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Curcumin is a lipid dependent inhibitor of the Na,K-ATPase that likely interacts at the protein-lipid interface

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ABSTRACT

Curcumin is an important nutraceutical widely used in disease treatment and prevention. We have previously suggested that curcumin interferes with K^+ binding to pig kidney Na,K-ATPase by interaction with its extracellular domains. The aim of this study was to further characterize the site of curcumin interaction with the ATPase. We have performed pair inhibitor studies and investigated the sided action of curcumin on pig kidney Na,K-ATPase reconstituted into lipid vesicles of defined composition. An addition of curcumin to either the intracellular or extracellular domains of the Na,K-ATPase produced similar inhibition. The lipid environment and temperature strongly influenced the potency of the drug. Curcumin inhibition decreased following insertion of the ATPase in sphingomyelin-cholesterol 'raft' domains and fully abolished following treatment with non-ionic detergents. The drug induced cross-linking of membrane embedded domains of the Na,K-ATPase. We conclude that curcumin interacts with Na,K-ATPase at the protein-lipid interface. Non-annulus lipids likely participate in this interaction. These results provide new information on the molecular mechanism of curcumin action and explain (at least partly) the ambiguous effectiveness of this polyphenol in the different systems.

1. Introduction

Scientific interest in ancient medicine has increased strongly during the last decade, and many efforts have been made to understand and explain the beneficial effects of many natural herbs. Curcumin (diferuloylmethane), the active constitute of *Curcuma longa* (commonly termed as tumeric), is one of the best studied natural molecules. Curcumin has been used as a spice [1] and food additive for centuries, but thereafter the anti-inflammatory, antiviral, anti-infectious and anti-carcinogenic effects of this molecule have become evident (for recent reviews see Refs. [2,3]). Of particular medical significance is the possible connection of curcumin intake with the treatment of several physiological disorders [4–7]. It is not until several years ago that curcumin was found to directly interact with a variety of enzymes: Curcumin has been shown to modulate the activity of protein kinases [8–10], mitochondrial F0F1-ATPases [11] and transcription factors [12]. This broad specificity suggests that the drug interacts with a common domain on these targets.

Curcumin also modulates the activity of transmembrane P-type pumps [13–16]. As an inhibitor of sacroplasmic reticulum Ca^{2+}-ATPase, curcumin was shown to be useful in the treatment of cystic fibrosis [5], although there have been some inconsistencies on this matter [5,17–20]. The activity of membrane proteins is modulated by lipids [21]. Indeed, several signaling cascades involve activation of membrane receptors that requires sequestration into specific lipid domains (rafts, for recent review see Ref. [22]). A typical example is the synchronization by lipid rafts of NFκB activation by interleukin 1β [23]. Hence, membrane-dependent events definitely play a substantial role in membrane protein function and regulation.

Understanding how curcumin interacts at its binding site(s) on target proteins is an important goal, as it will give information about its mechanism of action and allow us to understand how this molecule affect such large number of targets. We have been interested in the fact that curcumin modulates several proteins that are structurally and functionally unrelated but share the common property of being membrane-bound proteins, downstream proteins of membrane receptor-activated signaling cascades, or proteins that associate with cellular membranes upon activation (such as protein kinase C). This raises the possibility that curcumin interaction with its target proteins may be regulated by the lipid environment. Here, we address this issue by studying the sided action of curcumin on Na,K-ATPase reconstituted into lipid vesicles of defined lipid composition. We have found that the inhibitory effect of curcumin, including its strong effect on K^+ affinity, was independent of whether curcumin binds to the Na,K-ATPase from the extracellular- or the cytoplasmic-side, suggesting that this molecule

Abbreviations: BS^3, Bis-(sulfosuccinimidyl)suberate; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EC_{50}, ligand concentration producing half-maximum effect; Na,K-ATPase, sodium and potassium activated adenosine triphosphatase; PBS, phosphate-buffered saline; PVDF, Polyvinylidene fluoride; Tricine, N-[2-hydroxy-1,1 bis(hydroxymethyl)ethyl]glycine

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interacts from within the membrane area. Moreover, the membrane structure was found to be an important determinant of curcumin potency. The interaction of curcumin with transmembrane domains of membrane proteins in a lipid dependent manner seems to be a general mechanism that needs auxiliary attention to understand the pharmacological properties of this polyphenol.

2. Methods

2.1. Na,K-ATPase preparation and hydrolytic activity

Pig kidney membrane fragments rich in Na,K-ATPase was prepared as previously described [24] but with little modifications as follows: the outer renal medulla tissue was homogenized in 30 mM histidine buffer (pH 7.4 at 20 °C) containing 250 mM sucrose and 1 mM EDTA (HSE buffer). The microsomal fraction was opened and peripheral contaminating proteins removed by incubation with a low concentration of SDS (estimated from calibration curves in which increasing SDS concentrations were employed) and the washed protein was finally suspended in HSE buffer. The maximum hydrolytic activity was around 1400 $\mu mol^{-1} h^{-1} mg^{-1}$ at 37 °C. The activity was determined by measuring ouabain-dependent ATP hydrolysis after incubation of the enzyme with substrate ions, MgATP, and inhibitors in the presence of either DMSO (dimethyl sulfoxide) vehicle or curcumin (details are in separate figure legends). In the case of measuring ATPase activity in the presence of the non-ionic detergent $C_{12}E_{10}$, the ATPase assay was performed exactly as above but in the presence of increasing detergent concentrations.

