Regulation of Na\(^+\),K\(^+\)-ATPase by the Endogenous Sodium Transport Inhibitor from Hypothalamus

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SUMMARY We characterized the effect of a small, nonpeptidic molecule isolated from bovine hypothalamus on mammalian Na\(^+\),K\(^+\)-adenosine triphosphatase (ATPase). This hypothalamic factor has been shown to inhibit ATPase activity of purified dog kidney enzyme reversibly with high affinity. This report reviews the mechanism of inhibition. Hypothalamic factor inhibits Na\(^+\),K\(^+\)-ATPase only from the extracellular surface. It prevents the phosphorylation from magnesium and inorganic phosphate of the active site aspartate residue of Na\(^+\),K\(^+\)-ATPase and stabilizes the enzyme in an E\(_2\) conformation, preventing a sodium-induced shift from E\(_2\) to E\(_1\). Binding and dissociation reactions of hypothalamic factor in cultured renal tubular epithelial cells show a time frame different from that in isolated membranes and consistent with physiological relevance. A possible mechanism for the physiological regulation of Na\(^+\),K\(^+\)-ATPase, including a cycle of binding and rapid dissociation in intact renal tubular cells, is discussed. (Hypertension 10 [Suppl I]: 1-61-1-66, 1987)

KEY WORDS • Na\(^+\),K\(^+\)-ATPase inhibitor • hypothalamus • endogenous digitalis • essential hypertension

SEVERAL known endogenous substances have been implicated in the regulation of Na\(^+\),K\(^+\)-adenosine triphosphatase (ATPase) activity. Catecholamines,\(^1\)-\(^3\) thyroid hormone,\(^4\) and vanadium\(^5\) have all been linked to either direct or indirect effects on enzyme activity. In recent years, however, a body of experimental evidence has been accumulating that favors the existence of a presumed unique and as yet structurally uncharacterized regulator of Na\(^+\),K\(^+\)-ATPase that functions in an inhibitory mode. In this, as well as other ways, it functionally resembles the cardiac glycosides.

A circulating inhibitor(s) of Na\(^+\),K\(^+\)-ATPase has been found in the plasma of both animals with experimental volume-expanded hypertension\(^6\) and human patients with essential hypertension.\(^7\) Other biochemical evidence exists for the effects of a circulating sodium transport inhibitor in human essential hypertension.\(^8\)-\(^9\) Suppression of sodium pump activity in cardiovascular muscle has been related to the genesis of the hypertensive disease in these situations.\(^7\)-\(^10\)-\(^12\) In the case of hypertensive animals it appears that the hypothalamus is required for either the elaboration or activation of the circulating inhibitor.\(^13\)-\(^14\) We previously documented the presence of an acid-stable substance extractable from bovine hypothalamus that reversibly blocks the active step in transepithelial sodium transport across anuran membranes and that demonstrates ouabainlike activity by its ability to inhibit Na\(^+\),K\(^+\)-ATPase from mammalian kidney directly and to inhibit the binding of labeled ouabain to its cellular receptor in frog urinary bladder.\(^15\)

Although several endogenous factors have been characterized for their ability to inhibit ouabain binding,\(^16\)-\(^21\) this assay tells little about the binding site or the mechanism of inhibition. Ouabain binding is inhibited by monovalent cations, lipophilic drugs, metal chelators, and a host of other agents that affect phosphorylation of the Na\(^+\),K\(^+\)-ATPase.\(^22\) For a "new" compound to be considered a serious candidate for a physiological regulator of Na\(^+\),K\(^+\)-ATPase, several criteria must be met: high binding affinity, reversibility of binding and inhibition, specificity for the target enzyme or receptor (in this case Na\(^+\),K\(^+\)-ATPase), and a plausible biochemical mechanism to account for inhibitory effects that translate into a physiological action.

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Thus it was of interest not only to compare the inhibitory mechanism of the natural factor from hypothalamus with the inhibitory mechanism of the potent plant-derived cardiac glycosides, but also to determine if the hypothalamic factor (HF) could satisfy the above set of biochemical criteria, which would be consistent with physiological relevance.

