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Energetics of Na^+-dependent amino acid co-transport in Ehrlich ascites tumor cells

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The energy available from the Na^+ electrochemical potential gradient ($\Delta\tilde{\mu}_{Na}$) has been evaluated in Ehrlich ascites tumor cells during accumulation of 2-aminoisobutyric acid. Cells were incubated in media of varying [Na^+] (25–154 mM) in the presence of 0.25 mM 2-aminoisobutyric acid to establish maximum steady-state accumulation of the amino acid. Membrane potential (V_m) and intracellular Na^+ activity (a_{Na}) were estimated using standard electrophysiological techniques. In physiological saline ([Na^+] = 154 mM) a_{Na} is 4.4 ± 0.6 mM, giving an apparent Na^+ activity coefficient (γ_{app}) in the cytoplasm of 0.17 ± 0.02. V_m under these conditions is -20.8 ± 2.1 mV. From these values, $\Delta\tilde{\mu}_{Na} = 9.9 \pm 0.8$ kJ/mol. Concomitant determinations of 2-aminoisobutyric acid (AIB) accumulation show an energy requirement ($\Delta\mu_{AIB}$) of 8.5 ± 0.5 kJ/mol. Stepwise reductions in extracellular [Na^+] give parallel reductions in a_{Na}, V_m and 2-aminoisobutyric acid accumulation. However, under all conditions tested the energy available from the Na^+ electrochemical potential gradient exceeds that needed to drive 2-aminoisobutyric acid uptake. The effects of 2-aminoisobutyric acid on V_m have also been determined. Addition of AIB (10 mM) to steady-state cells leads to membrane depolarization (resting $V_m = -22.1 \pm 1.3$ mV; plus AIB $V_m = -16.2 \pm 1.2$ mV) within 1 min. Subsequent repolarization of the membrane to resting levels occurs within 10 min. The repolarization phase is blocked in the presence of ouabain (2 mM). The results establish that the energy available from the Na^+ gradient is sufficient to serve as a source for 2-aminoisobutyric acid accumulation.

Introduction

The Na^+ gradient hypothesis [1,2] proposes that in animal cells the electrochemical potential gradient for Na^+ ($\Delta\tilde{\mu}_{Na^+}$) serves as the sole energy source for driving the accumulation of some organic solutes. Indeed, investigations of the role of ion gradients in metabolically inhibited Ehrlich ascites tumor cells have removed any doubt that cation gradients can be used to at least partially energize amino acid transport [3,4]. The major controversial point is whether the energy available from $\Delta\tilde{\mu}_{Na^+}$ is sufficient to completely account for amino acid accumulation. Uncertainties with respect to intracellular ion activities, cellular compartmentation of the solutes and the membrane potential during amino acid accumulation have prevented an evaluation of the energy balance, even in normally energized and steady-state cells (cf. Refs. 5 and 6).

Previous comparisons of the corresponding amino acid energy gradients ($\Delta\mu_A$) and $\Delta\tilde{\mu}_{Na^+}$ have demonstrated a consistently larger energy

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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requirement that can be provided by $\Delta\tilde{\mu}_{Na^+}$ [6–9]. These investigators recognized, however, that the studies did not account for possible reductions in cytoplasmic Na^+ activity which may result from intracellular compartmentation or binding. In one case, it has been calculated that if cytoplasmic Na^+ activity is reduced by 60% compared to chemical estimates, $\Delta\tilde{\mu}_{Na^+}$ could provide the needed energy [9]. We have recently estimated intracellular ion activities in these cells using ion-selective glass microelectrodes [10]. The apparent activity coefficients for Na^+ and K^+ in the cytoplasm are 0.18 ± 0.02 and 0.41 ± 0.05, respectively. These are significantly lower than the activity coefficients expected for the ions in physiological salt solutions (0.71 and 0.73, respectively). Our finding that the apparent Na^+ activity is only about 25% of that expected from chemical measurements emphasized the need to re-examine the energy balance between Na^+ and amino acid transport.

