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# Curcumin modulation of Na,K-ATPase: phosphoenzyme accumulation, decreased $K^+$ occlusion, and inhibition of hydrolytic activity

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**1** Curcumin, the major constitute of tumeric, is an important nutraceutical that has been shown to be useful in the treatment of many diseases. As an inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, curcumin was shown to correct cystic fibrosis (CF) defects in some model systems, whereas others have reported no or little effects on CF after curcumin treatment, suggesting that curcumin effect is not due to simple inhibition of the $Ca^{2+}$-ATPase.

**2** We tested the hypothesis that curcumin may modulate other members of the $P_2$-type ATPase superfamily by studying the effects of curcumin on the activity and kinetic properties of the Na,K-ATPase.

**3** Curcumin treatment inhibited Na,K-ATPase activity in a dose-dependent manner ($K_{0.5} \sim 14.6\ \mu M$). Curcumin decreased the apparent affinity of Na,K-ATPase for $K^+$ and increased it for $Na^+$ and ATP. Kinetic analyses indicated that curcumin induces a three-fold reduction in the rate of E1P → E2P transition, thereby increasing the steady-state phosphoenzyme level. Curcumin treatment significantly abrogated $K^+$ occlusion to the enzyme as evidenced from kinetic and proteolytic cleavage experiments. Curcumin also significantly decreased the vanadate sensitivity of the enzyme.

**4** Thus, curcumin partially blocks the $K^+$ occlusion site, and induces a constitutive shift in the conformational equilibrium of the enzyme, towards the E1 conformation.

**5** The physiological consequences of curcumin treatment previously reported in different epithelial model systems may, at least in part, be related to the direct effects of curcumin on Na,K-ATPase activity.
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**Keywords:** Curcumin; Na,K-ATPase; P-type ATPases; ouabain; oligomycin; nutraceuticals

**Abbreviations:** CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; E1, enzyme conformation that has high affinity for sodium ions; E2, enzyme conformation that has high affinity for potassium ions; ECL, enhanced chemiluminescence; ENaC, epithelial sodium channel; EP, phosphoenzyme; $K_{0.5}$, ligand concentration producing half-maximum effect; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SERCA, sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tween-20, polyoxyethylene sorbitan monolaurate

## Introduction

The Na,K-ATPase is a member of the $P_2$-type ATPase super family, which includes gastric H,K-ATPases and sarco(endo)-plasmic reticulum $Ca^{2+}$-ATPases (SERCAs) (Kühlbrandt, 2004). The Na,K-ATPase is a heterodimeric membrane protein that pumps three $Na^+$ ions out and two $K^+$ ions into the cell, at the expenditure of hydrolysis of one ATP molecule. The Na,K-ATPase establishes and maintains the resting membrane potential that is vital for cellular homeostasis in eukaryotes. The Na,K-ATPase is also involved in many specialized tissue functions such as transepithelial $Na^+$ transport, muscle and nerve excitability, as well as secretory and signal transduction processes. The enzyme consists of a catalytic $\alpha$-subunit that contains substrates and inhibitor-binding sites and a glycosylated $\beta$-subunit that is important for proper expression and function of the overall Na,K-ATPase activity (for reviews, see Kaplan, 2002; Kühlbrandt, 2004). The Na,K-ATPase is also associated with proteins of the FXYD family, which regulate Na,K-ATPase activity in a tissue-specific manner (Cornelius & Mahmmoud, 2003).

The Post-Albers mechanism (Scheme 1) illustrates the Na,K-ATPase reaction cycle. Enzyme in the E1 conformation undergoes a $Na^+$- and $Mg^{2+}$-dependent phosphorylation from ATP and subsequent $Na^+$ occlusion. Phosphorylated enzyme with occluded $Na^+$, $E1P(Na^+)_3$, undergoes a conformational change to the $E2P(Na^+)_3$ conformation, associated with the release of $Na^+$ at the extracellular side, and followed by dephosphorylation and $K^+$ occlusion to form $K^+$-occluded enzyme, $E2(K^+)_2$. ATP catalyzes the release of $K^+$ to the intracellular side and transition to the E2 form, where phosphorylation can proceed in the presence of $Na^+$ and ATP. In the absence of $K^+$, the E2P phosphoenzyme (EP)

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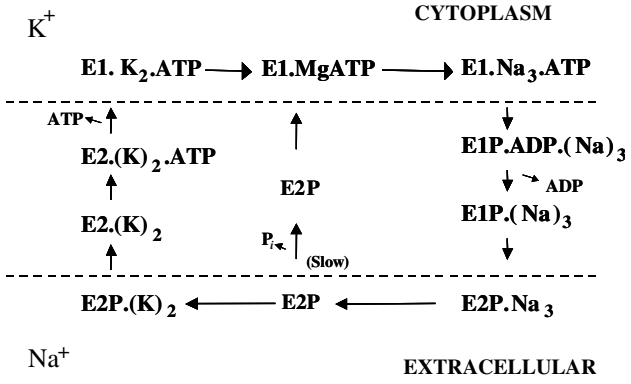


**Scheme 1** Minimum reaction cycle of Na,K-ATPase.

intermediate hydrolyzes at a slow rate to form the E2 form, where phosphorylation can be initiated in the presence of $Na^+$ and ATP ($Na^+/Na^+$ exchange).

P-type ATPases are indeed targets for many drugs. Of particular importance is the specific inhibition of the Na,K-ATPase with digitalis (Hansen, 1984). The positive inotropic effect of digitalis is linked to inhibition of myocardial Na,K-ATPase, with subsequent rise in $[Ca^{2+}]_i$ and increase in cardiac cell contractility (Dostanic *et al*., 2003). The most water-soluble digitalis, ouabain, is a potent inhibitor of Na,K-ATPase activity that binds to the enzyme from the extracellular side of the plasma membrane (Schultheis *et al*., 1993). Exposure of cultured rat cardiac myocytes to pharmacological doses of ouabain was shown to result in interaction of Na,K-ATPase with adjacent proteins, leading to activation of multiple signal transduction pathways, regulation of growth-related genes, and hypertrophy (Kometiani *et al*., 1998; Xie *et al*., 1999; Liu *et al*., 2000). Furthermore, partial inhibition of Na,K-ATPase with ouabain in renal epithelial cells has been shown to induce $Ca^{2+}$ oscillations and subsequent activation of the transcription factor NF-$\kappa$B (Aizman *et al*., 2001). Thus, treatment with drugs that modulate the housekeeping Na,K-ATPase may result in significant consequences on cellular physiology.

