Increased Membrane Permeability of Skin Fibroblasts from the Spontaneously Hypertensive Rat

HIROKAZU TAMURA, MINORU KINO, AIJIROH TOKUSHIGE, BERNARD M. SEARLE, AND ABRAHAM AVIV

SUMMARY  Recently, we have demonstrated several abnormalities in Na+ and K+ homeostasis in cultured vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR). To study whether similar defects can be identified in other cells of this rat strain, 86Rb and 22Na flux experiments as well as measurements of intracellular Na+ and K+ levels were performed in cultured skin fibroblasts of SHR and normotensive Wistar-Kyoto rats (WKY). The efflux rate constant (kj for Rb+ (K+ analogue) was higher (p<0.001) in fibroblasts of SHR than in those of WKY (2.11 ± 0.03 and 1.66 ± 0.02 × 10⁻²/min; mean ± SEM). The ouabain-insensitive influx rate constant (kj) for Rb+ was also higher (p<0.001) in fibroblasts of SHR than in those of WKY (13.26 ± 0.41 and 10.71 ± 0.27 × 10⁻²/min). On the other hand, the activity of the Na+–K+ pump of cells of SHR (44.81 ± 0.81 × 10⁻⁵/min) was not different from that of cells of WKY (44.72 ± 0.47 × 10⁻⁵/min). This parameter was obtained by calculating the ouabain-sensitive Rb+ influx rate constant. There was also no difference in the Na+ uptake (in the presence of ouabain) between cells of the two rat strains. Although there was no statistically significant difference in the measured intracellular total K+ levels between the two groups, on the basis of equilibrium distribution of ⁸⁶Rb+, we calculated a significantly lower (p<0.001) level of exchangeable intracellular K+ in fibroblasts of SHR (98.2 ± 1.2 mEq/L) as compared with cells of WKY (115.3 ± 1.5 mEq/L). These findings indicate increased membrane permeability to K+ in fibroblasts of SHR and that this defect is likely to be innate to their membrane structure. (Hypertension 7: 300-305, 1985)

KEY WORDS  • ⁸⁶Rb • ²²Na • ion flux • Na+-K+ pump • intracellular Na+ and K+

ALTHOUGH an increase in peripheral vascular resistance is a common feature in established human essential hypertension, the exact cellular abnormalities responsible for this phenomenon are not entirely understood. Considerable evidence has been accumulated indicating that alterations in cellular ion regulation may contribute to the pathogenesis of hypertension. These include alterations in passive cation movements and the Na+–K+ pump activity, as demonstrated in blood cells of patients with essential hypertension. Similar defects also have been observed in erythrocytes as well as in vascular tissues of spontaneously hypertensive rats (SHR). It has been suggested that if these abnormalities occur in the vascular smooth muscle, they may result in a finite increase in cytosolic Na+ and consequently Ca2+, which in turn, will increase the vascular tone and predispose to hypertension.

Recently, we have demonstrated (unpublished data, 1984) that serially passed, cultured vascular smooth muscle cells (VSMCs) obtained from SHR exhibit abnormalities in their Na+ and K+ homeostasis (i.e., increased membrane permeabilities to Na+ and K+ associated with a higher Na+–K+ pump activity). As these alterations were observed in cultured cells, it may be assumed that they reflect innate defects in the VSMC membrane of the SHR rather than a secondary manifestation of the elevated arterial pressure or the effects of humoral factors. The present study was performed to determine whether some of these abnormalities of VSMCs of SHR are generalized and can be demonstrated in other cultured cells.
Materials and Methods

Fibroblasts from male SHR and normotensive Wistar-Kyoto rats (WKY), 14–17 weeks old, were studied. Systolic blood pressure was measured in conscious animals with tail-cuff plethysmography (IITC Inc., Landing, NJ).