2.2. Reconstitution of Na,K-ATPase

Pig kidney Na, K-ATPase was reconstituted into lipid vesicles of dipalmitoyl phosphatidylcholine (PC) containing 30 mol% cholesterol (Chol), as described previously [25]. In some experiments, 5 mol% phosphatidyl serine (PS) was included. In this artificial system active Na,K-ATPase molecules are oriented either inside-out (the cytoplasmic domains are facing the extra-liposomal medium), right-side out (the cytoplasmic domains are oriented inside the liposomal lumen), or non-oriented (enzyme molecules are randomly adsorbed on liposome surface). The reconstitution buffer contained 20 mM histidine, pH 7.05, 2 mM $MgCl_2$, and 260 mM sucrose. Reconstitution was performed in the absence or in the presence of 25 μM curcumin. Extra-vesicular curcumin was in some cases removed from the external medium by passing the liposome suspension through Benefsky columns [26] just before use in hydrolytic assays. Controls were prepared in exactly the same way, except that curcumin was replaced by the same volume of DMSO. Prior to the ATPase test, liposomes was preincubated in a buffer containing 1 mM Na_2HPO_4 and 1 mM ouabain to inhibit the non-oriented Na,K-ATPase with no effect on inside-out pumps [27]. Potassium activation of ATP hydrolysis by inside-out Na,K-ATPase in liposomes was measured in the presence of 100 mM NaCl, 2 mM $MgCl_2$ (final concentration), 1 mM ouabain, 20 μM ATP (containing 10^4 cpm ^{32}P-[ATP]) and different concentrations of potassium chloride. Several experiments were performed to confirm that curcumin does not increase the permeability of the membrane to ions or ATP, thereby activating the right-side out oriented pumps (see later discussion). In order to prepare liposomes containing raft domains, reconstitution was performed by mixing PC, Chol, sphingomyelin (SM), brain cerebrosides (CB) at a mole ratio of 50/30/10/10 mol%, respectively, essentially as previously described [28].

2.3. Proteolytic cleavage

Extensive proteolytic cleavage of membrane-bound Na,K-ATPase was performed using trypsin and ended up with the "19 kDa membranes", i.e., membranes with Na,K-ATPase lacking all cytoplasmic domains and containing intact transmembrane domain fragments connected with hairpin loops [29,30]. One mg of purified protein was incubated with 0.2 mg trypsin at either 24 °C (liposomes) or 37 °C (native membranes) for 1 h, in the presence of 20 mM histidine, pH 7.0, 5 mM EDTA and 25 mM KCl. The post-tryptic membranes were washed at least two times in cold imidazole 25 mM buffer (pH 7.0) containing 10 mM KCl. Finally, the membranes were washed twice in histidine buffer, finally homogenized in 20 mM histidine containing 25% glycerol, and immediately used for cross-linking experiments.

2.4. Effect of curcumin on membrane permeability

We performed a simple experiment to confirm that curcumin does not increase liposome membrane permeability. First, inside-out- and non-oriented Na,K-ATPase were proteolytically degraded by incubation with trypsin at 24 °C for 30 min in the presence of 20 mM histidine, pH 7.0, 3 mM EDTA and 20 mM K^+ [29,30]. This treatment results in the complete loss of ATPase activity of these two populations while preserving the integrity of right-side out pumps because trypsin has no access to the internal lumen of the vesicles. Only right-side out oriented Na,K-ATPase molecules remain intact, but they are not active unless they have access to Na^+, K^+, and MgATP. Vesicles containing only functional right-side out pumps were then incubated with the above mentioned substrates, plus different curcumin concentrations. Destabilization of the membrane by curcumin would result in increase in membrane permeability and activate right-side out pump molecules to hydrolyse ATP. Hence, ouabain-dependent ATP hydrolysis was monitored at different periods of time up to 2 h. Positive control experiments were performed in which $C_{12}E_{10}$ or alamethicin were used. Curcumin concentrations up to 200 μM did not lead to increased ATP hydrolysis by right-side out oriented pumps. On the other hand, addition of 1 $\mu g\ ml^{-1}$ alamethicin (making the vesicles leaky) or 2 mM $C_{12}E_{10}$ (opening the vesicles) permits access to Na^+ and ATP of the cytoplasmic-side of the enzyme and resulted in a 100-fold increase in ATP Hydrolysis. It should be mentioned that the lack of activation of right-side out Na,K-ATPase following curcumin treatment is most likely a consequence of membrane stability and not an inhibition of Na,K-ATPase by curcumin. This is because is it established that curcumin even at relatively high concentrations (~200 μM), does not produce full inhibition of pig kidney Na,K-ATPase (see Fig. 1 in Ref. [16]). Hence, if curcumin increases membrane permeability for Na,K-ATPase substrates, even a small increase in ATP hydrolysis must have been observed, which was not the case.

2.5. Cross-linking

Cross-linking of membrane imbedded transmembrane domains of the Na,K-ATPase α-subunit in the absence or in the presence of curcumin was performed by incubating 100 μg protein with either DMSO or 30 μM curcumin in the presence of 20 mM Hepes-KOH, pH 6.8, 20 mM KCl, and 1 mM EDTA. Cross-linking was also carried out by incubating the membranes with 50 μM Bis-(sulfosuccinimidyl)suberate (BS^3) in the presence of 20 mM HEPES, pH 6.5, and 5 mM EDTA. The reaction was allowed to proceed for 20 min at 23 °C, and terminated by addition of SDS sample buffer [31] followed by loading to SDS gels. BS^3 cross links primary amines and it possibly cross links lysine residues located close to the inner face of the plasma membrane in the M5M6 transmembrane domains and in the C-terminal 19 kDa fragment (unpublished data).

2.6. Gel electrophoresis and immunoblotting

Proteins were separated using Tricine-based SDS-Polyacrylamide Gel Electrophoresis, as previously described [32,33]. For immunoblotting,

proteins were transferred to Polyvinylidene fluoride (PVDF) membranes, then washed three times with phosphate-buffered saline (PBS) containing 5% Tween-20, and incubated over night 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 at room temperature. After washing, protein fragments were detected using enhanced chemiluminescence (ECL) reagent. For the detection of the 19 kDa C-terminal fragment, a specific C-terminal α-antibody, α 1002–1016, was used (a generous gift from Dr. J. V. Møller, Department of Physiology and Biophysics, University of Aarhus).