In the present study we have re-evaluated the adequacy of the Na^+ electrochemical gradient in Ehrlich ascites tumor cells to energize the uptake and accumulation of the non-metabolized 2-aminoisobutyric acid (AIB). Ion-selective microelectrodes were used to measure intracellular Na^+ activity in cells which have maximally accumulated 2-aminoisobutyric acid. Concomitant measurements of the membrane potential (V_m) permitted evaluation of $\Delta\tilde{\mu}_{Na^+}$. Results of this study demonstrate that $\Delta\tilde{\mu}_{Na^+}$ exceeds $\Delta\mu_{AIB}$ over the range of $[Na^+]$ tested (extracellular [Na] = 25–154 mequiv./l). These findings are consistent with the hypothesis that $\Delta\tilde{\mu}_{Na^+}$ is adequate to serve as the sole energy source for amino acid uptake.

Materials and Methods

Cell suspension. Ehrlich ascites tumor cells (Lettré strain; hyperdiploid) were maintained by weekly peritoneal transplantation in Ha/ICR male mice. Tumor-bearing animals with growths between 8 and 11 days were used. Cells were removed by aspiration and washed free of ascitic fluid. The wash medium was either physiological saline ($[Na^+] = 154$ mM; $[K^+] = 6$ mM; $[Cl^-] = 150$ mM; $[Ca^{2+}] = 2$ mM; and $[Mg^{2+}] = 0.2$ mM) or Na^+-free medium in which Na^+ was replaced by K^+. Each solution contained 10 mM Hepes buffer to yield a final pH of 7.4 and 290–300 mosM. Cells were finally resuspended 1 : 10 (v/v) in mixtures of physiological saline or Na^+-free saline to yield five experimental media in which $[Na^+]$ varied over the range 25–154 mM. The activity coefficients (γ_{ion}) for the cations in the incubation media were 0.71 and 0.73 for Na^+ and K^+, respectively [11]. Cell suspensions were incubated in Erlenmeyer flasks under an air atmosphere on a gyrorotary shaker set for 48 oscillations per min. All experiments were performed at 21–23°C.

Intracellular cation contents and amino acid accumulation. The methods used to determine intracellular Na^+ and K^+ contents by flame photometry have been previously described [12]. Briefly, 1.0 ml aliquots of the cell suspensions (50 mg cell wt/ml cell suspension) were centrifuged at $15\,000 \times g$ for 1.0 min, the pellets were carefully aspirated and extracted with 1.0 ml of 1% perchloric acid. The Na^+ and K^+ contents of the perchloric acid extract were determined with a Beckman Klina flame photometer using Li^+ as an internal standard. The cellular accumulation of the Na^+-dependent, non-metabolizable amino acid, 2-aminoisobutyric acid, was followed using 2-amino[^{3}H]isobutyric acid (ICN, Irvine, CA) as a tracer. 2-Amino[^{3}H]isobutyric acid (0.08 Ci/mol) was added to the cell suspensions and the radioactivity associated with the perchloric acid extracts and supernatant determined as a function of time and [2-aminoisobutyric acid]. All estimates of intracellular ion contents and radioactivity were corrected for trapped extracellular fluid using a regression line relating [^{3}H]methoxyinulin space to the wet weight of the cell pellet. Cellular water content was determined from wet and dry weights of the pellets [12].

Measurement of the membrane potential. The membrane potential of Ehrlich ascites tumor cells was measured using glass microelectrodes filled with 300 mM potassium acetate as previously described [10,13]. Steady-state cell suspensions were diluted 60 : 1 with the appropriate saline medium (with or without added 2-aminoisobutyric acid) and added to the four quadrants of an X-plate. The cells were allowed to settle and at-

tach to the bottom of the plate. The X-plates containing diluted cell suspensions were placed on a modified microscope stage and observed with 100 × magnification. The potential difference between cell cytoplasm and medium was recorded as previously described [12]. Our criteria for the validity of potential measurements have been discussed in detail [10]. These include consideration of the characteristics of the recordings upon insertion and withdrawal of the electrode, as well as attainment of a stable recording for at least 30 s. Approx. 60% of our attempts at cell impalement yield responses which are consistent with these criteria.

