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Partial Purification and Characterization of Natriuretic Factor from Rat Kidney (40831)

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The first experimental evidence that a natriuretic hormone might exist came from deWardener *et al.* (1) who showed that infusion of large amounts of saline into a donor dog cross-circulated with a recipient dog produced significant natriuresis in the donor dog and a lesser, but still significant, natriuresis in the recipient animal. Since that time, many investigators have attempted to show that the natriuresis which follows volume expansion is related to either changes in peritubular physical factors or to the presence of a circulating humoral substance capable of inhibiting tubular reabsorption of sodium. In the majority of studies, plasma or urine has been employed as the source of hormone activity. In our laboratory, we have used kidney tissue from volume-expanded rats as a source of natriuretic factor, reasoning that the principal target organ might serve as an effective trap for the circulating hormone (2, 3). This factor not only causes natriuresis when injected into test animals, but also decreases frog skin short circuit current and renal Na-K-ATPase activity in a dose-dependent manner, indicating that it acts as an inhibitor of active transport. In contrast, kidney tissue from nonexpanded rats yields no significant natriuretic, antinatriferic or Na-K-ATPase inhibitory activity.

Most of the investigations to date have employed Sephadex or Biogel chromatography for the initial isolation. Only Bricker and his associates (4, 5) and Gruber and Buckalew (6) have attempted further purification with the use of ion-exchange chromatography or high pressure liquid chromatography. In this communication, we report the partial purification of rat kidney natriuretic factor by acrylamide gel electrophoresis. This factor has also been further characterized by its inhibitory effects on Na-K-ATPase derived from various sources and by its inactivation by selected proteolytic enzymes.

Methods and materials. In each experiment, boiled homogenates of whole kidneys from two volume-expanded rats were subjected to Sephadex G-25 gel chromatography and natriuretic material contained in the post-salt fraction was lyophilized and kept at −20°C as described by Hillyard *et al.* (3). This lyophilized material was stable for at least 6 weeks when it was tested after acrylamide gel electrophoresis.

Approximately 400 μg of the lyophilized material was dissolved in Tris buffer (0.01 *M*, pH 7.2), with a drop of bromphenol blue as a marker, then 50 μg applied on each of six 7% acrylamide gel tubes, and run up to 10 cm length. Four equal 2.5-cm segments were sliced from the gel and each segment tested for antinatriferic activity, employing both the frog skin short-circuit current (SCC) assay and the Na-K-ATPase assay previously described (3). In five preliminary experiments, when 100 μg/ml of Lowry-reactive material was added to the test system, only the third segment from the top (5.0 to 7.5 cm) was found to have significant antinatriferic or Na-K-ATPase inhibitory activity (i.e., greater than 10% decrease from control). For all later studies, the third segments from six tubes were pooled, the biologically active material was eluted from the acrylamide gel by homogenization with distilled water and centrifugation at 13,000 rpm; the supernatant was then lyophilized for subsequent assays.

In each such experiment the lyophilized material represented 15 to 20% of the Sephadex G-25 post-salt fraction obtained from two rat kidneys. The protein content of this material was measured by the method of Lowry *et al.* (7). A dose–response curve was established with material from five separate volume expansion studies, using both inhibition of frog skin SCC and inhibition of Na-K-ATPase activity as assays for natriuretic factor activity, described earlier by Hillyard *et al.* (3). For the dose–response curve, lower concen-

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trations of natriuretic factor were obtained by appropriate dilutions with distilled water. Three different sources of Na-K-ATPase were employed, namely whole homogenate of rat kidney, microsomal fraction of rat kidney, and purified Na-K-ATPase from hog cerebral cortex. The rat kidney microsomal fraction was prepared in our laboratory by differential centrifugation and contained the light and heavy microsomes (8). Purified Na-K-ATPase from hog cerebral cortex was purchased from Sigma Chemical Company, St. Louis, Missouri. For determination of total ATPase activity in the first two sources of enzyme, incubation tubes contained 0.5 ml of substrate solution and provided final concentrations of 1 m*M* ATP, 1 m*M* Mg^{2+}, 10 m*M* imidazole–HCl buffer, pH 7.2, 100 m*M* Na^+, and 20 m*M* K^+, plus 0.1 ml of 5 m*M* ethylene glycol bis(β-aminoethyl ether)-*N*,*N*^{1}-tetraacetic acid (EGTA); 0.3 ml of material from each fraction dissolved in distilled water was added to each assay tube, and the tube was placed in a water bath at 37°C. A control was prepared in the same manner, but 0.3 ml of distilled water was substituted for the kidney fraction. The reaction was started by adding 0.1 ml of the enzyme preparation and was arrested after 10 min by adding 1 ml of ice-cold 10% trichloroacetic acid (TCA). After centrifugation of the mixture at 1700*g* for 5 min, 1 ml of the supernatant fluid was assayed for inorganic phosphate as described earlier (3). Mg-ATPase activity was assayed with 1 m*M* ouabain in the incubation medium, and Na-K-ATPase activity was calculated as the difference between total ATPase and Mg-ATPase activities. Protein determinations were obtained for each enzyme preparation by the method of Lowry *et al.* (7) and ATPase activity was then expressed as micromoles of inorganic phosphate liberated per milligram of protein per hour. On the other hand, when the highly purified hog cerebral cortex Na-K-ATPase was employed as the enzyme source, only total ATPase activity was measured as it was observed that 1 m*M* ouabain inhibited this enzyme completely. In all instances, percentage inhibition of Na-K-ATPase activity was calculated by comparing the Na-K-ATPase activity before and after incubation with the natriuretic factor.

