



How Might 12 (R) HETE Cause the Inhibition of Na,K-ATPase?

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Purpose: 12 (R) hydroxy 5,8,10,14-eicosatetraenoic acid [12 (R) HETE] is a potent inhibitor of Na,K-ATPase. This study was an attempt to determine how the eicosanoid might inhibit the enzyme by using molecular modeling.

Methods: Models were generated using the program HyperChem 2.0 for Windows. Models of 12 (R) HETE, 12 (S) HETE (the "S" isomer of 12 (R) HETE), and 8 (R) hydroxy-hexadecatrienoic acid [8 (R) HHDTrE, a catabolic isomer of 12 (R) HETE] were formed and docked with phosphatidyl choline and the H3-H4 peptide of the alpha-subunit of Na,K-ATPase. In addition, models of 12 (R) HETE, and related compounds, were formed and complexed with calcium, and then docked with phosphatidyl choline. The energies of stabilization were calculated for each optimal docking.

Results: Optimal steric fitting and calculated energies of stabilization indicated that 12 (R) HETE and 8 (R) HHDTrE had the best fits when bound to the fatty acid portions of phosphatidyl choline. However, when Ca-HETE complexes were modeled, it was found that they formed even more stable complexes when bound to phosphatidyl choline. Calculated energies of 12 (S) HETE, whether complexed to calcium or not, were less favorable than the other HETE compounds.

Conclusions: The results of the study indicate that plasma membrane lipids rather than Na,K-ATPase itself are more likely to be bound by 12 (R) HETE and its related compounds. Moreover, it was found that the calcium complexes of 12 (R) HETE and 8 (R) HHDTrE are even more likely to dock with plasma membrane lipids. This suggests that such complexes may be able to transport calcium into the cell and make it available for the inhibition of Na,K-ATPase at the enzyme's sodium binding site.

The eicosanoid compound 12 (R) hydroxy 5,8,10,14-eicosatetraenoic acid [12 (R) HETE] has been shown to be a potent inhibitor of the plasma membrane enzyme: sodium/potassium-stimulated adenosine triphosphatase [Na,K-ATPase; E.C. 3.6.1.37] (9,21,22,25,30). This eicosanoid can be formed in the corneal epithelium upon stimulation with vasopressin (6) and after short-term contact lens wear (5). In the anterior segment, the presence of 12 (R) HETE has been associated with corneal swelling (5) and the lowering of intraocular pressure (20). Both of these physiological changes are related to a decrease in Na,K-ATPase activity (13,34).

In an effort to determine how 12 (R) HETE might inhibit Na,K-ATPase, molecular models of 12 (R) HETE, its closely related eicosanoids, as well as the membrane phospholipid phosphatidyl choline, and the H3-H4 sequence (with its exterior loop) of the alpha-subunit of Na,K-ATPase were modeled and selectively docked to determine optimal binding energies and conformational compatibilities of the molecules. Besides 12 (R) HETE, the congeners 12 (S) HETE, the "S" stereoisomer that does not inhibit Na,K-ATPase (9), and 8 (R) hydroxy-hexadecatrienoic acid [8 (R) HHDTrE], a metabolite that also inhibits Na,K-ATPase, were chosen (35). Phosphatidyl choline was selected to represent a plasma membrane molecule since it is one of the chief glycerophospholipids found in plasma membranes (19). The H3-H4 sequence of the alpha-

subunit of Na,K-ATPase was chosen as an area of the enzyme to model since it represents one of the most likely extramembrane regions of the enzyme to be bound by an inhibitor. This has been shown by studies with ouabain (2,15). It was hypothesized that the results would indicate that either the plasma membrane lipid or the enzyme itself would show more favorable binding to 12 (R) HETE. The study, however, revealed a more interesting finding that suggests the actual mechanism of inhibition.

METHODS

Models—Models were generated of 12 (R) HETE, 12 (S) HETE, 8 (R) HHDTrE, phosphatidyl choline (containing one 18:0 fatty acid, n-octanoate, on C-1 of the glycerol moiety and one 18:2 Δ 9,12 fatty acid, alpha-linoleate, on C-2 of the glycerol moiety), and the H3 helix-intervening exterior loop H4 helix sequence (17) of the alpha-subunit of Na,K-ATPase. These models were used for the first part of the investigation.