Curcumin (diferuloylmethane) is a dietary polyphenolic that received attention during the last few years due to its antioxidant properties (for reviews, see Gescher *et al*., 1998; Joe *et al*., 2004). Curcumin treatment has been shown to have various beneficial effects on cellular physiology, including anti-inflammatory, antiviral, anti-infectious, and anticarcinogenic effects (Li *et al*., 1993; Singh & Aggarwal, 1995; Kang *et al*., 1999). Treatment of cystic fibrosis (CF) cells with SERCA inhibitors (including the potent inhibitor thapsigargin) was shown to induce functional surface expression of the most common CF transmembrane conductance regulator (CFTR) mutation, $\Delta$F508 (Egan *et al*., 2002). Since curcumin is a mild SERCA inhibitor (Bilmen *et al*., 2001; Logan-Smith *et al*., 2001; Sumbilla *et al*., 2002) and has a very low level of toxicity (Cheng *et al*., 2001), it was shown to be efficient in correcting $\Delta$F508-CFTR misfolding in CF cells and animal models (Egan *et al*., 2004). However, results from other laboratories have shown that curcumin had no or little effects on CF cells as well as in animal models (Dragomir *et al*., 2004; Loo *et al*., 2004; Song *et al*., 2004). It is clear that investigations should be carried out to uncover the mechanism of action of curcumin and its pharmacological properties before being used clinically.

Despite the accumulating interest in the mechanism of curcumin action, little is known about the cellular targets where curcumin directly interact. The proposed role of curcumin as a corrector of CFTR defects was apparently based only on its ability to inhibit SERCA. Since the SERCA inhibitor thapsigargin has been shown to modulate trafficking of the $K^+$ channel KCNH2 (Delisle *et al*., 2003), the possibility exists that curcumin, like thapsigargin, may modulate the function of more than one transport protein in the cell. It is also unclear how curcumin is transported to the SR membrane and whether or not curcumin has variable effects on SERCA in different cell types.

The Na,K-ATPase is a member of $P_2$-type ATPases, to which SERCA belongs. Given the important effects curcumin has on membrane transport processes in epithelial cells (Egan *et al*., 2004) and its profound effects on SERCA (Bilmen *et al*., 2001; Logan-Smith *et al*., 2001; Sumbilla *et al*., 2002), we studied the effects of curcumin on the homologous Na,K-ATPase from shark rectal glands. We show that curcumin binding to the Na,K-ATPase strongly shifts the conformational equilibrium of the enzyme toward the E1 conformation with the accumulation of the E1P EP intermediate. Curcumin also interfered with $K^+$ occlusion to the enzyme, suggesting that this molecule possibly binds to the enzyme at the extracellular side of the plasma membrane. Thus, Na,K-ATPase could be a target for curcumin action *in vivo*.

## Methods

### *Materials*

Curcumin, which is obtained by isolation from *Curcuma longa* (commonly termed tumeric), was purchased from Sigma-Aldrich, St Louis, MO, U.S.A. (Catalog number; C1386). Natural curcumin mixture rather than synthetic curcumin was employed because it is the natural compound that is used as a healthy food additive, as well as it is readily available and affordable, and thus would more likely be the one to be used as a human drug. The naturally occurring ratio for curcuminoids is $\sim$5% bisdesmethoxycurcumin, $\sim$15% desmethoxycurcumin, and $\sim$80% curcumin (Ireson *et al*., 2001). The enhanced chemiluminescence (ECL) reagents, polyvinylidene fluoride (PVDF) membranes, and $AT^{32}P$ were purchased from Amersham Biosciences, Buckinghamshire, U.K. All other chemicals were of the highest analytical grade available.

### *Na,K-ATPase purification and measurement of hydrolytic activity*

Highly purified, plasma membrane-bound Na,K-ATPase from the rectal glands of the shark *Squalus acanthias* was prepared as described previously (Skou & Esmann, 1979). The ouabain-dependent ATP hydrolysis was measured at 24°C as described previously (Mahmmoud *et al*., 2000; 2003). In all experiments described in this study (except for the data in Figure 5b), the ionic strength was kept constant at 150 mM (NaCl, KCl, and choline chloride). All data are expressed as percentage of control, measured in the absence of curcumin. All experiments shown are representative of at least three independent measurements, each performed in duplicate.

### *Phosphorylation*

The phosphorylation reaction was performed by diluting the enzyme at 0°C with 10-folds of an ice-cold mixture containing 20 mM histidine pH 7.0, 5 mM EGTA, NaCl (see legends to figures), 1 mM $MgCl_2$, and 10 $\mu$M Tris–ATP (containing ~20.000 c.p.m. $^{32}$P-[ATP]). The EP was quenched after 10 s with ice-cold mixture containing 5% TCA, 3 mM ATP, and 2 mM $Na_2HPO_4$, as described previously (Daly *et al*., 1996). Phosphorylation in the presence of curcumin or oligomycin was performed by pre-incubating the enzyme with the drug for 5 min at 22°C, followed by chilling on ice for few minutes prior to phosphorylation. The acid-stable EP was collected and its radioactivity determined using scintillation counting. All phosphorylation data were expressed as EP formed in the presence of NaCl minus EP formed in the presence of KCl (replacing NaCl). All phosphorylation data are presented as mean ± s.e.m. of triplicate measurements. Experiments were repeated at least two times and gave similar results each time.

### *The E1P/E2P distribution and E1P → E2P transition*

The fractional amounts of E1P and E2P were estimated essentially as described previously (Vilsen, 1997). Control- and curcumin-treated enzymes were phosphorylated as described above. Dephosphorylation was then studied at 0°C by chasing the EP into 1 mM ADP and 1 mM ATP, followed by acid quenching after several time intervals. Data were analyzed using two-phase exponential decay function, which gave the rate coefficients present in Figure 5a.

The E1P → E2P transition was measured by first phosphorylating the enzyme in the presence of 600 mM NaCl to accumulate the E1P form (using such high $Na^+$ concentration is necessary in order to prevent spontaneous transition of E1P to E2P, see, for example, Vilsen, 1997). The EP was then chased with a solution producing final concentrations of 120 mM NaCl, 2 mM ATP, and 5 mM KCl (to insure rapid dephosphorylation of E2P), followed by acid quenching after several time intervals. Data were analyzed using one-phase exponential decay function, which gave the rate coefficients present in Figure 5b.

### *Dephosphorylation*

The spontaneous dephosphorylation of E2P (E2P → E1) was performed by first phosphorylating the enzyme as described above. Dephosphorylation was studied at 0°C by diluting the EP into 1.5 mM Tris–ATP and 3 mM $MgCl_2$ (chasing solution), followed by acid quenching after several time intervals (Figure 6a). $K^+$-induced dephosphorylation was studied in the same way, except that 1 mM $K^+$ was present in the chasing solution (Figure 6b). Data were analyzed using one-phase exponential decay function, which gave the rate coefficients described in Figure 6.

