Increased Cytosolic Sodium and Reduced Na,K-ATPase Activity in Transgenic Rats

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Abstract The transgenic rat TGR(mRen2)27 is a new monogenetic model in hypertension research that develops fulminant hypertension after the mouse Ren-2 renin gene has been integrated into its genome. To evaluate the molecular mechanism of development of hypertension in this animal model, we measured cytosolic free sodium concentration in intact lymphocytes from seven transgenic rats and eight age-matched normotensive Sprague-Dawley rats using the novel sodium-sensitive fluorescent dye sodium-binding benzofuranisophthalate. Resting cytosolic sodium was significantly higher in transgenic rats compared with Sprague-Dawley rats (31.7 ± 2.2 versus 18.2 ± 0.4 mmol/L, mean ± SEM, P < .001). Inhibition of Na,K-ATPase by 0.5 mmol/L ouabain for 5 minutes significantly increased lymphocytic cytosolic sodium in Sprague-Dawley rats to 36.5 ± 3.4 mmol/L (P < .001 compared with resting value), whereas no significant change could be observed in transgenic rats (35.4 ± 0.6 mmol/L), indicating that Na,K-ATPase is less responsive in transgenic rats. The Na,K-ATPase activity from erythrocytes was measured with an enzyme-linked assay. Na,K-ATPase activity was significantly reduced in transgenic rats compared with Sprague-Dawley rats (4.0 ± 0.3 versus 8.1 ± 0.6 U/L, P < .001). We concluded that reduced Na,K-ATPase activity leads to elevated cytosolic sodium in this model of genetic hypertension. (Hypertension. 1994;23[suppl I]:I-198-I-202.)

Key Words • sodium • hypertension, sodium-dependent • lymphocytes • animals, transgenic • Na'−K'−transporting ATPase • fluorescent dyes

Intracellular sodium has been shown to be involved in the pathogenesis of hypertension and to be a marker of hypertension.1,2 There are several reports on abnormal sodium handling in erythrocytes, leukocytes, and other tissues in animal models of hypertension.3-7 Recently, a new monogenetic model of hypertension, the transgenic rat strain TGR(mRen2)27, has been established and characterized.8,9 In this strain, an additional mouse Ren-2 gene has been integrated into its genome, and TGR(mRen2)27 rats show a sustained increase in blood pressure up to 300 mm Hg.8,9 Most of the previous studies determining intracellular sodium content were carried out using destructive techniques in lysed cells. The techniques based on newly developed sodium-sensitive fluorescent indicators, such as sodium-binding benzofuran-isophthalate, now allow study of intact cells and thus may be more sensitive in detecting minor changes of cytosolic free sodium concentration ([Na⁺]) and sodium transport systems. To evaluate whether development of hypertension in TGR(mRen2)27 rats is associated with abnormalities of [Na⁺], in the present study we investigated [Na⁺] in intact lymphocytes using a novel fluorescent dye technique. For further characterization of the major sodium transport systems, Na,K-ATPase activity was measured in erythrocytes using an enzyme-linked assay. Blood cells were used because a systemic disturbance of sodium transport may be assumed in the transgenic rat strain. On the other hand, Na,K-ATPase measurements in lymphocytes are subject to a greater variability than those in erythrocytes, which follow well-established techniques.

Methods

Preparation of Lymphocytes

Lymphocytes were obtained from heparinized blood according to previously described methods.10,11 Briefly, blood was centrifuged at 240g for 10 minutes, and the plasma of the supernatant was removed. The lymphocytes were isolated by layering 5 mL of diluted blood (1:1 with isotonic NaCl) on 3 mL of lymphoprep (Boehringer, Mannheim, Germany; 5.6% wt/vol Ficoll; density, 1.077 g/mL) and centrifugation at 240g for 20 minutes. The lymphocyte interphase was carefully aspirated, washed three times in isotonic NaCl by centrifugation at 400g for 5 minutes, and resuspended in Hanks' balanced salt solution containing (mmol/L): NaCl, 136; KCl, 5.4; KH₂PO₄, 0.44; Na₂HPO₄, 0.34; CaCl₂, 1.0; d-glucose, 5.6; and HEPES, 10, pH 7.4. The lymphocyte viability was greater than 95% as determined by the trypan blue exclusion test.