Estimation of intracellular Na^+ activity. Na^+ activity (a_{Na^+}) in the cytoplasm was estimated from direct measurements using Na^+-selective microelectrodes. Detailed descriptions of the preparation and use of these electrodes have been previously given [10]. Glass capillaries identical to those used for conventional microelectrodes (borosilicate microfilament capillaries; A-M Microsystems) were used for fabrication of the Na^+-selective electrodes. The electrode tips were siliconized by filling with 0.025–0.1% Dow-Corning 1107 silicone in acetone, followed by curing overnight at 100–120°C. The tips could then be filled with Na^+ Ligand I (Fluka, Switzerland) dissolved in 3-nitro-*o*-xylene (50 mg Ligand I in 177 µl solvent) containing 2.5 mg sodium tetraphenylborate. The barrels of the electrodes were subsequently filled with 1.0 M NaCl. The resultant electrodes were highly Na^+ selective (selectivity range: 33–50 compared to K^+) and yielded 55–60 mV changes in output for a 10-fold change in $[Na^+]$ in the absence of other cations (theoretical response = 58 mV for an ideal electrode).

For calibration of the electrodes and measurement of cytoplasmic a_{Na^+} we have adopted the method of mixed solutions [10,14]. A typical calibration curve is shown in Fig. 1. Each electrode was calibrated by determining its response to varying a_{Na^+} (range: 2.0–21.0 mM) in the presence of 80 mM KCl. This (K^+) was selected to mimic that estimated for the Ehrlich cell cytoplasm [10]. Prior to calibration, each electrode potential was set to 0 mV, when immersed in the saline solution to be used for cell incubation. In this way, deviations from an ideal response would be automatically included in the calibration curve for the electrode [14]. Furthermore, inclusion of 80 mM KCl in the calibration solutions serves to correct for contributions from cytoplasmic K^+ upon cell impalement. Use of these electrodes and the mixed solution calibration for estimating a_{Na^+} is also demonstrated in Fig. 1 (for theoretical details, see Ref. 10). Briefly, the total electrode response (ΔV_{Na^+}) can be associated with the calibration curve in order to estimate a_{Na^+}.

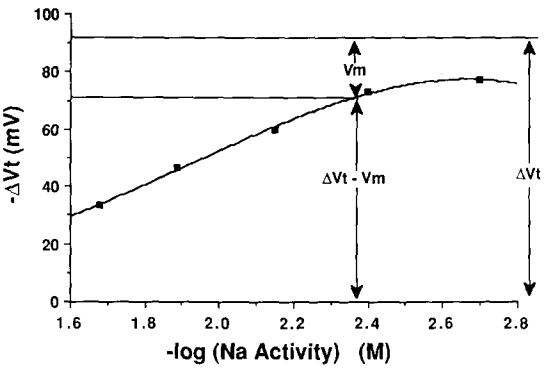

Fig. 1. Na^+-selective electrode calibration and use for intracellular ion activity estimates. For calibration, the electrodes were first immersed in the saline bathing solution and the electrode output was taken as zero. Electrode output was then determined in test solutions of known a_{Na} (2.0 to 21.0 mM) in the presence of 80 mM K^+ to mimic the intracellular a_K. The calibration curve is shown. Insertion of the electrode into a cell yields a potential change (ΔV_t) reflecting both the intracellular a_{Na} and V_m. Correction of the response for the contribution from V_m permits a_{Na} corresponding to ΔV_{Na} to be read directly from the calibration curve. In this case, $\Delta V_t = 91.0$ mV, $V_m = -20.5$ mV. Intersection of ΔV_{Na} ($= \Delta V_t - V_m$) with the calibration curve occurs where $a_{Na^+} = 4.3$ mM.

