The Search for a Hypothalamic Na⁺,K⁺-ATPase Inhibitor
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SUMMARY Accumulating experimental evidence suggests that natriuresis in response to intravascular volume expansion is promoted by an endogenous regulator of Na⁺,K⁺-adenosine triphosphatase (ATPase). Efforts to purify this substance by a number of laboratories have as yet been unsuccessful. The properties of partially purified inhibitors from plasma, urine, and tissue often fail to possess the characteristics thought to be consistent with those of a physiological regulator. These include potency (Kᵢ of approximately 1 nM), reversibility of inhibition, specificity for Na⁺,K⁺-ATPase, and responsiveness to relevant physiological stimuli. Two rather different candidate substances, extracted from urine and hypothalamus, have been purified to a high degree. Neither is a peptide, and both are of low molecular weight and resistant to acid hydrolysis. The substance from urine is rather nonpolar and interacts with digoxin-specific antibodies, while that from hypothalamus is polar and does not appear to share epitopes with the cardiac glycosides. On the serosal surface of the toad urinary bladder, the hypothalamic substance causes a reversible inhibition of Na⁺ transport, inhibits rubidium uptake in red blood cells by acting on the membrane's exterior surface, inhibits binding of ouabain to purified Na⁺,K⁺-ATPase, and reversibly inhibits hydrolysis of adenosine 5'-triphosphate by the enzyme with a Kᵢ of 1.4 nM. The hypothalamic inhibitor may be differentiated from ouabain by their respective ionic requirements for optimal inhibition of enzymatic activity, and although both ouabain and the hypothalamic inhibitor fix Na⁺,K⁺-ATPase in its Eᵢ conformation, the hypothalamic inhibitor does not promote phosphorylation of the enzyme by inorganic phosphate in the presence of Mg²⁺. Ionic requirements for inhibition also differentiate the hypothalamic inhibitor from vanadate ion, as does the inhibitor's activity in the presence of norepinephrine. Further enzymological and physiological studies will be facilitated by structural characterizations of the inhibitory substances and by the availability of a method to measure their concentrations in physiological fluids. (Hypertension 9: 315-324, 1987)

KEY WORDS • sodium transport • endogenous glycoside • endogenous digitalis • natriuretic hormone • essential hypertension • hypothalamus

AMONG the myriad herbal extracts and nostrums that have arisen in the long history of folk medicine, a few, such as atropine, digitalis, opium, aspirin, and ephedrine, are remarkable for exceptional potency. Each has been shown to interact with a specific receptor or enzyme. Since the discovery of endogenous analogues of opium, the endorphins and enkephalins,1,2 it has been tempting to postulate that each of these plant alkaloids may mimic an intrinsic regulator of vertebrate physiology.

A series of experimental observations has suggested that there may indeed be an endogenous analogue of digitalis, a potent inhibitor of membrane Na⁺,K⁺-adenosine triphosphatase (ATPase). In a provocative series of experiments, de Wardener et al.3 suggested that intravascular volume expansion caused natriuresis and that this was mediated in part by a humoral substance. Later, Overbeck et al.4 showed that volume expansion was associated with the elaboration of a heat-stable substance in plasma that inhibited cell membrane Na⁺ transport in vascular muscle. Brody et al.,5 seeking a locus of anatomical control, demonstrated that lesions in the region of the anteroven tral third ventricle of the brain prevented the hypertension of volume expansion. Songu-Mize et al.6 and Bealer et al.7 subsequently showed that these lesions prevented the secretion of the Na⁺ transport inhibitor.