### *$K^+$-occlusion*

$K^+$-occlusion was performed essentially as described previously (Daly *et al*., 1996). Briefly, control- and curcumin-treated enzymes were incubated with different $K^+$ concentrations at 24°C to form the $K^+$-occluded enzyme. Oligomycin (10 $\mu$g) was added to the mixture and after 2 min incubation the samples were chilled on ice. Phosphorylation of the $K^+$-occluded enzyme was performed as described above. $K^+$ occlusion was measured as the difference between $E^{32}P$ obtained following incubation in the absence of $K^+$ and $E^{32}P$ obtained following incubation in the presence of $K^+$.

### *Proteolytic cleavage*

The '19 kDa membranes' were produced essentially as described previously (Karlish *et al*., 1990). Briefly, samples containing 50 $\mu$g of control- or curcumin-treated protein were incubated with 10 $\mu$g trypsin at 37°C for different periods of time in the presence of 20 mM histidine buffer, pH 7.0, 2 mM EDTA, and 20 mM KCl. The proteolytic reactions were started with the addition of trypsin, allowed to proceed for the time intervals indicated in Figure 7b, and terminated with an equal volume of sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970).

### *Gel electrophoresis and immunoblotting*

Proteins were separated using *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)-based SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 3% stacking gel, 9% intermediate, and 16% resolving gel) as described previously (Mahmmoud *et al*., 2000). About 10 $\mu$g of the tryptic products was loaded onto each lane in the SDS gel and electrophoresis was allowed to proceed overnight.

For immunoblotting, proteins were transferred to PVDF membranes, then washed for 1 h with phosphate-buffered saline (PBS) containing 5% polyoxyethylene sorbitan monolaurate (Tween-20), and incubated overnight at room temperature with the primary antibody. The PVDF membranes were washed again with PBS and incubated with goat anti-rabbit antibody for 2 h. After washing, the protein fragments were detected using ECL reagents. For the detection of the 19 kDa fragment, a specific C-terminal $\alpha$-antibody, $\alpha$1002–1016 (a generous gift of J.V. Møller, Institute of Physiology and Biophysics, Aarhus University), was used.

## Results

### *Effects of curcumin on Na,K-ATPase hydrolytic activity*

The physiological consequences of curcumin treatment are apparently difficult to measure *in vivo*. Suspected curcumin targets can be identified using *in vitro* systems where direct effects on purified proteins can be measured. We investigated the functional effects of curcumin on Na,K-ATPase in a cell-free system by measuring the ouabain-dependent ATP hydrolysis for control- and curcumin-treated membranes. Treatment with curcumin of membrane-bound Na,K-ATPase from shark rectal glands resulted in significant (55–60%) inhibition of hydrolytic activity (Figure 1). Data analysis using a sigmoid dose–response curve gave a $K_{0.5}$ for curcumin inhibition of 14.64 ± 1.12 $\mu$M. To investigate the effect of curcumin on the affinity of the enzyme for its substrates, the ouabain-dependent ATPase activity at different concentrations of $Na^+$, $K^+$, or ATP was measured for control- and curcumin-treated enzymes. Curcumin-treated Na,K-ATPase had a small but significant increase in the apparent affinity for $Na^+$ when

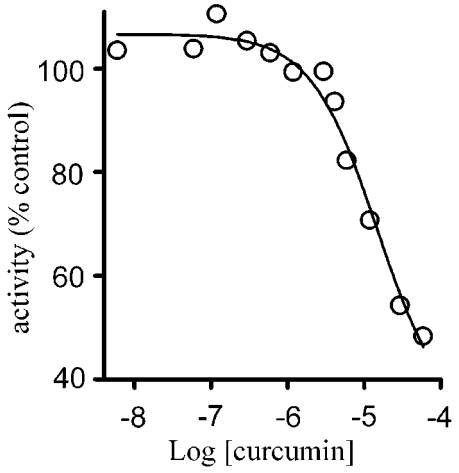


**Figure 1** Curcumin inhibition of ATPase activity. The ouabain-dependent Na,K-ATPase activity was measured in the presence of 20 mM histidine buffer, pH 7.0, 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 3 mM ATP, and the indicated concentrations of curcumin. Data are expressed as percentage of control, measured in the absence of curcumin. A sigmoid dose–response equation was fitted to the data, giving a $K_{0.5}$ for curcumin inhibition of $14.64 \pm 1.12\,\mu$M. Data are mean ± s.e.m. of duplicate measurements. Representative of three independent experiments is shown, and each experiment gave similar result.

