Biochimica et Biophysica Acta, 367 (1974) 59–66

BBA 76732

CHARACTERIZATION OF THE STEADY STATE IN MOUSE LYMPHOBLASTS CULTURED IN HYPOTONIC MEDIUM

BRENDA BUCKHOLD SHANK and NANCY EGAN SMITH

Physiology Department, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, N. J. 08854 (U.S.A.)

(Received May 2nd, 1974)

SUMMARY

1. Mouse lymphoblasts (L5178-Y), cultured in hypotonic medium, grow at a slower rate than in their normal culture medium.

2. Cells grown in 70 % (v/v) medium for several days have a nearly normal cell volume, lower intracellular K^+ (79 % of normal), Na^+ (64 %), and Cl^- concentrations (63 %).

3. Utilizing the equations based on constant-field assumptions, the permeability ratio, P_{Na^+}/P_{K^+}, remains constant at 0.54. $Na^+ : K^+$ pump ratios are 1 : 1 for cells in the steady state in both 70 % (v/v) and 100 % (v/v) medium. Passive ionic fluxes in 70 % (v/v) medium have decreased to 74 % of control levels for both Na^+ and K^+.

4. $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) activity remains at the control level of 2.0 mM $P_i/10^8$ cells per h for cells in the hypotonic environment.

5. These results are compatible with pump regulation by means of intracellular Na^+ concentration rather than an alteration of number of pump sites.

INTRODUCTION

Previous publications [1–4] have described the phenomenon of osmotic adaptation in cultured mammalian cells, in which cells suspended in hypotonic medium at 37.5 °C first swell rapidly and then shrink in about 10 min toward their original volume. The shrinking phase has been shown to be dependent upon an active Na^+ pump [2] and the ionic mechanism has been described [3]. Mouse lymphoblasts have been shown to grow in their usual isotonic medium at a normal rate after a 10-min hypotonic shock in 35 % medium–65 % water (35 % (v/v) medium) and even grow at a reduced rate after a 10-min shock in 20 % (v/v) medium [1]. In addition, cells may grow continuously [5, 6] in medium as hypotonic as 60 % (v/v) medium.

In this publication, we demonstrate that, in the steady state, growth in hypotonic medium is associated with reduced intracellular Na^+ and K^+ concentrations, constant $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) activity, reduced passive fluxes of Na^+ and K^+, and as a result, reduced active transport of Na^+ and K^+. This is compatible with pump regulation in vivo through regulation of enzyme activity by intracellular Na^+,

without an alteration in enzyme levels per se. $Na^+ : K^+$ pump ratios were 1 : 1 for cells in the steady state in hypotonic and normal isotonic medium.

MATERIALS AND METHODS

Cells

L5178-Y mouse lymphoblasts, grown at 37.5 °C in Fischer's medium (Flow Laboratories) supplemented with 10 % horse serum (Flow Laboratories), were used in these experiments. Normal Na^+ concentration in the cell medium was 140 mequiv/l and K^+ concentration was 5.0 mequiv/l. Cells were cultured routinely in screw-top Falcon plastic tubes and in screw-top glass Erlenmeyer flasks for experiments, with caps loose in a 7 % CO_2 environment.

Cell growth in dilute medium

Standard cell medium was diluted with sterile distilled water for the appropriate final tonicity and cells were added.

Cells were grown for 2–3 days (3–5 generations) in these solutions, with aliquots taken for cell counts and determinations of cell volume. Cell counts and median cell volume were measured in the appropriate solution tonicity on an electronic particle counter (Coulter, Model Z). Absolute volumes were determined by comparison with standard 9.7 μm polystyrene divinylbenzene particles (Particle Information Service). There is an apparent cell-volume shift due to the concomitant conductivity change in hypotonic solutions; this was corrected by a factor determined from the apparent volume shifts of the standard particles in hypotonic solutions.

