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Sodium transport inhibitor from bovine hypothalamus

(active sodium transport/natriuresis/Na^+,K^+-ATPase/ouabain)

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ABSTRACT **A low molecular weight, basic, nonpeptidic factor that possesses sodium transport inhibitory properties has been prepared from bovine hypothalamus by acid/acetone extraction and gel filtration. Concentration and partial purification was achieved by ion-exchange chromatography. This substance inhibits active Na^+ transport across anuran membranes, inhibits ouabain binding to frog urinary bladder, and directly inhibits renal Na^+,K^+-ATPase. This substance thus possesses the putative characteristics of a natriuretic factor of hypothalamic origin.**

The control of renal sodium handling is not fully explained by presently identified hormones or hemodynamic factors. Since the classic cross-circulation experiments of de Wardener *et al.* (1) numerous investigators have been involved in attempts to isolate and identify a humoral factor, other than aldosterone, that influences urinary sodium excretion in response to extracellular fluid volume manipulation. Physiologic studies provided considerable evidence for existence of such a "third factor" capable of modulating urinary sodium excretion independently of filtered sodium load and mineralocorticoid activity (2–4). Assays *in vitro* of plasma and urine extracts from subjects and animals with expanded extracellular fluid volume (5, 6), certain chronic uremic patients (7), and animals in mineralocorticoid "escape" (8) confirmed the biological effect of sodium transport inhibition across living cell membranes. Several investigators (9–11) have provided experimental evidence supporting the view that the brain is the source of the natriuretic factor(s). Despite the substantial indirect evidence for a circulating natriuretic substance, its precise chemical nature, source of production, and mechanism of action have remained obscure.

We report here the partial purification of a low molecular weight substance of hypothalamic origin that inhibits active sodium transport in toad bladder, inhibits ouabain binding in frog bladder, and appears to mediate these effects through direct inhibition of Na^+,K^+-dependent ATPase.

MATERIALS AND METHODS

Preparation of Tissues. Pieces of bovine brain encompassing the hypothalamic region and excluding the pituitary were dissected from freshly slaughtered cattle (Snider Brothers, Wilkinsonville, MA), frozen immediately on dry ice, and maintained frozen until 9 kg of tissue was obtained. Then 4.5 kg of tissue was thawed, homogenized in 4 M acetic acid, and extracted with acetone and petroleum ether by the method of Carraway and Leeman (12).

Gel Chromatography. Initial fractionation of the extract was carried out on a 20-liter column of Sephadex G-25 equilibrated in 0.1 M acetic acid, pH 4. Eluates were collected in 400-ml fractions, and aliquots were lyophilized and screened for Na^+ transport inhibitory activity on toad bladder.

Ion-Exchange Chromatography. When necessary, gel filtration fractions were desalted by a batch technique utilizing Amberlite IR-120. Aliquots were added to resin equilibrated in water (1:0.5 vol/vol) and shaken for 30 min. The supernatant was discarded and the resin was washed with 3 vol of deionized water, then resuspended in an equal volume of deionized water; the pH was adjusted to 9 with concentrated ammonium hydroxide. After serial passages, the final supernatant was collected and lyophilized several times, resuspended in a small volume of deionized water, and assayed for activity on toad bladder. Adequate lyophilization is required to remove ammonium ion, which has known membrane effects (13).

Biological Studies. The bioassay used for routine checking of sodium transport inhibition is the toad bladder preparation, as described by Leaf *et al.* (14). Transepithelial sodium transport is measured as the short-circuit current (SCC), and conductance measurements are made at 10-sec intervals throughout the study (15) with adequate controls for comparison. For an experiment to be considered positive, the SCC must return to basal levels after the membrane is bathed with fresh buffer. One unit (U) of activity is defined as the amount of activity producing a 50% decrease in SCC when added to the serosal side of the membrane.

Inhibition of ouabain binding to frog urinary bladder (*Rana catesbeiana*) was studied by the method of Mills *et al.* (16). Natriuretic factor was applied to the serosal surface of the experimental chamber. An equal volume of Ringer's solution was applied to the control chamber. After 15 min [^{3}H]ouabain was applied to both sides in final concentrations of 0.4 and 2.9 μM and allowed to incubate for 30 min. After processing as described (16), liquid scintillation counting was performed and ouabain binding in pmol/mg of wet tissue was computed.