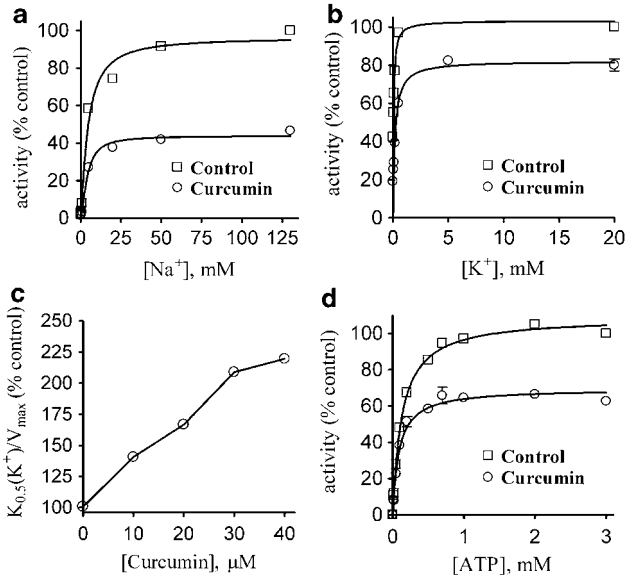


**Figure 2** Substrate-dependent ATPase activity of control- and curcumin-treated enzymes. Ouabain-dependent ATPase activity was measured in the presence of 20 mM histidine buffer, pH 7.0, 3 mM $MgCl_2$ without or with 25 $\mu$M curcumin. Data are mean ± s.e.m. of duplicate measurements and expressed as percentage of 100% control. Representative of three independent experiments is shown, and each experiment gave similar result. (a) $Na^+$-stimulated ATP hydrolysis in the presence of 10 $\mu$M ATP, in the absence of $K^+$, and in the presence of the indicated $Na^+$ concentrations is shown. Analysis of the data using a sigmoid dose–response curve gave a $K_{0.5}(Na^+) = 4.52 \pm 1.10$ mM for control- and $4.08 \pm 1.06$ mM for curcumin-treated enzyme, $P$-value $<0.0001$. Curcumin treatment resulted in ~60% inhibition of hydrolytic activity. (b) $K^+$-stimulated ATP hydrolysis measured in the presence of 130 mM $Na^+$, 10 $\mu$M ATP, and the indicated $K^+$ concentrations. Analysis of the data using a hyperbolic function gave a $K_{0.5}(K^+) = 51.8 \pm 1.0\,\mu$M for control- and $177 \pm 2.0\,\mu$M for curcumin-treated enzyme, $P$-value $<0.0001$. Curcumin treatment resulted in ~21% inhibition of hydrolytic activity. (c) The effect of curcumin on the $K_{0.5}(K^+)/V_{max}$ is shown. The $K_{0.5}(K^+)/V_{max}$ was estimated from $K^+$-activation curves measured at the indicated curcumin concentrations. (d) ATP-stimulated ATP hydrolysis measured in the presence of 130 mM $Na^+$, 20 mM $K^+$, and the indicated ATP concentrations. Analysis of the data using a Michaelis–Menten equation gave a $K_{0.5}(ATP) = 130.0 \pm 0.006\,\mu$M for control- and $90 \pm 0.009\,\mu$M for curcumin-treated enzyme, $P$-value $<0.05$. Curcumin treatment resulted in ~40% inhibition of hydrolytic activity.

compared to control ($K_{0.5}(Na^+) = 4.52 \pm 1.10$ mM for control- and $4.08 \pm 1.06$ mM for curcumin-treated enzyme, $P$-value $<0.0001$, Figure 2a). On the other hand, curcumin-treated enzyme had a significant three-fold reduction in $K^+$ affinity when compared to control ($K_{0.5}(K^+) = 51.8 \pm 1.0\,\mu$M for control- and $177 \pm 2.0\,\mu$M for curcumin-treated enzyme, $P$-value $<0.0001$, Figure 2b). The significant increase in $K_{0.5}$ for $K^+$ was accompanied with a parallel decrease in $V_{max}$, suggesting that curcumin directly interferes with $K^+$ interaction. As proposed earlier (Cleland, 1963), the $K_{0.5}$ is a poor indicator of the substrate-binding affinity when $V_{max}$ is subject to significant changes. However, the value $K_{0.5}(K^+)/V_{max}$ gives a better estimate of whether or not a ligand directly interacts with the substrate-binding site. We have therefore determined the effect of various curcumin concentrations on the $K_{0.5}(K^+)/V_{max}$ by measuring the effect of different concentrations of curcumin on the $K_{0.5}(K^+)$ and the corresponding $V_{max}$ values, showing that curcumin treatment significantly increases $K_{0.5}(K^+)/V_{max}$ (consistent with a decrease in the affinity of $K^+$) (Figure 2c). The increase in the apparent affinity for $Na^+$ seems to be indirect, by inducing a shift towards the E1 conformation, since the $K_{0.5}/V_{max}$ for $Na^+$ was not significantly changed after curcumin treatment (data not shown).

Curcumin treatment was also found to increase the apparent affinity for ATP when compared to control ($K_{0.5}(ATP) = 130.0 \pm 0.006\,\mu$M for control- and $90 \pm 0.009\,\mu$M for curcumin-treated enzyme, $P$-value $<0.05$, Figure 2d). The level of inhibition by curcumin was not reduced at low ATP concentrations (10 $\mu$M, not shown), indicating that curcumin inhibition of Na,K-ATPase is not due to a simple interference with ATP binding when the ATP concentration is limited. Interestingly, the $K_{0.5}$ for curcumin inhibition of Na,K-ATPase from pig kidney was ~6.5 $\mu$M, and was associated with about 80% inhibition of ATPase activity (unpublished data), suggesting isoform- and/or species-specific effects.

### *The E1/E2 equlibrium*

An increase in the apparent affinity of the enzyme for $Na^+$ and ATP and a decrease in its affinity for $K^+$ would most likely reflect a shift from the $K^+$-form (E2) to the $Na^+$-form (E1). The vanadate sensitivity of control- and curcumin-treated enzymes was measured to test this possibility. Vanadate binds preferentially to the E2 conformation (Segall *et al*., 2002; Mahmmoud *et al*., 2003) and a decrease in vanadate sensitivity reflects a shift to the E1 conformation. As seen in Figure 3, curcumin treatment resulted in a significant four-fold decrease in vanadate sensitivity ($K_{0.5}(vanadate) = 3.25 \pm 0.015\,\mu$M for control- and $13.16 \pm 0.017\,\mu$M for curcumin-treated enzyme, $P$-values $<0.01$), indicating that curcumin induces a shift in the conformational equilibrium of the enzyme toward E1.

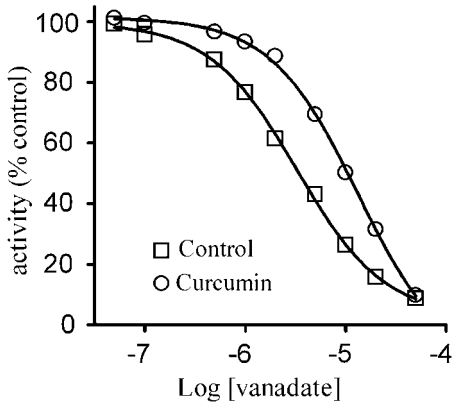


**Figure 3** Vanadate sensitivity of control- and curcumin-treated enzymes. The hydrolytic activities of control- and curcumin-treated enzymes were measured in the presence of 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1 mM ATP, and the indicated vanadate concentrations. Data are normalized to 100% control (in the absence of curcumin), and expressed as percentage of the activity measured in the absence of vanadate. Data are mean ± s.e.m. of duplicate measurements. Representative of two independent experiments is shown, and each experiment gave similar result. A sigmoid dose–response curve was fitted to the data points, which gave a $K_{0.5}$ for vanadate inhibition of 3.26 ± 0.015 $\mu$M for control-, and 13.16 ± 0.017 $\mu$M for curcumin-treated enzyme (*P*-value <0.001).