2.7. Isolation of low density membranes from 'total' pig kidney membranes

Low density membranes were isolated from total plasma membranes by the detergent-free method introduced by Song et al. [34]. Briefly, the membranes were suspended in 20 mM HEPES containing 500 mM sodium carbonate, (adjusted to pH 9 with HCl). The sonication step employed by Song et al. was replaced with 10 successive up and down homogenization steps using a motor driven tight Teflon homogenizer running at a speed of ~2500 rpm. The homogenate was loaded onto a continuous sucrose density gradient (sucrose concentrations from top to bottom ranging between 4% and 40% sucrose, dissolved in 20 mM HEPES pH 7.4, 1 mM EDTA, and 150 mM NaCl) and centrifuged for 12 h at 100,000 g (4 °C) in a swing-out rotor SW28. Following centrifugation, 2.5 ml aliquots from the top of the tube were aspirated (total of 10 fractions). Low density membranes are localized near the top of the tube whereas heavy membranes were localized near the bottom. Finally, aspirated samples were diluted in 20 mM imidazole buffer, pH 7.0, and centrifuged for 50 min at 200,000 g (4 °C). The different fractions were tested for protein concentration, the presence of ouabain-dependent Na,K-ATPase activity, and by immunoblotting.

3. Results

3.1. Paired inhibitor studies

Using pairs of pump inhibitors, indirect information about the binding site of an inhibitor can be obtained, provided that the binding site of another inhibitor is known. If binding of inhibitor I decreases binding of inhibitor II in a competitive manner, then the binding sites of both inhibitors are (either directly or indirectly) related. On the other hand, if binding of inhibitor I has no effect on the inhibition by inhibitor II, then the two inhibitors bind at different sites on the ATPase. Consequently, we have compared the effect of curcumin on purified enzyme treated with Na,K-ATPase inhibitors that have been estimated to bind to known sites on the Na,K-ATPase. Hence, inhibition curves by oligomycin or ouabain have been measured in the presence of different curcumin concentrations, and the relation of the amount of added curcumin to the apparent affinity of oligomycin or ouabain have been determined. Data have been conveniently normalized such that 100% activity represents the activity in the absence of inhibitor. As seen in Fig. 1A, the apparent affinity for oligomycin decreased strongly in a linear fashion by the addition of increasing curcumin concentrations (the EC_{50} for oligomycin increased from 1.5 ± 0.18 μM in the absence of curcumin to 3.9 ± 0.12 μM in the presence of 40 μM curcumin), demonstrating that curcumin decreases the inhibitory potency of oligomycin. This is possibly due to competition for the Na,K-ATPase α-subunit. Using ouabain instead of oligomycin, we could demonstrate that curcumin treatment did not significantly affect the EC_{50} of ouabain (Fig. 1B), indicating that curcumin and ouabain interact at different sites on the ATPase. Indeed, it has been found that the inhibition by curcumin and ouabain is additive, this is not the case with oligomycin (data not shown).

3.2. The sided action of curcumin on reconstituted Na,K-ATPase

Based on studies in which pig renal membrane fragments were used, we have previously proposed that curcumin binds at the extracellular domains of the Na,K-ATPase, as evidenced from its inhibitory effect that was associated with a significant decrease in K^+ interaction [16]. As a continuation to those studies, we have compared the effect of curcumin on pig kidney Na,K-ATPase in native membranes (see Ref. [16]) and Na,K-ATPase reconstituted into tight vesicles of defined lipid composition (this study). To further characterize the binding site of curcumin on the Na,K-ATPase, we investigated the functional effects of curcumin allowed to interact with the cytoplasmic domains of the Na,K-ATPase. This was achieved by measuring the activity of reconstituted inside-out Na,K-ATPase, the function of which can be dissected by pre incubation with ouabain in the presence of magnesium and inorganic phosphate. This incubation results in full inhibition of non-oriented pumps with no effect on inside-out pumps (see Ref. [27] for details). If curcumin interacts at the extracellular domains of the α-subunit, it will not inhibit the

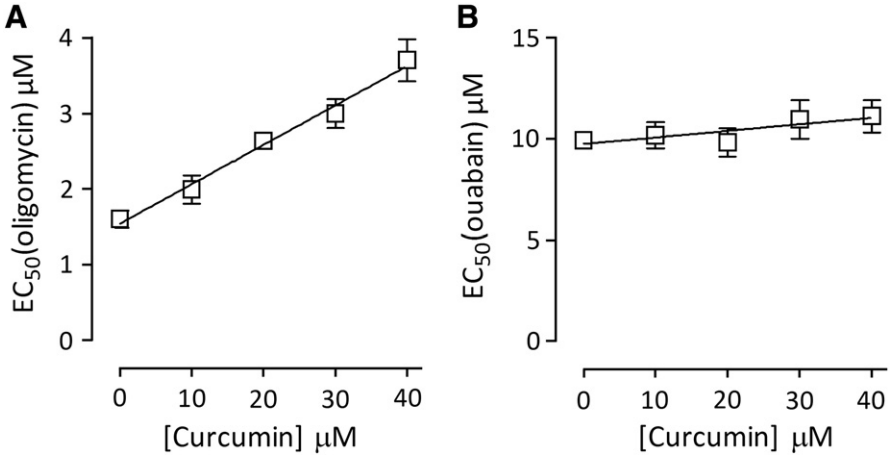

Fig. 1. Effect of curcumin on the apparent affinity for other sodium pump inhibitors. In these experiments purified membrane-bond Na,K-ATPase was used. Inhibition curves by oligomycin or ouabain were measured in the presence of curcumin concentrations ranging from 0 to 40 μM. Data were analyzed using dose–response function: Y = bottom + [(top-bottom)/(1 + $10^{X-Log\ EC50}$)]. The different EC_{50} values for oligomycin (Panel A) or ouabain (panel B) were blotted as a function of the curcumin concentration used, as indicated. The ATPase assays contained 20 mM histidine buffer, pH 7.0, 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 3 μg pig kidney Na,K-ATPase, and several oligomycin (Panel A) or ouabain (Panel B) concentrations. The strong increase in the EC_{50} for oligomycin following curcumin treatment implies that the interaction of oligomycin with Na,K-ATPase is abrogated by curcumin, and hence the two inhibitors share the same binding site. This is not the case for ouabain. ATPase activity was measured at 24 °C.

