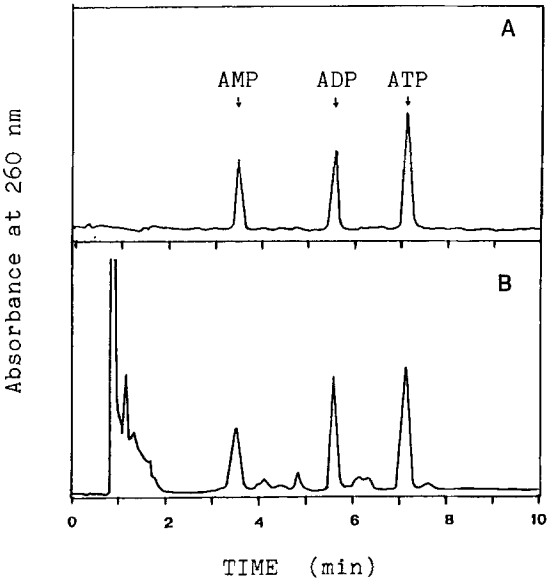

Separation of adenine nucleotides from a standard mixture (A) and from an extract of digestive gland of mussel (B). Injection volume: 10 µl. Experimental conditions as described in the text.

nucleotides in the TCA extract was checked by analyzing the same extract, maintained at −20 °C over a period of two months several times; no significant variation was observed.

The values of AEC calculated from the results obtained utilizing this procedure (Mytilus digestive gland AEC = 0.67; Palaemon muscular tissue = 0.78) are in good agreement with the data reported in the literature[5,8].

In conclusion, the method described here, based on TCA extraction and anion exchange HPLC, allows a simple, rapid, and accurate determination of adenine nucleotides. Therefore, it represents a very useful tool for routine determination of the adenylate energy charge in marine organisms, and thus for the assessment of pollution stress in environmental monitoring programs.

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Comparative effects of ouabain, natriuretic factor and ammonium chloride in the toad urinary bladder

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Summary. Electrical changes and direct effects on Na-K ATPase activity induced by an endogenous digitalis-like natriuretic factor (NF), NH_4Cl and ouabain were studied in toad bladders. NF inhibited the SCC and the Na-K ATPase activity in a similar manner to ouabain, but induced a greater increase in calculated direct current resistance (R) ($p < 0.05$). NH_4Cl was a weak inhibitor of Na-K ATPase activity, although it produced steeper SCC inhibition slopes than those observed with ouabain or NF ($p < 0.01$). The data suggested the same mechanism of action of NF and ouabain on the sodium pump, with an additional effect of the former on apical sodium permeability of the cells and/or closure of paracellular routes leading to an increased tissue resistance. In contrast, the effects of NH_4Cl were mostly compatible with intracellular inhibition of apical sodium entry into the cell.
Key words. Bladder; cell membrane permeability; Na-K ATPase; digitalis; natriuretic factor; ouabain; ammonium chloride.

In the 1970s, NH_4Cl and a natriuretic factor (NF) isolated from the chromatographed urine of salt-loaded normal men were reported to have ouabain-like electrical properties in frog skin preparations[1]. Ouabain is known to be a specific and powerful inhibitor of the sodium pump[2] and the Na-K activated ATPase of cell membranes, and to inhibit the short-circuit current (SCC) in a toad bladder preparation mounted in an Ussing-chamber, and the transepithelial sodium flux through various epithelia and cells.

NH_4Cl was shown to inhibit the SCC in toad bladder[3] and frog skin preparations[4], and to suppress ouabain-binding to isolated Na-K ATPase receptors of rabbit kidney preparations at $5 \cdot 10^{-1}$ M (unpublished observations). NF inhibited the SCC in toad bladder[5] and rat colon[6] in vitro preparations, and induced a natriuresis when injected into rats[5]; its activity was shown to be closely related to the sodium balance and the urinary sodium elimination in the rat[7]; it displaced ouabain bound to Na-K ATPase receptors of rabbit kidney at concentrations thought to be physiological[8].

Our purpose was to compare the respective modes of action, at the cellular level, of NF and NH_4Cl on those cellular events which are known to be induced by ouabain.