It now seemed possible to explain a number of puzzling physiological phenomena, including both the natriuresis and hypertension of volume expansion, on the basis of a circulatory substance released by the midbrain in response to volume expansion. The postulated
mechanism of this substance’s action was the modulation of renal tubular Na reabsorption and vascular smooth muscle tone by regulation of $Na^+\cdot K^+$-ATPase activity. Indeed, the exogenous analogue of this putative regulator, digalitsa, a potent inhibitor of $Na^+\cdot K^+$-ATPase, had been demonstrated to cause both natriuresis and an increase in vascular resistance, although these are not its major pharmacological effects. A number of intriguing clinical observations supported this hypothesis. A natriuretic factor was found in the urine of volume-expanded, but not volume-depleted, uremic patients. A decrease in $Na^+\cdot K^+$ pump activity was shown in uremia, which may reflect a decrease in pump velocity in some patients or a decreased number of pump sites in others. The increased vascular reactivity observed in many hypertensive patients found an interesting parallel in the enhanced responsiveness to vasoconstrictor substances documented in digoxin-treated normal humans. Patients with essential hypertension also appeared to have an elevated concentration of a circulating pump inhibitor, which raised the very provocative possibility that an abnormality in $Na^+\cdot K^+$-ATPase regulation played a role in a prevalent human disease.

Although there was no reason to assume a structural identity between this postulated endogenous $Na^+\cdot K^+$-ATPase inhibitor and the digitalis glycosides, the widespread availability of the digoxin radioimmunoassay stimulated the measurement of immunoreactivity in plasma in situations where the inhibitor might be elevated. Klingmuller et al. postulated that the material was a peptide; an inhibitory constant could not be measured. Klingmuller et al., who, as mentioned previously, had identified digoxinlike immunoreactivity in human urine, effected a partial purification utilizing the binding properties of digitalis antibody. A detailed characterization of this material is not yet available, although it is suspected to be a polypeptide. A disturbing ob-

Purification Efforts

Various laboratories have used plasma, urine, or tissue as potential sources of the Na$, K^+$-ATPase inhibitor. Most of these efforts are at a preliminary stage. None has yielded molecular characterization, and few have addressed the essential tests just discussed. Conclusions as to the chemical nature of the material are often contradictory.

Plasma

Gruber et al. used high performance liquid chromatography (HPLC) to partially purify a substance from dog plasma that exhibited immunological cross-reactivity with digitalis. The same fractions inhibited Na$, K^+$-ATPase, and concentration varied with volume expansion, as would have been predicted by the earlier experiments of de Wardener et al. Gruber et al. postulated that the material was a peptide; an inhibitory constant could not be measured. Klingmuller et al., who, as mentioned previously, had identified digoxinlike immunoreactivity in human urine, effected a partial purification utilizing the binding properties of digitalis antibody. A detailed characterization of this material is not yet available, although it is suspected to be a polypeptide. A disturbing ob-
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servation in this more recent work is the lack of correlation between the digoxin radioimmunoassay and either Na intake or total natriuretic activity by bioassay. Apparently, while these investigators believe that the natriuretic substance binds to digoxin antibody, they suggest that there are also irrelevant substances in urine that interfere with a radioimmunoassay that uses the same digoxin antibody. The results of Gault et al.,31 however, suggest that radioimmunoassay results on plasma extracts with digoxin-specific antibody in untreated hypertensive subjects do correlate with Na loading. This group has most recently suggested that dehydroepiandrosterone accounts for some of the digoxin immunoreactivity in plasma and that this steroid also is a Na⁺,K⁺-ATPase inhibitor.32 Further confirmation of this observation is awaited with interest. Crabos et al.33 also used HPLC to fractionate plasma and urine in normal and hypertensive subjects. They found several peaks of Na⁺,K⁺-ATPase inhibitory activity, some of which cross-reacted in a digoxin radioimmunoassay and were present in greater amounts in hypertensive subjects than in normal subjects. The same group34 then proceeded to purify the urinary Na⁺ transport inhibitor. Preliminary structural analysis by nuclear magnetic resonance and mass spectroscopy suggests that the compound is not peptide. It may be steroidal, with a molecular mass of 431 daltons. Information on its affinity constant, substrate specificity, and binding characteristics has not yet been reported.