Results

Effects of amino acids on the membrane potential

Models describing mechanisms utilizing $\Delta\tilde{\mu}_{Na}$ for the accumulation of amino acids universally propose a coupled entry of the two substrates. A number of studies have demonstrated increased net Na^+ uptake in ouabain-treated cells and acceleration of unidirectional Na^+ influx with the addition of Na^+-dependent amino acids [1,15]. In this case the co-transport is expected to be rheogenic, with the addition of amino acid leading to membrane depolarization. Several studies, using

potential-sensitive fluorescence dyes to monitor V_m, support this possibility [7–9,16]. The addition of test amino acids to suspensions of Ehrlich ascites cells gives fluorescent changes which depend on $[Na^+]$, amino acid concentration and the extent of Na^+-dependence shown by the test amino acid. However, there are significant qualitative and quantitative differences in the results of the previous studies. Eddy and co-workers have estimated the resting V_m of these cells to be -50 to -60 mV [7,9]. The test amino acids lead to depolarizations of 10–40 mV which are stable. In contrast, Laris and his colleagues report a much reduced resting V_m (-20 to -35 mV; [8]) and a transient depolarization in response to 2-aminoisobutyric acid. Subsequent repolarization returns the V_m to near-rest values.

In the present study, we have used direct electrophysiological methods to evaluate the response of the membrane potential to added 2-aminoisobutyric acid. It is possible to achieve membrane potential measurements which are stable for as long as 15 min from steady-state Ehrlich cells [10,13]. The average steady-state potential of all cells sampled is -21.9 ± 1.1 mV (S.E.; $n = 53$).

The effects of 2-aminoisobutyric acid on V_m were determined with [2-aminoisobutyric acid] = 10 mM, a concentration chosen to approximate saturation of the co-transport system [17]. We utilized two different protocols. First, we tested the effects on the average V_m of the population of cells. Cells were placed in the impalement chamber and the steady-state V_m of a number of cells was determined. Stock 2-aminoisobutyric acid (300 mM) was then added to the chamber to give a final [2-aminoisobutyric acid] of 10 mM. Impalements were then continued for at least 10 min. The results of these studies are shown in Fig. 2, giving the averages for 2-min intervals after 2-aminoisobutyric acid addition. V_m for the steady-state cells is -22.1 ± 1.3 mV (S.E.; $n = 41$). During the initial 2 min after 2-aminoisobutyric acid addition, the average V_m falls to -16.2 ± 1.2 mV. Subsequently, the average V_m increases toward the steady-state value. For longer times ($t > 10$ min), repolarization returns the average V_m to -20.8 ± 2.1 mV (S.E.; $n = 35$), not significantly different from the steady state V_m in the absence of 2-aminoisobutyric acid.

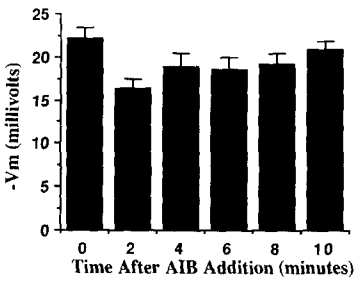

Fig. 2. Time-course of the effect of added 2-aminoisobutyric acid on the average membrane potential of Ehrlich ascites tumor cells. The V_m of steady state cells in physiological saline was determined by impalements using glass microelectrodes (Time = 0). Stock 2-aminoisobutyric acid (AIB, 300 mM) was added to yield a final concentration of 10 mM. Impalements were continued over 10 min. Each column of the histogram gives the average V_m for cells impaled during the specified 2-min interval. The number of measurements are given for each period. Error bars represent the standard errors of the mean (S.E.).