The fibroblasts were obtained from abdominal skin patches. The patches (approximately 2 cm²) were aseptically removed while the rats were under pentobarbital anesthesia. They were immediately immersed in Dulbecco’s Modified Eagles Medium (DMEM, GIBCO Laboratories, Grand Island, NY) containing 150 μg of penicillin/ml and 150 μg of streptomycin/ml and were dissected into small pieces. Subsequently, the pieces were incubated in the same medium plus 0.25% collagenase for 1.5 hours at 37°C in 5% CO₂:95% air. The dispersed cells were added to flasks (75 cm²), and all cultures were left undisturbed in a growth medium at 37°C and 5% CO₂:95% air for 12 to 14 days. The cells were fed twice weekly with a growth medium consisting of DMEM plus 292 μg/ml of l-glutamine with antibiotics (50 μg of penicillin/ml, 50 μg of streptomycin/ml) and 10% heat-inactivated fetal calf serum. Cells from passages 5 through 7 were studied.

For the ion flux experiments, aliquots of 6 × 10⁶ cells were inoculated into each well of Nunc-24-well plates (Interlab, Thousand Oaks, CA). Growth medium without antibiotics was used to grow the cells in the wells. The experiments were performed on the third day after inoculation, when the cell layers were confluent. After removing the growth medium, the cells were washed twice with 0.75 ml of DMEM and aliquots (0.5 ml) of DMEM containing 145 mM Na⁺, 5 mM K⁺, and either ⁸⁶Rb (2.0 μCi/ml; 0.1 mM RbCl) or ⁴²Na (0.7 μCi/ml) were added to the wells. Rb⁺ was used as a K⁺ analogue. For ⁸⁶Rb and ⁴²Na uptake experiments, the cells were incubated at 37°C in 5% CO₂:95% air for up to 180 minutes and 120 minutes respectively. Ouabain (1 mM) was used to inhibit the Na⁺-K⁺ pump. The experiments were terminated by aspirating the media and then rapidly washing the cells four times with 0.75-ml aliquots of ice-cold 0.1 M MgCl₂. The cells were then extracted for 1 hour with 5% trichloroacetic acid, and their ⁸⁶Rb or ⁴²Na activities were determined. The Rb⁺ influx rate constant (kᵢ) values were calculated with a two compartmental analysis, as described in the Appendix.

The Rb⁺ efflux experiments were performed as follows. The cells were preloaded with ⁸⁶Rb by incubation for 180 minutes in the same medium used for the Rb⁺ uptake studies. After the preloading, the cells were rapidly washed twice with 1 ml of ice-cold DMEM. The cells were then incubated in 1 ml of DMEM at 37°C in 5% CO₂:95% air for up to 60 minutes. The efflux experiments were terminated by aspirating the media, which was followed by washing the cells as described in the previous paragraph. As the efflux of ⁸⁶Rb⁺ was monoexponential within the indicated time intervals, the efflux rate constant (kₑ) values were calculated from the formula $A = A₀e^{-kt}$, where $A$ = ⁸⁶Rb activities remaining in the cells at given incubation time; $A₀$ = ⁸⁶Rb activities at the initiation of the efflux experiments; $kₑ$ = efflux rate constant; $t$ = incubation time. The $kₑ$ values were also calculated from the Rb⁺ uptake experiments (see Appendix).

The intracellular Na⁺ and K⁺ levels were determined as follows. Aliquots of 6 × 10⁶ cells were inoculated into each well of Nunc-6-well (35 mm in diameter) tissue clusters. On the third day after the inoculation, the growth medium was removed and the cells were washed five times with 1 ml of ice-cold 0.1 M MgCl₂. One milliliter of distilled deionized water was added to each well, the cells were frozen in dry ice for 30 minutes, and then they were thawed at room temperature. Aliquots of the solutions were measured for Na⁺ and K⁺ in a Perkin-Elmer atomic absorption spectrometer (Norwalk, CT).