In testing for susceptibility to destruction of natriuretic factor activity by proteolytic enzymes, the semipurified material isolated following acrylamide gel electrophoresis from five separate experiments was tested on the frog skin SCC assay system before and after incubation for 1 hr at room temperature with the following proteolytic enzyme sources: (i) 1.0 mg of purified trypsin (Sigma), (ii) 1.0 mg of purified chymotrypsin (Sigma), and (iii) fresh whole kidney homogenate (0.1 ml of 10% homogenate). In each instance, the amount of natriuretic factor was that contained in 50 μg of Lowry protein. The reaction mixture was boiled at the end of the 1 hr incubation period for 15 min in order to destroy residual enzyme activity.

Results. Inhibition of SCC produced by natriuretic factor after sequential Sephadex G-25 gel chromatography and acrylamide gel electrophoresis is shown in Fig. 1. This dose–response curve shows the mean and SE of five separate experiments at each dose range, represented by the natriuretic factor activity contained in 5, 50, and 100 μg/ml of Lowry protein, respectively. At these dosages, SCC was inhibited by 20.2, 37.9, and 54.4%, respectively. The dose–response effects of natriuretic factor on Na-K-ATPase activity from rat kidney whole homogenate, microsomal fraction and hog cerebral cortex are shown in Fig. 2. This figure also shows the mean and SE for five separate experiments at each dose

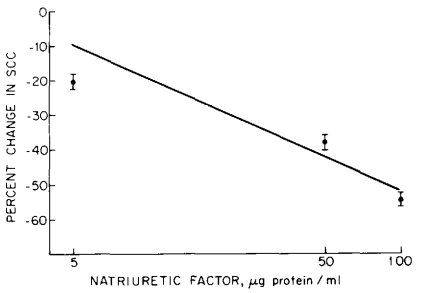

FIG. 1. Inhibition of short-circuit current (SCC) produced by varying concentrations of semipurified natriuretic factor from volume-expanded rat kidneys. Each point is the mean of five experiments and the vertical bar represents SEM.

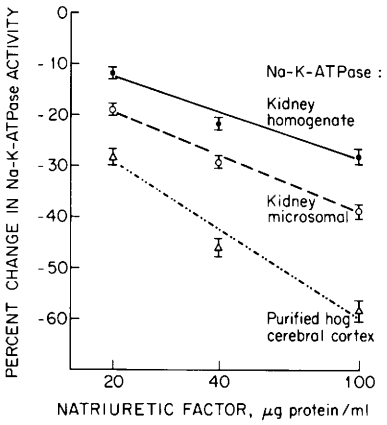

FIG. 2. Inhibition of different preparations of Na-K-ATPase by varying concentrations of natriuretic factor from volume-expanded rat kidneys. Each point is the mean of five experiments and the vertical bar represents SEM.

range, represented in this instance by the natriuretic factor activity contained in 20, 40, and 100 μg/ml of Lowry protein, respectively. When whole kidney homogenate was used as the enzyme source, inhibition of activity was 11.4, 21.1, and 28.0%, respectively. When the microsomal fraction was used as the enzyme source, inhibition was 18.7, 29.1 and 38.7%, respectively. Finally, when purified hog cerebral Na-K-ATPase was used as the enzyme source, inhibition was 27.3, 46.0, and 58.6%, respectively.