We have also tested the response of individual tumor cells. In these studies, a cell was first impaled and the stable resting V_m was recorded. 2-Aminoisobutyric acid was carefully added to the chamber and the response of the single cell followed. A typical recording is shown in Fig. 3. Depolarization occurs soon after 2-aminoisobutyric acid is added, with maximum depolarization obtained in less than 1 min. Repolarization follows within 3 min. In some cases, the onset of the response was delayed by as much as 1 min after

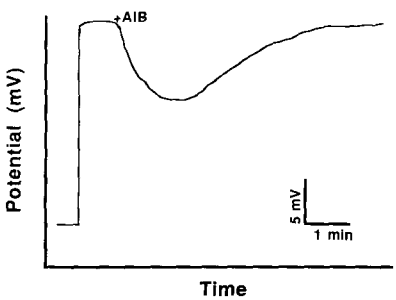

Fig. 3. Effect of 2-aminoisobutyric acid (AIB) (10 mM) on the V_M of an Ehrlich ascites tumor cell. The record is a reproduction of an oscilloscope tracing of the potential measurement from a single cell. After a stable value of the potential had been achieved, stock 300 mM 2-aminoisobutyric acid was added (+AIB). In this case, on-set of depolarization was rapid and reached a maximum value in about 1 min. Repolarization was complete by 10 min.

2-aminoisobutyric acid addition. We interpret this to represent delayed mixing of 2-aminoisobutyric acid with the incubation medium, since its addition directly to the cell frequently resulted in termination of the impalement. However, in all of the cells tested the depolarization-repolarization cycle was similar. The average pre-impalement $V_m = -21.5 \pm 0.5$ mV (S.E.; $n = 12$). The average maximum depolarization is 8.5 ± 0.6 mV. This exceeds that measured in the population studies (5.9 ± 1.8 mV). The difference probably reflects our inability to monitor the maximum depolarization in the cell population. However, the time-course of the transient response is the same in the single cells and the cell population.

The depolarization we find is consistent with enhanced, rheogenic entry of Na^+ coupled to 2-aminoisobutyric acid uptake. Since this entry has also been shown to result in net Na^+ accumulation in ouabain-treated cells [1], we have tested the effects of 2-aminoisobutyric acid on V_m in cells pre-incubated with ouabain (2 mM). A typical response is shown in Fig. 4. Again, the addition of 2-aminoisobutyric acid (10 mM) induces depolarization. However, in this case there is no repolarization. In eight cells tested, the average pre-impalement V_m was 20.9 ± 1.7 mV (S.E.). 2-Aminoisobutyric acid depolarized the cells to -11.5 ± 2.3 mV. Repolarization was not observed during any of these measurements.

Amino acid gradients

The ultimate accumulation capacity of Ehrlich

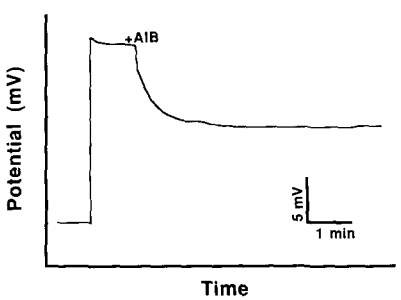

Fig. 4. Effect of 2-aminoisobutyric acid (AIB) (10 mM) on the V_m of an Ehrlich ascites tumor cell pre-treated for 45 min with ouabain (2 mM). The record is a reproduction of an oscilloscopic tracing from a single cell. After a stable V_m was achieved, stock (300 mM) 2-aminoisobutyric acid was added (+AIB). Depolarization was irreversible.

cells for 2-aminoisobutyric acid depends on several factors, in addition to the energy source for uptake. For example, if non-concentrative, Na^+-independent transport pathways for 2-aminoisobutyric acid operate in parallel with the Na^+-dependent co-transport, then this pathway will serve as an 2-aminoisobutyric acid 'leak' limiting the accumulation capacity [9]. Furthermore, the magnitude of the 'leak' can be expected to reflect a transmembrane difference in [2-aminoisobutyric acid], while the accumulation capacity reflects a measure of the [2-aminoisobutyric acid] ratio. Even in the simple case where 2-aminoisobutyric acid entry occurs only by Na^+-dependent co-transport and its exit only by a simple diffusional 'leak,' the accumulation capacity would be a function of extracellular [2-aminoisobutyric acid].