The intracellular ion levels were expressed in terms of intracellular water volume. This parameter was obtained by the intracellular distribution of 3-0-methyl-d-glucose as described elsewhere. Statistical methods utilized Student’s unpaired t test and linear regression analysis. Two parameter nonlinear regression analysis was used for the total Rb⁺ uptake experiments. This was performed by an NLIN procedure for statistical analysis system (SAS; SAS Institute Inc., Cary, NC) with an IBM 3033-U computer. Data are presented as means ± SEM.

Results

The respective systolic blood pressure values of SHR and WKY were 218.5 ± 2.4 (n = 7) and 116.5 ± 3.8 (n = 8) mm Hg.

Figure 1 and Table 1 summarize the results of the Rb⁺ efflux experiments. Both in the presence and absence of ouabain, the Rb⁺ $kₑ$ values of cells of SHR were significantly higher than those of cells of WKY (p < 0.001). In the presence of ouabain, substantial increases in $kₑ$ values were observed in both groups (37% increase, p < 0.001 for fibroblasts of SHR; 24% increase, p < 0.001 for cells of WKY). Among other factors, these increases may relate to the detergentlike effect of ouabain on the cell membrane or to an increase in intracellular Na⁺ concentration, which leads to a rise in cytosolic Ca²⁺. A rise in cytosolic Ca²⁺ has been shown to increase K⁺ permeability.

Figure 2 depicts the Na⁺ uptake as a function of incubation time. The Na⁺ uptake experiments were carried out only in the presence of ouabain in the medium. There were no demonstrable differences in the uptake of Na⁺ between fibroblasts of the SHR and WKY.

The results of the Rb⁺ uptake experiments are shown in Figure 3 and Table 2. When the Na⁺-K⁺ pump was active, intracellular Rb⁺ levels reached a plateau after 120 to 150 minutes of incubation. On the other hand, when ouabain was present in the media, a new steady state for Rb⁺ was reached after 90 minutes of incubation. It is of interest that in the absence of ouabain the mean value of the intracellular Rb⁺ concentration of cells of SHR at the plateau phase was
Figure 1. Rb⁺ efflux from fibroblasts obtained from SHR (●) and WKY (•) in the absence (A) and the presence (B) of 1 mM ouabain. Correlation coefficients of each line are: -0.988 for SHR and -0.988 for WKY (A); -0.973 for SHR and -0.985 for WKY (B). A = ^Rb activity remaining in the cells at given incubation time; A₀ = ^Rb activity at the initiation of the incubation. Vertical lines denote the SEM. When the vertical line is absent, the SEM is equal to or less than the space of the symbol. Each symbol represents the mean of eight observations.

TABLE 1. Rb⁺ Efflux Rate Constant Values (kₑ, 10⁻²/min)

<table>
<thead>
<tr>
<th>Condition</th>
<th>SH</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ouabain</td>
<td>2.11 ± 0.03* (32)</td>
<td>1.66 ± 0.02 (32)</td>
</tr>
<tr>
<td>1 mM ouabain</td>
<td>2.89 ± 0.06* (32)</td>
<td>2.06 ± 0.03 (32)</td>
</tr>
</tbody>
</table>

Numbers of observations are indicated in parentheses.
*Significant at p < 0.001 as compared with WKY.

lower than that of cells of WKY (i.e., respectively, 1.96 ± 0.02 mEq/L, n = 38, and 2.31 ± 0.03 mEq/L, n = 31; p < 0.001). The computer-predicted curves, which were obtained from the model described in the Appendix, are depicted in Figure 3. The observed data closely fit these curves. Table 2 presents the kₑ values obtained from Figure 3. Ouabain-sensitive kₑ values, which represent the Na⁺-K⁺ pump activity, were computed from the difference between total and ouabain-insensitive kₑ values. The total and ouabain-insensitive kₑ values of cells of SHR were significantly higher than those of cells of WKY (p < 0.001); however, the difference in the total kₑ value was minimal (4.8%). Furthermore, there was no difference in the ouabain-sensitive kₑ between fibroblasts of the two rat strains.

