameters listed in Table 1.

inhibitory dissociation constant for diethylstilbesterol of 5.1 ± 1.3 μM, equal to the value observed in the absence of curcumin (5.2 ± 1.2 μM) (Figure 5).

Ellagic acid also inhibits ATPase activity following an initial small stimulation (Figure 6, Table 1). The affinity of the ATPase for ellagic acid is increased by the presence of either BHQ or curcumin (Figure 6, Table 1). From eq 9, the affinities of BHQ and curcumin for ellagic acid-bound ATPase are 3.4- and 6.8-fold higher, respectively, than for unbound-ATPase (Table 2). The effect of nonylphenol on ATPase activity also consists of an initial stimulation followed by inhibition (Figure 7). However, inhibition shows a steeper dependence on nonylphenol concentration than would be expected from binding at a single inhibitory site, and the fit to a model with a single inhibitory binding site is poor (Figure 7). The data were therefore fitted to a stimulatory/multiple inhibitory site model in which the inhibitor binds to *n* identical sites on the protein, binding not affecting the affinity for the inhibitor. The fraction of enzyme without bound inhibitor was described by the equation

$$F = \frac{1}{(1 + [\mathrm{I}]/K_{\mathrm{I}})^n} \qquad (10)$$

Equally good fits to the data could be obtained for a range of values for *n* greater than 3. Since the important parameter for our experiments was not the value of *n* but the value for the inhibitor dissociation constant, the value of *n* was fixed at 3, giving values for the dissociation constants for nonylphenol of 11.1 ± 1.9 μM and 6.76 ± 1.55 μM in the absence and presence of 0.4 μM BHQ, respectively (Figure 7, Table 1), corresponding to a 1.9-fold higher affinity for BHQ for nonylphenol-bound ATPase than for unbound ATPase (Table 2).

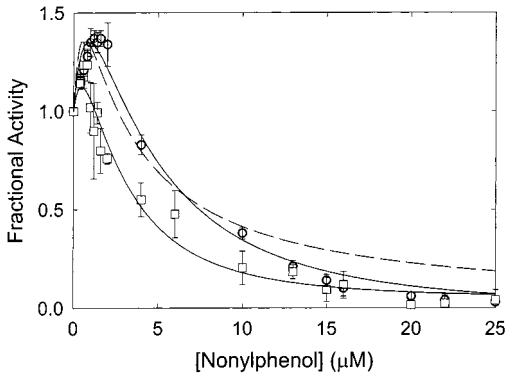


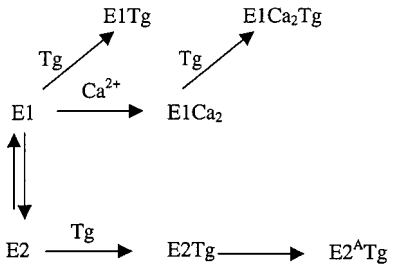
FIGURE 7: Effect of nonylphenol on the activity of the ATPase in the presence or absence of BHQ. Activities were measured in the presence of the given concentrations of nonylphenol in the absence (○) or presence (□) of 0.4 μM BHQ. Activities are expressed as a fraction of the activity measured in the absence of nonylphenol. The solid lines show fits to the stimulatory/multiple inhibitory binding site model, with the number of inhibitor binding sites (*n*) fixed at 3, giving the parameters listed in Table 1. The broken line shows the best fit to a two-site model for stimulation/inhibition with the affinity of the stimulatory site fixed at 1 μM.

DISCUSSION

The transmembrane region of the Ca^{2+}-ATPase consists of a bundle of 10 α-helices (*32*). The two Ca^{2+} ions to be moved across the membrane bind between transmembrane α-helices M4, M5, M6, and M8. An unexpected feature of the crystal structure of the Ca^{2+}-ATPase is that no clear pathway is visible leading from the pair of Ca^{2+} binding sites to the lumenal side of the membrane (*21*, *32*). The implication is that release of Ca^{2+} from the pair of binding sites involves a significant change in the packing of the transmembrane α-helices, possibly corresponding to an opening up of the interface between helical bundles M1−M4 and M5−M10, allowing access from the binding sites to the lumen (*21*). The transmembrane α-helices of the Ca^{2+}-ATPase on the lumenal side of the membrane are linked by small loops, these loops making little contact with each other (*33*). Thus the lumenal part of the Ca^{2+}-ATPase provides relatively few constraints to changes in helix packing.

Young et al. (*22*) suggested that the binding site for thapsigargin is on the lumenal side of the membrane, between loops M3−M4 and M7−M8. This is consistent with cross-linking studies with an azido derivative of thapsigargin, which found labeling of a fragment of the ATPase corresponding to helices M3 and M4 and the connecting loop (*25*). Young et al. (*22*) suggested that thapsigargin could bridge between the M3−M4 and M7−M8 loops, preventing the change in transmembrane α-helical bundle packing necessary to bind Ca^{2+}. Mutation of residues in the S3 stalk helix on the cytoplasmic side of the membrane, close to the membrane surface, reduces the affinity of the ATPase for thapsigargin without preventing inhibition at high concentrations of thapsigargin (*23*, *24*). Together, these results are consistent with the proposal that binding of thapsigargin results in global changes in the structure of the Ca^{2+}-ATPase

Scheme 5



(*23*, *25*), with binding on the lumenal side of the membrane being linked to changes on the cytoplasmic side of the membrane (*22*).