Ion concentration measurements

Cells grown in hypotonic and in normal medium were centrifuged, washed rapidly with an ice-cold balanced salt solution with LiCl substituted for NaCl and glucose added, concentrated, lysed in 98 % distilled water, and analyzed for Na^+ and K^+ flame photometrically, using the same amount of the LiCl solution in the Na^+ and K^+ standards and cell samples as an internal standard.

Intracellular ion concentration $[X]_c$ (in mequiv/l) was calculated by the following equation:

$$f[X]_c+(1-f)[X]_s = [X]_t$$

or rearranged,

$$[X]_c = \frac{[X]_t-(1-f)[X]_s}{f} \qquad (1)$$

where $[X]_t$ = the total concentration of X, in mequiv/l, of the cells in suspension, f = the fractional volume of the cells relative to the whole suspension, and $[X]_s$ = concentration of X in mequiv/l, in the suspending solution. For Na^+ and K^+ determinations, $[X]_s$ is zero in the LiCl solution, so the calculation is simply

$$[X]_c = \frac{[X]_t}{f}$$

and f is merely

$\overline{V}_c \cdot c$

where $\overline{V}_c$ = median cell volume (ml/cell) and c = the cell concentration in the suspension (cells/ml). Both of these parameters were determined on a diluted aliquot of the cells concentrated in the LiCl-balanced salt solution, using the electronic particle counter described above.

For Cl^- determinations, cells were highly concentrated in their own medium so that $f > 0.12$, i.e. at least 12 % of the suspension volume consisted of cell volume. One aliquot was lysed in the usual acid reagent for measurement of Cl^- concentration, $[X]_t$, on a Buchler Chloridometer. The Cl^- concentration in cell medium per se, $[X]_s$, was also measured. An aliquot of the suspension was diluted, then counted and sized, so that f might be determined and intracellular Cl^- calculated from Eqn 1.

$(Na^+ + K^+)$-ATPase determination

Each culture (100 ml) was grown for 2 days, to a level of approx. $2 \cdot 10^5$ cells/ml.

Cultures were then centrifuged, washed once in 20 ml serum-free medium of the appropriate tonicity, centrifuged again and quantitatively transferred with a wash of 10 ml 0.05 M Tris–EDTA (pH 7.4 at 37.0 °C) to a 13-ml graduated pointed centrifuge tube, and centrifuged again. The volume of the button remaining in the tube (approx. 0.1 ml) after pouring off the wash liquid was estimated by using the 0.1-ml graduation mark. The maximum error in such an estimation is 0.02 ml as tested independently, but is usually only 0.01 ml or less. Based on this estimate, an amount of Tris–EDTA was added by pipetting to yield a total volume of 1.2 ml. This was then well mixed and pipetted into a Duall tissue homogenizer where the cells were homogenized to a uniform solution with 15–20 strokes. Two 0.5-ml samples were taken out for the ATPase determinations in the presence and absence of ouabain. In several of the experiments, 0.09 ml or less was removed for a protein determination by the Hartree modification of the Lowry method [7]. The incubation mixtures for the reaction based on the methods of Ismail-Beigi and Edelman [8] are shown in Table I, unless otherwise stated. The enzyme was preincubated in the reaction mixtures without ATP for 10 min. The reaction was initiated by the addition of ATP and terminated 40 min later by the addition of 1.0 ml cold 6 % $HClO_4$. This was

TABLE I

REACTION MIXTURES FOR $(Na^+ + K^+)$-ATPase ASSAY (FINAL CONCENTRATIONS)

For details, see Materials and Methods.