Kelly et al.35 fractionated deproteinized human plasma by hydrophobic gel chromatography followed by reverse-phase HPLC, using different elution protocols as well as two different supports. Four fractions were isolated that cross-reacted with digoxin-specific antibodies. It is of interest that no cross-reaction was noted with a ouabain antiserum. These fractions were resistant to acid hydrolysis and enzymatic proteolysis and were of low molecular weight. Three of the fractions inhibited pump activity in human erythrocytes and displaced ouabain from Na⁺,K⁺-ATPase. These fractions also stimulated sarcoplasmic reticulum Ca²⁺-ATPase. The fourth fraction, although cross-reactive with digitalis-specific antibody, was not an ATPase inhibitor. On further analysis, each of these fractions was found to contain either nonesterified fatty acid or lysophospholipid, both of which may well be nonspecific inhibitors of Na⁺,K⁺-ATPase in human plasma.

If antibodies specific for the digitalis glycosides are indeed capable of binding the natriuretic substance, one would expect that the administration of high affinity antibodies in vivo would inhibit the activity of the natriuretic factor in the same way that such antibodies inhibit the pharmacological effects of digoxin.37 In a hypertensive model, in which rats were hemiphrectomized and then treated with deoxycorticosterone and salt, Kojima et al.38 showed that the administration of anti-digoxin antibody caused a marked decrease in blood pressure. Because whole antibody was used in this study rather than Fab fragments, the possibility that the fall in blood pressure was due to immune-complex-mediated release of vasodilators cannot be excluded. Control animals, nevertheless, did not manifest a similar hypotensive response when infused with the same antibody solution. Later, Kojima39 showed that digoxinlike immunoreactivity as well as Na⁺,K⁺-ATPase binding activity was present in the plasma of these hypertensive animals. Fractionation by gel filtration (but no further chemical characterization) has been reported.

Extracts from Tissue

Because the midbrain has been implicated in the control of circulating Na⁺,K⁺-ATPase inhibitors, the brain has been a favored source of tissue for study. Haupert and Sancho40 extracted material from the hypothalamus. This work has now progressed considerably and will be discussed in detail. Fishman41 prepared a fraction of guinea pig brain that inhibited [³H]ouabain binding to brain microsomes and the uptake of ⁸²Rb⁺ into human erythrocytes. Lichtstein and Samuelov42 also extracted a low-molecular-weight "ouabain-like compound" from whole rat brain. This material inhibited [³H]ouabain binding to rat brain synaptosomes and Na⁺,K⁺-ATPase activity. Material prepared from sheep brain by the same procedure influenced electrical membrane potential in chick embryonic fibroblasts in a manner parallel to that of ouabain.43 This brain fraction was further purified by HPLC and shown to exert positive inotropic effects in sheep cardiac muscle.44 Preliminary characterization efforts suggested that the material was a low-molecular-weight, polar, nonpeptidic molecule, in agreement with the findings of Haupert and Sancho.45 Akagawa et al.46 also used bovine hypothalamus to obtain a fraction that, after gel filtration and ion-exchange chromatography, inhibited Na⁺,K⁺-ATPase activity and specific ouabain binding to rat brain microsomes in an apparently competitive manner. This activity was destroyed in part by a proteolytic enzyme, indicating that at least some of the inhibition was due to a peptide.

Although endogenous Na⁺ transport inhibitory activity need not be restricted to the central nervous system, most investigators have been unable to find it in other organs. One exception is that of De Pover et al.,47 who extracted inhibitory activity from guinea pig heart. On the other hand, Alaghband-Zadeh et al.,48 using an indirect assay of Na⁺,K⁺-ATPase activity, processed numerous organ tissues from the rat (including cerebral cortex tissue) and found inhibitory activity only in extracts from the hypothalamus. Two additional lines of evidence suggest that the brain is an important source of this inhibitory factor: two groups (Halperin et al.49 and Lichtstein et al.40) have isolated "ouabain-like" compounds from human cerebrospinal fluid. Since the material has not been substantially purified by these investigators, both specificity of ATPase inhibition and estimates of concentration are in doubt. Morgan et al.49 have recovered and partially characterized a Na⁺,K⁺-ATPase inhibitor from rat hypothalamic cells in culture. This latter substance is completely destroyed by several proteolytic enzymes, which indicates that it is a peptide and is thus chemical-
ly different from the nonpeptidic substances reported by most investigators who have processed whole brain or hypothalamus. Finally, Jandhyala and Ansari have recently reported that perfusion of cerebral ventricles in the dog with artificial cephalic cerebral fluid containing an elevated Na concentration leads to release into the circulation of a Na⁺,K⁺-ATPase inhibitor with resulting inhibition of Na⁺-K⁺ pump activity in the animal’s blood vessels. To this point the bulk of evidence suggests that the brain, and more specifically, the hypothalamus, represents an enriched source of the endogenous inhibitor of Na⁺,K⁺-ATPase, if not the site of production.

