Problems and Pitfalls in the Isolation of an Endogenous Na⁺,K⁺-ATPase Inhibitor

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SUMMARY Plasma from volume-expanded and salt-loaded hypertensive animals and from patients with essential hypertension has been reported to inhibit Na⁺,K⁺-adenosine triphosphatase (ATPase). Inhibition of the sodium pump in vascular smooth muscle caused by such a circulating factor could increase vascular tone and sensitivity to vasoactive agents, and thereby result in arterial hypertension. Numerous efforts in the past failed to isolate the putative factor from urine and plasma. Recent studies have suggested that the hypothalamus is an important source of an endogenous Na⁺,K⁺-ATPase inhibitor, but its isolation from the tissue extracts has been rendered difficult by the presence of other cellular constituents that cause artifactual interference with the assays and purification procedures. Using an alternative approach of isolating the inhibitor from culture medium, we found that dispersed fetal rat hypothalamic neurons in a capillary culture system release a heat-stable, peptidic, low-molecular-weight, active sodium transport inhibitor that causes a reversible increase in vascular tone, sensitizes vascular smooth muscle to the vasoactive effect of norepinephrine, and possesses several characteristics of the putative endogenous digitalislike factor. This inhibitor may be a chemical mediator linking kidney, brain, and cardiovascular system in the genesis of experimental volume-expanded and salt-loaded hypertension and human essential hypertension. (Hypertension 10 [Suppl I]: I-57-I-60, 1987)

KEY WORDS • hypothalamus • sodium transport • hypertension • digitalis • vasoreactivity

DURING the last 25 years evidence has been accumulating to suggest that endogenous humoral factor(s) may be involved in regulating sodium transport in vital tissues, as well as sodium excretion by the kidney and vascular reactivity in arterioles. In 1961 de Wardener et al.1 demonstrated in dogs that an infusion of saline caused a rise in urinary sodium excretion even when the glomerular filtration rate and renal blood flow were experimentally reduced. Dahl et al.2 placed their salt-resistant rat in parabiosis with their salt-sensitive rat and found that hypertension developed in the resistant rat when both animals were fed salt. Under these experimental conditions a humoral hypertensive agent must have passed from the salt-sensitive to the salt-resistant rat. Many investigators confirmed these results and further showed that plasma extracts from saline-loaded and volume-expanded dogs have natriuretic, pressor, vascular sensitizing, and digitalislike activities.3-10

Considerable evidence from various laboratories is in favor of the existence of a common humoral factor that inhibits Na⁺,K⁺-adenosine triphosphatase (ATPase) and sodium transport, properties that could make it an endogenous diuretic as well as implicate it in certain forms of hypertension. Blaustein11 proposed that inhibition of Na⁺,K⁺-ATPase in the smooth muscle of the arteriole increases intracellular sodium, which increases intracellular calcium concentration and thereby the arteriolar tone. This hypothesis is supported by numerous experimental12-14 and clinical studies.15-18 Poston et al.16 showed that, after incubation with the plasma of patients with essential hypertension, normal leukocytes had low active sodium efflux and high intracellular sodium concentrations. Hamlyn et al.,15 MacGregor et al.,17 and Devynek et al.18 found that the plasma from hypertensive patients contained an inhibitor of Na⁺,K⁺-ATPase.

A digoxinlike immunoreactive substance was demonstrated in the amniotic fluid of hypertensive pregnant women19 and in the plasma of preeclamptic pregnant patients.20 Although these studies support the contention that an endogenous sodium transport inhibitor may be involved in the pathogenesis of hyper-

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tension, the nature of this hormone and its precise chemical composition, source, and mechanism of action remain obscure.