activity of inside-out reconstituted Na,K-ATPase (i.e., behaving essentially as ouabain, which does not inhibit inside-out Na,K-ATPase). Results presented in Fig. 2A (squares) demonstrate that curcumin, added to the extra-vesicular medium, inhibit inside-out Na, K-ATPase in the same manner as it does with open membrane fragments [16], albeit the affinity of inhibition was considerably higher (see later discussion). To test the effect of curcumin interacting from the extracellular side of the pump, inside-out and non-oriented pumps were first deactivated by treatment with trypsin followed by washing through Penefsky columns to remove trypsin from the medium [26], as described in Methods section. Subsequently, the cytoplasmic domains of right-side out pumps inside the liposomal lumen were given access to substrates by the addition of 1 μg ml^{-1} alamethicin. This makes the liposome membrane leaky to medium components. As seen in Fig. 2A (circles), addition of curcumin to the medium resulted in inhibition of the right-side out pumps. The inhibition was identical to that obtained with the inside-out pumps.

To further investigate the sided action of curcumin on Na,K-ATPase, we have measured the effect of a single curcumin concentration on K^+ activation of either inside-out or right-side out pumps. Curcumin was added to the medium to allow interaction with cytoplasmic domains of inside-out Na,K-ATPase or added to leaky vesicles after deactivation of inside-out pumps by proteolytic degradation to allow for interaction with the extracellular domains of right-side out pumps, as described above. In this experiment the effect of curcumin on the affinity of K^+, measured as $K_{0.5}(K^+)/V_{max}$ was studied [16]. As seen in Fig. 2B, the curcumin induced inhibition and decrease in the apparent affinity of the pump for K^+ were almost the same in both cases. Interestingly, the effect of curcumin on the apparent affinity of reconstituted Na,K-ATPase for K^+ was found to be much more significant than early observed in the native enzyme. Thus, in native membrane-bound enzyme, treatment with 25 μM curcumin increased the $K_{0.5}$ for K^+ by three fold compared to the control [16]. On the other hand, in the reconstituted system, the $K_{0.5}$ for K^+ increased by almost 10 fold following treatment with the same concentration of curcumin (the $K_{0.5}$ for K^+ was 65.7 ± 4.6 μM, 637.5 ± 38.3 μM, and 703.0 ± 42.1 μM for the reconstituted control, inside-out pumps, and right-side out pumps, respectively). It is not likely that the observed difference in K^+ affinity is due to limited K^+ equilibration across the liposomal membrane, since the addition of nigericin rapidly compensate for K^+ depletion from the vesicle lumen. Rather, the membrane lipid structure seems to be important for the modulation of the interaction of curcumin with the enzyme. Finally, we have performed several control experiments to make sure that curcumin does not affect membrane permeability (see Methods section).

3.3. Curcumin potency depends on specific lipid molecules in the membrane and on temperature

To investigate the effect of membrane surface charge on curcumin interaction, we have studied the effect of co-reconstitution of PS on curcumin inhibition of Na,K-ATPase. Control liposomes and liposomes containing 5 mol% PS was incubated with increasing curcumin concentrations and the EC_{50} of inhibition was determined by fitting sigmoid dose–response function to the data. As seen in Fig. 3, in the presence PS in the bilayer, the EC_{50} for curcumin inhibition decreased by almost three fold, which is consistent with an increased affinity for inhibition. Thus, curcumin inhibited Na,K-ATPase in PC-cholesterol vesicles with an EC_{50} of 15.7 ± 0.55 μM. Following insertion of the ATPase in PC–Chol–PS vesicles, the EC_{50} decreased to 5.19 ± 0.26 μM.

Rafts are lipid domains enriched in sphingolipid and cholesterol that have been shown to participate in several cellular functions and are known to contain several marker proteins important for raft function [22]. We have been interested in testing how lipid rafts would affect the potency of curcumin against one of its targets. Hence, the effect of curcumin on Na,K-ATPase reconstituted in lipids enriched in cholesterol and sphingolipids was investigated. Previous studies have documented that rafts isolated from native membranes and that prepared from the mixing of synthetic raft components have similar physical behavior [28] and this allowed us to investigate a possible relation between raft membrane association and curcumin potency. Na,K-ATPase was reconstituted in PC–Chol alone or in combination with sphingomyelin and cerebrosides, and curcumin inhibition curves were measured in both cases. As seen in Fig. 4A, reconstitution of the ATPase in raft containing membranes strongly decreased the potency of curcumin. Fitting a sigmoid dose–response function to the data showed that curcumin inhibited Na,K-ATPase in PC-cholesterol