To do so, we first studied the electrical effects of NF and NH_4Cl in terms of short-circuit current (SCC), potential difference (PD) and calculated direct-current resistance (R) obtained in toad bladders mounted in chambers. Secondly, we examined the direct effects of NF and NH_4Cl on the activity of the Na-K ATPase isolated from toad bladder cells. The phenomena observed in the presence of NF and NH_4Cl were compared with those produced by ouabain.

Material and methods. SCC, PD, and R measurements. Hemibladders from Dominican *Bufo marinus* female toads were mounted in a conventional Ussing-chamber[5] with an exposed surface area of 2 cm^2. Both surfaces of the bladders were bathed with 5 ml of Ringer's solution (110 mM, NaCl; 2.5 mM, KCl; 2.0 mM, $MgCl_2$; 1.5 mM, $CaCl_2$; 10 mM, glucose; 5.0 mM, $NaHCO_3$). The solutions were gassed with an O_2 95%–CO_2 5% mixture. SCC and PD values were automatically recorded at 5-min intervals[8].

No membrane was employed for experiment unless the SCC and

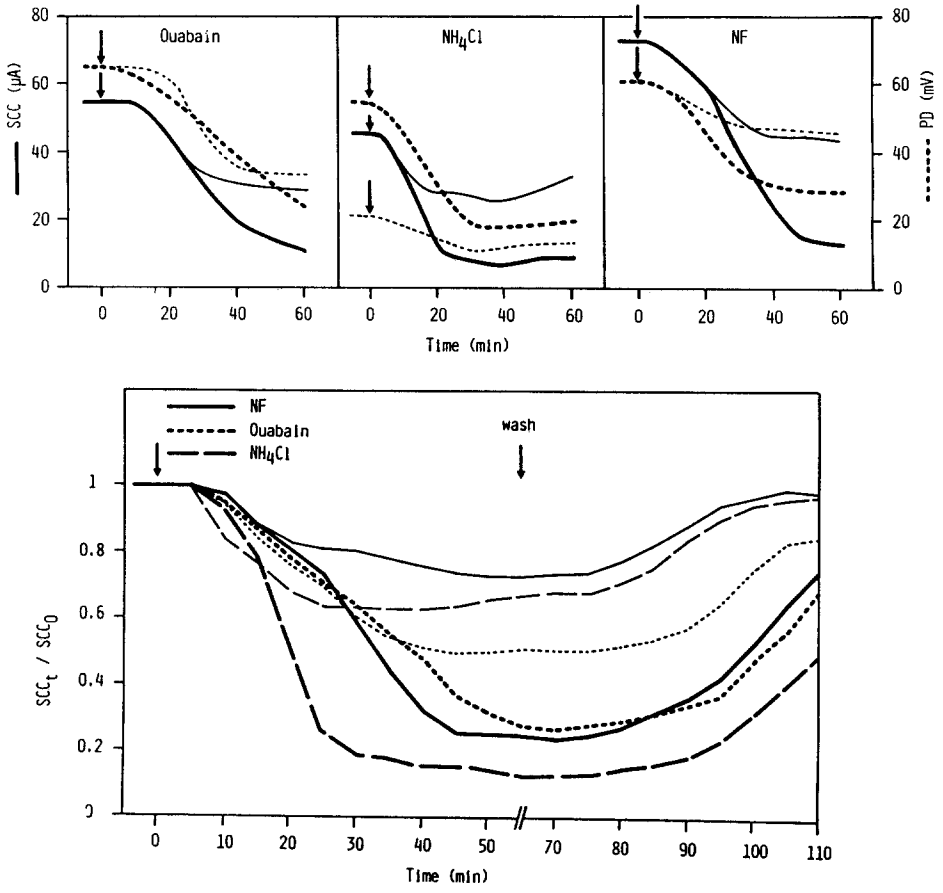

Figure 1. Time-dependent representative inhibition of the short-circuit current (SCC) and potential difference (PD) in absolute (top panel) and relative values for SCC (lower panel). Arrows (at t_o) indicate addition to the serosal bath of either: natriuretic factor (NF) (thin lines: 0.5 mg/ml, n = 3; thick lines: 1 mg/ml, n = 6), or ouabain (thin lines: 10^{-4} M, n = 5; thick lines: 10^{-3} M; n = 6), or NH_4Cl (thin lines: 10^{-3} M, n = 6; thick lines: 10^{-2} M; n = 6). Lower panel: reversion of SCC after replacement of serosal solution with fresh Ringer's. All curves returned toward base-line; thick lines indicate the higher, thin lines the lower concentration; number of experiments and molarity in top panels.

