Ouabain-like activity in human cerebrospinal fluid

(Ja+/K+-ATPase/Na+/K+-ATPase inhibitor/Na+ transport inhibitor/natriuretic factor)

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ABSTRACT Human cerebrospinal fluid has been found to mimic the effect of ouabain on net Na+ efflux and 86Rb+ influx across erythrocyte membranes and on the in vitro activity of a purified Na+/K+-ATPase (ATP phosphorylasease, EC 3.6.1.3) derived from canine kidney. These results indicate the possible existence in human cerebrospinal fluid of an endogenous factor with ouabain-like activity, which might be linked to sodium metabolism.

Since the classic cross-circulation experiments of de Wardener et al. (1) and Dahl et al. (2), numerous investigators have provided experimental evidence supporting the view that a humoral factor could influence urinary sodium excretion and vascular responsiveness by inhibition of the Na+/K+ pump (3, 4). Although the chemical nature and site of production of this putative hormone have remained obscure, some data suggest the brain as the possible source (5).

Neurochemical analysis of cerebrospinal fluid (CSF) is recognized as a method that can provide valid information about the metabolism of neurotransmitters and hormones produced in the central nervous system of living subjects (6). We report here the presence of an endogenous "ouabain-like" activity in human cerebrospinal fluid.

MATERIAL AND METHODS

CSF Samples. CSF was obtained by lumbar puncture from healthy female and male donors to whom lumbar myelographies were performed as a diagnostic procedure for possible spinal-root compression. Informed consent was obtained in all cases. CSF samples were clear and colorless and had normal biochemistry and cell count. No erythrocytes were detected.

The Na+ and K+ contents of each CSF sample were determined by flame photometry. The final concentration of both ions was adjusted to a value similar to the control medium of the respective experiment by adding the required amount of a 1 M NaCl or KCl solution.

CSF Ca2+ and Mg2+ contents were determined on an ACA II analyzer (DuPont), and vanadium was determined by flameless atomic absorption spectrometry (Varian GTA-95 Atomizer coupled to a Varian 575 spectrometer; maximum sensitivity, 25 ppb).

Assays for Ouabain-like Activity. Intracellular Na+ concentration of unloaded human erythrocytes. Freshly drawn human venous blood, collected in heparin-treated tubes, was centrifuged (1,700 × g for 10 min), and the plasma anduffy coat were removed. The erythrocytes were then washed three times with 10 volumes of ice-cold 110 mM MgCl2 and were resuspended in the same solution to obtain a hematocrit of 0.600. The erythrocyte suspension was distributed in duplicate tubes at a final hematocrit of 5% in a volume of 250 μl of either (i) control medium (150 mM NaCl/2.8 mM KCl/1 mM MgCl2/10 mM glucose/5 mM sodium phosphate, pH 7.4 at 37°C) with and without ouabain (0.1 mM) or (ii) CSF (with adjusted Na+, K+, and buffer concentrations similar to those of the control medium). An aliquot of the cell suspension was set aside for measurement of the basal intracellular Na+, hemoglobin, and hematocrit.

Samples were incubated for 1, 2, and 3 hr at 37°C and then were washed three times with 10 volumes of isotonic solution of choline chloride (150 mM choline chloride/10 mM Tris-HCl, pH 7.4 at 37°C) with ouabain (0.02 mM). After the last wash, the pellet was hemolyzed with 2.5 ml of Acetonex (0.02%) for hemoglobin determination and Na+ measurement in the same sample.

Na+ was measured by atomic absorption spectrometry (Techtron AA4, Melbourne, Australia) and hemoglobin was spectrophotometrically determined as oxyhemoglobin (541 nm). Intracellular Na+ concentrations were calculated in mmol/liter of erythrocytes and expressed as the ratio of postincubation to preincubation values (relative variation).