Table 1 summarizes the efforts of a number of laboratories working in this field. The tabulation indicates the continuing uncertainty about the molecular identity of the inhibitor(s) and underscores the relative lack of critical biochemical data, such as the demonstration of high binding affinity, necessary to support the notion that a given substance may be a physiologically relevant regulator of the Na⁺-K⁺ pump.

**Hypothalamic Na⁺,K⁺-ATPase Inhibitor**

Haupert and colleagues began a serious effort to isolate and characterize the Na⁺ transport inhibitory activity of hypothalamic extracts. The hypothalamus was chosen because it is a rich source of other important regulatory hormones. Bovine hypothalami were homogenized and extracted, and the extraction products were subjected to gel filtration, ion-exchange chromatography, and lipophilic gel chromatography. When small amounts of these partially purified extracts were applied to the serosal surface of the toad urinary bladder, a reversible, nontoxic inhibition of active Na⁺ transport across the membrane could be documented. The inhibited Na⁺ transport was accompanied by an increase in membrane resistance, indicating that the inhibition occurred through the active transport pathway and not because of a toxic effect on the preparation. Hypothalamic inhibitor (HI) was effective only from the serosal (circulatory) side of the membrane. When applied to frog urinary bladder, a tissue that binds ouabain tightly, HI caused inhibition of the hypothalamus, which is dissected fresh and quick-frozen on dry ice. Following homogenization in methanol, lipids are extracted and discarded and the remaining aqueous phase, which contains the inhibitor, is desalted and then further purified by lipophilic gel and ion-exchange chromatography. HI has been shown to be a low-molecular-weight nonpeptidic substance, since it elutes on a Sephadex G-25 column in the included volume and is resistant to acid hydrolysis. Activity is lost after ashing or base hydrolysis. The molecule appears to act as a zwiterion. At acid pH it behaves as a weak base and can be absorbed and eluted from strong cation exchange resins. At neutral pH it appears to be anionic by its retention on anion exchange resins. The anionic behavior may be due to the presence of a carboxylic acid group, since the biological activity is lost after methylation by diazomethane and can be restored by gentle acid hydrolysis (as would be expected for a methyl ester). A complete understanding of molecular structure awaits complete purification.

**Inhibition of Purified Renal Na⁺,K⁺-ATPase**

The effects of partially purified HI were assessed on purified canine kidney Na⁺,K⁺-ATPase activity, using a coupled enzyme assay. This method links the hydrolysis of adenosine 5'-triphosphate (ATP) to a regenerating system in which pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate, with the consequent conversion of the adenosine 5'-diphosphate (ADP) produced by ATP hydrolysis back to ATP. In this manner, constant ATP concentrations are maintained and the accumulation of ADP, which is an ATPase inhibitor, is prevented. The reaction is conveniently monitored by conversion of pyruvate to lactate, catalyzed by lactic dehydrogenase, which converts the reduced form of nicotinamide adenine dinucleotide (NADH) to the oxidized form (NAD⁺). The former is a chromophore, the concentration of which can be quantitated spectrophotometrically (NADH degradation is stoichiometrically linked to the hydrolysis of ATP). Figure 1 shows the inhibition of Na⁺,K⁺-ATPase by HI in the coupled enzyme assay. Nearly complete inhibition can be demonstrated. Studies of the ligand requirements for binding (see the following sections) indicated that binding of the inhibitor to purified enzyme was facilitated by Mg²⁺ and inhibited by Na⁺. By adjusting the concentration of these ligands in the binding assay, it was possible to show the reversibility of binding in vitro, which is consistent with earlier findings of the reversibility of transport inhibition in toad bladder and thus satisfies one of the essential criteria for a physiological inhibitor.
### Table 1. Summary of Efforts to Isolate and Characterize an Endogenous Na⁺,K⁺-ATPase Inhibitor