In the proteolytic enzyme study, the mean baseline inhibition of SCC produced by semipurified natriuretic factor from the third segment of the acrylamide gel, containing 50 μg/ml of Lowry protein, was 32.6 ± 0.8%. In five studies in which purified trypsin was used as the source of proteolytic enzyme, inhibition of SCC was reduced to 5.1 ± 0.6%. In five studies in which purified chymotrypsin was used, inhibition of SCC was 31.7 ± 0.9%. Finally, in five studies which employed fresh whole kidney homogenate as the source of enzyme, inhibition of SCC was reduced to 3.8 ± 0.6%. Changes in SCC after incubation with trypsin or whole kidney homogenate were significant at the $P < 0.001$ level, whereas changes in SCC after incubation with chymotrypsin were not significant. As control, the same proteolytic enzymes were added to 50 μg/ml of Lowry protein from the first, second, and fourth segments of the acrylamide gel. SCC was not significantly affected by the original material or the material following incubation with proteolytic enzyme.

Discussion. The results of the present study indicate that approximately a 25-fold purification of the Sephadex gel-isolated natriuretic factor may be achieved by acrylamide gel electrophoresis. This calculation derives from comparison of the dose response curves of the present study with those presented in our earlier communication (3), in which the natriuretic factor was tested immediately after Sephadex gel chromatography, and assumes a conversion factor of 0.85 μg Lowry protein per microgram of lyophilized material. Examining each assay separately, the SCC results indicate a 50-fold purification, whereas the Na-K-ATPase inhibition assay indicates a 10-fold purification. In our first study, 500 μg/ml natriuretic factor inhibited SCC by 22.7% and 1000 μg/ml natriuretic factor inhibited whole kidney homogenate Na-K-ATPase by 25.3%. By comparison, in the present study 5 μg/ml natriuretic factor inhibited SCC by 20.2% and 40 μg/ml natriuretic factor inhibited kidney homogenate Na-K-ATPase by 21.1%.

There were striking differences in the susceptibility of Na-K-ATPase to inhibition by natriuretic factor, depending on the source used. Enzyme derived from whole kidney homogenate was inhibited to a lesser degree than enzyme from kidney microsomal fraction, while maximum susceptibility to inhibition was seen with the highly purified Na-K-ATPase from hog cerebral cortex. We might speculate that a number of naturally-occurring endogenous Na-K-ATPase inhibitors, such as monovalent and divalent cations (9) and a variety of organic substances (10), are present in whole kidney homogenate, where they could interfere with the demonstration of an exogenously-added inhibitor, such as natriuretic factor. Such impurities would be removed to a large degree by differential centrifugation and isolation of the microsomal fraction of kidney homogenate,

where Na-K-ATPase activity is maximal (11), and even further by the lengthy purification process used in extracting the hog cerebral cortex enzyme (12).

An additional important observation is that natriuretic factor is capable, at least *in vitro*, of inhibiting Na-K-ATPase from a source other than kidney. Whether Na-K-ATPase inhibition in tissues other than kidney also occurs *in vivo* following volume expansion would presumably depend on the presence of receptor-ligands for natriuretic factor in these other tissues. Pamnani *et al.* (13) have demonstrated a humoral agent in plasma of volume-expanded rats, which inhibits $Na^+ - K^+$ pump activity in tail arteries of control animals, as measured by reduction in ouabain-sensitive ^{86}Rb uptake. In a recent review article (14), the same group has suggested that this humoral agent might be natriuretic factor and that the mode of action is via inhibition of Na-K-ATPase activity. In a similar vein, Nizet (15) has demonstrated that humoral material released from kidneys of volume-expanded dogs is capable of inhibiting sodium transport across jejunal mucosa *in vivo*. Thus, it appears likely that natriuretic factor has several potential target organs.

The inactivation of natriuretic factor by proteolytic enzymes has been reported earlier by several groups of investigators (16–18). When trypsin, chymotrypsin, kidney aminopeptidase, or pepsin were incubated with natriuretic factor, complete or partial loss of activity was found, although results have been variable depending upon the source or molecular weight species of the natriuretic factor studied. The semipurified low molecular weight factor in the present study was inactivated by trypsin and by fresh kidney homogenate but not by chymotrypsin. These results are compatible with the suggestion that natriuretic factor may be a peptide.

Summary. A partial purification of natriuretic factor from volume-expanded rat kidney has been achieved by Sephadex gel chromatography and acrylamide gel electrophoresis. This factor inhibits frog skin SCC and Na-K-ATPase from different sources in a dose-dependent manner. Activity is destroyed by incubation with trypsin or fresh kidney homogenate but not chymotrypsin.

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