Using highly purified canine renal Na\(^+\),K\(^+\)-ATPase, we determined that the apparent affinity for inhibition of the enzyme by HF was in a physiological range (affinity = 1.4 nM) and that this in vitro inhibition was reversible,\(^2\) just as had been the inhibition of actual sodium pumping studied in toad urinary bladder.\(^5\) In collaboration with Carilli et al.,\(^2\) we further determined that HF could inhibit sodium pumping in a human cell (erythrocyte), and that inhibition was specific for Na\(^+\),K\(^+\)-ATPase, since at concentrations of HF that inhibited Na\(^+\),K\(^+\)-ATPase by 75%, other ATPases in the human erythrocyte plasma membrane were not affected.\(^2\) Thus, three of the cardinal criteria for a physiologically relevant inhibitor of Na\(^+\),K\(^+\)-ATPase — affinity, reversibility, and specificity — were satisfied by the endogenous inhibitor extracted from hypothalamus.

The mechanism by which HF inhibits enzyme activity (an in vitro observation) and active sodium pumping in intact transporting epithelia (an activity that translates enzyme inhibition into a relevant physiological function) required further study. To address this question, it is useful to briefly review the biochemical events thought to account for the hydrolysis of adenosine 5'-triphosphate (ATP) by Na\(^+\),K\(^+\)-ATPase, and the resulting transport of sodium and potassium ions across the plasma membrane. Figure 1 represents a model that provides a working hypothesis and incorporates much of the current thinking for the mechanism of sodium and potassium transport by Na\(^+\),K\(^+\)-ATPase.

The model was discussed in detail and critically evaluated by Cantley,\(^2\) but the salient features can be summarized as follows. The enzyme is thought to exist in two major conformational states, E\(_1\) and E\(_2\). The E\(_1\) conformation binds and is stabilized by sodium and ATP, and the E\(_2\) conformation by potassium or a covalently bound phosphate. In the presence of sodium, magnesium, and ATP, E\(_1\) becomes phosphorylated (B to C in Figure 1). The phosphorylated protein relaxes into the E\(_2\) state, the change in conformation resulting in the outward transport of three sodium ions. The outwardly directed cation binding sites now possess a higher affinity for potassium than for sodium, and the resulting potassium binding catalyzes dephosphorylation of the enzyme (D to F in Figure 1). The model proposes that during this process the potassium ions become trapped at their binding site and are released to the interior of the cell only after a shift in conformation from E\(_2\) back to E\(_1\), and this shift is accelerated by the presence of ATP inside the cell (F to G in Figure 1). The driving of the enzyme from E\(_2\) to E\(_1\) after ATP binding (G to B in Figure 1) releases two potassium ions inside the cell and reestablishes the conformation that permits phosphorylation of the enzyme in the presence of sodium. Thus perpetuation of the cycle results in directed, active sodium and potassium transport.

The model permits us to ask several questions regarding the biochemical interaction of HF with the cycling enzyme: 1) Does HF bind to the inside (like vanadate) or the outside (like ouabain) of the cell? 2) Does binding occur to or stabilize the E\(_1\) conformation (like sodium and ATP), or the E\(_2\) conformation (like potassium)? 3) Could the inhibitory effects of HF be related to enzyme phosphorylation events? 4) Is the reversibility of binding of HF related in some way to the cycling of Na\(^+\),K\(^+\)-ATPase through E\(_1\) and E\(_2\)?

To determine sidedness of HF binding, inside-out vesicles were prepared from human erythrocytes, and the ATPase activity of these vesicles was determined in the presence of HF.\(^2\) Results showed that HF had no effect on the Na\(^+\),K\(^+\)-ATPase activity of intact vesicles but completely inhibited this activity in vesicles made permeable by detergent, thus exposing a binding site on the extracellular surface.\(^2\) In human erythrocytes, therefore, HF acts only from the extracellular surface of the cell. This finding is consistent with previous experiments that showed that HF inhibited active sodium transport in toad urinary bladder only when applied to the serosal (blood) side of the membrane.\(^5\)

The two conformational states of Na\(^+\),K\(^+\)-ATPase, E\(_1\) and E\(_2\), can be distinguished fluorometrically after labeling the purified enzyme by fluorescein 5'-isothiocyanate (FITC).\(^2\) The conformational state can be manipulated in vitro by altering monovalent cations in the bathing buffer: potassium stabilizes the E\(_2\) conformation, while sodium shifts the conformation to E\(_1\), as indicated by an increase in relative fluorescence.\(^2\)