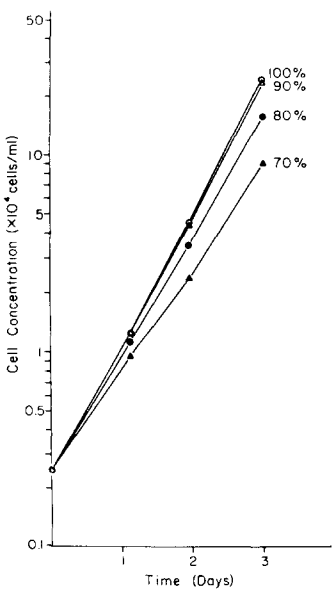
	I (mM)	II (mM)
Na^+	120	120
Mg^{2+}	2	2
Cl^-	170.5	170.5
N_3^-	5	5
K^+	12.5	—
Ouabain	—	5
Tris	50	50
ATP	5	5

centrifuged and phosphate was then determined on the supernatant by the Lowry modification of the Fiske–Subbarow method [9, 10] with colorimetric readings at 6 min. $(Na^+ + K^+)$-ATPase activity is taken to be the difference between the ATPase activity in the presence and absence of ouabain.

RESULTS

Cell growth

A typical growth curve for lymphoblasts in several dilutions of cell medium is shown in Fig. 1. Growth rate as a function of tonicity is plotted for a number of experiments in Fig. 2, where the decrease in growth rate with more dilute medium is illustrated by the ratio of the control generation time to the generation time of cells in hypotonic medium. The average generation time for control cells was 11.4 h while cells in 70 % (v/v) medium had a generation time of 15.2 h corresponding to growth at 75 % of the control rate. Median cell volume during log growth essentially paralleled each other, although the cells in 70 % (v/v) medium tended to have a slightly smaller volume on the average than the control cells in normal 100 % (v/v) medium. In cells in hypotonic solution, a lag in growth (2 h) was noted when more aliquots were taken shortly after the initial rapid volume adaptation previously described (1].



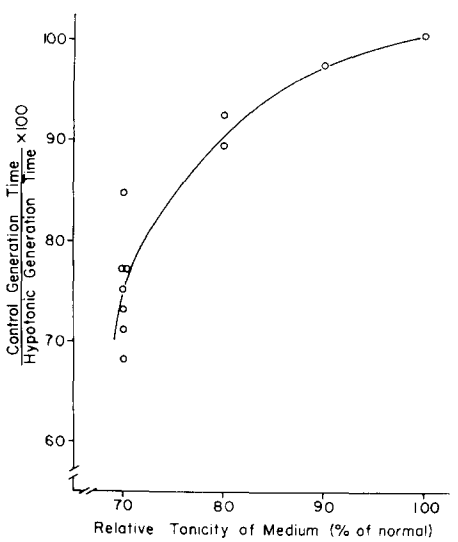


Fig. 1. Cell growth in medium of varying tonicities (% = v/v).

Fig. 2. Relative growth rate of cells in medium of varying tonicity.

Intracellular ion concentrations

In Table II are listed the intracellular concentrations of the most prevalent cations, Na^+ and K^+, and of Cl^-, after log growth for 3 days in 70 % (v/v), or 100 % (v/v), i.e. normal isotonic medium.

TABLE II

INTRACELLULAR ION CONCENTRATIONS AND NERNST POTENTIALS

	After growth in 70 % (v/v) medium	After growth in 100 % (v/v) medium
Cell $[Na^+]$ (mequiv/l)	18±2 (4)*	28±2 (4)
Medium $[Na^+]$ (mequiv/l)	98	140
E_{Na+} (mV)	+45	+43
Cell $[K^+]$ (mequiv/l)	157±9 (7)	198±8 (7)
Medium $[K^+]$ (mequiv/l)	3.5	5.0
E_{K+} (mV)	−101	−97
Cell $[Cl^-]$ (mequiv/l)	32±6 (2)	51±7 (2)
Medium $[Cl^-]$ (mequiv/l)	94	136
E_{Cl-} (mV)	−29	−26

* Mean ± S.E. No. of observations in parentheses.

The Nernst potential for each ion is also tabulated, calculated from these ion concentrations.