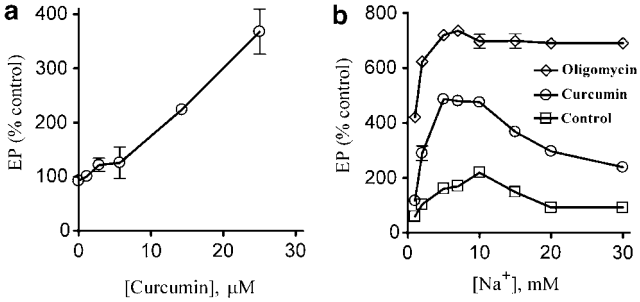


**Figure 4** Curcumin- and $Na^+$-dependent accumulation of EP. (a) Steady-state EP level was measured in the presence of 20 mM $Na^+$ and the indicated concentrations of curcumin, as described in Methods. Data are expressed as percentage of control, measured in the absence of curcumin. (b) Phosphorylation was measured in the presence of the indicated concentrations of $Na^+$ plus either DMSO (control), 25 $\mu$M curcumin, or 25 $\mu$M oligomycin, as indicated in the figure. Data are expressed as percentage of control, measured in the presence of 1 mM NaCl, and in the absence of curcumin and oligomycin. Fitting of the initial $Na^+$-dependent stimulation of EP accumulation using a hyperbolic function gave maximum steady-state phosphorylations of 288.7 ± 32.3, 664.3 ± 52.37, and 798.0 ± 21.9% for control-, curcumin-, and oligomycin-treated enzymes, respectively. The $K_{0.5}$'s for the initial $Na^+$ activation were 3.915 ± 1.07, 2.805 ± 0.627, and 0.742 ± 0.11 mM for control-, curcumin-, and oligomycin-treated enzymes, respectively (*P*-value <0.0001). Data are mean ± s.e.m. of triple determinations. Representative of three independent experiments is shown, and each experiment gave similar result.

### *Phosphorylation*

The curcumin and $Na^+$ dependencies of EP formation were measured to study the effect of curcumin on the phosophorylation reaction. Curcumin treatment resulted in a significant increase in the steady-state EP level (Figure 4a) in such a way that treatment with 25 $\mu$M curcumin increased the EP level by more than three orders of magnitude. This could either be due to an increase in the rate of phosphorylation (i.e., increasing the rate of the reaction; E1 → E1P → E2P), a decrease in the rate of dephosphorylation (i.e., decreasing the rate of the reaction; E1P → E2P → E1), or both.

The $Na^+$-dependent phosphorylation of control- and curcumin-treated Na,K-ATPase is depicted in Figure 4b, showing a significant increase in EP accumulation after curcumin treatment, with maximum effect measured at $Na^+$ concentrations of 5–10 mM. The steady-state level of EP was found to markedly decrease at higher $Na^+$ concentrations, in such a way that the EP level measured at 20 mM was ~55% of that measured at 5 mM $Na^+$, suggesting an increase in the dephosphorylation rate. To account for this $Na^+$-dependent decrease in the EP level in curcumin-treated enzyme, we measured the $Na^+$-dependent EP formation in Na,K-ATPase treated with the antibiotic oligomycin as well. Oligomycin blocks the E1P → E2P transition and inhibits dephosphorylation (Fahn *et al*., 1966; Hobbs *et al*., 1983; Arato-Oshima *et al*., 1996), giving rise to maximum EP levels. As expected, the steady-state EP level of oligomycin-treated enzyme increases to maximum at about 5–10 mM $Na^+$, but remained at a constant level at $Na^+$ concentrations up to 30 mM, reflecting blocking of dephosphorylation. Thus, during the course of the steady-state phosphorylation reaction, curcumin-treated enzyme exhibits a slower rate of dephosphorylation when compared to control enzyme, but a faster rate of dephosphorylation when compared to oligomycin-treated enzyme.

The EP consists of two species, E1P and E2P. E1P reacts with ADP to form dephosphorylated enzyme plus ATP. E2P is ADP insensitive but reacts rapidly with $K^+$ to form dephosphorylated enzyme plus inorganic phosphate. The E1P/E2P distribution was investigated by measuring the EP decay after chasing with ADP for short periods of time at 0°C. As seen in Figure 5a, curcumin-treated enzyme is much more sensitive to ADP compared to control, indicating that curcumin treatment decreases the E1P → E2P transition. In order to give an estimate of the initial amount of E2P, the slow-decaying component of the graph was analyzed using linear regression model, which gave an intercept with the ordinate axis of 74.48 ± 6.88 for control and 55.43 ± 5.94 for curcumin-treated enzyme. Thus, the distribution of E1P and E2P was altered after curcumin treatment, in favor of E1P.

The rate of E1P → E2P was also directly measured by phosphorylating the enzyme in the presence of 600 mM NaCl to accumulate the E1P form (Vilsen, 1997). Dephosphorylation of the enzyme was then initiated by adding a chase solution producing 2 mM ATP and 5 mM $K^+$ to insure rapid hydrolysis of E2P. Since dephosphorylation of E2P is fast in the presence of $K^+$, the rate of EP decay will reflect a transition from E1P to E2P. As seen in Figure 5b, curcumin treatment induces a three-fold decrease in the rate of E1P → E2P transition (the rate constant for dephosphorylation was 1.99 ± 0.05 and 0.68 ± 0.001 $s^{-1}$ for control- and curcumin-treated enzymes, respectively). Thus, the increase in the steady-state EP level may be a consequence of a decrease in the rate of E1P → E2P transition.

### *Dephosphorylation*

The dephosphorylation of control- and curcumin-treated enzymes was measured in order to gain information about

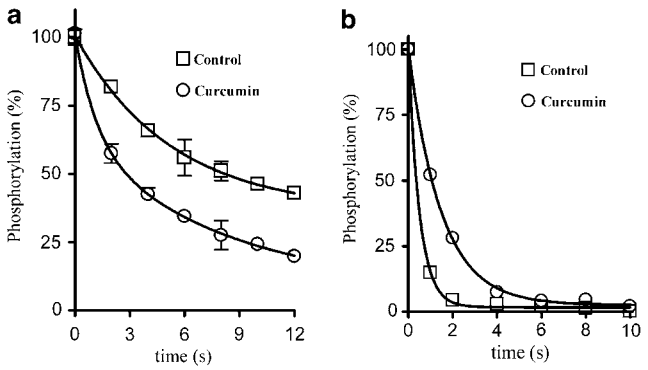


**Figure 5** The E1P→E2P reaction in control- and curcumin-treated enzymes. (a) Phosphorylated enzyme was chased at 0°C with 1 mM ATP and 1 mM ADP, followed by acid quenching at the indicated time intervals. A two-phase exponential decay function was fitted to the date points, giving the rate constants corresponding to the slow and fast components as follows: control, $0.04 \pm 0.01$ and $0.23 \pm 0.10\,s^{-1}$; curcumin-treated, $0.01 \pm 0.003$ and $0.83 \pm 0.22\,s^{-1}$. The slow-decaying component was analyzed using linear regression model to estimate the initial amount of E2P, giving intercept with the ordinate axis of $74.48 \pm 6.88$ for control- and $55.43 \pm 5.94$ for curcumin-treated enzyme, corresponding to the initial fractional amounts of E2P phosphoform as percentage of total. (b) Dephosphorylation of enzyme phosphorylated at 600 mM $Na^+$ (see Methods) was initiated at 0°C by chasing with 2 mM ATP and 5 mM KCl, followed by acid quenching at the indicated time intervals. A monoexponential decay function was fitted to the data points, giving rate constants of $1.99 \pm 0.05\,s^{-1}$ for control-, and $0.68 \pm 0.001\,s^{-1}$ for curcumin-treated enzyme. Data are mean ± s.e.m. of triple determinations. Representative of two independent experiments is shown, and both experiments gave similar results.