PD values had been stable for a minimum of 20 min and unless SCC exceeded 30 μA/2 cm^2. At t_0, 1 ml of Ringer's solution containing one of the following substances: 0.5 or 2 mg natriuretic factor; 10^{-3} M or 10^{-2} M NH_4Cl; 10^{-4} M or 10^{-3} M ouabain was added to the serosal bath and 1 ml of Ringer's solution was added to the mucosal side.

Na-K ATPase activity measurements. ATPase from toad bladders was prepared following the method described by Cortas et al.[9], modified for Na-K ATPase activity measurements by Jorgensen[10]. Empty open bladders were soaked at 4 °C for 10 min in a solution containing 1 M NaI (Merck) and 65 mg/100 ml Tris (Merck) pH 7.3 ± 0.2, for removal of mucosa from submucosa. The mucosa was then scraped with a glass slide. Scrapings, collected in a chilled centrifuge tube, were washed 3 times with 2 ml ice-cold 15 mM Tris solution, 5 mM EDTA (Sigma) pH 7.0, and 3 times with 2 ml 50 mM Tris, pH 7.0. The scrapings were then homogenized (B. Braun No. 1469) with 2 ml, 5 mM EDTA, 50 mM Tris, 30 mM histidine-HCl (Merck), 250 mM sucrose (Merck), 0.1% of deoxycholic acid (Merck), adjusted to pH 7.6; homogenization was performed at 650 rev/min. The homogenate was then diluted to 2.0 ml with the same solution. Protein concentration ranged between 1 and 1.5 mg/ml. After 1 h of incubation at 4 °C, the solution was immediately assayed. The Na-K ATPase activity was considered to be the rate of release of inorganic phosphate (Pi) in one ml of solution 1 (3 mM, $MgCl_2$; 10 mM, NaCl; 20 mM, Na N_3; 20 mM, KCl; 30 mM, histidine, (Merck); pH 7.5), minus the rate of release of Pi in solution 2 (solution 1 without KCl and NaCl), which corresponded to the Mg-ATPase. 18 mg ± 6 mg (SD) of enzyme protein were incubated in solution 1 for 10 min at 25 °C, the reaction was started by addition of 50 μl 3 mM Na_2ATP (Boehringer Mannheim), and stopped after 6 min by adding 1 ml TCA 10% (Merck). After 10 min of centrifugation, Pi was determined[11]. Mg-ATPase activity was 21.5 ± 4.7 (SD, n = 15) μmoles Pi/mg protein/h, which corresponded to 65% ± 7.5% (SD) of the total ATPase. NF, ouabain and NH_4Cl were solubilized in solution 1, and results expressed as percent of Na-K ATPase inhibition after having subtracted the activity due to the Mg-ATPase.

Natriuretic factor preparation. NF was isolated from a pool of urine provided by normal men receiving 100 mM of sodium (Slow sodium, Ciba) a day in addition to a normally salted diet (about 150 mmol Na a day) and a free intake of water during a 10-day-period. Urine, after lyophilization, was chromatographed through a 80 × 8 cm Sephadex G-25 column and eluted with 0.1 M of acetic acid. From every second 10 ml fraction following the salt peak, 2 ml were sampled and tested for Na-K ATPase receptor binding affinity following a method previously described[8]. The remaining 8 ml of each fraction able to inhibit at least 50% of the ouabain-binding was taken, pooled and lyophylized; the product was proven to be natriuretic when injected to rats and termed NF. 1.5 mg of NF (dry weight of lyophylisate) contained: 7.7 mmoles Na; 0.29 mmoles K; 0.12 mmoles Ca; 0.35 mmoles Pi; 66 nmoles ammonium; 1.8 μmoles urea; 0.2 nmoles vanadium as measured with a Perkin Elmer apparatus for atomic absorption and 1.5 pmoles of active substance, as estimated by the method of Scatchard previously reported[8]. There was no detectable prostaglandin E_2. When this material was solubilized in the 5 mM $NaHCO_3$ Ringer's at the concentra-

tion of 1.5 mg/ml, the final solution had a pH of 7.6 and an osmolality ranging between 230 and 240 mosmoles/kg. Thus, none of these substances is likely to have biased the observed effects of NF.