An analysis of the effects of thapsigargin and other sesquiterpene lactones on Ca^{2+} binding to the Ca^{2+}-ATPase suggested that thapsigargin bound with equal affinity to the E1, $E1Ca_2$, and E2 conformations of the ATPase but that the E2−thapsigargin complex E2Tg that was initially formed could undergo a further conformational change to a modified $E2^ATg$ state, as shown in Scheme 5 (*5*). The initial binding event to give E1Tg, $E1Ca_2Tg$, or E2Tg could correspond to binding of Tg to just one of the two key lumenal loops, most likely the M7−M8 loop (Met-857 to Glu-895) since this loop is considerably longer than the other lumenal loops. The conformational change to the $E2^ATg$ conformation would then correspond to interaction of the bound thapsigargin with the short M3−M4 loop, an interaction that, according to Scheme 5, would only be possible in the E2 conformation of the ATPase.

In previous spectroscopic studies it has been shown that the mechanism of inhibition of the ATPase by 1,4-dihydroxybenzenes such as BHQ is similar to inhibition by thapsigargin, again involving an $E2I \rightleftharpoons E2^AI$ conformation change on the ATPase, despite the fact that BHQ and thapsigargin bind to different sites on the ATPase (*12*). The similar mechanisms of inhibition by two inhibitors of very different structure suggest that the conformational changes on the ATPase on inhibitor binding cannot be just local effects restricted to the immediate environment of an inhibitor binding site but must reflect a more global change in conformation for the ATPase. The range of these global changes cannot, of course, be determined directly from binding experiments. The two inhibitor binding sites could be very widely separated on the ATPase, in which case the conformational changes on the ATPase linking the sites would have to be long range. Alternatively, the two inhibitor binding sites could be close together on the ATPase, in which case the conformational changes linking the sites would be shorter range.

Here we show that kinetic methods can also be used to determine whether pairs of inhibitors bind to a single site on the ATPase and can be used to detect global conformational changes on the ATPase. When two inhibitors bind to the same site on the ATPase, the presence of one of the inhibitors will decrease the affinity of the ATPase for the other (Scheme 2). This is what is observed for mixtures of the 1,4-dihydroxybenzenes BHQ and PHQ (Figure 2) suggesting that these two inhibitors compete for binding to the same site on the ATPase, as expected from their similar structures. In contrast, the presence of BHQ increases the affinity of the ATPase for curcumin and vice versa (Figures 3 and 4, Table 1). Thus not only must BHQ and curcumin bind to separate sites on the ATPase but the binding of one inhibitor must increase the affinity of the ATPase for the other. Similar effects are seen with mixtures of BHQ and ellagic acid, diethylstilbesterol, or nonylphenol; the presence of BHQ increases their affinities by a factor of between 2 and 6 (Table 2). Similarly, ellagic acid increases the affinity of the ATPase for curcumin by a factor of about 7. However, the presence of 3 μM curcumin had no effect on the apparent affinity for diethylstilbesterol (Table 2), showing that curcumin and diethylstilbesterol must bind to separate sites on the ATPase with the affinity for curcumin being the same for diethylstilbesterol-bound and free ATPase.

An increase in affinity for one inhibitor on binding a second inhibitor is consistent with the proposal (*5*) that binding of inhibitor to E2 leads to a conformation change to a new state $E2^AI$:

$$E2 + I \rightleftharpoons E2I \rightleftharpoons E2^AI$$

In terms of this model $E2^AI$ would have a higher affinity for the second inhibitor than E2. An increase in affinity for BHQ of about 5-fold caused by a second inhibitor such as curcumin corresponds to an increase in free energy $\Delta G°$ of BHQ binding of about 4 kJ mol^{-1}, compared to free energy changes for the initial binding event (E2 + BHQ ⇌ E2.BHQ) and for the conformational change E2·BHQ ⇌ $E2^A$·BHQ of about 40 and 8 kJ mol^{-1}, respectively (*12*). Thus the $E2^A$·curcumin state formed by binding curcumin does not express the full increase in affinity for BHQ observed when the $E2^A$·BHQ state is formed. That is, superimposed on the global conformation change observed on binding inhibitor are other, more local, inhibitor-specific changes. In terms of the binding model proposed above, binding of curcumin could bridge the M3−M4 and M7−M8 loops leading to an increased affinity for BHQ but with a conformation not as favorable for interaction with BHQ as that formed when BHQ alone bridges the two loops.

The only obvious similarity between inhibitors of the ATPase such as curcumin, BHQ, and diethylstilbesterol is the presence of two −OH groups and the absence of any charged groups. These −OH groups and their position are essential for activity. Reversing the positions of the −OH and methoxy groups in curcumin leads to total loss of activity (*17*). Similarly, an epoxide derivative of curcumin shows very low inhibitory potency (*17*). This compares with the 1,4-dihydroxybenzenes where the two −OH groups at the 1 and 4 positions were found to be essential for activity (*12*). The structure of nonylphenol is significantly different from that of the other inhibitors in that nonylphenol possesses only a single −OH group and inhibition of the ATPase by nonylphenol is different in that the concentration dependence of inhibition suggesting that inhibition results from binding to more than one site on the ATPase (Figure 7). The hydrophobicity of the inhibitors suggests that the inhibitors bind near the membrane surface so that they can penetrate, at least in part, into the hydrophobic transmembrane α-helical bundle of the ATPase.

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