In the second part of the study, models of Ca [12 (R) HETE]₂, Ca [12 (S) HETE]₂, and Ca [8 (R) HHDTrE]₂ were additionally formed. These were essentially dimers of each HETE molecule bridged by calcium. The models used were based on a study by DeLeers et al (7).

The program used for model construction was HyperChem (version 2.0 for Windows, Autodesk, Inc., Sausalito, CA). After the initial geometrical formation of the 3D models for the HETE molecules (12 (R) HETE, 12 (S) HETE, and 8 (R) HHDTrE) and phosphatidyl choline, the models were refined

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by geometric optimization selecting the geometric forms with minimal potential surface energy using a steepest descent algorithm (11). Models of the H3-outer membrane loop-H4 sequence of the alpha-subunit of Na,K ATPase were formed from amino acid databases, inherent in the program, with standard alpha-helical and looping bond angles specified for each joining residue (23,27).

Docking— Docking or overlay of the models was performed in the combinations shown in Table 1. The optimal amounts of steric fit, ionic attraction, hydrogen bonding, hydrophobic interaction, and resonance synchronization were attempted with each docking in order to obtain the highest energies of stabilization with the best steric fit.

Calculated energies of stabilization— Where optimal steric fit and bonding occurred with molecular overlays, the energies of stabilization were calculated as the sum of the kinds and numbers of ionic and weak bonds involved in the overlay (10,12,32). Published average values of stabilization energies (kJ/mol) were used (18). If a bond occurred within an overlay or, in the case of ionic bonds, very close to the overlay, its energy of stabilization was included in the summation.

RESULTS

Models of the HETE molecules, phosphatidyl choline, and the H3-H4 sequence of Na,K-ATPase— Models of the HETE molecules are shown in Figure 1. The structural differences between 12 (R) HETE and 12 (S) HETE are the relative 120 degrees angled positions of the hydroxy group. By contrast, 8 (R) HHDTrE has a truncated chain (by four carbons) adjacent to its carboxylate group. These characteristically bent eicosanoid structures conform to those shown by Deleers et al

(7). Phosphatidylcholine is shown in Figure 2. Three characteristic regions of the molecule may be seen. The hydrophilic region (also known as the polar head group) is seen at the top and contains ester bonds, charged phosphate oxygen, and a charged ammonium group. This region is located at the cell plasma membrane surface. The straight chain hydrocarbon region (containing the saturated fatty acid) is seen as the lower left leg of the figure. The twisted hydrocarbon region (containing the unsaturated fatty acid) is shown as the right leg of the figure. Both legs of the phospholipid bury themselves deep in the plasma membrane.

Complete amino acid sequences of Na,K-ATPase have been thoroughly documented and entered in the GenBank/EMBL library. The H3-extracellular loop-H4 sequence of the alpha-subunit of Na,K-ATPase is illustrated in Figure 3. The H3 sequence prior to the loop [left arrow] is an alpha-helix contained within the cell's plasma membrane as is the H4 sequence following the loop [right arrow] (3). The highly conserved loop sequence itself (EYTWLEA) contains charged amino acid residues capable of binding to cations such as K⁺. Indeed, this is a predominant region on the enzyme that is considered to be a binding site for the inward transport of K⁺ as well as the binding of the blocking (inhibiting) substance ouabain (24). It is, therefore, an important candidate site for Na,K-ATPase inhibition.

Docking of HETE molecules with phosphatidylcholine versus the H3-H4 loop of Na,K-ATPase— When each of the HETE models was docked with phosphatidylcholine, two manners of docking were possible: (a) the HETE could be associated with each upper leg of the fatty acid (Figure 4a); and (b) the HETE could be associated with a portion of the polar head group (Figure 4b). Association of the HETE



Figure 1a. Model of 12 (R) HETE. In this isomer, the hydroxy group is indicated by an arrow and is located behind its attached carbon (C-12). A color legend is shown below.

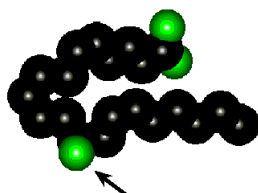


Figure 1b. Model of 12 (S) HETE. This isomer has its hydroxy group (arrow) located to the front of its attached carbon (C-12) as viewed. A color legend is shown below.