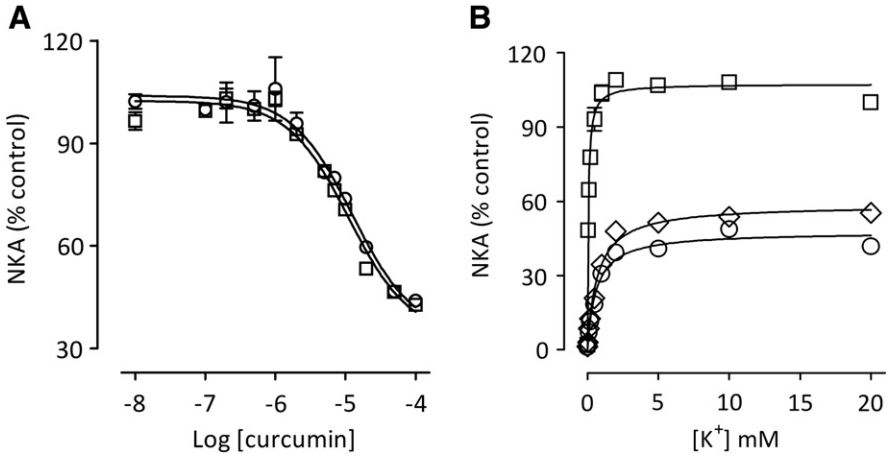

Fig. 2. Effect of curcumin on reconstituted pig kidney Na,K-ATPase. A. The activity of inside-out pumps (□) was measured at 24 °C following pre incubation in 1 mM ouabain and 1 mM inorganic phosphate to inhibit the non-oriented pumps. In this particular experiment the ouabain concentration in the ATPase reaction was kept constant at 1 mM ouabain to prevent reactivation of non-oriented pumps. The activity of right-side out pumps were measured by first inhibiting inside-out and non-oriented pumps by trypsin followed by incubation of the liposomes in ATPase assays containing 1 μg.μl^{-1} alamethicin to make the vesicles leaky to ions and ATP (O). The ATPase assay contained 20 mM histidine, pH 7.0, 130 mM Na^+, 20 mM K^+, 2 mM $MgCl_2$, 20 μM ATP, 0.2 μg nigericin (K^+ congener), and the indicated curcumin concentrations. Analysis of the data using a sigmoid dose–response curve gave an EC_{50} for curcumin inhibition of 10.16 ± 1.15 and 9.5 ± 1.21 for inside-out (□) and right-side out (O) pumps, respectively. B. The ATPase activity of inside-out Na,K-ATPase reconstituted into liposomes was measured at 24 °C in the absence of curcumin (□), in the presence of 25 μM curcumin added in the extra-liposomal medium (O), or in the presence of 25 μM curcumin loaded into liposomes (◇). The reaction mixture contained 100 mM Na^+, 20 mM K^+, 2 mM $MgCl_2$, 20 μM ATP, 0.2 μg nigericin, and the indicated curcumin concentrations. Analysis of the data using a hyperbolic function gave a $K_{0.5}$ (K^+) of 65.7 ± 4.6 μM (□), 637.5 ± 38.3 μM (O), and 703.0 ± 42.1 μM (◇), respectively. Note that the $K_{0.5}(K^+)$ in the absence of curcumin is not appreciably changed after reconstitution (see Ref. [16]).

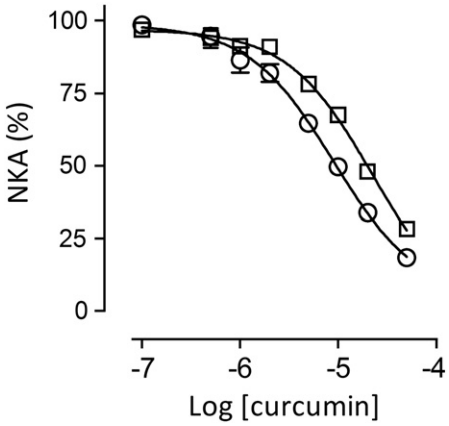

Fig. 3. Effect of co-reconstitution of PS on curcumin inhibition of Na,K-ATPase. The activity of PC–Chol reconstituted inside-out pumps was measured at 24 °C following pre incubation in 1 mM ouabain and 1 mM inorganic phosphate to inhibit the non-oriented pumps. In this particular experiment the ouabain concentration in the ATPase reaction was kept constant at 1 mM ouabain to prevent reactivation of non-oriented pumps (□). The same experiment was performed with the exception that 5 mol% PS was included (O) as described in Methods section. The ATPase reaction contained 20 mM histidine, pH 7.0, 130 mM Na^+, 20 mM K^+, 2 mM $MgCl_2$, 20 μM ATP (containing 10^4 cpm $AT^{32}P$-[ATP] per reaction), 0.2 μg nigericin, and 25 μM curcumin. Data were analyzed using dose–response function which gave an EC_{50} for inhibition of 15.7 ± 0.55 μM and 5.19 ± 0.26 μM for the PC–Chol mixture and the PC–Chol–PS mixture, respectively.

vesicles with an EC_{50} of 9.7 ± 0.34 μM. Following insertion of the ATPase in raft vesicles, the EC_{50} increased to 25.9 ± 0.52 μM.

We have also attempted to answer the question of whether native pig renal membranes contain Na,K-ATPase molecules resident in raft-like domains. Rafts are characterized by their association with low density fractions in continuous sucrose density gradients following equilibrium centrifugation [34]. Consequently, we have submitted native membrane-bound Na,K-ATPase onto a continuous sucrose density gradient followed by equilibrium centrifugation in a swing-out rotor. Indeed, we have demonstrated the separation of the membranes into several membrane fractions, including low density membrane (LDM)- and high density membrane (HDM) fractions. As shown in Fig. 4B, upper panel, Western blot experiments have revealed the occurrence of Na,K-ATPase in both the LDM and the HDM fractions. Subsequently, both membrane types were tested in terms of their susceptibility to curcumin. Curcumin inhibition curves, a representative of which is shown in Fig. 4B, lower panel, confirmed the odd drug potency found above with the reconstituted system: curcumin inhibited Na,K-ATPase in the light fraction with a much lower potency. Thus, EC_{50} values for curcumin inhibition were calculated to 8.1 ± 0.46 μM and 15.8 ± 0.96 μM for the heavy and light fractions, respectively. There is no evidence for the existence of different Na,K-ATPase isoforms in renal medulla membranes. Results in Fig. 4C demonstrate that Na,K-ATPase in both the light and heavy membrane fractions are inhibited similarly with the water soluble inhibitor ouabain.