Source of Sodium Transport Inhibitor

During the last 6 years many investigators have attempted to identify the source of this inhibitory factor. Brain extract, after various steps of purification, was found to have a digitoxislike activity. 19,22 Pampani and co-workers 23, 24 showed that in the volume-expanded rat a lesion in the anteroventral third ventricle decreased the level of Na⁺,K⁺-ATPase inhibitor. Haupert and Sancho 24 used a more specific approach and found that bovine hypothalamic extract contained a factor that reduced sodium transport across anuran membranes and inhibited canine renal Na⁺,K⁺-ATPase. Using a cytochemical assay as a marker of Na⁺,K⁺-ATPase inhibitory activity, Alaghband-Zadeh et al. 25 tested a variety of rat tissues and found that the inhibitory activity in acetone extract of hypothalamus, which was six times more active than the corresponding plasma. Studies from our laboratory 26 have shown that hypothalamic neurons in culture release an active sodium transport inhibitor (ASTI), and it seems that the hypothalamus may be one of the principal sources of ASTI.

Problems in Extracting Sodium Transport Inhibitor

Attempts to isolate ASTI from plasma and urine of volume-expanded animals have been unsuccessful mainly because of artificial interferences caused by enzymes, peptides, proteins in the plasma, and waste products and high concentration of cations in the urine. On boiling, plasma releases free fatty acids that inhibit Na⁺,K⁺-ATPase. 27,28 The use of urine as a source of ASTI introduces an additional problem, since ASTI, like many circulating factors in plasma, may be modified (i.e., conjugated, detoxified, etc.) before being excreted.

In an effort to look for a more reliable source of ASTI, various investigators turned their attention to the brain and hypothalamus, but the extraction procedures used generated their own artifacts. Fishman 29 reported that an acetone extract of mammalian brain inhibited ouabain binding to brain microsomes, but Whitmer et al. 29 demonstrated that these results were due to cations and phospholipids in the extract. Haupert and Sancho 24 subjected bovine hypothalamic extract to chromatography and found that a low-molecular-weight fraction inhibited sodium transport and Na⁺,K⁺-ATPase. Kracke, 30 however, pointed out that Haupert and Sancho had failed to identify and separate the salt peak, and the results, in part, were due to ionic interference. Brain and hypothalamic extracts contain high concentrations of neurotransmitters, peroxidized lipids, lysophospholipids, phospholipids, peptides, and cations, all likely to interfere with any assay system used to isolate the putative hormone. 29,31

In their recent reports Haupert et al. 32 modified their isolation procedures to accommodate the criticism directed at their earlier study. 29,30 However, these authors have not used any specific measures to protect a possible peptide of hypothalamic origin against nonspecific peptidases released from pulverized hypothalamus.

Culture Medium as a Source of Sodium Transport Inhibitor

We attempted to overcome these problems by employing an alternative approach of using the culture medium of hypothalamic neurons as a source of ASTI. Culture medium is free from contamination by hypothalamic cell extract debris and other cellular constituents of high molecular weight, which can be excluded by using a capillary perfusion system (with a molecular weight cut-off of 100,000 or less), so that relatively pure hypothalamic secretions can be obtained.

Some of these well-known hypothalamic products can be used as markers to follow the unknown hypothalamic secretions through various purification procedures. In our earlier study, 26 we used somatostatin as a marker, and found that potassium depolarization in the presence of calcium increased the release of ASTI twofold and that of somatostatin fourfold. Similarly, luteinizing hormone-releasing hormone increased from 30 pg/10⁻⁷ cells to 80 pg/10⁻⁷ cells, and thyrotropin-releasing hormone increased fourfold during potassium depolarization (S.M. Foord, unpublished data, 1985).

One of the problems associated with plasma, urine, or tissues used as sources is that despite large initial quantities, only small, partially purified fractions are obtained, which are insufficient for detailed structural analysis. The use of a culture system provides large quantities of medium through continual perfusion of intact cells, which can be stimulated to increase the yield by altering the perfusion conditions, for example, by potassium depolarization. Using standard culture techniques we were able to isolate inhibitin, a sodium-sodium exchange inhibitor, in microgram quantities from promyelocyte culture medium. 33 Data obtained from two groups of investigators working in this field who tested our culture material on their assay systems suggested that 20 ml of culture medium was several times more potent than urine and intact rat hypothalami (H.E. de Wardener, personal communication, 1986), and as active as the extract from three bovine hypothalami (G.T. Haupert, personal communication, 1985).