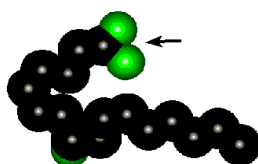


Figure 1c. Model of 8 (R) HHDTrE. This degradation product of 12 (R) HETE is shortened by 3 carbons (arrow) from the carboxylate end of the molecule. A color legend is shown below.

Black = Carbon, Blue = Nitrogen, Green = Oxygen, Red = Phosphorus, Yellow = Sulfur, Lavender = Calcium

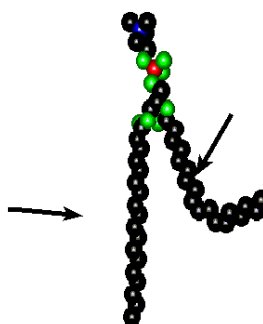


Figure 2. Model of phosphatidylcholine. A model of phosphatidylcholine showing one saturated (left arrow) and one monounsaturated (right arrow) fatty acid ester. The polar head group is at the top and contains a phosphorus atom. A color legend is shown to the left below.

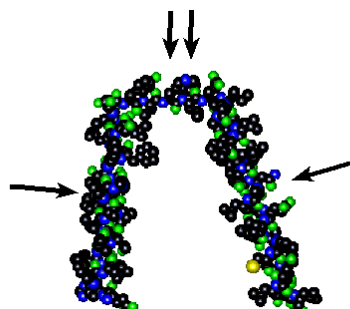


Figure 3. Model of helices number 3 and 4 with their attached outer loop from the alpha-subunit of Na,K-ATPase. The helices (single arrows) transverse the cell plasma membrane while the outer loop (double arrow) possesses a binding area for K⁺ ions and the inhibitor ouabain. A color legend is shown to the left.

molecules with the upper legs of the fatty acids of phosphatidylcholine (first manner) produced the most compatible steric and weak bond fits. In the second manner of docking, in which fits were attempted with the polar head group, a non-polar portion of the HETE always extended into an aqueous environment (Figure 4b, right hand arrow). All the fittings were similar for each HETE except that for 8 (R) HHDTrE when it was fitted onto the polar head group (not shown). In that case, a non-polar portion of the molecule still extended into an aqueous environment, but the entire molecule had to be rotated 180 degrees.

As each of the HETE models was docked with the H3-H4 loop of Na,K ATPase, it was possible to locate the HETE in a hydrophobic region of a helix just beneath one of the charged amino acids in the loop. Only the docking for 12 (R) HETE with the H3-H4 loop is shown (Figure 5). However, each docking configuration had to be different in order to obtain an optimal fit. The 8 (R) HHDTrE molecule gave a fit which was closest to a negatively charged glutamate residue in the outer loop. None of the HETE molecules could be reasonably fit to the actual loop residues themselves without having some portion of the HETE molecule extend into the aqueous environment which would have been energetically unfavorable.

Calculation of energies of stabilization after docking—The energies of stabilization for the docking processes were obtained based on the summation and kinds of bonds produced. In the case of docking to the phosphatidyl-choline head group region, correction was made for the hydrocarbons extending into the aqueous environment by subtracting the appropriate destabilizing energies of 6 kJ per carbon for the carbons extending into that region (1). These energies are shown in Table 2.

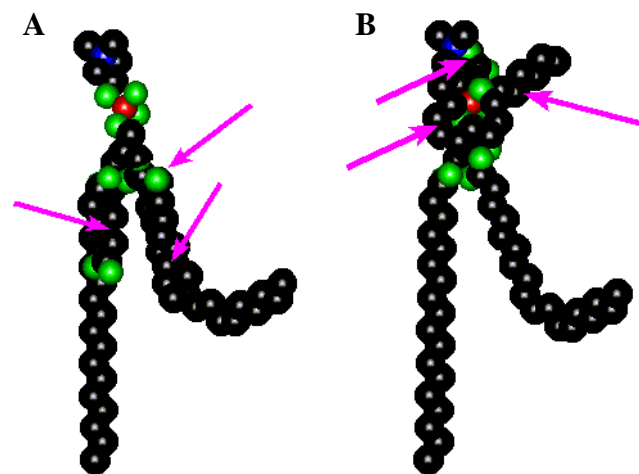


Figure 4. Models of 12 (R) HETE docked with phosphatidylcholine. Model of two possible configurations for docking 12 (R) HETE with phosphatidylcholine. In Figure 4a, the 12 (R) HETE is docked at the fatty acid ester region of phosphatidylcholine (arrows). In Figure 4b, the 12 (R) HETE is docked at the polar head group region of phosphatidylcholine (arrows). The right hand arrow indicates the non-polar portion of the 12 (R) HETE molecule that extends into the aqueous environment. An isolated phosphatidylcholine molecule is shown in Figure 2 for comparison. Colors are as shown in Figure 1.