<table>
<thead>
<tr>
<th>Source</th>
<th>Extraction</th>
<th>Assay</th>
<th>Affinity (Kᵦ)</th>
<th>Chemical nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine plasma²⁹</td>
<td>Deproteinized</td>
<td>Digoxin RIA, ATPase activity</td>
<td>NR</td>
<td>? peptidic</td>
</tr>
<tr>
<td></td>
<td>Diafiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human plasma¹⁸</td>
<td>Deproteinized</td>
<td>ATPase activity</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Hog plasma²⁸</td>
<td>Acetone/methanol</td>
<td>ATPase activity</td>
<td>100 μM</td>
<td>Oleic acid, Linoleic acid</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Ouabain binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digoxin RIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human plasma³⁶</td>
<td>Deproteinized</td>
<td>ATPase activity</td>
<td>20 μM</td>
<td>Nonesterified fatty acids, Lysophospholipid</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Ouabain binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digoxin RIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine plasma⁵¹</td>
<td>Methanol</td>
<td>Ouabain binding</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>Sep-Pak (C18)</td>
<td>ATPase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human urine³⁰</td>
<td>Gel filtration</td>
<td>Natriuresis (rat)</td>
<td>NR</td>
<td>Peptidic</td>
</tr>
<tr>
<td></td>
<td>Ion exchange</td>
<td>Digoxin RIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human urine³⁶</td>
<td>Ion exchange</td>
<td>ATPase activity</td>
<td>NR</td>
<td>Nonpeptidic, ? steroidal</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Ouabain binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digoxin RIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine hypothalamus⁵²</td>
<td>Methanol</td>
<td>Na⁺ transport (toad bladder, human RBCs)</td>
<td>1.4 nM</td>
<td>Hydrophilic, Nonpeptidic, Zwitterionic</td>
</tr>
<tr>
<td></td>
<td>Ion exchange</td>
<td>ATPase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipophilic gel</td>
<td>Ouabain binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain (rat,⁵² sheep²⁹)</td>
<td>Acid/acetone</td>
<td>ATPase activity</td>
<td>NR</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>Ouabain binding</td>
<td></td>
<td>Nonpeptidic</td>
</tr>
<tr>
<td></td>
<td>Gel filtration</td>
<td>Cardiac muscle contractility</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>HPLC</td>
<td></td>
<td></td>
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<tr>
<td>Bovine hypothalamus⁴⁴</td>
<td>Acid/acetone</td>
<td>ATPase activity</td>
<td>NR</td>
<td>Peptidic</td>
</tr>
<tr>
<td></td>
<td>Gel exchange</td>
<td>Ouabain binding</td>
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<tr>
<td></td>
<td>TLC</td>
<td></td>
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<tr>
<td>Hypothalamic cell culture supernatants⁴⁴</td>
<td>Deproteinized (heat)</td>
<td>ATPase activity</td>
<td>NR</td>
<td>Peptidic</td>
</tr>
<tr>
<td></td>
<td>Gel filtration</td>
<td>Ouabain binding</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Na⁺ transport (human RBCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CSF⁴⁷</td>
<td>None</td>
<td>ATPase activity</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>Na⁺ transport (human RBCs)</td>
<td>ATPase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CSF⁴⁸</td>
<td>Methanol</td>
<td>ATPase activity</td>
<td>0.5 μM</td>
<td>Nonpeptidic</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Ouabain binding</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ATPase activity = inhibition of Na⁺⁺,K⁺⁺-ATPase; CSF = cerebrospinal fluid; HPLC = high performance liquid chromatography; NR = not reported; RIA = radioimmunoassay; RBC = red blood cell; TLC = thin layer chromatography.