The function of membrane proteins strongly depends on membrane fluidity [35], which is in turn a function of temperature. Hence, we also looked at the effect of temperature on curcumin potency. As demonstrated in Fig. 5, the potency of curcumin is significantly modulated by temperature: the EC_{50} values for curcumin inhibition of native pig kidney membranes were 19.58 ± 0.28, 11.69 ± 0.16, and 3.8 ± 0.11 at 37 °C, 30 °C, and 20 °C, respectively.

3.4. Curcumin possibly interacts with transmembrane domains of the pump and is dissociated by detergent

To the best of our knowledge, all known sodium pump inhibitors tightly bind to the ATPase even after complete solubilization of the membrane with detergent (however it is expected that a given detergent may only mildly affect the potency of an inhibitor, see Ref. [36], but full abrogation of the inhibitor effect following detergent solubilization is never expected). We have investigated the effect of

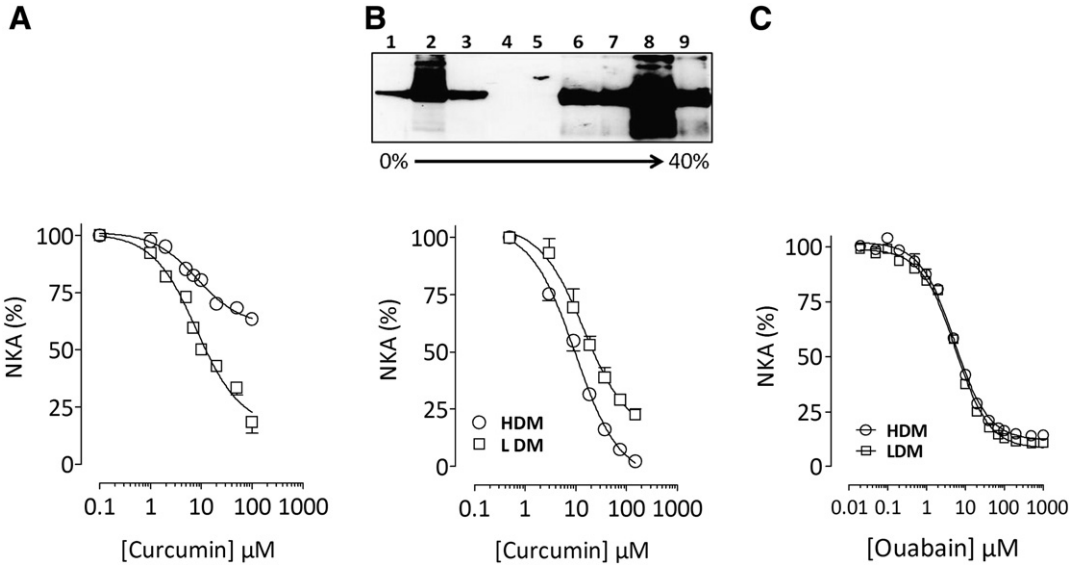

Fig. 4. Effect of cholesterol-sphingolipid on curcumin inhibition of Na,K-ATPase. A. The activity of inside-out pumps was measured at 24 °C following pre incubation in 1 mM ouabain and 1 mM inorganic phosphate to inhibit the non-oriented pumps. In this particular experiment the ouabain concentration in the ATPase reaction was kept constant at 1 mM ouabain to prevent reactivation of non-oriented pumps (□). The same experiment was performed with the exception that sphingomylein and cerebrosides were included together with PC–Chol (raft membranes, O) as described in Methods section. The ATPase reaction contained 20 mM histidine, pH 7.0, 130 mM Na^+, 20 mM K^+, 2 mM $MgCl_2$, 20 μM ATP (containing 10^4 cpm $AT^{32}P$-[ATP] per reaction), 0.2 μg nigericin, and 25 μM curcumin. Data were analyzed using dose–response function which gave an EC_{50} for inhibition of 9.7 ± 0.34 μM and 25.9 ± 0.52 μM for the PC–Chol mixture and the PC–Chol–SM–SB mixture, respectively. B. Upper panel. Western blot demonstrates the presence of raft-like domains in native pig renal medulla membranes (fractions 1–3). Heavy membranes are associated with fractions 6–9. Lower panel. Shown is the effect of curcumin on pig renal membranes fractions 2 (LDM, □) and 8 (HDM, O), as indicated. The ATPase mixture was identical to that in panel A, except that nigericin was omitted and that the reaction contained 3 mM $MgCl_2$ as well as 3 mM ATP. Data were analyzed using dose–response function which gave an EC_{50} for inhibition of 8.1 ± 0.46 μM and 15.8 ± 0.96 μM for fractions 2 and 8, respectively. C. Shown is the effect of ouabain on pig renal membranes fractions 2 (Low Density Membranes, □) and 8 (High Density Membranes, O), as indicated. The ATPase mixture was identical to that in panel B. The EC_{50} for ouabain inhibition was calculated by fitting a sigmoid dose–response function to the data, which was 5.2 ± 1.1 μM and 5.5 ± 0.9 μM for the LDM and HDM, respectively.