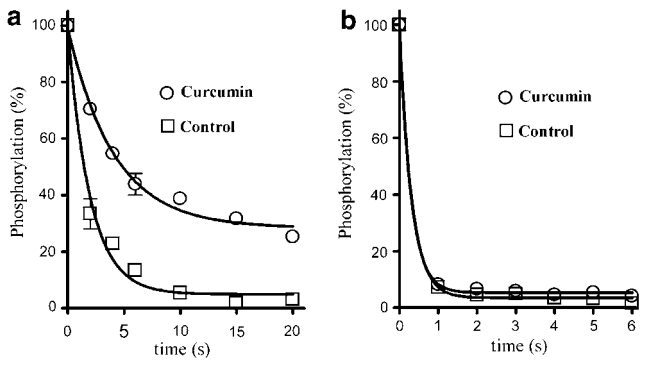


**Figure 6** Spontaneous and $K^+$-dependent dephosphorylation of control- and curcumin-treated enzymes. (a) Spontaneous dephosphorylation of E2P (E2P→E1) was performed by first phosphorylating the enzyme as described in Methods. Dephosphorylation was studied at 0°C by diluting the EP into 1.5 mM Tris–ATP and 3 mM $MgCl_2$, followed by acid quenching at the indicated time intervals. Analysis of the data using a monoexponential decay function gave rates of EP decay of $30.44 \pm 1.8$ and $14.58 \pm 1.21\,min^{-1}$ for control- and curcumin-treated enzyme, respectively. (b) $K^+$-induced dephosphorylation was studied at 0°C by diluting the EP into 1.5 mM Tris–ATP and 1 mM $K^+$, followed by acid quenching at the indicated time intervals. Analysis of the date using a monoexponential decay function gave a rate of EP decay of $193.86 \pm 13.10$ and $206.94 \pm 11.34\,min^{-1}$ for control- and curcumin-treated enzyme, respectively. Data are mean ± s.e.m. of triple determinations. Representative of two independent experiments is shown, and each experiment gave similar result.

the E2P→E1 transition, which occurs slowly in the absence of $K^+$ and very fast in its presence. The spontaneous ($K^+$-independent) dephosphorylation was determined by chasing phosphorylated enzyme with unlabeled ATP and $Mg^{2+}$ and measuring the rate of EP decay. Figure 6a depicts a representative experiment showing that the rate of spontaneous dephosphorylation was reduced about two-fold after curcumin treatment (the rate of EP decay was $30.44 \pm 1.80$ and $14.58 \pm 1.21\,min^{-1}$ for control- and curcumin-treated enzyme, respectively). On the other hand, $K^+$-induced phosphorylation (Figure 6b) in curcumin-treated enzyme was not significantly different from control (the rates of EP decay were $193.86 \pm 13.10$ and $206.94 \pm 11.34\,min^{-1}$ for control- and curcumin-treated enzyme, respectively). Thus, curcumin does not seem to affect the dephosphorylation rate under physiological conditions.

### *$K^+$-occlusion*

The occlusion of $K^+$ to control- and curcumin-treated enzymes was estimated from the difference between $E^{32}P$ obtained following incubation in the absence of $K^+$ and $E^{32}P$ obtained following incubation in the presence of $K^+$ (Daly *et al*., 1996). Control- and curcumin-treated enzymes were pre-incubated with different concentrations of $K^+$ and the enzyme was phosphorylated by $AT^{32}P$ in the presence of 35 mM $Na^+$ (a concentration at which the effect of curcumin on the steady-state EP level is diminished, see Figure 4b). As seen in Figure 7a, for control enzyme maximum occlusion occurs after pre-incubation with 0.5 mM $K^+$ whereas curcumin-treated enzyme required ~1.5–2 mM $K^+$, reflecting approximately four-fold reduction in $K^+$ occlusion.

In the presence of occluded $K^+$ ($Rb^+$), trypsin cleaves the α-subunit at 37°C to form the '19 kDa membranes', which comprises the α-subunit lacking cytoplasmic domains and contains transmembrane segments with occluded $K^+$ (Karlish *et al*., 1990). Incubation of Na,K-ATPase with trypsin in the absence of $K^+$ results in further tryptic cleavage of the 19 kDa fragment (Karlish *et al*., 1990; Mahmmoud & Cornelius, 2002). Domains of the 19 kDa membranes form a complex that is stabilized (and protected against trypsin) upon occlusion of $K^+$. Upon displacement of occluded $K^+$, domains of the 19 kDa membranes relax, allowing for inactivation of $K^+$ occlusion, release of the M5M6 transmembrane hairpin (Lutsenko *et al*., 1995), loss of transmembrane domain interactions, and subsequent trypsin attack (Shainskaya & Karlish, 1994; Shainskaya *et al*., 1998). This is also evidenced from the fact that $Ca^{2+}$ ions bind at the $K^+$-binding sites, but fails to be occluded allowing for degradation of the 19 kDa fragment by trypsin (Shainskaya *et al*., 1998). Based on the above observations, it is expected that incubation of Na,K-ATPase with ligands that abrogate $K^+$ occlusion would result in proteolytic degradation of 19 kDa fragment even in the presence of $K^+$. To test whether curcumin affects $K^+$ occlusion to the enzyme, an experiment was carried out in which equal amounts (50 μg) of control- and curcumin-treated membranes were incubated with 20 mM KCl followed by incubation with trypsin at 37°C for different periods of time. The time-dependent accumulation of the 19 kDa fragment was monitored with a C-terminal specific antibody after SDS–PAGE, as described in Methods. As seen in Figure 7b, enzyme pre-incubated with curcumin shows a significant less protection against trypsin, such that after 40 min the 19 kDa