Isolation and Purification

Most of the artificial problems known to have hindered progress in this field appear during various purification procedures. In addition to the numerous substances in brain and hypothalamic extracts that interfere with the assays used to isolate ASTI, some purification procedures may destroy the putative factor and generate other non-native compounds. Unless deliberate measures are taken to protect peptides (the likely secretory products of the hypothalamus) from nonspecific peptidases, the harsh mechanochemical
isolation procedures required to purify ASTI are likely to destroy the native peptide.

Culture medium is unlikely to contain peptidases that are released, usually from disrupted cells, during pulverization. We collect perfusate from capillary culture units in 1 N acetic acid, which ensures stability during purification.\(^{34}\) In addition, the presence of somatostatin and thyrotropin-releasing hormone, used as markers during Sephadex chromatography, suggests that other secretory products of hypothalamic neurons are likely to be intact.

**Controls**

The use of suitable controls is one of the most critical issues in this field. Investigators who used urine or plasma from hypertensive subjects\(^{15,20}\) after volume expansion\(^{35}\) or after salt loading\(^{36}\) had the option of using normal urine or plasma. Workers selecting the hypothalamus\(^{24,25}\) may use the cerebral cortex on the assumption that the hypothalamus is the principal, if not the sole, source of ASTI. The tedious and effort-intensive procedures of isolation and purification are tempting deterrents against the use of controls, which double the effort, time, and frustrations. Past experience in this field suggests that suitable controls are important safeguards against the creation of artifacts.

**Assays**

Some of the previously encountered problems have come from the fact that various investigators used only one or two assays to test for the presence of ASTI in tissue, plasma, and urine extracts. Every assay has its limitations, and the use of several assays eliminates most of the false-positive results.

**Current Perspective**

Twenty-six laboratories in various countries of the world are known to be working in this field. Little agreement exists among various investigators about the origin and nature of ASTI. Because of the many similarities between the actions of the endogenous ASTI and digitalis, some investigators\(^{37,38}\) suggested that the putative compound is a steroid. On the other hand, Kramer et al.\(^{39}\) consistently reported that the factor in the urine of salt-loaded subjects is a peptide. Of the four groups\(^{25,32,34,35}\) known to be using the hypothalamus as their source of the sodium transport inhibitor, however, only two\(^{34,39}\) have presented evidence to show that it is a peptide. Haupert et al.\(^{32}\) and de Wardener's group\(^{40}\) isolated a nonpeptidic factor that is resistant to boiling hydrochloric acid. Two investigators,\(^{41,42}\) using plasma as their source of the endogenous digitalis-like factor, found that the effects attributed to the endogenous inhibitor were caused by unsaturated fatty acids.

Recent studies in our laboratory showed that ASTI has a dose-dependent vasoconstrictor effect on rabbit aortic strips denuded of endothelium (Figure 1). This effect is well sustained, is greater than that achieved with 10^{-4} M ouabain, and can be reversed by washing the preparation. A significantly greater vasoconstrictor response to nonepinephrine was seen in the strips that had been exposed previously to ASTI than in control preparations. ASTI-containing fractions, which inhibited Na^+\cdot K^+\cdot ATPase activity and displaced [^3H]ouabain from its binding site, failed to cross-react with a variety of polyclonal and monoclonal digoxin antibodies (R.A. Kelly, personal communication, 1985).

Further work is in progress to sequence this peptide; however, progress is likely to be slow as novel solutions bring new problems.

Despite these and the several pitfalls described, the enthusiasm of many investigators in this field remains unabated, and the structure of the putative endogenous sodium transport inhibitor will be known before long. The conflicting results about the nature of ASTI suggest that more than one factor exists. One suspects that the discovery of the structure of a single factor will not deter others from pursuing their factors faithfully with their methods.

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