Intracellular Na+ concentration of preloaded erythrocytes. Washed erythrocytes were loaded with Na+ by incubation at 4°C for 2 hr in a loading solution (60 mM NaCl/200 mM choline chloride/3 mM KCl/1 mM MgCl2/2.5 mM sodium phosphate/1 mM EGTA/0.02 mM p-chloromercuribenzenesulfonate, pH 7.2 at 4°C) and then were incubated at 37°C for 1 hr in a recovery solution (150 mM NaCl/1 mM MgCl2/5.4 mM sodium phosphate/4 mM cysteine/2 mM adenosine/3 mM inosine/1 mM EGTA/10 mM glucose, pH 7.2 at 37°C) as described by Dagher and Garay (7). After recovery, the erythrocytes were washed three times with a cold solution of 110 mM MgCl2, resuspended in the same solution to an hematocrit of ~0.600, and distributed in control, ouabain-containing, or CSF media for further processing as described above.

An aliquot of the loaded-cell suspension together with an aliquot of cells untreated with p-chloromercuribenzenesulfonate were set aside to measure intracellular Na+, hemoglobin, and hematocrit. Intracellular Na+ concentrations were calculated in mmol/liter of erythrocytes and were expressed as the relative variation with respect to the initial (preload) cellular Na+ content.

Rubidium-86 uptake into human erythrocytes. Rb+ is an ion handled similarly to K+, and therefore 86Rb+ uptake by erythrocytes has been used to give a measure of the K+ influx into erythrocytes. Washed erythrocytes were resuspended at a final hematocrit of 5% in a volume of 200 μl of either (i) control medium (150 mM NaCl/5 mM KCl/10 mM Tris-HCl, pH 7.4 at 37°C) with and without ouabain (0.1 mM) or (ii) CSF (with adjusted Na+, K+, and buffer concentrations similar to those of the control medium). Triplicate aliquots were incubated at 37°C

Abbreviation: CSF, cerebrospinal fluid.
for 1 and 2 hr with 10^6 cpm of 86Rb^+ (New England Nuclear; specific activity, 1.09 mCi/mg; 1 Ci = 37 GBq) and then washed three times with 10 volumes of ice-cold isotonic choline chloride. Radioactivity was eluted with 300 μl of 5% trichloroacetic acid and assayed in 5 ml of isotonic saline in a Searle liquid scintillation spectrometer (8). Results were calculated in cpm per 10^6 cells.

**Assay for Na^+−K^+-ATPase inhibition.** The Na^+−K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity was estimated by the rate of [γ-32P]ATP hydrolysis. Highly purified canine kidney Na^+−K^+-ATPase (grade IV, lot 22F, 9523), free of Mg^2+- ATPase activity, was purchased from Sigma. It was dissolved in 20 mM Tris-HCl, pH 7.4/1 mM EDTA at a protein concentration of 6.1 mg/ml. For each assay the enzyme was further diluted 1:10 in 20 mM Tris-HCl, pH 7.4/5.2 mM MgCl₂/0.2 mM EDTA. The enzyme (50 μl) was assayed in a final volume of 1 ml of either (i) control medium (140 mM NaCl/20 mM KCl/5.2 mM MgCl₂/1 mM EDTA/50 mM Tris-HCl, pH 7.4 at 37°C) with and without 0.1 mM ouabain or (ii) CSF (with adjusted Na^+, K^+, and buffer concentrations similar to those of the control medium). After 20 min of preincubation at 37°C, the reaction was started by adding [γ-32P]ATP (enzymatically prepared as described by Walsh and Johnson (9)) dissolved in 20 mM Tris-HCl with 40 mM ATP (vanadium free, Sigma) to a final concentration of 2 mM ATP and 250,000 cpm in the reaction mixture. Aliquots of 0.1 ml were separated at 5, 10, 15, and 20 min and immediately diluted in 0.9 ml of ice-cold 20 mM Tris-HCl (pH 8) containing activated charcoal (2 mg/ml); samples were then centrifuged for 10 min at 1,700 × g, and 0.5 ml of the supernatant fluid was carefully removed for assay of radioactivity in a Searle scintillation spectrometer (Cerenkov radiation) to determine the liberated 32P. [γ-32P]ATP spontaneous hydrolysis represented <4% of the total counts added. Results were calculated as the percentage of radioactivity (cpm) present in the supernatant fluid with respect to the total radioactivity added and were expressed as the percentage [γ-32P]ATP hydrolysis.