The model described in the Appendix also renders it possible to calculate the kₑ values from the Rb⁺ uptake experiments. The kₑ value calculated for fibroblasts of SHR was 2.89 ± 0.01 x 10⁻²/minute (n = 107) whereas that of fibroblasts of the WKY was 2.38 ± 0.01 x 10⁻²/minute (n = 110). The difference was significant at p < 0.001. It is noteworthy that these values are somewhat higher than those documented by direct measurements of Rb⁺ efflux (Table 1). The discrepancy between the kₑ values calculated from the uptake and efflux experiments may have resulted from the different treatments of the cells. Nonetheless, the difference between the fibroblasts of SHR and WKY was consistent.

The basal intracellular Na⁺ and K⁺ concentrations (Table 3) and the intracellular water volumes were not statistically different between fibroblasts of SHR and WKY. The intracellular water volume of the cells of SHR was 2.01 ± 0.06 µL/10⁶ cells (n = 23) compared with 1.94 ± 0.08 (n = 23) µL/10⁶ cells for cells of WKY.

Discussion

The central finding of the present study is that of increased K⁺ permeability in cultured skin fibroblasts
of the SHR. Abnormalities of cellular Na⁺-K⁺ homeostasis in hypertensive patients have been documented, although the results are not always consistent. For instance, the activity of the Na⁺-K⁺ co-transport in erythrocytes of hypertensive patients has been shown to be lower, higher, and unchanged compared with that of normotensive controls. Another example relates to the activity of the Na⁺-K⁺ pump, which has been shown to be lower, higher, and not different compared with normotensive erythrocytes of hypertensive individuals versus their normotensive counterparts. Similar discrepancies have been shown in vascular tissues of laboratory animals with experimentally induced hypertension. Differences in experimental conditions may explain some of the discrepancies. It should be noted, however, that studies in red blood cells or blood vessels can be performed only within a short time after obtaining these tissues. Thus, circulating extracellular factors operating in the organism in vivo may exert lasting effects while the tissues are examined in vitro. For example, several hormones and the natriuretic factor can affect cellular Na⁺-K⁺ regulation. The effects of these factors may also render it difficult to determine whether the abnormalities identified are primary or secondary. In contrast, abnormalities demonstrated in serially passed cultured cells are unquestionably innate to the structure of the cells, though it is essential to show that these defects have not been acquired by the cells owing to the in vitro conditions. As a higher permeability to K⁺ has been demonstrated in erythrocytes and other tissues of the SHR, it is likely that our finding of K⁺ "leakiness" in the cultured fibroblasts of the SHR represents an innate defect of these cells and not an acquired phenomenon relating to the in vitro conditions. We have reached the same conclusions with regard to cultured VSMCs obtained from the SHR.

The higher $k_e$ value for Rb⁺ in fibroblasts of the SHR cells indicates increased membrane permeability to K⁺. This increased permeability of the fibroblasts of SHR is associated with the same activity of the Na⁺-K⁺ pump as that of cells of WKY. It is therefore expected that compared with the latter, cells of SHR should demonstrate either augmented, nonactive K⁺ influx or lower intracellular concentrations of K⁺. The total $k_r$ for Rb⁺ was higher in fibroblasts of the SHR than in those of WKY; however, this increase (4.8%) cannot compensate for the 27% higher $k_e$ values in the cells of SHR. The data also indicate that the mean intracellular K⁺ level is lower in fibroblasts of SHR than in those of WKY, yet this difference is not statistically significant. One possible reason for the discrepancy of the results may be the fact that we measured the total and not the free intracellular K⁺. One may assume that at the plateau phase of the Rb⁺ uptake (Figure 3), the exchangeable intracellular Rb⁺/K⁺ ratio is equivalent to the ratio between the respective ions in the incubation medium. Using such an argument, we have calculated that the intracellular exchangeable K⁺
would be $98.2 \pm 1.2$ mEq/L ($n = 38$) for fibroblasts of SHR and $115.4 \pm 1.5$ mEq/L ($n = 31$) for cells of WKY. This difference is statistically significant at $p < 0.001$. It concurs with findings by Hermsmeyer, who measured membrane potentials in vascular tissue of SHR and WKY and calculated lower intracellular K+ concentrations in vessels of the SHR. The values calculated for the exchangeable over total intracellular K+ (69% for SHR, 76% for WKY) are in agreement with other observations pertaining to the free intracellular concentrations of this ion made in different species and tissues.