The table indicates that binding of 12 (R) HETE and 8 (R) HHDTrE to phosphatidyl choline in the upper fatty acid regions are the most favored by ~1.6 to 1 over binding to the enzyme and by more than eight fold over binding to the head group region. In the most favored site, 12 (R) HETE and 8 (R) HHDTrE are attracted to the binding site by a factor of ~2 to 1 over that of 12 (S) HETE.

Calcium formation of HETE dimers (models)—Deleers et al (7) have called attention to evidence that metabolites of cyclooxygenase and lipoxygenase are able to act as calcium ionophores in cell membranes. They have formed models of several eicosanoids complexed with Ca having an eicosanoid to Ca ratio of two-to-one. These models had a similar ligand to Ca ratio to that of the well known ionophore A23187 (31). We made similar calcium bound models of the HETE molecules used in this study. They are shown in Figure 6. All of the models shown indicate that the calcium moiety is borne

Table 1. Docking Combinations Studied.

Combination	Molecule 1	Molecule 2
membrane - HETE	phosphatidyl choline	12 (R) HETE
membrane - HETE	phosphatidyl choline	12 (S) HETE
membrane - HETE	phosphatidyl choline	8 (R) HHDTrE
enzyme - HETE	H3-loop-H4 sequence	12 (R) HETE
enzyme - HETE	H3-loop-H4 sequence	12 (S) HETE
enzyme - HETE	H3-loop-H4 sequence	8 (R) HHDTrE
membrane - Ca [HETE]2	phosphatidyl choline	12 (R) HETE
membrane - Ca [HETE]2	phosphatidyl choline	12 (S) HETE
membrane - Ca [HETE]2	phosphatidyl choline	8 (R) HHDTrE

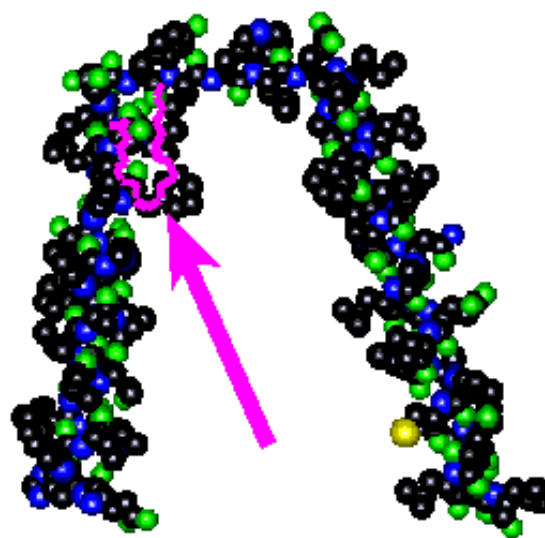


Figure 5. Model of 12 (R) HETE docked with the alpha-subunit of Na,K-ATPase. The arrow shows where the 12 (R) HETE is docked with the H3-H4 region of the alpha-subunit of Na,K-ATPase. A model of an isolated molecule of the H3-H4 region of the alpha-subunit of Na,K-ATPase is shown in Figure 3 for comparison. A color legend is shown in Figure 1.

at the center of two twisted HETE arms. However, the Ca [12 (S) HETE]₂ dimer has arms that are not axially aligned as is that of Ca [12 (R) HETE]₂ while the arms of Ca [8 (R) HHDTrE]₂ are similar to that of Ca [12 (R) HETE]₂.