Measurement of [Na⁺]

Fluorescence measurements of [Na⁺] in intact lymphocytes were performed using sodium-binding benzofuran-isophthalate-acetoxy-methyl ester (SBFI-AM, Calbiochem, Bad Soden, Germany) according to recently published methods.12,13 A stock solution of SBFI-AM (1 mmol/L final concentration) was prepared in dimethyl sulfoxide. To 5 mL of lymphocyte suspension (1×10⁴ cells/mL), 30 μL of the membrane-permeant SBFI-AM at a final concentration of 6 μmol/L and 5 μL of the nonionic detergent Pluronic F-127 (Molecular Probes, Eugene, Ore) at a final concentration of 0.1% wt/vol were added. Lymphocytes were incubated with the dye for 60 minutes at 37°C. After centrifugation at 400g for 5 minutes to remove extraneous dye, the lymphocyte pellet was resuspended in fresh Hanks' balanced salt solution, pH 7.4. The fluorescence intensity of 1000 μL suspension of SBFI-loaded...
lymphocytes (1 × 10⁶ cells/mL) in a thermostated quartz cuvette with constant stirring was measured in a fluorescence spectrophotometer (model F-2000, Hitachi Ltd, Tokyo, Japan) using the ROM board (251-0250) (Hitachi Ltd). The light source used was a 150-W xenon lamp with ozone self-dissociation function. Monochromators were large stigmatic concave gratings with 900 lines per millimeter used on both excitation and emission sides. The wavelength drive motors and slit control motors were operated by the computer. The wavelength accuracy was better than ±5 nm. Output signals from the monitor detector and fluorescence detector (photomultiplier) were processed via the A/D convertor. Data sampling interval was 0.5 seconds with alternate excitation wavelengths of 340 and 385 nm (bandwidth, 10 nm), and emission was collected at 500 nm (bandwidth, 10 nm). The fluorescence intensity (F) at 340 and 385 nm excitation decreased by approximately 10% within 1000 seconds. The decrease in fluorescence had been attributed to bleaching and leakage of the fluorescent dye. In contrast, the fluorescence excitation ratio at F340/F385 was calculated and remained constant during the measurements.

The complete hydrolysis of SBFI-AM to SBFI was judged by changes in the excitation and emission spectra. The auto-fluorescence of lymphocytes was more than 110 times lower than the fluorescence of the SBFI-loaded lymphocytes. The auto-fluorescence was subtracted from the observed fluorescence values before the fluorescence excitation ratio was calculated. Measurements were undertaken at 22°C to minimize temperature-dependent dye leakage from lymphocytes. For assessment of leakage of SBFI from the cells, the lymphocyte suspension was centrifuged at 11 000g for 3 minutes, and the fluorescence intensity of the supernatant was measured and subtracted from the observed values of the lymphocyte suspension. The fluorescence intensity of the supernatant was only less than 5% of the fluorescence intensity of the whole lymphocyte suspension. Calibration of the F340/F385 excitation ratio in terms of [Na⁺], was performed in situ on each lymphocyte preparation. SBFI-loaded lymphocytes were added to solutions of known extracellular sodium concentration that were made by appropriate mixtures of high-sodium and high-potassium solutions in the presence of 5 μmol/L monensin and 5 μmol/L nigericin (Sigma, Deisenhofen, Germany). When equilibrations of intracellular and extracellular sodium concentrations were performed by 1 μmol/L gramicidin (Sigma), similar results were obtained. The high-sodium solution contained (mmol/L): sodium gluconate, 110; NaQ, 30; CaCl₂, 1.2; MgCl₂, 0.6; and Na-HEPES, 10. The high-potassium solution was identical except for complete replacement of sodium by potassium.