The activity of the Na+-K+ pump has been examined in vascular tissues and erythrocytes of the SHR. Using a variety of methods, many investigators have demonstrated higher Na+-K+ pump activity in this rat strain as compared with normotensive controls. Recently, we have shown the same phenomenon in cultured VSMCs of this rat strain (unpublished data, 1984). In the same study we speculated that the most likely cause of the augmented Na+-K+ pump activity in VSMCs of SHR was the increased membrane permeability to Na+ that was readily demonstrated by Na+ flux experiments. In the present study no apparent differences in Na+ uptake curves or cytosolic Na+ concentrations were shown between cells of SHR and WKY. This finding may well be the reason for the equal Na+-K+ pump activities of these cells. As the Na+ uptake curves incorporated both influx and efflux of the ion, and as outward non-active transport of Na+ has not been examined in the present study, the possibility of an increase in Na+ permeability of fibroblasts of SHR cannot be entirely excluded.

**Conclusion**

Our results indicate that compared with the cultured VSMC of the SHR, the cultured skin fibroblasts of SHR demonstrate K+ "leakiness" but not an increase in the passive Na+ uptake or augmented activity of the Na+-K+ pump. This defect in K+ permeability is likely to represent an innate structural abnormality in the fibroblast of the SHR.

**Appendix**

Analysis of a two-compartment model is applied to describe the Rb+ distribution across the cell membrane.

$$K_e + Rb_e = K_i + Rb_i$$

where $K_e$ = extracellular K+ concentration

$Rb_e$ = initial extracellular Rb+ concentration

$K_i$ = intracellular K+ concentration

$Rb_i$ = intracellular Rb+ concentration

$k_i$ = Rb+ influx rate constant

$k_e$ = Rb+ efflux rate constant

The rate of Rb+ influx is described by:

$$d(K_i + Rb_i)\over dt = k_i \cdot (K_e + Rb_e - Rb_{i,m}) - k_e \cdot (K_i + Rb_i)$$

where $Rb_{i,m} = \text{the amount of Rb+ per unit volume of incubation medium that enters the cells.}$

In case of steady state for $K_i$,

$$dK_i\over dt = 0$$

and

$$k_i \cdot K_e = k_e \cdot K_i$$

Furthermore,

$$Rb_{i,m} = Rb_e \cdot V$$

where $V = \text{intracellular water volume per unit volume of incubation medium.}$

Thus,

$$dRb_i\over dt = k_i \cdot (Rb_e - Rb_i \cdot V) - k_e \cdot Rb_i$$

Integration of Equation 6 results in

$$Rb_i = Rb_{i,m} \cdot \frac{k_i}{k_e \cdot V + k_i} \cdot [1 - e^{-k_e \cdot V \cdot \frac{k_i}{k_e} t}]$$

In the presence of ouabain, Equations 3 and 4 are not fulfilled until a new steady state condition is reached with respect to intracellular Rb+. This shortcoming renders Equation 7 inapplicable. When the Na+-K+ pump is blocked, however, a new steady state is reached for Rb+ within 90 minutes of incubation (Figure 3). Under these circumstances, solution of Equation 6 results in

$$Rb_{i,a} = Rb_e \cdot \frac{k_i}{k_e \cdot V + k_i}$$

where $Rb_{i,a}$ = intracellular Rb+ concentration at the new steady state.

The $k_i$ can be calculated by using the $k_i$ values obtained from the efflux experiments:

$$k_i = \frac{Rb_{i,m} \cdot k_e}{Rb_e - Rb_{i,a} \cdot V}$$

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