When these manipulations were carried out in the presence of HF, it was found that the inhibitor stabilized the enzyme in an E\(_2\) conformation and prevented the

**Figure 1.** A kinetic model for the mechanism of ATP hydrolysis by Na\(^+\),K\(^+\)-ATPase and the transmembrane active transport of sodium and potassium ions. See text for explanation. The stoichiometries for sodium- and potassium-binding sites are omitted. Reproduced from Cantley\(^2\) with permission.
shift to $E_1$ ordinarily caused by addition of sodium ions.\textsuperscript{24}

The active site aspartate residue of Na\textsuperscript{+},K\textsuperscript{+}-ATPase can be phosphorylated in two ways: from ATP, magnesium, and sodium; or by forming a covalent bond with inorganic phosphate in the presence of magnesium, a reaction that is supported by ouabain and results in a protein conformation similar but not identical to the $E_2$ state.\textsuperscript{23} HF was found to block this phosphorylation of the enzyme from inorganic phosphorus and magnesium\textsuperscript{24}; and HF prevented ouabain from supporting this phosphorylation, consistent with earlier findings that HF blocks ouabain binding to Na\textsuperscript{+},K\textsuperscript{+}-ATPase.\textsuperscript{15}

With reference to the model in Figure 1, the findings from these experiments permit the following hypothesis: HF binds with high affinity to the extracellular surface of the cell, consistent with the concept of a circulating inhibitor of the sodium pump. HF may inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by influencing the phosphorylation site on the enzyme, and may alter active sodium transport by "locking" the enzyme in $E_2$, thus preventing the cycling of the enzyme between $E_1$ and $E_2$ with the attendant interruption of the translocation of sodium and potassium ions across the plasma membrane.

Kinetic analysis of the binding of HF to purified Na\textsuperscript{+},K\textsuperscript{+}-ATPase\textsuperscript{23} raised an additional conceptual problem for HF as a physiological regulator in vivo. Like ouabain, on and off rates for HF binding to purified membrane preparations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase were relatively long (off rates = 60 minutes). Since HF has been proposed as a natriuretic hormone, and since such a slow rate of dissociation after binding might seem inconsistent with short-term regulation of renal tubular sodium handling, it was of interest to determine whether binding reactions in intact cells would show a time frame more consistent with physiological regulation in vivo. Using ouabain-sensitive $\textsuperscript{86}Rb^+$ uptake as a measure of sodium pump activity, we have begun to study the kinetics of binding and release of HF to cultured pig kidney epithelial cells, LLC-PK\textsubscript{1}. We find that HF acts on renal cells as a potent regulator of Na\textsuperscript{+},K\textsuperscript{+}-ATPase with a time frame quite different from that found in isolated membranes, and consistent with physiological relevance. Recent experiments support the possibility that the rapid dissociation of HF from intact cells may be due to shifts in enzyme conformation produced by intracellular accumulation of sodium and ATP after HF binding and pump inhibition.

**Methods**

**Isolation of Hypothalamic Factor**

The low-molecular-weight, acid-stable sodium transport inhibitor was extracted, partially purified, and concentrated as previously described.\textsuperscript{22-24} One unit of HF activity is defined arbitrarily as the amount of the inhibitor producing a 50\% inhibition of ouabain-sensitive $\textsuperscript{86}Rb^+$ uptake into human erythrocytes after 3-hour incubation.\textsuperscript{24}

**Coupled-Enzyme Assay**

Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity was measured in a spectrophotometer as the initial rate of hydrolysis of ATP after the addition of purified canine renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase to a kinetic coupled-enzyme assay system as previously described.\textsuperscript{23}