The excitation spectra of SBFI-loaded lymphocytes titrated in the presence of monensin and nigericin with increasing sodium concentrations in the external buffer are shown in Fig 1. With increasing sodium concentrations, the excitation peak was shifted to shorter wavelengths, a pattern that has been shown to be characteristic for SBFI. The observed calibration curves of [Na⁺]₁, did not show significant differences between the two rat strains studied (Fig 2). Linear regression lines were y=0.07x+4.15 (r=.974) for TGR(mRen2)27 rats and y=0.08x+4.40 (r=.992) for Sprague-Dawley rats, respectively. [Na⁺]₁, was also measured after inhibition of Na,K-ATPase by 0.5 mmol/L ouabain (Sigma), a concentration showing maximal effect on [Na⁺]₁, in rat lymphocytes, or after activation of calcium-sodium exchange by specific inhibitors, nystatin (Calbiochem) or thapsigargin (Calbiochem). Addition of ouabain or thapsigargin did not change the auto-fluorescence or the pH of the suspension.

**Measurement of Na,K-ATPase Activity in Erythrocytes**

Na,K-ATPase activity in erythrocytes was measured using an enzyme-linked assay essentially according to the method described by Hamlyn et al. In that assay, the regeneration of enzymatically hydrolyzed ATP is coupled to the oxidation of NADH, so that the rate of ATP turnover can be monitored by recording the absorbance of NADH at 340 nm. The membrane preparation was identical for both rat strains. After centrifugation of hemeporized blood, the erythrocytes were washed three times with isotonic NaCl solution and then frozen at −18°C. Three freeze-thawing cycles were performed to disrupt the cell membranes. One hundred microliters of the hemolysate was weighed and subsequently diluted to 50-fold of the observed weight with H₂O, thereby adjusting the amount of erythrocyte membranes in all samples. The assay was initiated by rapid addition of 200 μL of that hemolysate suspension to 800 μL of the ATPase cocktail, containing (final concentrations) 100 mmol/L NaCl, 20 mmol/L KCl, 4.5 mmol/L MgSO₄, 5 mmol/L ethyleneglycoltetraacetic acid, 3 mmol/L Na₂-ATP, 0.25 mmol/L NADH, 1.2 mmol/L phosphoenolpyruvate, 1.2 U/L lactate dehydrogenase, 1.2 U/L pyruvate kinase, and 40 mmol/L trimethylamino-ethanesulphonic acid-Tris, pH 7.4. Day-to-day variability of the assay system was less than 3%. Control experiments showed that Na,K-ATPase activity did not decrease significantly with storage time.

**Statistics**

Data are presented as mean±SEM. Where error bars do not appear on figures, errors are within the symbol size. For...
Results

\([\text{Na}^+]\), in resting lymphocytes from hypertensive TGR(mRen2)27 rats was significantly higher compared with normotensive Sprague-Dawley rats (31.7±2.2 versus 18.2±0.4 mmol/L, \(P<.001\); Fig 3).

Fig 4 shows the time course of lymphocytic \([\text{Na}^+]\), after inhibition of Na,K-ATPase by 0.5 mmol/L ouabain. Five minutes after addition of ouabain, \([\text{Na}^+]\), in lymphocytes from TGR(mRen2)27 rats was not significantly different compared with resting values (35.4±0.6 mmol/L, \(P=27\)). On the other hand, 5 minutes after addition of ouabain, a significant \([\text{Na}^+]\), increase could be observed in lymphocytes from Sprague-Dawley rats (36.5±3.4 mmol/L, \(P<.001\) compared with resting values).