Statistical Analysis. Non-parametric Mann-Whitney tests were performed for statistical analysis of the data. Means and standard deviation are given throughout.

Results. Comparative electrical effects. Figure 1 shows representative time-course inhibition curves with both absolute and relative values for SCC, and absolute values for PD, following the addition to the serosal bath, at 2 different concentrations, of either ouabain, NF or NH_4Cl. The SCC inhibition started after a lag period of 10 min. The inhibition was observed with the highest concentration of each substance studied. Removal of the three inhibitors from the bath allowed the SCC to return towards the baseline value (fig. 1, lower panel).

Time-dependent decrease in activity, calculated by the method of least squares, induced by NF and ouabain were comparable (slope (−b) 60 ± 9.6 vs 66 ± 4.1) and differed significantly ($p < 0.01$) from those obtained in the presence of NH_4Cl (40 ± 3.0).

PD decreased to a lesser extent than SCC in the presence of each of the 3 agents tested. The calculated direct-current resistance was larger with larger concentrations of each substance (fig. 2).

Effects on isolated Na-K ATPase. An approximately 50% inhibition of the Na-K ATPase isolated from toad bladder was obtained with 10^{-4} M ouabain, 10^{-9} M NF and 10^{-1} M NH_4Cl. Increasing concentrations of NF and ouabain inhibited the Na-K ATPase activity in a similar dose-response relationship (linear with log-dose) although the slope was smaller with ouabain than with NF. The effect of NH_4Cl occurred at much higher concentrations and was not linear with log-dose (fig. 3).

Discussion. Toad urinary bladder is representative of the distal segment of the nephron and of tight epithelia, characterized by high electrical resistance and low passive permeability of junctions. SCC is currently assumed to be accounted for by transepithelial sodium transport generated by the ouabain-sensitive basolateral sodium pump[10]. However, other routes are involved in the sodium transport, i.e. apical sodium permeability and paracellular routes. Using three substances, known to have an inhibitory effect on the SCC; NF, ouabain and NH_4Cl[2,3,5], the relative importance of the sodium pump and of those other

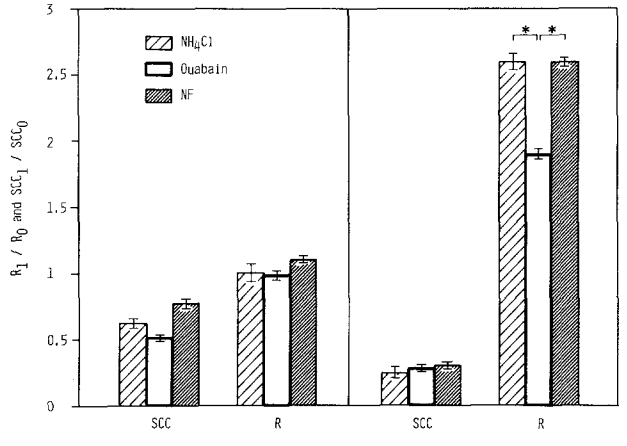

Figure 2. Effects of the natriuretic factor (NF), ouabain and NH_4Cl on SCC and resistance (R). The left panel demonstrates that R_1/R_0 ratio did not significantly change when either NF ($5 \cdot 10^{-10}$ M, n = 3), ouabain (10^{-4} M, n = 5) or NH_4Cl (10^{-3} M, n = 8) was added to the serosal bath. The right panel evidences the characteristic changes in R_1/R_0 ratio produced by higher concentrations of either NF (10^{-9} M, n = 6), ouabain (10^{-3} M, n = 6) and NH_4Cl (10^{-2} M, n = 6). Asterisks indicate statistical significant (*$p < 0.05$) differences of changes in R between the effect of ouabain and NF and NH_4Cl, respectively.