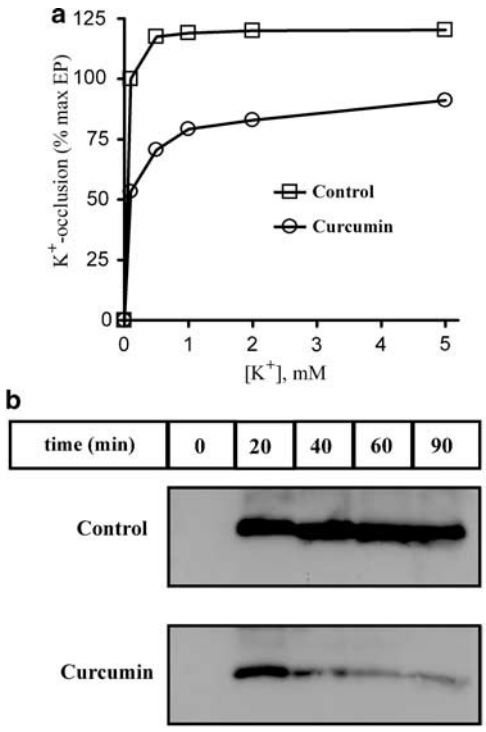


**Figure 7** $K^+$ occlusion in control- and curcumin-treated enzymes. (a) $K^+$ occlusion was measured as described in Methods. Data are expressed as percentage of maximum EP level, as previously described (Daly *et al*., 1996). (b) Immunoblot showing time-dependent accumulation of the '19 kDa fragment' in control- and curcumin-treated membranes, as indicated. Control- and curcumin-treated membranes were incubated with $K^+$, submitted to trypsin treatment for the indicated time intervals, loaded on SDS gels, and blotted using antibody specific for the C-terminus of the α-subunit, as described previously (Mahmmoud *et al*., 2000).

fragment was almost completely degraded. This is in contrast to control enzyme, where the 19 kDa fragment was stable for at least 90 min.

## Discussion

### *ATPase activity*

In this study, we report direct inhibition of Na,K-ATPase with curcumin treatment and further investigated the steps in the reaction cycle of Na,K-ATPase that are affected by curcumin binding. The Na,K-ATPase reaction cycle (Scheme 1) involves ion-dependent phosphorylation and dephosphorylation processes, associated with the occlusion of $Na^+$ and $K^+$, respectively. The E1 conformation has a high affinity for $Na^+$ (and low affinity for $K^+$), whereas the E2 conformation has a high affinity for $K^+$ (and low affinity for $Na^+$). The measured apparent ion affinity is thus an overall value that depends on ion binding, occlusion, and deocclusion, and a decrease in the apparent affinity for the ion after treatment with a given ligand does not necessarily mean that all three steps are affected. A modulator can directly affect the ion-binding affinity, by direct interaction with the ion-binding and/or occlusion site. On the other hand, modulators can also affect the ion affinity by inducing indirect effects on cytoplasmic regions of the protein that are controlling the E1/E2 conformational equilibrium, thus leading to indirect change in ion affinity. Kinetic analysis indicated that curcumin interferes directly with $K^+$-interaction and not indirectly by inducing a shift to the E1 conformation. This is evidenced from the significant increase in the $K_{0.5}(K^+)/V_{max}$ in enzyme treated with different curcumin concentrations (Figure 2c), reflecting a curcumin-dependent reduction in $K^+$ interaction (Cleland, 1963). Another evidence for the $K^+$-specific effect was indicated from kinetic and proteolytic experiments, in which curcumin induced a four-fold reduction in $K^+$ occlusion (Figure 7a), resulting in destabilization of fragments of the '19 kDa membranes' and their subsequent tryptic cleavage in the presence of $K^+$ (Figure 7b). A shift to the E1 conformation of the enzyme is also indicated from results shown in Figure 3, where curcumin treatment induced a four-fold reduction in the sensitivity to vanadate. At this stage, we cannot rule out the possibility that curcumin interacts with transmembrane domains that are involved in the occlusion of $K^+$ (see below).

The inhibition of Na,K-ATPase activity by curcumin seems to be controlled with the equilibrium between the E1P and the E2P phosphoforms. Thus, in the absence of $K^+$, there are approximately comparable amounts of both E1P and E2P (see also Figure 5a), which, together with the slow rate of the spontaneous hydrolysis of E2P, produce a drastic reduction in Na-ATPase activity ($V_{max}$ of Na-ATPase was reduced by 60% after curcumin treatment, Figure 2a). On the other hand, however, the rapid hydrolysis of E2P in the presence of $K^+$ ensures continuous disappearance of E2P and thus an increase in the rate of E1P→E2P transition, thereby shifting the conformational equilibrium toward E2P, allowing for an increase in the turnover rate ($V_{max}$ of Na,K-ATPase was reduced by only 21% after curcumin treatment, Figure 2b and see below).

Is curcumin a specific inhibitor of $P_2$-type ATPase superfamily? The results of the present investigation also point out to the interesting possibility that curcumin may modulate other members of the $P_2$-type ATPase superfamily, which share significant homology (Kühlbrandt, 2004). The possibility that curcumin affects the activity of the gastric H,K-ATPase is currently under investigation.

### *Phosphorylation/dephosphorylation*

The Na,K-ATPase is phosphorylated from ATP in the presence of $Na^+$ and the EP level under steady-state conditions will depend on the rate of dephosphorylation. At low $Na^+$ concentrations, treatment with curcumin resulted in significant increase in the EP level (Figure 4). Curcumin seems to do so by decreasing the rate of formation of E2P from E1P (Figure 5b). An increase in $Na^+$ concentration above 15 mM was accompanied by a decrease in the steady-state EP level of curcumin-treated enzyme, and when the $Na^+$ concentration exceeds 30 mM the steady-state EP level of curcumin-treated enzyme was comparable to that of control. It is concluded that the accumulation of E1P induced by curcumin treatment may stimulate a spontaneous shift to the E2P form, allowing for an increase in the dephosphorylation rate. This is in great contrast to the Na,K-ATPase inhibitor oligomycin, where the EP level of oligomycin-treated enzyme was found to be stable at $Na^+$ concentrations up to 30 mM, reflecting blocking of

dephosphorylation (Figure 4b). In this regard, curcumin is thus considered as a mild blocker of the E1P→E2P transition, when compared to oligomycin.

By measuring the $Na^+$-dependent EP formation under steady-state conditions (Figure 4b), indirect information about the rate of dephosphorylation can be obtained. The EP level of control enzyme increased slightly at 10 mM $Na^+$ but rapidly decreased when the $Na^+$ concentration is increased (Figure 4b), suggesting a significant increase in the rate of dephosphorylation. After curcumin treatment, the EP level drastically increased at $Na^+$ concentrations up to 10 mM, indicating that curcumin-treated enzyme has a lower rate of dephosphorylation when compared to control. As mentioned above, the EP level formed after oligomycin treatment was almost stable at $Na^+$ concentrations up to 30 mM. The conclusion is, however, that enzyme treated with curcumin has a slower rate of dephosphorylation when compared to control enzyme and a higher rate of dephosphorylation when compared to oligomycin-treated enzyme. Figure 6a confirms the data in Figure 4b, showing that curcumin-treated enzyme has a two-fold lower rate of spontaneous dephosphorylation. This significant decrease in the rate of spontaneous dephosphorylation is probably secondary to an initial decrease in the E1P→E2P transition (Figure 5b).