**Measurement of Active Sodium Transport in Cultured Porcine Renal Tubular Epithelial Cells**

Pump activity was measured as ouabain-sensitive $\textsuperscript{86}Rb^+$ influx into the LLC-PK\textsubscript{1} cells;\textsuperscript{26} 5 to 10 x 10\textsuperscript{6} cells were incubated in buffer containing glucose for varying times with HF (20 U/ml) or ouabain (0.5 mM) and then cooled to create conditions for maximal pump activation. Cells were removed from the cold and placed at 37°C, and $\textsuperscript{86}Rb^+$ was added to make 0.012 uCi/\mu l each tube. At the end of 10 minutes the reaction was quenched with cold buffer and the unincorporated counts were removed by spinning through 1:1 silicone/phalate oil. Cell pellets were counted for gamma emission. Trapped counts in the pellet were below 1\%, as judged by $\textsuperscript{14}C$-inulin. Uptake in the presence of 0.5 mM ouabain was subtracted to determine ouabain-sensitive $\textsuperscript{86}Rb^+$ influx. In some experiments cells were quickly washed twice in cold buffer after the incubation with HF or ouabain, with subsequent addition of the $\textsuperscript{86}Rb^+$ to run the flux (washout experiments).

**Results**

**Effects of Hypothalamic Factor on Sodium Pump Activity in Renal Epithelial Cells**

Previous studies of HF binding to purified membrane preparations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase showed relatively slow off rates (60 minutes\textsuperscript{22}). It was of interest to determine whether different binding and dissociation reactions more consistent with physiological regulation in vivo might obtain in intact cells. The cultured porcine renal epithelial cells, LLC-PK\textsubscript{1}, were chosen since they are of renal tubular origin and contain a large number of sodium pump sites (10\textsuperscript{6} sites/cell), and 80 to 90\% of potassium transport is through Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Pump activity was measured as ouabain-sensitive $\textsuperscript{86}Rb^+$ influx, a marker for potassium transport. As with membranes, 60-minute incubation with HF inhibited Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity ($\textsuperscript{86}Rb^+$ influx) in LLC-PK\textsubscript{1} (Figure 2). In contrast to membranes, no prior incubation with LLC-PK\textsubscript{1} was needed. Saturating HF produced an immediate 33\% reduction of $\textsuperscript{86}Rb^+$ influx. A 60-minute incubation of cells with HF followed by washout (see Methods) showed rapid reversal of inhibition and provoked a doubling of potassium influx (see Figure 2). Ouabain-binding studies in HF-treated LLC-PK\textsubscript{1}, before and after washout show that the enhanced transport is due to an increase in pump velocity and not to recruitment of additional pump units into the cell membrane (data not presented). Note in the right-hand portion of Figure 2 that ouabain does not demonstrate this rapid reversal of binding in LLC-PK\textsubscript{1} cells.
Dissociation of Binding of Hypothalamic Factor to Na⁺,K⁺-ATPase by Sodium and ATP

Our earlier studies of ligand requirements for binding of HF to membrane preparations of Na⁺,K⁺-ATPase showed that certain combinations of ligands could diminish or prevent inhibition of Na⁺,K⁺-ATPase by the factor. The most effective of these was the combination of sodium, magnesium, and ATP. As discussed above, this combination stabilizes the E₂ conformation and HF apparently binds to the E₂ conformation. Therefore it seemed possible that the rapid reversal of binding seen in a sided preparation (intact cells, LLC-PK₁) but not in membrane preparations of Na⁺,K⁺-ATPase might be due to a shift in enzyme conformation resulting from intracellular accumulation of sodium and ATP subsequent to pump inhibition caused by HF. We therefore studied the ability of the combination of sodium plus ATP to reactivate enzyme activity after inhibition by HF. Purified canine renal Na⁺,K⁺-ATPase was incubated for 30 minutes with HF in the presence of magnesium. Control tubes were left in the binding buffer while ATP or sodium plus ATP were added to experimental tubes. Aliquots of enzyme were removed at various times and assayed for Na⁺,K⁺-ATPase activity in the coupled-enzyme assay (see Methods). As shown in Figure 3, HF-treated enzyme kept in the Tris-MgCl₂ remained inhibited over the course of the experiment. Addition of ATP alone did not reverse enzyme inhibition by HF, but addition of sodium plus ATP completely reversed the inhibitory effects of HF by 30 minutes.