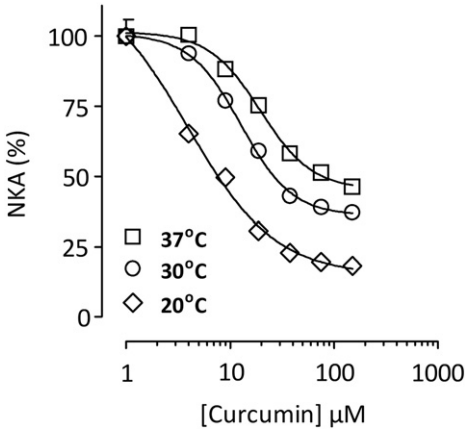

Fig. 5. The effect of temperature on curcumin inhibition. In these experiments purified membrane-bond Na,K-ATPase was used. The ATPase assays contained 20 mM histidine buffer, pH 7.0, 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 3 μg pig kidney Na,K-ATPase, and the indicated curcumin concentrations. The reactions were performed at 20 °C, 30 °C, or 37 °C, as indicated. Data were analyzed using sigmoid dose–response function, giving the EC_{50} values for curcumin inhibition of native pig kidney membranes of 19.58 ± 0.28 μM, 11.69 ± 0.16 μM, and 3.8 ± 0.11 μM at 37 °C, 30 °C, and 20 °C, respectively.

curcumin on detergent solubilized Na,K-ATPase and it was indeed unanticipated to establish that addition of detergent robustly decreases the inhibitory potency of curcumin. As demonstrated in Fig. 6, addition of increasing detergent concentrations to ATPase mixtures containing 50 μM curcumin resulted in concomitant increase in ouabain-dependent ATP hydrolysis, most likely because of the dissociation of bound curcumin. At 2 mM detergent the inhibitory effect of curcumin is fully abolished.

In an attempt to unravel the effect of curcumin on the conformation of the Na,K-ATPase α-subunit, proteolytic cleavage have been used. We have previously shown that curcumin destabilizes the conformation of the 19 kDa C-terminal domain of the α-subunit owing to decreased K^+ binding [16]. In the course of our experiments in this direction we could repeatedly demonstrate that

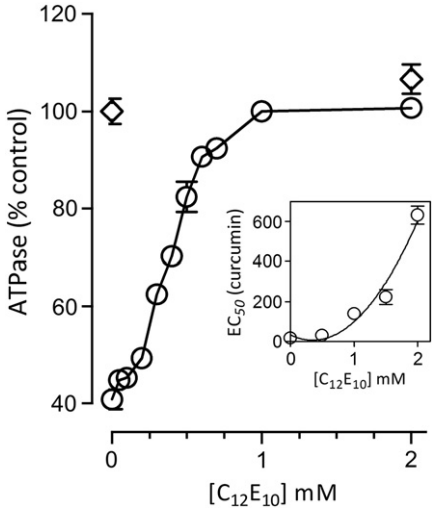

Fig. 6. Effect of $C_{12}E_{10}$ on curcumin inhibition of Na,K-ATPase. 50 μM curcumin was used to inhibit pig kidney Na,K-ATPase and aliquots of the inhibited enzyme was transferred to ATPase assays containing 50 μM curcumin, 20 mM histidine, pH 7.0, 130 mM Na^+, 20 mM K^+, 3 mM $MgCl_2$, 3 mM ATP, and the indicated $C_{12}E_{10}$ concentrations. Diamonds indicate the ATPase activity in the absence of curcumin and either in the absence or in the presence of 2 mM $C_{12}E_{10}$. Inset. Curcumin inhibition curves were measured in the presence of different $C_{12}E_{10}$ concentrations and the EC_{50} for curcumin was blotted against the corresponding $C_{12}E_{10}$ concentration. At $C_{12}E_{10}$ concentrations > 2 mM is was not possible to precisely calculate EC_{50}. Data are expressed as percentage of control, measured in the absence of curcumin.

incubation of Na,K-ATPase lacking its cytoplasmic domains with curcumin induces cross-linking of several transmembrane domains of the ATPase (Fig. 7). For comparison, cross-linking with the water soluble BS^3 is shown, which is believed to take place between stalk segments M5 and M7 (unpublished data). The cross-linking fragments induced in the presence of curcumin are stable in SDS gels (although the cross-linking efficacy was only 20%, as indicated from intensity measurement of the 19 kDa and the higher molecular mass cross-linked fragments), indicating the tight association facilitated by this polyphenol (see Discussion section).

4. Discussion

4.1. Curcumin does not seem to bind at the extracellular side of the Na,K-ATPase

Ouabain has been shown to inhibit the ATPase by binding to a cavity between transmembrane helices M1, M2, M4, M5, and M6 near the K^+-binding sites. In this position the drug is well inserted between transmembrane domains of the catalytic subunit [37] with no apparent interaction with the lipid bilayer. We have found that treatment with curcumin did not modify the apparent affinity for ouabain (Fig. 1B). As ouabain binds strongly to (i.e., inhibits) the detergent solubilized ATPase whereas curcumin does not (see later discussion), it is likely that the interaction site of curcumin is located at the protein-lipid interface rather than at the extracellular side or between transmembrane helices. This is in consistence with our paired inhibitor studies showing that ouabain and curcumin bind to the ATPase at different sites. Furthermore, the inhibition by oligomycin is strongly diminished by curcumin treatment, suggesting that both inhibitors share a common binding site on the protein. Indeed, previous studies have indicated that oligomycin binds and dissociates from within the membrane phase [38] consistent with its interaction with transmembrane domains of the pump. This is in line with our results in which curcumin is suggested to interact with transmembrane domain(s) of the α-subunit and explains the observation that addition of curcumin strongly decreases the inhibition produced by oligomycin (Fig. 1). It is noteworthy to mention that both oligomycin and curcumin produce similar modifications to the kinetic properties of the Na,K-ATPase [16], with