Docking of calcium HETE dimers to phosphatidylcholine—We attempted to dock the calcium HETE dimers to one molecule of phosphatidylcholine based on the docking that was accomplished with (monomer) HETE molecules previously. The limitations of the program used did not allow docking the dimers to two molecules of phosphatidylcholine simultaneously. However, due to the symmetry of some of the dimers, simultaneous docking could be presumed. The results for Ca [12 (R) HETE]₂ and phosphatidylcholine are shown in Figure 7. Since the arms of [12 (S) HETE]₂ were not axially aligned, we considered that hydrophobic binding of this complex with phosphatidylcholine was limited to a single arm. This was important in the calculation of stabilization energies for each complex. No attempt was made to dock these dimers to the H3-loop-H4 sequence of the alpha-subunit of Na,K-ATPase since the previous stabilization energies found with the mono HETE compounds were not as favorable as those Table 2. Calculated stabilization energies for the binding of HETEs to Na,K-ATPase and phosphatidyl choline.

HETE docked	to H3-H4 sequence of Na,K-ATPase	to phosphatidyl choline fatty acid region	to phosphatidyl choline head group region*
12 (R) HETE	31 kJ/mol	51 kJ/mol	6 kJ/mol
12 (S) HETE	27 kJ/mol	24 kJ/mol	6 kJ/mol
8 (R)HHDTrE	29 kJ/mol	45 kJ/mol	-4 kJ/mol

*corrected for destabilization energies of exposure to aqueous environment. See text.

Table 3. Calculated stabilization energies for the binding of calcium-HETE dimers and HETEs (monomers) to phosphatidyl choline (PC).

HETE docked to PC	as Ca [HETE] ₂	as HETE*
12 (R) HETE	88 kJ/mol	51 kJ/mol
12 (S) HETE	24 kJ/mol	24 kJ/mol
8 (R) HHDTrE	72 kJ/mol	45 kJ/mol

*data in the right column taken from Table 2

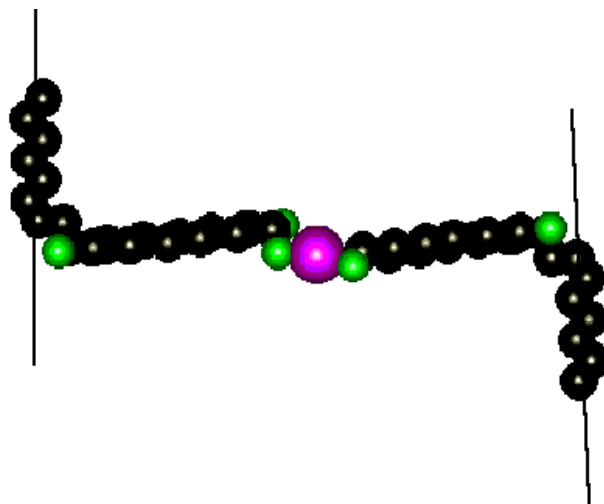


Figure 6a. Model of Ca [12 (R) HETE]₂. Each arm of 12 (R) HETE is located to the right and left and is bound to the calcium. Note that the configuration of each 12 (R) HETE is substantially altered upon binding to the calcium ion and represents a more open structure. Each hydrocarbon segment, beyond its hydroxy group, is able to interact (form a hydrophobic bond) with the hydrocarbon chain of a plasma membrane fatty acid ester. In this way two hydrophobic bondings may form simultaneously along each chain. Note that the axis lines for those hydrocarbon segment are nearly parallel. A color legend is shown in Figure 1.

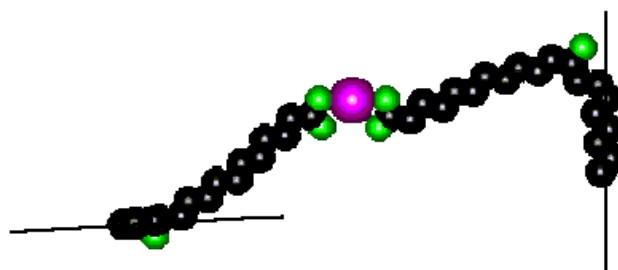


Figure 6b. Model of Ca [12 (S) HETE]₂. The twist of the arms of the 12 (S) HETE isomer, although also more open, do not allow the furthest hydrocarbon segments to be lined up in parallel. This feature of the molecule may make it more difficult for two fatty acid esters to form hydrophobic bonding groups at the same time. A color legend is shown in Figure 1.