**RESULTS**

**Unloaded Erythrocytes.** As expected, the cellular Na^+ concentration remained constant when human erythrocytes were incubated in control medium. Incubation of erythrocytes either with CSF or with 0.1 mM ouabain significantly increased their intracellular Na^+ concentration with respect to the initial Na^+ content (Fig. 1a).

**Preloaded Erythrocytes.** When erythrocytes were incubated in a solution containing p-chloromercuribenzenesulfonate, their Na^+ concentration increased 1.35 times (from 9.8 to 13.5 mmol per liter of erythrocytes; see Fig. 1b). These Na^+-loaded erythrocytes recovered their preload Na^+ content after incubation for 3 hr in control medium. The preloaded erythrocytes incubated in CSF or ouabain-containing media not only failed to recover the initial Na^+ concentration but also further increased their intracellular Na^+ level.

**56Rb^+ Uptake into Human Erythrocytes.** 56Rb^+ uptake into human erythrocytes was significantly inhibited by incubation of erythrocytes in CSF or in a medium containing 0.1 mM ouabain (Fig. 2). The Rb^+ (K^+) influx calculated from data shown in Fig. 2 was 2.8 mmol/liter of erythrocytes per hr.

**Na^+/K^+-ATPase Inhibition.** The Na^+/K^+-ATPase activity is expressed as the rate of [γ-32P]ATP hydrolysis, which remained constant during the experimental time (Fig. 3). The ATPase activity was completely suppressed by 0.1 mM ouabain and was depressed in a dose-dependent manner by incubation in CSF (Fig. 3 Inset). CSF diluted 1:8 showed no inhibitory effect. This inhibition was not due to (i) calcium ions (because Ca^2+ concentration in the CSF samples used ranged between 0.9 and 1.1 mM, and the addition of 1 mM EGTA did not result in a sig-
FIG. 2. Inhibition of $^{86}\text{Rb}^+$ uptake into erythrocytes by human CSF. Human erythrocytes were incubated at 37°C for 1 and 2 hr in control (△), 0.1 mM ouabain-containing (○), and CSF (□) media with $\approx 10^8$ cpm of $^{86}\text{Rb}^+$; $^{86}\text{Rb}^+$ uptake into erythrocytes was calculated in cpm per 10$^9$ cells. Data are reported as the mean ± SEM of the number of experiments, n.

significant variation of the CSF inhibitory effect or (ii) the presence of vanadium ions (because V was not detected in all of the CSF samples investigated by flameless atomic absorption spectrometry).

FIG. 3. In vitro inhibition of a purified Na$^+$/K$^+$-ATPase by human CSF. Na$^+$/K$^+$-ATPase activity was assayed by the rate of [$\gamma$-32P]ATP hydrolysis in control (○), ouabain-containing (△), and CSF (□) media. Results are expressed as the percentage [$\gamma$-32P]ATP hydrolysis with respect to the total radioactivity added to the reaction mixture. (Inset) Percent inhibition of the Na$^+$/K$^+$-ATPase activity produced by different CSF concentrations.

Table 1. Effect of human CSF on erythrocyte intracellular Na$^+$ and $^{86}\text{Rb}^+$ uptake