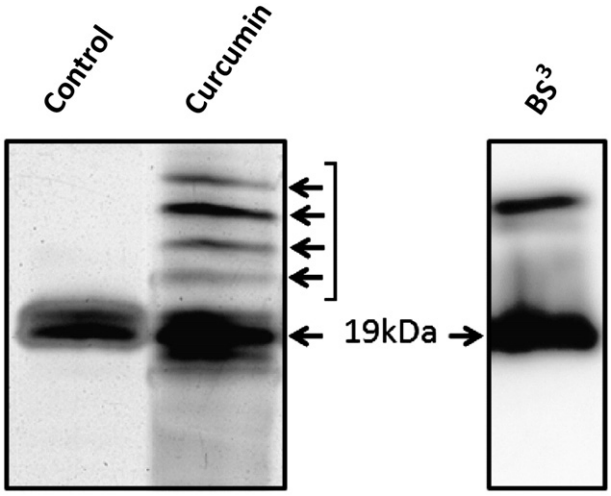

Fig. 7. Cross-linking of transmembrane domains of the pump. Incubation with curcumin or BS^3, SDS-PAGE, and immunoblotting was performed as described in Methods section. In the absence of cross-linking agents the C-terminal fragment is stained with α-antibody (left panel, left lane) migrates at about 19 kDa on the PVDF membrane. Cross links appear following treatment with curcumin (right lane) or BS^3 (right panel). All stained fragments stained with the α-antibody involve cross-linking of the 19 kDa fragment to itself or to other transmembrane hairpins.

curcumin being less effective as a stabilizer of the E_1P conformation (and hence inhibitor of the reaction cycle) of the Na,K-ATPase.

4.2. Membrane lipids modulate curcumin potency

The increased affinity of inhibition by curcumin of reconstituted Na,K-ATPase suggests that interaction of the drug with the ATPase is facilitated by the PC–Chol membranes more than the native renal medulla embranes. Thus, a change in the membrane structure (and hence, physical properties) is important in determining the potency of the drug (see later discussion). In addition, a negatively charged membrane phospholipid increases the effectiveness of the drug (Fig. 3). Since PS is believed not to modify membrane fluidity, the enhancement of curcumin inhibition mediated by this phospholipid is most likely a consequence of an increased membrane surface charge [39] which may facilitate curcumin interaction with the ATPase. PS has previously been found to modulate the interaction of the Na,K-ATPase with one of its regulatory proteins [40]. Insertion of curcumin to a model membrane has been shown to decrease membrane fluidity and thickness [41,42]. These significant effects on the membrane's physical properties might be indicative of indirect effect on the Na,K-ATPase through a membrane-mediated mechanism.

Components known to sequester in liquid-ordered phases decrease the potency of curcumin inhibition of Na,K-ATPase (Fig. 4). Rafts are highly ordered membrane domains that contain sphingolipid and cholesterol in tightly packed states. In these domains lipid–lipid interactions are much more stable relative to lipid–detergent interactions and hence the membranes are insoluble in Triton X-100 under conditions of low temperatures [43]. It is therefore expected that curcumin insertion into these domains is rate limiting, so as its interaction with proteins in these domains (Fig. 4). Indeed, we have previously determined that the EC_{50} for curcumin inhibition of shark Na,K-ATPase is 15.8 ± 1.12 μM against 5.24 ± 1.0 μM for the pig kidney enzyme [16]. Shark enzyme is known to contain much more cholesterol than the pig kidney membranes [44], demonstrating that the potency of the drug is a function of the membrane structure.

The inhibition by curcumin was found to increase with decreasing temperature (Fig. 5). The fluidity of biological membranes is described by the rate of movement of lipid and protein molecules within the membranes. Increased lipid packing and decrease in membrane fluidity is thus consistent with enhanced interaction of the drug with the pump and can be explained in terms of a decreased rate of dissociation of the drug from its binding site on the ATPase.

4.3. Interaction of curcumin possibly occurs at the protein-lipid interface

As the inhibitory effect of curcumin is fully abolished following solubilisation with detergent, it is likely that curcumin interaction with the ATPase is supported by non-annulus lipids at the lipid-protein interface. Another possibility is that curcumin is simply displaced from its binding site up on detergent treatment. This makes curcumin the first known sodium pump inhibitor that absolutely requires the membrane for interaction with the pump. We demonstrated also that curcumin addition to isolated transmembrane domains of the Na,K-ATPase α-subunit resulted in several cross links on SDS gels (Fig. 7). The cross links seems to involve interaction between the C-terminal 19 kDa fragment and other transmembrane hairpins M1M2, M3M4, and M5M6 [45]. The low cross-linking efficiency observed here (about 20%) may indicate that cross-linking does not correlate with inhibition of ATPase activity. However, we cannot rule out the possibility that inhibition of the pump is related to cross-linking of the pump, since a partial cross-linking efficacy could be an intrinsic property of the cross-linking reaction *in vitro* due to conformational fluctuations of the pump [46]. More reliable biophysical experiments would be necessary to study the interaction of curcumin with the ATPase in the presence of membranes. While this report was in preparation, Bernard et al. published results on the cross-linking of peptides of the cystic fibrosis transmembrane conductance regulator [47], indicating that curcumin may act by a general mechanism on several integral membrane proteins. Further studies are necessary to uncover the nature and possible importance of the cross-linking induced by curcumin.

5. Conclusion

We present evidence for the lipid dependent interaction of curcumin with the Na,K-ATPase. The lipid dependence possibly plays a substantial role in the determination of the potency of this drug. Since solubilisation with low concentrations of $C_{12}E_{10}$ produces active Na,K-ATPase and concurrently abolishes curcumin inhibition, we conclude that curcumin may interact with the Na,K-ATPase at the protein-lipid interface, with non-annulus lipid being necessary for this interaction. Hence, hydrophobic drugs may differentially affect targets inserted in different lipid domains.

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