$(Na^+ + K^+)$-ATPase activity

For cells grown in 70 % (v/v) or 100 % (v/v) medium, there was no significant difference in $(Na^+ + K^+)$-ATPase activity to the 99 % confidence interval by a *t*-test comparison for paired observations. The individual data, including the non-specific ATPase (ouabain-insensitive ATPase), are listed in Table III. $(Na^+ + K^+)$-ATPase

TABLE III

Experiments 1 and 2 had 12.5 mM K^+ in the reaction mixture with ouabain present at a level of 0.5 mM. Independent tests showed that complete inhibition of $(Na^+ + K^+)$-ATPase activity was not always obtained under these conditions. If one neglects these two less sensitive experiments, the means are still found to have no significant difference for the two growth conditions.

Expt	After growth in 70 % (v/v) medium			After growth in 100 % (v/v) medium		
	Total ATPase (A)	Ouabain-Insensitive ATPase (B)	$(Na^+ + K^+)$-ATPase (A–B)	Total ATPase (A)	Ouabain-Insensitive ATPase (B)	$(Na^+ + K^+)$-ATPase (A–B)
1	12.0	10.6	1.4	11.4	9.6	1.8
	11.8	10.6	1.2	11.9	10.9	1.0
2	6.3	5.3	1.0	5.9	5.0	0.9
	6.3	5.4	0.9	7.3	6.1	1.2
3	10.2	5.7	4.5	10.1	5.5	4.6
	12.3	7.0	5.3	9.8	5.3	4.5
4	6.6	4.7	1.9	6.8	5.4	1.4
	6.0	4.8	1.2	7.0	5.3	1.7
5	4.7	3.4	1.3	6.1	4.2	1.9
	4.2	3.2	1.2	4.8	3.7	1.1
Mean	8.11	6.12	1.99	8.11	6.10	2.01

activity was 34 % of total ATPase activity when complete inhibition was assured (Expts 3–5). The mean $(Na^+ + K^+)$-ATPase activity for cultures grown in 70 % (v/v) medium and control cultures grown in 100 % (v/v) medium, was 2.0 mM $P_i/10^8$ cells per h. For a 95 % confidence interval, the limits for the difference between these two means is 0–0.3 mM $P_i/10^8$ cells per h. Assuming spherical cells, activity per unit area in these mouse lymphoblasts would be $1 \cdot 10^{-9}$ M P_i/cm^2 per s which compares with the highest activity found in a biological system by Bonting and Caravaggio [11]. The non-innervated membrane of the electroplax in an electric eel had an activity of $0.4 \cdot 10^{-9}$ M P_i/cm^2 per s. Such a high activity in these lymphoblasts is understandable when one considers the very high $[K^+]_i$ maintained in these cells which have a large surface–volume ratio.

In the studies in which protein determinations were also done as a check on the method, $(Na^+ + K^+)$-ATPase activity was found to average 195 μmoles P_i/mg protein per h for cultures grown in 70 % (v/v) or 100 % (v/v) medium. A further check on the methodology was inherent in the ATPase method; the non-specific ATPase (ouabain-insensitive), which was not expected to demonstrate any change, was the same for cells placed in hypotonic medium, relative to controls in 100 % (v/v) medium.

DISCUSSION

Cell growth in hypotonic cell medium was similar to that recently described by Wotring Roti Roti et al. [6] in this cell line.

The membrane potential is assumed to be equal to the Cl^- Nernst potential in these studies, an assumption which is always open to question, but which has, however, proven valid in many studies, e.g. those of Lassen et al. [12] and certainly useful for investigating cells which do not attach to glass and are as small (10 μm diameter) as the L5178-Y mouse lymphoblasts used in this investigation. The value of −26 mV is similar to values obtained for a variety of non-excitable cells, tabulated by Williams [13] and by Hause et al. [14].

Volume regulation is considered to be an end result of the regulation of ion distributions across the cell membrane, determined by the balance between passive leaks and an active pump. It has been determined that the initial rapid volume adjustment which occurs after osmotic swelling in a hypotonic solution is the net result of an initial rapid passive loss of K^+ and a pumping out of Na^+ which was gained during the swelling process [3].