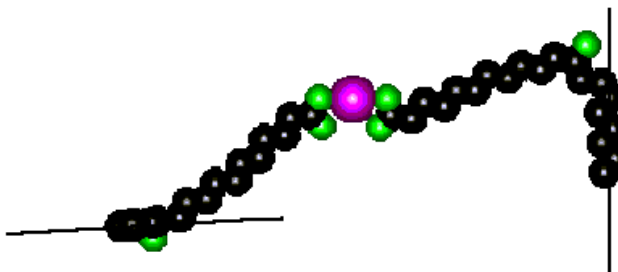


Figure 6c. Model of Ca [8 (R) HHDTrE]₂. This structure is similar to Ca [12 (R) HETE]₂. Note that the distal hydrocarbon chains from each arm of the 8 (R) HHDTrE form nearly parallel axes allowing for the simultaneous formation of hydrophobic bonds between each hydrocarbon and a phospholipid fatty acid esters. A color legend is shown in Figure 1.

bound to phosphatidylcholine.

Calculation and comparison of energies of stabilization of calcium HETE dimers— Calculations were made on the basis of hydrophobic interactions of each axial arm with a straight chain fatty acid of phosphatidylcholine. If axial alignment was possible, then the results were doubled. The results are shown in Table 3 where they are compared with those for the HETE (monomer) compounds.

The data, therefore, suggests that Ca [12 (R) HETE]₂ and Ca [8 (R) HHDTrE]₂ are the most stable forms of the HETE molecules examined and the most likely to be involved in binding to the plasma membranes of the cell.

DISCUSSION

The first part of the study suggests that plasma membranes, rather than Na,K-ATPase, are more favorable sites for binding of HETE molecules to the cell. Furthermore, the data is indicative that the straight chain fatty acid groups of the phospholipids in the membranes are best suited for association with HETE molecules. Three kinds of HETE molecules were examined and, of those, 12 (R) HETE was energetically the most stable when bound to the straight chain fatty acid of the phospholipid. However, 8 (R) HHDTrE, a metabolite of 12

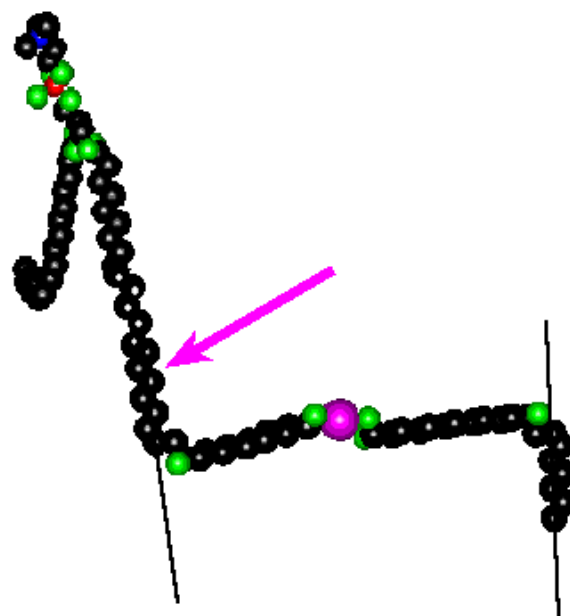


Figure 7. Model of Ca [12 (R) HETE]₂ docked with phosphatidylcholine. Model of Ca [12 (R) HETE]₂ with one hydrocarbon arm docked with a fatty acid ester of phosphatidylcholine (arrow). The parallel axes of the hydrocarbon distal arms of Ca [12 (R) HETE]₂ are indicated by lines that demonstrate how two phospholipids could make hydrophobic contact (bonding) with the calcium dimer simultaneously. A color legend is shown in Figure 1.