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>n</th>
<th>Intraellular Na$^+$ increase, %</th>
<th>$^{86}\text{Rb}^+$ Uptake, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Control/0.1 mM ouabain</td>
<td>4</td>
<td>18 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>CSF (nondiluted)</td>
<td>16</td>
<td>13.8 ± 1.5</td>
<td>45.1 ± 4</td>
</tr>
<tr>
<td>CSF (diluted 1:2)</td>
<td>6</td>
<td>6.4 ± 1.9</td>
<td>70.6 ± 3</td>
</tr>
<tr>
<td>CSF (diluted 1:4)</td>
<td>6</td>
<td>2.2 ± 0.7</td>
<td>92.5 ± 3</td>
</tr>
<tr>
<td>CSF (diluted 1:8)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CSF/0.1 mM ouabain</td>
<td>6</td>
<td>17.4 ± 2.8</td>
<td>18 ± 2.5</td>
</tr>
<tr>
<td>CSF (boiled)$^\dagger$</td>
<td>4</td>
<td>14.1 ± 2.3</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>CSF (trypsin)$^\dagger$</td>
<td>4</td>
<td>14.1 ± 2.2</td>
<td>45 ± 2.8</td>
</tr>
</tbody>
</table>

$^\dagger$ Intraellular Na$^+$ concentration after 3 hr was calculated in mmol of erythrocytes per liter, and the results are expressed as the percentage variation with respect to the initial Na$^+$ content (9.8 ± 0.7 mmol/liter of erythrocytes).

$^{86}\text{Rb}^+$ uptake after 2 hr was calculated in cpm per 10$^9$ cells, and the results are expressed as the percentage uptake during incubation in control medium, which was taken as 100%.

$^\dagger$ CSF samples were incubated for 1 hr at 37°C with or without trypsin (1 mg/ml, Sigma). After the incubation period, all reaction mixtures were boiled for 15 min and then assayed for ouabain-like activity.

As shown in Table 1, the CSF also affected the erythrocytes intracellular Na$^+$ concentration and $^{86}\text{Rb}^+$ uptake in a dose-dependent manner. The CSF effect on the ion fluxes was not additive with the action of 0.1 mM ouabain and remained unaltered after boiling or trypsin digestion.

**DISCUSSION**

The effect of the CSF on the intracellular Na$^+$ concentration in Na$^+$-loaded and -unloaded erythrocytes suggests the possible inhibition by CSF of the uphill extrusion of internal Na$^+$, catalyzed by the Na$^+$/K$^+$ pump. The Na$^+$/K$^+$ pump exchanges internal Na$^+$ for external K$^+$; therefore, if this pump is inhibited by CSF, a reduction in the K$^+$ influx also should be expected upon incubation of erythrocytes in CSF. That this seems to be the case is strongly suggested by the ability of CSF to inhibit the $^{86}\text{Rb}^+$ uptake into human erythrocytes. As expected, ouabain (0.1 mM) inhibits both Na$^+$ extrusion and $^{86}\text{Rb}^+$ uptake.

The above-mentioned CSF effect on erythrocyte ion fluxes was not additive with the action of a maximal inhibitory concentration of ouabain (0.1 mM), indicating that, under our experimental conditions, CSF suppresses the ouabain-sensitive ion fluxes across human erythrocyte membranes.

Both ouabain-sensitive Na$^+$ efflux and $^{86}\text{Rb}^+$ uptake provide an index of the Na$^+$/K$^+$ pump activity (10) and its enzymatic machinery, the Na$^+$/K$^+$-ATPase that is specifically inhibited by the cardiac glycoside ouabain. That the reduced pump activity shown after incubation of erythrocytes with CSF may be secondary to the suppression of the Na$^+$/K$^+$-ATPase is strongly supported by the inhibitory effect of human CSF on a highly purified Na$^+$/K$^+$-ATPase system (Fig. 3).

The results of the experiments reported here clearly suggest the existence in human CSF of an endogenous factor that could be similar to the ouabain-like compound recently extracted from mammalian brain (11–13) or postulated to be present in the plasma of volume-expanded animals (14–16) and some hypertensive subjects (17, 18). Like these compounds, the CSF factor seems to have, at least in vitro, ouabain-like activity on Na$^+$/K$^+$-ATPase from different sources.

The inhibition of the Na$^+$/K$^+$-ATPase by the digitalis glycosides is thought to be responsible for their pharmacologic properties, such as the positive inotropic action on cardiac mus-
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