Direct inhibition of Na^+,K^+-ATPase activity was studied by using a modification of the method of Silva *et al.* (17). A microsomal preparation of rabbit renal outer medulla was used as the enzyme source. Inhibition of enzyme activity by ouabain and various doses of natriuretic factor was investigated.

RESULTS

Bovine Hypothalamic Extracts. The tissue extracts were fractionated in a preliminary gel filtration step. The profile of the later portion of the eluate is shown in Fig. 1. Sodium transport inhibitory activity was limited to the pooled fractions eluting with the first portion of the salt peak. The activity of such a fraction was approximately 0.2 U/ml of crude extract. It was found that the active material of these fractions could be desalted and concentrated by batch extraction using Amberlite IR-120 (see *Materials and Methods*). When the activity

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Abbreviation: SSC, short-circuit current.

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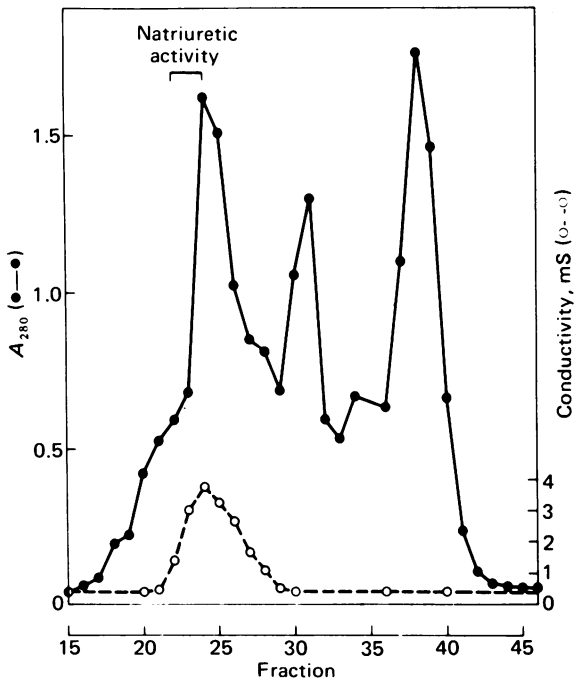

FIG. 1. Gel chromatography of bovine hypothalamic extract on Sephadex G-25. Sample, acid acetone extract representing 4.5 kg (wet weight) of tissue; column size, 14 × 150 cm (resin volume 20 liters); fraction size, 400 ml; eluent, 0.1 M acetic acid. Bracketed fractions contain natriuretic activity, and were eluted in the salt peak.

was adsorbed and eluted serially by this technique, a 20-fold concentration was achieved with complete elimination of salts.

Activity on Toad Bladder. Inhibition of active sodium transport across the isolated toad bladder was reflected in the drop in SCC when the activity was applied to the serosal side of the membrane (Fig. 2). Addition of the activity to the mucosal side produced no decrease in baseline SCC. The response was dose related, with a 250-μl aliquot of the partially purified material in 4 ml of amphibian Ringer's solution producing a mean reduction in SCC of 50%. The decrease in sodium transport was invariably accompanied by a simultaneous decrease in conductance across the bladder, an observation consistent with this material causing an inhibition of sodium transport through the active transport pathway. Gentle rinsing of the serosal membrane with fresh buffer resulted in a return of sodium transport to the baseline level (Fig. 2).

Table 1. Binding of ouabain to frog bladder

Ouabain, μM	Binding, pmol ouabain/mg (wet wt) tissue: Ouabain	Ouabain + 2 U natriuretic factor	Δ binding*	P†
2.9 (n = 5)	0.623	0.442	0.181 ± 0.031	<0.01
0.4 (n = 4)	0.186	0.125	0.061 ± 0.012	<0.02

*Mean difference ± SEM.
†Probability that Δ binding = 0.

Ouabain Binding. Application of natriuretic activity to the serosal surface of the frog urinary bladder produced the anticipated decrease in SCC. After addition of [^{3}H]ouabain to both chambers, a further drop in SCC did not occur on the experimental side, and ouabain binding was diminished compared with binding to the control half of the bladder that had not been pretreated with the natriuretic activity (Table 1).

Action on Renal Na^+,K^+-ATPase. The direct effect on Na^+,K^+-ATPase from the outer medulla of the rabbit kidney was studied by using ouabain and various doses of natriuretic factor. The factor significantly reduced the activity of this enzyme in a dose-related manner. When submaximal doses of ouabain and natriuretic factor were incubated with the enzyme, inhibition was additive, the combination effect being significantly greater than effects of either ouabain or the factor alone (Fig. 3).