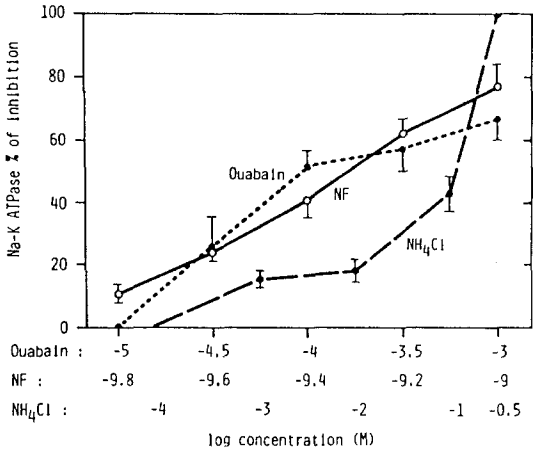

Figure 3. Inhibition of the Na-K ATPase activity produced by increasing amounts of natriuretic factor (NF, n = 6), ouabain (n = 6) and NH_4Cl (n = 6).

routes of transepithelial sodium transport were evaluated for each substance by comparing their effects, at concentrations which submaximally (80%) inhibit the SCC, on the Na-K ATPase activity. Electrical measurements showed that ouabain, NH_4Cl and NF inhibit the SCC in a dose-related manner (fig. 1). After washing with fresh Ringer's this inhibition was reversible (fig. 1). Ouabain and NF induced a similar time-dependent decrease in SCC activity; in contrast, the NH_4Cl-induced time-dependent decrease in SCC activity was steeper. Calculated direct-current resistance increased only at submaximal SCC inhibition with the greatest effect being observed in the presence of NF and NH_4Cl (fig. 2). Time-dependent decrease in activity suggest that NF could alter the transepithelial transport of the sodium through an inhibition of the sodium pump, as established for ouabain[2]. In contrast NH_4Cl could exert its inhibitory effect on the sodium transport through different mechanisms. The former suggestion is supported by the study of the effect of each of the three substances on the Na-K ATPase activity (fig. 3). When the activity of this enzyme was measured in the presence of each of the three substances at concentrations which had submaximally inhibited the SCC, an 80% reduction was observed with NF and ouabain, contrasted with a 20% inhibition with NH_4Cl. Thus NF and ouabain regulate the transepithelial sodium transort, mostly through their action on the sodium pump; in contrast, the 20% inhibition of the enzyme activity observed after adjunction of NH_4Cl at 10^{-2} M cannot explain the inhibitory effect of that substance on the SCC, suggesting its non-specific action on the enzyme. The 20% inhibition of the membrane enzyme speaks for a competition between NH_4^+ ions and K^+ ions for the potassium site of the enzyme[12]. However, at a higher concentration (5.10^{-1} M), NH_4Cl completely abolished the enzyme activity (fig. 3). In agreement with this finding in toad bladder is our observation of a 20% inhibition of binding of ouabain to the Na-K ATPase receptors of the rabbit kidney with NH_4Cl, and a 100% inhibition at 5.10^{-1} M. Thus there is evidence for a direct inhibitory effect of NH_4Cl on the ouabain-sensitive receptors only at higher concentrations than that needed to suppress the SCC, suggesting the involvement of mechanisms other than sodium pump inhibition.

Epithelial resistance in bladders mounted in chambers is usually thought to be largely artefactual because of significant damage at the edges[13]. Such damage may account for variations of the basal resistance observed between bladders, but not for the dose-dependent rise in tissue resistance observed in the presence of the three agents under investigation (fig. 2). According to the literature, changes in tissue resistance may be the consequence of an inhibition of the apical sodium entry into the cell or of the closure of paracellular shunts[14]. These phenomena can depend

on direct effects of the inhibitory substances on these routes or be secondary to the inhibition of the sodium pump.
Both ouabain and NF were shown to raise the intracellular sodium activity, accounted for by the inhibition of the sodium pump[14] which activates sodium feedback mechanisms involving the apical membrane, and hence, reduces the apical entry of sodium. NF and ouabain inhibited the sodium pump to the same extent[15], but had different effects on tissue resistance (fig. 2). These differences may reflect a greater inhibitory effect by the former on apical sodium entry and/or on paracellular pathways. To conclude, the present data demonstrate that, in toad bladder preparations, ouabain and NF act mostly by inhibiting the sodium pump; NF has an additional inhibitory effect more pronounced than that of ouabain on apical permeability to sodium and/or on the paracellular pathways for sodium transport, and NH_4Cl exerts its effect mostly on the apical membrane permeability.