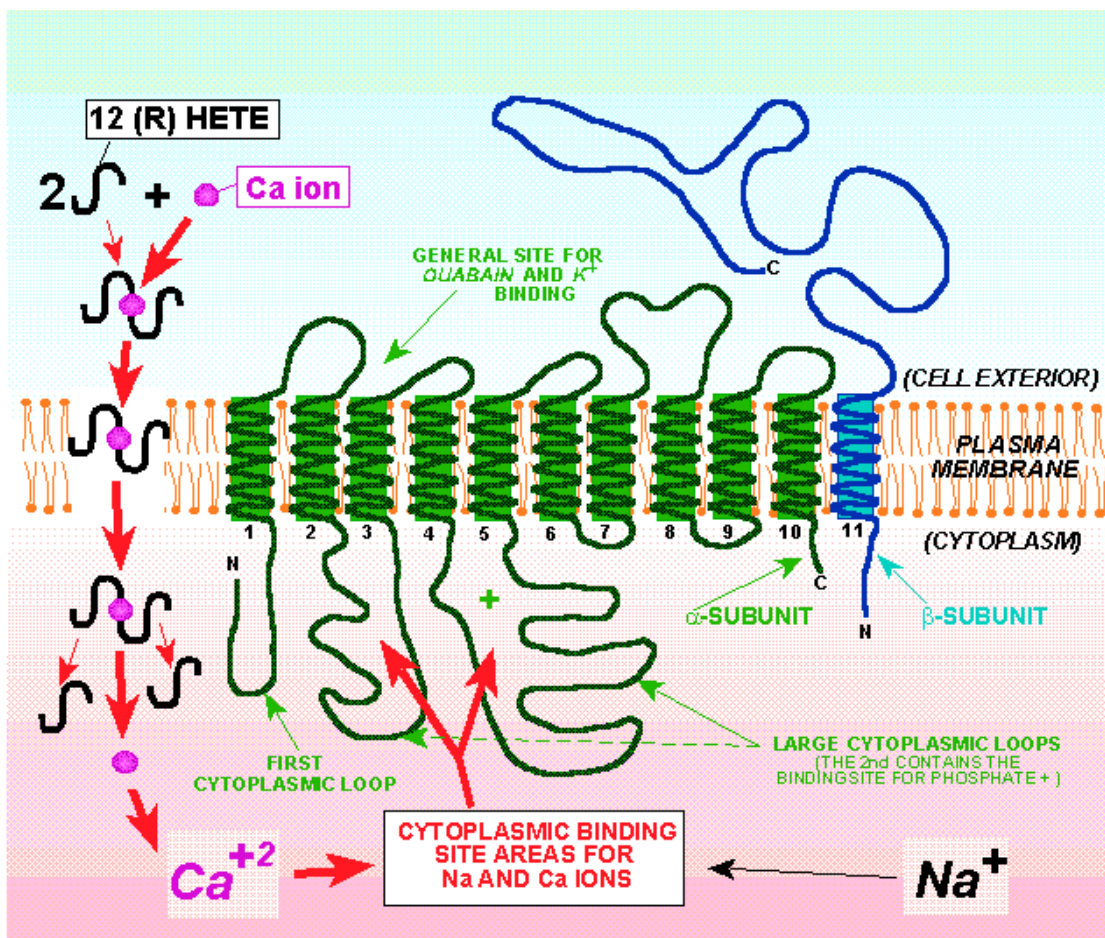


Figure 8. Proposed mechanism of Na,K-ATPase inhibition by 12 (R) HETE. Twelve (R) HETE forms a dimer with Ca²⁺ at the plasma membrane outer surface and transports calcium intracellularly. Upon dissociation of the dimer, Ca²⁺ competes with Na⁺ for the sodium binding site (or its adjacent area) preventing Na⁺ binding and, thereby, inhibits the enzyme.

(R) HETE, was almost as stable. Previously it was found by Woods et al (35) that 12 (R) HETE and 8 (R) HHDTrE are comparable in inducing corneal swelling and, therefore, in causing the inhibition of Na,K-ATPase.

Is it possible that 12 (R) HETE and 8 (R) HHDTrE, in their original molecular forms, cause the inhibition of Na,K-ATPase by binding to the plasma membrane phospholipids and affecting a membrane disruption sufficient to inhibit the enzyme? This seems unlikely given that Jorgensen (16) has stated that there are more than enough phospholipids in cell plasma membranes to sustain normal Na,K-ATPase activity. Since HETE molecules are only present at a maximal stimulated concentration of 525 ng/mg protein in the corneal epithelium (6), the binding of a few of the membrane fatty acids by HETE molecules should not significantly inhibit enzyme activity. These HETE molecules are equivalent to less than 2% of the available membrane fatty acids. (This is based on 0.35%-5.7% of homogenate proteins consisting of plasma membrane proteins in the corneal endothelium and a protein:lipid plasma membrane ratio of 1:1 (4,26,36).)