Other Characteristics. The substance(s) is of low molecular weight (<2500) as judged by ultrafiltration membranes. The activity survives, and indeed appears enhanced, after acid hydrolysis. It is very stable and bears a net positive charge at acid pH. It affects sodium transport in toad bladder quite differently from vanadium, which is present in mammalian tissues and known to inhibit Na^+,K^+-ATPase.

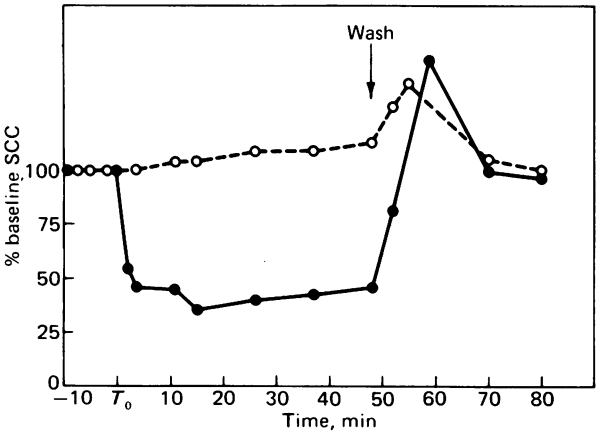

FIG. 2. Inhibition of SCC (active Na^+ transport) in isolated toad bladder. The 250-μl sample of natriuretic factor (●—●) was applied to 4 ml of amphibian Ringer's solution bathing serosal surface at T_0. The control (○- - -○) was 250 μl of Ringer's solution. Serosal surfaces were rinsed with fresh Ringer's solution at 48 min. Natriuretic factor produced a prompt and sustained inhibition of active sodium transport, reversible with rinsing.

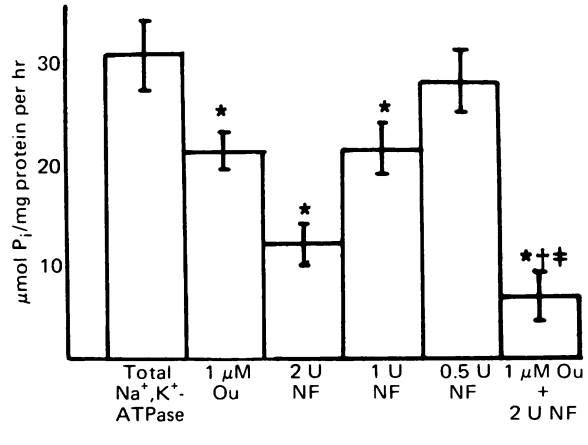

FIG. 3. Inhibition of Na^+,K^+-ATPase from rabbit renal outer medulla by ouabain (Ou) and various concentrations of natriuretic factor (NF); n = 6, error brackets indicate ±1 SEM. The effects of natriuretic factor were dose related. Natriuretic factor plus submaximal doses of ouabain produced additive inhibition.
* P <0.02–0.001 vs. total Na^+, K^+-ATPase.
† P <0.001 vs. 1 μM ouabain.
‡ P <0.02 vs. 2 U of natriuretic factor.

DISCUSSION

The present study documents the presence of a stable substance (or substances) extractable from bovine hypothalamus that produces the effects of a natriuretic factor. It reversibly inhibits sodium transport in toad and frog urinary bladder. It apparently acts by blocking the active step in transepithelial sodium transport as evidenced by decreases in membrane conductance and by its ability to inhibit the transport enzyme Na^+,K^+-ATPase *in vitro*. Because ouabain is known to block active Na^+ transport by specific inhibition of Na^+,K^+-ATPase, biological effects of the factor were studied in reference to this drug. With regard to the enzyme, inhibition of activity was found to be additive. In frog urinary bladder the factor inhibits binding of labeled ouabain to its cellular receptors. Such inhibition could occur through competition for ouabain-binding sites. Alternatively, direct effects of the factor on $Na,^+$-K^+-ATPase, perhaps mediated through a separate receptor, could secondarily reduce ouabain binding, because binding of this drug to its receptors in cultured pig kidney cells (18) and sheep and human erythrocytes (19) appears to be modulated as a function of the rate of turnover of the Na^+ "pump."

This substance thus possesses the putative characteristics of a natriuretic factor of hypothalamic origin. Its ouabain-like properties thus far mimic those of a substance extracted from whole brain and reported in the accompanying communication (20).

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