Na,K-ATPase was measured in erythrocytes using an enzyme-linked assay. Na,K-ATPase activity was significantly reduced in erythrocytes from TGR(mRen2)27 rats compared with Sprague-Dawley rats (4.0±0.3 versus 8.1±0.6 U/L, \(P<.001\)). Specific inhibition of Ca-ATPase by thapsigargin increased cytosolic free calcium and thereby activates sodium-calcium exchange.19-20 The thapsigargin-induced \([\text{Na}^+]\), increase was mediated by calcium-sodium exchange. In the absence of external calcium, thapsigargin increased \([\text{Na}^+]\), in lymphocytes from Sprague-Dawley rats by 13.2±1.5 mmol/L.

Discussion

In the present study, \([\text{Na}^+]\), was significantly higher in lymphocytes from hypertensive TGR(mRen2)27 rats compared with normotensive Sprague-Dawley rats. \([\text{Na}^+]\), in intact lymphocytes from normotensive rats was

\[18 \text{ mmol/L}, \text{ a value that is in agreement with } [\text{Na}^+], \text{ measured in several cells using the fluorescent dye SBFI. Harootunian et al}^{13} \text{ showed } [\text{Na}^+], \text{ of approximately } 10 \text{ mmol/L in Jurkat tumor lymphocytes. In vascular smooth muscle cells, } [\text{Na}^+], \text{ of approximately 14 mmol/L}^7 \text{ and approximately 24 mmol/L have been reported.}^{21} \text{ Donoso et al}^{12} \text{ found } [\text{Na}^+], \text{ of approximately 11 mmol/L in rat cardiac myocytes.}

Measurement of [Na\(^+\)], in intact cells may be influenced by the number of cells, incomplete hydrolysis of the dye, intracellular microviscosity, calibration of the fluorescence signal, and dye leakage from the cells. In the present study, these problems were considered and addressed by the following points. The number of intact washed lymphocytes was adjusted to 1×10\(^6\)/mL, and the calibration of the fluorescence signal was performed in situ on each preparation using the same cell count. The dual-wavelength excitation method with alternate excitation wavelengths of 340 and 385 nm is a prerequisite for sodium measurements using SBFI.\(^{13-15}\) The complete hydrolysis of the dye was judged by changes in the excitation and emission spectra.\(^2\) Intracellular microviscosity may contribute to the fluorescence intensity of intracellular trapped SBFI.\(^{13} \text{ Therefore, it is necessary to calibrate the fluorescence signal in terms of } [\text{Na}^+], \text{ in each cell preparation in situ by suspending dye-loaded lymphocytes in buffer with known extracellular sodium concentrations in the pres-}

**Fig 3.** Bar graph shows cytosolic free sodium concentration ([Na\(^+\)]) in lymphocytes from hypertensive transgenic rats (TGR) and normotensive Sprague-Dawley rats (SD). Values are mean±SEM. ***\(P<.001\) between groups.

**Fig 4.** Line plot shows effect of inhibition of Na,K-ATPase by ouabain on cytosolic free sodium concentration ([Na\(^+\)]) in lymphocytes from hypertensive transgenic rats (TGR) and normotensive Sprague-Dawley rats (SD). Fluorescent dye-loaded lymphocytes from TGR (●) and SD (○) were exposed to 0.5 mmol/L ouabain, and [Na\(^+\)] was evaluated.

**Fig 5.** Graph shows effect of inhibition of Ca-ATPase by thapsigargin on cytosolic free sodium concentration ([Na\(^+\)]) in hypertensive transgenic rats (TGR(mRen2)27) and normotensive Sprague-Dawley rats (SD). A, Lymphocytic [Na\(^+\)] after addition of 5 μmol/L thapsigargin at the indicated time (arrow); B, addition of 6 mmol/L NiCl\(_2\); C, addition of 6 mmol/L NiCl\(_2\) before 5 μmol/L thapsigargin. Tracings are representative of at least four separate experiments.

