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

Martin Tepel, Gregor Theilmeier, Jürgen Bachmann, Michael Barenbrock, Claus Spiekermann, Detlev Ganten, Karl-Heinz Rahn, Walter Zidek

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 benzofuran-isophthalate. 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[6]: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+]i) and sodium transport systems. To evaluate whether development of hypertension in TGR(mRen2)27 rats is associated with abnormalities of [Na+]i, in the present study we investigated [Na+]i 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+]i

Fluorescence measurements of [Na+]i in intact lymphocytes were performed using sodium-binding benzofuran-isophthalate-acetoxyhemethylster (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 (1×10^6 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. The 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 static 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 340/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 autofluorescence of lymphocytes was more than 110 times lower than the fluorescence of the SBFI-loaded lymphocytes. The autofluorescence 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 11 000 g 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. The autofluorescence of the supernatant was less than 5% of the fluorescence intensity of the whole lymphocyte suspension.

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statistical evaluation of the data, the Wilcoxon test was used, and two-tailed values of P<.05 were considered significant. The original experimental tracings shown in the figures were computed by locally weighted scatterplot smoothing (GraphPad Software Inc, San Diego, Calif).

Results

[Na\(^+\)]\(_i\), 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 [Na\(^+\)]\(_i\), after inhibition of Na,K-ATPase by 0.5 mmol/L ouabain. Five minutes after addition of ouabain, [Na\(^+\)]\(_i\) 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 [Na\(^+\)]\(_i\) 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 13.2±1.5 mmol/L, P<.001).

Specific inhibition of Ca-ATPase by thapsigargin increases cytosolic free calcium and thereby activates sodium-calcium exchange. The thapsigargin-induced [Na\(^+\)]\(_i\), increase was mediated by calcium-sodium exchange. In the absence of external calcium, thapsigargin increased [Na\(^+\)]\(_i\) in lymphocytes from Sprague-Dawley rats to 49.4±12.8 mmol/L (P=.11 compared with resting values) and in lymphocytes from Sprague-Dawley rats to 47.8±3.8 mmol/L (P<.001). NiCl\(_2\) has been shown to inhibit sodium-calcium exchange. The thapsigargin-induced [Na\(^+\)]\(_i\) increase could be prevented by addition of 6 mmol/L NiCl\(_2\) (Fig 5), indicating that thapsigargin-induced [Na\(^+\)]\(_i\) increase was mediated by calcium-sodium exchange. In the absence of external calcium, thapsigargin increased [Na\(^+\)]\(_i\) in lymphocytes from Sprague-Dawley rats by 13.2±1.5 mmol/L.

Discussion

In the present study, [Na\(^+\)]\(_i\), was significantly higher in lymphocytes from hypertensive TGR(mRen2)27 rats compared with normotensive Sprague-Dawley rats. [Na\(^+\)]\(_i\), in intact lymphocytes from normotensive rats was 18 mmol/L, a value that is in agreement with [Na\(^+\)]\(_i\), measured in several cells using the fluorescent dye SBFI. Harootunian et al\(^{13}\) showed [Na\(^+\)]\(_i\), of approximately 10 mmol/L in Jurkat tumor lymphocytes. In vascular smooth muscle cells, [Na\(^+\)]\(_i\), of approximately 14 mmol/L and approximately 24 mmol/L have been reported.\(^{21}\) Donoso et al\(^{22}\) found [Na\(^+\)]\(_i\), of approximately 11 mmol/L in rat cardiac myocytes.

Measurement of [Na\(^+\)]\(_i\), 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.\(^{12-15}\) In the present study, these problems were considered and addressed by the following points. The number of intact washed lymphocytes was adjusted to 1×10^8/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.\(^{7}\) Intracellular microviscosity may contribute to the fluorescence intensity of intracellular trapped SBFI.\(^{13}\) Therefore, it is necessary to calibrate the fluorescence signal in terms of [Na\(^+\)]\(_i\), in each cell preparation in situ by suspending dye-loaded lymphocytes in buffer with known extracellular sodium concentrations in the pres-
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\(^+\)]\(_i\) did not show significant differences between the two rat strains studied, indicating that measurements of [Na\(^+\)]\(_i\) in lymphocytes are not biased by the rat strain per se. For assessment of the fluorescence intensity caused by dye leakage out of the lymphocytes, the fluorescence was also measured in the supernatant after centrifugation of the lymphocyte suspension and subtracted from the observed values.

Elevated [Na\(^+\)]\(_i\) values have long been implicated in the pathogenesis of hypertension. An increased [Na\(^+\)]\(_i\) value 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. The passive sodium permeability. The influx through the lymphocyte suspension and subtracted from the observed values.

In numerous studies, an elevation of cytosolic free calcium concentration was found in both essential hypertension. An increased [Na\(^+\)]\(_i\) 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 [Na\(^+\)]\(_i\) 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 to 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\(^+\)]\(_i\) may be 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\(^+\)]\(_i\) in TGR(mRen2)27 rats within 5 minutes, whereas ouabain significantly increased [Na\(^+\)]\(_i\) in Sprague-Dawley rats. Therefore, an increase of passive sodium permeability is not a likely explanation for elevated [Na\(^+\)]\(_i\) 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, and 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.

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