Immediately after the swelling and return to their original volume in hypotonic solution, cells have a greatly reduced K^+ concentration, e.g. half normal in 50 % (v/v) medium, while the Na^+ concentration is slightly greater than normal [3]. The net result is a reduced content of osmotically active substances, sufficient to allow the cell to shrink towards normal. The question then remains whether Na^+ and K^+ are at a steady state at this time and whether as the cells continue to grow in hypotonic medium, normal ionic permeabilities and/or concentrations are restored, and, finally, whether the Na^+ pump functions at the same level as in normal isotonic medium.

The results of this study indicate that both $[Na^+]_i$ and $[K^+]_i$ are at a reduced level relative to control cells in 100 % (v/v) medium after growth in hypotonic medium.

One may calculate the passive net flux for each ion by using the Goldman–Hodgkin–Katz constant-field equations [15]. For K^+,

$$\phi_{K^+} = P_{K^+} \frac{FE_m/RT}{e^{-FE_m/RT}-1} ([K^+]_i - [K^+]_0 e^{-FE_m/RT}).$$

The form is identical for Na^+. Assuming $E_m = E_{Cl^-}$ in these calculations, cells in normal isotonic medium (see Table II) have a passive K^+ flux at 37 °C of $-109 \cdot P_{K^+}$ mequiv/l, and a Na^+ flux of $203 \cdot P_{Na^+}$ mequiv/l. After complete adaptation in 70 % (v/v) medium, the passive K^+ flux is $-80 \cdot P_{K^+}$ mequiv/l, and the Na^+ flux is $151 \cdot P_{Na^+}$ mequiv/l.

Now, from the constant-field equation for membrane potential,

$$E_m = \frac{RT}{F} \ln \frac{P_{Na^+}/P_{K^+}[Na^+]_0 + [K^+]_0}{P_{Na^+}/P_{K^+}[Na^+]_i + [K^+]_i},$$

one may determine the relative ion permeabilities, P_{Na^+}/P_{K^+}, for the two different growth situations, 70 % and 100 % (v/v) medium, again assuming $E_m = E_{Cl^-}$. P_{Na^+}/P_{K^+} for growth in 100 % (v/v) medium was found to be 0.55 and for growth in 70 % (v/v) medium, this ratio was 0.53, essentially the same. These values are similar to the value of 0.57 obtained for cultured HeLa cells by Borle and Loveday [16] and the value of 0.57 obtained by Tosteson and Hoffman for HK sheep erythrocytes [17]. Thus, it would appear that the selective K^+ permeability increase (relative to Na^+) observed during osmotic swelling was only a transient one.

In 100 % (v/v) medium, the ratio of passive fluxes for Na^+ and K^+, $\phi_{Na^+}/\phi_{K^+} = 203 \cdot P_{Na^+}/109 \cdot P_{K^+}$, or 1.02, when $P_{Na^+}/P_{K^+} = 0.55$ is substituted in this equation. In the steady state with no net changes in Na^+ and K^+ concentrations, the ratio of active fluxes must be similarly related, i.e. a Na^+/K^+ pump ratio of 1 : 1 emerges from this study. In 70 % (v/v) media, similarly, $\phi_{Na^+}/\phi_{K^+} = 151 \cdot P_{Na^+}/80 \cdot P_{K^+}$, or 1.00, again indicating a Na^+/K^+ pump ratio of 1 : 1. This ratio applies only to these steady state conditions and, obviously, would have to be greater than 1 : 1 for the postulated volume regulation process occurring immediately after immersion in hypotonic solution [3].