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Kinins in cerebrospinal fluid: Reduced concentration in spontaneously hypertensive rats

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Summary. Rat cerebrospinal fluid contains peptides which displace radiolabeled bradykinin from its specific antibodies. Two peptides which showed the same retention time as kallidin and bradykinin in a reverse phase high pressure liquid chromatography system were detected in cerebrospinal fluid of rats. The concentration of radioimmunologically detected kinins in the cerebrospinal fluid of spontaneously hypertensive rats of the Okamoto strain was lower than that of the Wistar Kyoto control rats.
Key words. Kinins; bradykinin; kallidin; cerebrospinal fluid; HPLC; hypertension.

The cerebrospinal fluid (CSF) of normal rats contains a substance which has an immunoreactivity indistinguishable from that of kinins[1]. However, it is unknown whether bradykinin, kallidin or methionyl-lysyl-bradykinin is the peptide involved. To identify which kinin is present in rat CSF we used a high pressure liquid chromatography system (HPLC), in which the kinins as well as fragments of these peptides showed different retention times.
Although the i.v. (or i.a.) injection of kinins induces a fall in blood pressure, the administration of these peptides into the cerebral ventricles has the opposite effect[2]. In theory, constantly elevated levels of cerebral kinins could lead to a permanent rise of blood pressure and thus be involved in the pathogenesis of some forms of hypertension. To test this possibility we also measured the kinin concentration in the CSF of spontaneously hypertensive rats.
Materials and methods. Female normotensive Wistar Kyoto (WKR) and stroke-prone spontaneously hypertensive rats (SHRsp) of the Okamoto strain, weighing about 200 g were used. CSF (approximately 0.2 ml per rat) was collected under pentobarbital anesthesia by puncturing the Membrana atlanto-occipitalis. EDTA (12.5 mM) and o-phenanthroline (2.6 mM) were used (20 µl/ml CSF) to prevent peptide degradation.
The CSF from 25 animals was pooled and an aliquot (0.3 ml) was taken for measuring the basal kinin concentration by radioimmunoassay. The kinins, which were contained in 4.7 ml CSF, were prepurified and concentrated on octadecasylilsilica cartridges (3; Sep-Pak C_{18}, Waters, Eschborn, Federal Republic of Germany). Prior to sample application the cartridges were first rinsed with 3 ml methanol and then with 5 ml water. After the CSF was applied to the cartridges, these were rinsed with 9 ml 4% acetic acid in water. Then the peptides were eluted with 3 ml isopropanol. The isopropanol was removed from the eluate with a stream of nitrogen while the test tubes were immersed in a water bath at 40 °C. The remaining water phase was lyophilized. The freeze-dried residue was dissolved in 1 ml 0.001 N HCl. A sample of 0.3 ml was taken to assess the recovery of kinins, and the rest was applied to the HPLC column. The column was calibrated with synthetic kallidin and bradykinin. The peptides were detected by UV extinction at 220 nm and 0.1 absorbance units full scale.

The high pressure liquid chromatograph was carried out at room temperature on a 300 × 4 mm reverse phase C_{18} column (µ-Bondapak C_{18}, 10 µm, Waters). The mobilie phases were passed through a RC 55 membrane filter with 0.45 µm pore size (Schleicher and Schüll, Dassel, Federal Republic of Germany) and degassed under vacuum before use. The column was first rinsed with 40% acetonitrile in 0.08% (v/v) trifluoracetic acid (solution B), and then equilibrated with 20% acetonitrile in 0.08% trifluoracetic acid (solution A) for another 15 min. A linear gradient from solution A to solution B at a flow rate of 1 ml/min was started 3 min after sample application and completed 45 min later. Fractions of 0.5 ml were collected in polyethylene tubes. The samples were dried as described for the eluates of the Sep-Pak cartridges, and the residue dissolved in