**Inhibition of Red Blood Cell Rubidium Uptake**

It was of interest to determine whether inhibition of the Na⁺⁺-K⁺⁺ pump could be extended from the amphibian toad bladder to a mammalian cell. The uptake of K⁺ into human erythrocytes was thus studied using ⁸⁶Rb⁺ as a K⁺ analogue. Figure 2 shows that incubation of red blood cells with a partially purified preparation of HI caused 50% inhibition of ouabain-sensitive ⁸⁶Rb⁺ uptake. HI did not affect ouabain-resistant uptake, indicating that the inhibition is specific for K⁺ flux through the Na⁺⁺,K⁺⁺-ATPase.

**Comparison of the Properties of Hypothalamic Inhibitor with those of Ouabain and Vanadate Ion**

Both ouabain and vanadate ion are potent inhibitors of Na⁺⁺,K⁺⁺-ATPase, and it was important to establish whether they exhibited a similar mechanism of action. As shown in Figure 3, the ionic requirements for the action of the inhibitor were examined and compared with those of ouabain and vanadate ion. There are striking differences between the profiles of HI and ouabain or vanadate in this experiment. For ouabain, optimal inhibition of Na⁺⁺,K⁺⁺-ATPase activity oc-
Inhibition of purified Na⁺,K⁺-ATPase by increasing concentrations of hypothalamic inhibitor (HI) as measured in the coupled enzyme assay. Following a 30-minute incubation at 37°C, reaction mixtures were cooled on ice, diluted into assay buffer, and the rate of residual Na⁺,K⁺-ATPase activity was determined spectrophotometrically (see text). Fraction of enzyme remaining active was calculated by dividing initial velocity of HI samples by initial velocity of control samples. Data are means ± SE with n = 4 at each inhibitor level. (Reprinted from Haupert et al.52 with permission.)

Inhibition of human red blood cell ⁸⁶Rb⁺ uptake by hypothalamic inhibitor (HI). In this procedure, 150 µl of 50% (vol/vol) fresh red blood cells in Ringer's glucose was incubated with (●) or without (○) HI (10 units) at 37°C for 1 hour. In some suspensions, ouabain was added to a final concentration of 0.2 mM (dotted lines), and 0.6 µCi of ⁸⁶Rb⁺ was added to each tube at Time 0. The data plotted are averages of quadruplicate determinations. The standard deviation was ±5%. The initial rate of ⁸⁶Rb⁺-K⁺ uptake (assuming ⁸⁶Rb⁺ acts as a tracer for K⁺ uptake) was 70 nmol/10⁶ cells/hr. (Reprinted from Carilli et al.53 with permission.)

Affinity, Specificity, and Site of Action of Hypothalamic Inhibitor

Although HI has not been purified to homogeneity, its affinity constant for the enzyme can be determined if the assumption that there is only one binding site per Na⁺,K⁺-ATPase molecule is allowed. The basis of...
this calculation is that complete inhibition is not possible when enzyme concentration exceeds inhibitor concentration. Fifty percent inhibition occurs when enzyme concentration is approximately twice inhibitor concentration. This relationship is shown in Figure 4, where inhibitor concentration was fixed at 1 or 4 units and increasing concentrations of enzyme were added. The effect of the inhibitor clearly was diminished by increasing enzyme concentration; this effect was more pronounced at the lower inhibitor concentrations. If this were a low affinity inhibitor, present in a high multiple of the concentration of the enzyme, it would be impossible to titrate the effect in this manner. The shape of the curve is consistent with a simple one-to-one inhibitor-enzyme interaction. A nonlinear least-squares program was used to fit data from the experiment at both concentrations to the equation shown in the figure legend. The result was a $K_i$ value of 1.4 nM. An alternate computation made on kinetic grounds, which used the quotient of the on- and off-rate constants for inhibition and reversal of inhibition of the enzyme, gave the same result: a $K_i$ value of 1.4 nM. Thus, HI is a potent inhibitor of Na$^+$,K$^+$-ATPase and is somewhat more potent than ouabain (2 nM) or vanadate (6 nM). A potency of this order is consistent with a physiological inhibitor.