Wotring Roti Roti and Rothstein [6] concluded by $^{42}K^+$ efflux studies that the passive permeability for K^+ in cells grown for 5 h in hypotonic, i.e. 60 % (v/v) medium returned to the level found in normal control cells. If P_{Na^+}/P_{K^+} is also the same, as found in the present investigation, then the Na^+ permeability may be considered to be at the normal level as well. With constant P_{K^+}, the ratio of passive K^+ flux in 70 % (v/v) medium to that in 100 % (v/v) medium, $\phi_{K^+}{}^{70}/\phi_{K^+}{}^{100} = 80/109 = 0.73$; similarly $\phi_{Na^+}{}^{70}/\phi_{Na^+}{}^{100} = 151/203 = 0.74$. Therefore, the passive fluxes of Na^+ and K^+ are concomitantly reduced to a level about 74 % of that in normal control cells. In the steady state, the active fluxes must also be reduced by the same amount. These quantitative results support Wotring Roti Roti and Rothstein [4], whose data suggested a decreased active transport rate in fully adapted cells in hypotonic solution.

Since $[Na^+]_i$ has decreased in cells in 70 % (v/v) medium to 64 % of the value in 100 % (v/v) medium, the decreased pump rate could be readily explained by a regulation of the pump rate by $[Na^+]_i$ as suggested by Robinson [18] in a theoretical paper, and suggested by experimental results of several investigators (e.g., Hoffman, ref. 19). $(Na^+ + K^+)$-ATPase activity per se has remained constant, in these cells in hypotonic medium relative to controls, when tested in the same in vitro ionic environment. This indicates that there has been no cellular alteration of the amount of

enzyme produced in these two environmental conditions. Therefore, it is an attractive hypothesis that pump activity in these cells is regulated by kinetic alteration of the activity of $(Na^+ + K^+)$-ATPase through its ionic substrate, $[Na^+]_i$, rather than an eventual alteration of the amount of available enzyme per se.

ACKNOWLEDGEMENT

This work was supported in part by General Research Support Grant FR-05576 from the National Institutes of Health.

REFERENCES

1 Buckhold, B., Adams, R. B. and Gregg, E. C. (1965) Biochim. Biophys. Acta 102, 600–608
2 Rosenberg, H. M., Buckhold Shank, B. and Gregg, E. C. (1972) J. Cell. Physiol. 80, 23–32
3 Buckhold Shank B., Rosenberg, H. R. and Horowitz, C. (1973) J. Cell. Physiol. 82, 257–266
4 Wotring Roti Roti, L. and Rothstein, A. (1973) Exp. Cell Res. 79, 295–310
5 Buckhold, B. (1966) Mechanism of osmotic adaption in mammalian cells Ph. O. Thesis Case Western Reserve University, Ohio
6 Wotring Roti Roti, L. and Rothstein, A. (1973) Exp. Cell Res. 79, 311–317
7 Hartree, E. F. (1972) Anal. Biochem. 48, 422–427
8 Ismail-Beigi, F. and Edelman, I. S. (1971) J. Gen. Physiol. 57, 710–722
9 Lindberg, O. and Ernster, L. (1956) Methods of Biochemical Analysis III, (D. Glick, ed.), pp. 6–7, Interscience Publishers Ltd, London
10 Lowry, O. H. and Lopez, J. A. (1946) J. Biol. Chem. 162, 421–428
11 Bonting, S. L. and Caravaggio, L. L. (1963) Arch. Biochem. Biophys. 101, 37–46
12 Lassen, U. V. ,Nielsen, A.-M. T., Pape, L. and Simonsen, L. O. (1971) J. Memb. Biol. 6, 269–288
13 Williams, J. A. (1970) J. Theor. Biol. 28, 287–296
14 Hause, L. L., Pattillo, R. A., Sances, A., Jr. and Mattingly, R. F. (1970) Science 169, 601–603
15 Kotyk, A. and Janáček, K. (1970) Cell Membrane Transport Principles and Techniques, pp. 98–99, Plenum Press, New York
16 Borle, A. B. and Loveday, J. (1968) Cancer Res. 28, 2401–2405
17 Tosteson, D. C. and Hoffman, J. F. (1960) J. Gen. Physiol. 44, 169–194
18 Robinson, J. D. (1968) J. Theor. Biol. 19, 90–96
19 Hoffman, J. F. (1962) J. Gen. Physiol. 45, 837–859