Discussion

Several so-called endogenous factors that inhibit active sodium transport, inhibit Na⁺,K⁺-ATPase activity in vitro, or prevent ouabain binding to Na⁺,K⁺-ATPase have been extracted from amphibian and mammalian sources (for review see Reference 29). These substances have not, in general, been characterized with regard to the mechanism(s) of inhibition of enzyme activity or sodium transport, and the question of whether or not they are biochemically competent to assume a role in normal physiology has not been addressed. For substances thought to be regulatory in nature, the issues of binding affinity, reversibility (nontoxicity) of biochemical and physiological effects, and substrate specificity are of central importance. In addition, it is important to explore whether or not this factor(s) demonstrates a physiologically plausible mechanism of inhibition of Na⁺,K⁺-ATPase.

We previously addressed many of these issues with regard to the endogenous sodium transport inhibitor extractable from bovine hypothalamus. These studies showed that HF is both a high-affinity and reversible inhibitor of purified Na⁺,K⁺-ATPase in vitro. Its inhibitory effects are specific for the plasma membrane Na⁺,K⁺-ATPase. Like ouabain, HF inhibits ion flux only from the extracellular side of the cell. This is important, since a circulating inhibitor of Na⁺,K⁺-ATPase might explain certain effects associated with experimental and human essential hypertension and natriuretic effects associated with extracellular fluid volume expansion. Also like ouabain, HF inhibits one of the partial reactions of Na⁺,K⁺-ATPase, the hydrolysis of the pseudosubstrate p-nitrophenylphosphate, and stabilizes an E₂-like conformation of the purified enzyme as judged by fluorescence from FITC labeling. Significantly different from ouabain, HF does not support phosphorylation of Na⁺,K⁺-ATPase from inorganic phosphate and magnesium. Mechanistically speaking (see Figure 1), these data suggest that HF may inhibit Na⁺,K⁺-ATPase activity by influencing the phosphate-binding site on the enzyme and may alter active sodium transport by pre-
venting the cycling of the enzyme between the E₁ and E₂ conformations, thus preventing the translocation of sodium ions across the cell membrane.

An additional kinetic concern for HF as a physiological regulator of Na⁺,K⁺-ATPase was raised by the relatively slow binding and dissociation reactions of HF to purified membrane preparations. Since inhibition of enzyme activity in vitro does not necessarily translate to inhibition of pump activity (active sodium transport) in vivo, and since HF has been proposed as a natriuretic hormone, we studied the ability of HF to regulate sodium pump activity in the cultured renal tubular epithelial cell, LLC-PK₁. We found binding and dissociation reactions in the intact cells to be quite different from those in isolated membranes. Hypothalamic factor produced immediate inhibition of the sodium pump in the intact cells, and removal of HF after 1 hour of incubation showed rapid dissociation of the inhibitor and an actual stimulation of pump activity (see Figure 2). Studies of [³H]ouabain binding in the HF-treated cells under conditions of binding and washout indicate that the enhanced transport activity is due to an increase in pump velocity and not to an increase in pump number (manuscript submitted for publication). This pump stimulation is probably due to the accumulation of intracellular sodium during the period of inhibition by HF. Ouabain does not show the rapid dissociation of binding from the renal cells after washout (see Figure 2). This observation reveals a further mechanistic difference between ouabain and HF and would appear to indicate that HF behaves more physiologically than ouabain in regulating Na⁺,K⁺-ATPase activity in these cells.

Studies of ligands that facilitate and diminish HF inhibition of Na⁺,K⁺-ATPase, and the finding that HF binds to and stabilizes an E₂ form of the enzyme suggest a hypothesis to account for the rapid dissociation of HF from intact epithelial cells that did not occur in membrane preparations. The rapid reversal of HF binding in intact cells may be due to a shift to an E₁,E₃ form of the enzyme unfavorable for binding. This change in conformation would be produced by transient increased intracellular sodium and ATP concentrations, since the combination of these ligands is known to "pull" the enzyme into E₁, and previously was found to inhibit HF binding to purified enzyme in vitro. This explanation is supported by the finding that sodium and ATP, when added to incubates of HF-inhibited enzyme, displace the inhibitor as indicated by complete restoration of enzyme activity within 30 minutes (see Figure 3). Although the hypothesis remains to be explored fully, available data suggest that HF may generate its own feedback cycle of binding and dissociation reactions for regulating Na⁺,K⁺-ATPase in the plasma membrane of intact renal epithelial cells. These effects could play an important role in the transepithelial movement of cations in the kidney that would account for a natriuretic effect in vivo.

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