Is the HI uniquely specific for Na$^+$,K$^+$-ATPase? Table 2 compares its effect on several different cellular ATPases. Mg$^{2+}$-ATPase and Ca$^{2+}$-ATPase were unaffected under circumstances in which the Na$^+$,K$^+$-ATPase was inhibited by 75%. However, the Ca$^{2+}$-
ATPase of sarcoplasmic reticulum was inhibited significantly. This finding may be consistent with the structural homology between this enzyme and the Na⁺,K⁺-ATPase but also may not be of physiological relevance. It has been previously demonstrated that the inhibitor acts only from the serosal side of the toad bladder and is ineffective when applied to the mucosal surface. Thus, it may not penetrate the cell membrane, and any potential activity on sarcoplasmic Ca²⁺-ATPase may not be demonstrable in intact cells.

To further examine the location of the binding site of HI, inside-out human red blood cell vesicles were used to determine the sidedness of binding. HI had little effect on Na⁺,K⁺-ATPase activity of the inside-out vesicles but almost completely inhibited the Na⁺,K⁺-ATPase of detergent-permeabilized membranes. Na⁺,K⁺-ATPase activity of the intact vesicles was defined as that ATPase activity that is resistant to ouabain (which does not permeate the vesicles) but sensitive to strophanthidin (which is sufficiently hydrophobic to permeate the vesicles). Thus, HI was found, like ouabain, to inhibit Na⁺,K⁺-ATPase activity only from the extracellular surface. An action from the extracellular surface is consistent with the concept of a circulating regulator of the Na⁺⁺-K⁺ pump.

**Discussion**

While there has been a great deal of effort in many laboratories to identify and characterize a plasma, urinary, or tissue inhibitor of Na⁺,K⁺-ATPase, there has been a dearth of functional studies, and the definitive structure is not known. Haupert and colleagues have progressed significantly in identifying and characterizing a plasma, urinary, or tissue inhibitor of Na⁺,K⁺-ATPase, but it does inhibit ouabain-supported phosphorylation and ouabain binding. The HI also may be differentiated from ouabain in that its activity is not inhibited by a number of monoclonal or polyclonal antibodies specific for digoxin (G. T. Haupert, Jr., unpublished data). These functional studies diminish the likelihood that the inhibitory factor resembles an analogue of either digitalis or a bufodienolide. In addition to inhibiting the purified enzyme, HI also inhibits Na⁺⁺-K⁺ pump activity in both amphibian bladder and mammalian cells.

Initial chemical characterization of the hypothalamic Na⁺⁺,K⁺⁺-ATPase inhibitor indicates that it is of low molecular weight, zwitterionic, and stable to acid hydrolysis under conditions that exclude a peptide. Further physiological studies will be considerably facilitated by the structural characterization of the inhibitor and by the availability of a method to measure its concentration in physiological fluids.

How can one reconcile these characteristics with those of the other inhibitors discussed? To invoke more than one inhibitor is perhaps a simplistic evasion. To suggest that all the other activities described are manifestations of nonspecific inhibition by substances of low potency is an unwarranted anticipation of experiments not yet done or reported. Further structural and functional studies must be awaited before the role of an endogenous Na⁺⁺,K⁺⁺-ATPase inhibitor in electrolyte homeostasis can be assessed.

**Table 2. Effect of Hypothalamic Inhibitor on Various Membrane ATPases**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Control</th>
<th>0 min</th>
<th>30 min</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺⁺,K⁺⁺-ATPase</td>
<td>10.0±1.5*</td>
<td>10.0±1.5</td>
<td>3.1±1</td>
<td>70</td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>7.5±1</td>
<td>7.5±1</td>
<td>6.6±1</td>
<td>12</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>8.3±1</td>
<td>8.3±1</td>
<td>8.4±1</td>
<td>−1</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>2400*</td>
<td>2400</td>
<td>864</td>
<td>64</td>
</tr>
<tr>
<td>Ca²⁺-ATPase†</td>
<td>2400*</td>
<td>2400</td>
<td>1080</td>
<td>55</td>
</tr>
</tbody>
</table>

Values are means or means ± SEM. HI = hypothalamic inhibitor.

*The activities changed less than 10% during a 30-minute incubation in the same buffer without HI.
†Same as Ca²⁺-ATPase above except the preincubation buffer also contained 0.1 mM CaCl₂.
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