**Fig 4.** Unlabeled plot shows effect of inhibition of Na,K-ATPase by ouabain on cytosolic free sodium concentration ([Na\(^+\)]) in lymphocytes from hypertensive transgenic rats (TGR) and normotensive Sprague-Dawley rats (SD). Fluorescent dye-loaded lymphocytes from TGR (●) and SD (○) were exposed to 0.5 mmol/L ouabain, and [Na\(^+\)] was evaluated.
ence of ionophores to equilibrate extracellular and intracellular sodium content. The perturbing effect of compartmentation of the dye may be minimized (although probably not completely eliminated) by that calibration procedure, because the dye trapped in organelles should be nearly constant, so that it is approximately canceled out by the in situ calibration. The observed calibration curves of [Na\(^+\)]

have long been implicated in the pathogenesis of hypertension. An increased [Na\(^+\)]

in vascular smooth muscle cells may increase vascular tone first by increasing sensitivity to catecholamines, second by cell swelling with subsequent decrease of the vessel lumen, and third by inhibition of calcium extrusion by the sodium-calcium exchange.

To extrapolate the lymphocyte findings to vascular smooth muscle, a systemic alteration of sodium transport caused by alteration generalized in the genetic code must be assumed. Although in TGR(mRen2)27 rats an increased plasma aldosterone concentration has been described, increased resting [Na\(^+\)]

in TGR(mRen2)27 rats may not be due to hyperaldosteronism for several reasons. First, plasma potassium and sodium concentrations were not different between hypertensive TGR(mRen2)27 rats and normotensive Sprague-Dawley rats. Second, aldosterone has been linked to increased activity of the Na,K-ATPase in red blood cells, vascular smooth muscle cells, and rat tail arteries. In contrast, in the present study a reduced Na,K-ATPase activity was found in TGR(mRen2)27 rats. Third, reduced resting [Na\(^+\)]

in platelets has been reported recently in patients with primary aldosteronism.

In numerous studies, an elevation of cytosolic free calcium concentration was found in both essential hypertension and spontaneously hypertensive rats. Cytosolic calcium may be elevated by several mechanisms, including an increased calcium influx through calcium channels, a decreased Ca-ATPase activity, or a decreased calcium extrusion through the sodium-calcium exchange. It is the latter mechanism in which an elevation of [Na\(^+\)]

could be related to the observed changes in genetic hypertension.

Na,K-ATPase activity was decreased in erythrocytes of the transgenic rat strain. This may be an indirect indication that the increase in [Na\(^+\)]

due to decreased active sodium transport. This hypothesis is supported by the finding that inhibition of Na,K-ATPase by ouabain did not significantly increase [Na\(^+\)]

in TGR(mRen2)27 rats within 5 minutes, whereas ouabain significantly increased [Na\(^+\)]

in Sprague-Dawley rats. Therefore, an increase of passive sodium permeability is not a likely explanation for elevated [Na\(^+\)]

in TGR(mRen2)27 rats, because the sodium influx after ouabain is not accelerated in TGR(mRen2)27 rats compared with Sprague-Dawley rats, although that finding only allows an indirect and rough estimation of the passive sodium permeability. The influx through the sodium-calcium exchange should be increased by increasing cytosolic calcium by thapsigargin, which releases intracellular stored calcium. The inhibition of the thapsigargin-induced sodium increase by NiCl\(_2\)
supports the view that the sodium-calcium exchange is involved in that process, because NiCl\(_2\)
is known to inhibit that transport system. However, it cannot be ruled out that NiCl\(_2\)
may also inhibit calcium channels. The thapsigargin-induced [Na\(^+\)]

increase also was obtained in the absence of external calcium. This does not argue against an activation of the sodium-calcium exchange in the calcium extrusion mode, because the rise of cytosolic calcium after calcium release from intracellular stores may stimulate sodium influx by the sodium-calcium exchange.

In summary, the present study indicates that [Na\(^+\)]

is elevated in lymphocytes from TGR(mRen2)27 rats, probably because of reduced activity of Na,K-ATPase in this novel rat model of hypertension.

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References


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