Na,K-ATPase activity is profoundly affected, however, by the presence of other cations. On the extracellular surface: K^+ , Rb^+ , NH_4^+ , Cs^+ , and Tl^+ can all bind to the surface. On the intracellular surface, Ca^{+2} ions compete with the Na^+ ion binding site of Na,K-ATPase when there is an influx of Ca^{+2} from a transcellular transporting vehicle such as the ionophore A23187 (14). In the case of Ca^{+2} ion influx, enzyme activity is inhibited. A normal, basal intracellular Ca^{+2} concentration for many cells exists in the range of $0.2-5.4 \times 10^{-7}$ M (28). Na,K-ATPase is inhibited ~10% when the cytoplasmic Ca^{+2} is raised to 100×10^{-7} M and is inhibited more than 20% when cytoplasmic Ca^{+2} reaches 1000×10^{-7} M (33). Hootman and Ernst (14) suggested that ionophores such as A23187 may easily produce cytoplasmic levels of ionic calcium that can cause such inhibition. Accordingly, Ca^{+2} may be transported through plasma membranes in ionophores when that Ca^{+2} is bound to the negatively charged carboxylate groups of two ionophore molecules (8). The properties of such complexes have been described at both the lipid/ water interface (for uptake and release of calcium ions) and across the membrane itself (for the actual transport of calcium ions).

Calcium ion complexes are also known to form with arachidonic acid derivatives. Deleers et al (7) have described the molecular conformations of such complexes for prostaglandin and leucotriene compounds. Based on the studies of Deleers et al (7), we formed models of Ca^{+2} complexed to 12 (R) HETE; 12 (S) HETE; and 8 (R) HHDTrE. These were shown in Figure 6. Intuitively, if such Ca^{+2} ion complexes transport calcium inward, then they should all mediate the inhibition of Na,K-ATPase. Experimental evidence (9,13,34,35), however, has shown that although both 12 (R) HETE and 8 (R) HHDTrE both inhibit Na,K-ATPase, 12 (S) HETE does not do so. In addition, calculations of stabilization energies in this report indicate that the "R" compounds have three fold higher stabilization energies than the "S" compound when bound to calcium.

How can this be interpreted? The answer is suggested by

attempting to line up the axes of symmetry of these compounds with those of the inner fatty acid residues of the plasma membranes. For example, in attempting such an operation, a comparison of the models of Ca [12 (R) HETE]₂ and Ca [12 (S) HETE]₂ with phosphatidylcholine fatty acids (Figure 6a and 6b), indicates that only one arm of the 12 (S) HETE dimer can bind to a fatty acid hydrocarbon at one time while two arms of the 12 (R) HETE dimer can bind to two fatty acid hydrocarbons simultaneously. In the case of the 12 (R) HETE dimer, this would greatly facilitate stabilization and transport of Ca^{+2} ions. A similar case can be made for Ca [8 (R) HHDTrE]₂ (Figure 6c).

Based on the data reported in this study, we propose that 12 (R) HETE and 8 (R) HHDTrE cause the inhibition of Na,K-ATPase by acting as ionophores for Ca^{+2} ions. As such, it is Ca^{+2} that would serve as the actual inhibitor of Na,K-ATPase while the HETE compounds are transport mediators. This is diagrammed in Figure 8. Furthermore, it would seem that 12 (S) HETE could not be an effective ionophore for calcium since the hydrocarbon extensions from its calcium complex cannot form axes that line up well enough with plasma membrane fatty acids to efficiently transport calcium. The mediation of Ca^{+2} (due 12 (R) HETE) on other cellular functions is a further consideration, but it is outside the scope of this report. Nonetheless, it can be stated that protein mediated responses to the presence of increased cytoplasmic Ca^{+2} often require a second substance for activity such that Ca^{+2} may be a necessary, but insufficient ion for a specific physiological response (29). This does not seem to be the case for the inhibition of Na,K-ATPase.

Studies are currently underway to determine the normal cytoplasmic concentrations of Ca^{+2} in corneal endothelial cells and to assay Ca^{+2} cytoplasmic levels in the presence of 12 (R) HETE along with Ca^{+2} levels in the presence of its closely related eicosanoids.

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