The surprising finding that curcumin reduces $K^+$ occlusion (Figure 7) but not $K^+$-dependent dephosphorylation (Figure 6b) suggests that the site at which $K^+$ binds to induce dephosphorylation is distinct from the site at which $K^+$ occludes, as also previously suggested (Daly *et al*., 1996). A stimulatory effect of $K^+$ on the rate of dephosphorylation was described for SERCA (Andersen *et al*., 1985) and for the $H^+$-ATPase (Vara & Serrano, 1982). Indeed, Sørensen *et al*. (2004) have recently localized a $K^+$-binding site that is involved in accelerating the dephosphorylation of SERCA. This site is highly conserved among P-type ATPases and it was therefore suggested that it could be a general feature of all P-type ATPases. The interaction of $K^+$ ions with Na,K-ATPase seems to occur in two distinct steps: $K^+$ binding at the binding site which is curcumin insensitive (as evidenced from identical rates of $K^+$-dependent E2P→E1 transition in control and curcumin treated membranes, Figure 6b), followed by occlusion at the $K^+$ occlusion site, which is affected by bound curcumin (as evidenced from the effect of curcumin on $K^+$ occlusion, Figure 7). Furthermore, it was previously shown that, in the presence of $Ca^{2+}$ ions, $K^+$ occlusion is inactivated but the M5M6 transmembrane hairpin is not released (Shainskaya *et al*., 1998). This would indicate that $Ca^{2+}$ blocks the $K^+$-binding site, thus preventing the release of the M5M6 transmembrane hairpin, but it fails to be occluded at the $K^+$ occlusion site, thus resulting in further cleavage of the 19 kDa fragment, lending further evidence to the possibility that binding and occlusion are occurring at distinct sites.

In fact, earlier mutational (Vilsen, 1999 and references therein) and recent homology modeling (Ogawa & Toyoshima, 2002) studies have indicated the importance of transmembrane domains M4, M5, and M6 in $K^+$ binding and occlusion. It might be possible that curcumin binds, at the membrane phase, to one or more of these transmembrane domains that are participating in the occlusion of $K^+$ and thus would interfere with $K^+$ occlusion rather than $K^+$ binding. In this regard, the rate-limiting step of Na,K-ATPase treated with curcumin would be the rate of $K^+$ occlusion (which is also a function of extracellular $K^+$ concentrations or the E1/E2 conformational equilibrium) and the rate of formation of E2P from E1P.

### *Is there a link between inhibition of Na,K-ATPase activity and correction of CF defects?*

The reported functional effects of curcumin on SERCA are apparently due to binding at the cytoplasmic domains of this ATPase (Bilmen *et al*., 2001; Logan-Smith *et al*., 2001; Sumbilla *et al*., 2002). Curcumin was shown to decrease the slippage of SERCA (slippage is a consequence of high $Ca^{2+}$ levels in the lumen of SR, resulting in a high proportion of E2P($2Ca^{2+}$) that will result in releasing $Ca^{2+}$ ions into the cytoplasmic side rather than to the SR lumen) (Logan-Smith *et al*., 2001). Such decrease in slippage may be accounted for by a decrease in the EP level after curcumin treatment at a given $Ca^{2+}$ concentration in the SR lumen, in agreement with results showing that curcumin treatment of SERCA decreases the EP level (Bilmen *et al*., 2001; Sumbilla *et al*., 2002). This is in contrast to the effects of curcumin on Na,K-ATPase reported here, where curcumin treatment significantly increases the steady-state EP level. We believe that curcumin might bind to Na,K-ATPase from the extracellular side, as indicated from its effects on $K^+$ occlusion, and its functional similarity to oligomycin, which was proposed to bind from the extracellular side of the membrane (Arato-Oshima *et al*., 1996). However, a definite conclusion cannot be drawn unless more information has accumulated about the binding site for curcumin on these ATPases and more detailed results on the effects of curcumin on the kinetic properties of SERCA.

Curcumin was reported to correct CF defects in some model systems (Egan *et al*., 2004) but not others (Dragomir *et al*., 2004; Loo *et al*., 2004; Song *et al*., 2004). The fact that curcumin inhibits Na,K-ATPase may, at least in part, add more interpretations that could help explain this discrepancy and draw a number of speculations, some of which are as follows: (1) modulation of Na,K-ATPase by curcumin *in vivo* may lead to profound effects on membrane transport in some systems but not others, depending on physiological conditions that could affect curcumin action (e.g., $[Na^+]_i$, $[K^+]_o$ and existence of enzyme species with inherited slow rate of E1P→E2P transition (see below); (2) partial inhibition of the Na,K-ATPase by curcumin would result in modulation of numerous cell-specific signaling cascades, as previously reported for the inhibitor ouabain (Kometiani *et al*., 1998; Xie *et al*., 1999; Liu *et al*., 2000; Dostanic *et al*., 2003); and (3) a modulator of more than one transport protein would have differential effects depending on the number, selectivities, regulatory modes, and gating properties of these various transport proteins. The above speculations remain open until definite experiments are performed to uncover the effect of curcumin on membrane transport processes *in vivo*.

One of the above speculations is indeed supported by this and other studies. The pig kidney enzyme (α1 isoform) was found to be more sensitive to curcumin than the shark enzyme (α3 isoform) (unpublished data), indicating isoform- and/or species-specific effects. Furthermore, deletion of the N-terminus of the α-subunit, which has a highly dissimilar primary structure among different α-isoforms, has been shown to affect the rate of E1P→E2P transition (Segall *et al*., 2002). Thus, the rate constant for the E1P→E2P transition for α1 chimera lacking the N-terminal 32 amino acids (a chimera that

has the same kinetic properties as the α2 isoform) decreased six orders of magnitude compared to wild-type α1 (Segall *et al*., 2002), implying that the Na,K-ATPase α2 isoform may be less sensitive to curcumin.

Inhibition of Na,K-ATPase is actually consistent with the positive effects on CFTR cells. Studies on freshly excised and cultured normal and CF airway epithelial cells detected a raised apical membrane $Na^+$ conductance and a reduced or absent $Cl^-$ conductance in CF cells compared to control (Willumsen *et al*., 1989a, b). Chinet *et al*. (1994) later reported an increased activity of the epithelial sodium channel (ENaC) in CF cells and it was concluded that CFTR functions dually by increasing $Cl^-$ conductance and decreasing $Na^+$ conductance through interaction with ENaC. Inhibition of the basolateral Na,K-ATPase and the subsequent increase in $[Na^+]_i$ would also result in a similar decrease in ENaC activity. The inhibition of Na,K-ATPase cannot be used to draw a sequence of events regarding regulation of ion transport in a given system. This will need more investigations as to understand how inhibition of Na,K-ATPase activity could be related to variations in $Cl^-$ conductance under normal and pathological conditions.

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