We undertook preliminary experiments to evaluate the dependence of accumulation capacity ([2-aminoisobutyric acid]$_c$/ [2-aminoisobutyric acid]$_e$) on [2-aminoisobutyric acid]$_e$, where the subscripts c and e designate the cellular and extracellular compartments, respectively. 2-Amino[^{3}H]isobutyric acid was added to cell suspension in physiological saline to give an initial [2-aminoisobutyric acid]$_e$ over the range 0.25 mM to 1.6 mM. 2-Aminoisobutyric acid uptake was followed until steady-state cellular content was achieved (60 min). Cells at the lowest [2-aminoisobutyric acid] tested accumulated the amino acid 36.5 ± 3.3-fold. With increasing [2-aminoisobutyric acid]$_e$ accumulation capacity steadily decreased to 23.5 ± 2.8-fold at 1.6 mM. Consequently, for all subsequent studies directed to evaluating the adequacy of $\Delta\tilde{\mu}_{Na}$ for energizing 2-aminoisobutyric acid uptake we have used [2-aminoisobutyric acid]$_e$ = 0.25 mM. The molar free energy stored in the amino acid gradient is given by:

$$\Delta\mu_{AIB} = RT \ln\{[\text{2-aminoisobutyric acid}]_c / [\text{2-aminoisobutyric acid}]_e\} \quad (1)$$

where R is the universal gas constant and T the temperature (K).

Intracellular Na^+ activity (a_{Na}) and the electrochemical potential difference ($\Delta\mu_{Na}$)

The use of Na^+-selective microelectrodes to

estimate a_{Na} is illustrated in Fig. 1. Impalement of a cell with the selective electrode yields an immediate potential change (ΔV_t) with contributions from the a_{Na} difference between environment and cell (ΔV_{Na}), as well as the membrane potential difference (V_m). To isolate the change in response due to the difference in a_{Na}, V_m must be measured and subtracted from the total response (ΔV_t). The small size of Ehrlich cells makes it difficult to reliably measure ΔV_t and V_m in the same cell, since this would require impalement with separate electrodes. Thus, we measured the average V_m of the population of cells and used this to correct ΔV_t. Fig. 1 shows typical results. After ΔV_t is measured in 6–12 cells, a mean is calculated. This value is then corrected for the contribution of V_m. The intracellular activity corresponding to a_{Na} can be read directly from the calibration curve for the electrode.

We have evaluated cellular [Na^+], a_{Na} and V_m under conditions chosen to alter $\Delta\tilde{\mu}_{Na}$, with maximum accumulation capacity for 2-aminoisobutyric acid. The cell suspensions were incubated in media of varying [Na^+] in the presence of 0.25 mM 2-aminoisobutyric acid to permit establishment of steady-state 2-aminoisobutyric acid uptake. The results of these studies are summarized in Table I. In physiological saline ([Na^+] = 154 mM) cellular [Na^+], based on flame photometry measurements of total Na^+ and water contents, is 25.3 ± 0.5 mM. If the activity coefficient for Na^+ in the cytoplasm were the same as that in the physiological saline ($\gamma_{Na} = 0.71$), then a_{Na} should be about 18 mM. The measured a_{Na} is considerably lower than expected ($a_{Na} = 4.4 \pm 0.6$ mM). The resulting apparent activity coefficient for Na^+ ($\gamma_{app} = 0.17$) is consistent with the suggestion that approx. 25% of cellular Na^+ contributes to $\Delta\tilde{\mu}_{Na}$.

Reduction of [Na^+] to 25 mM in the suspension is accompanied by decreases in both cellular [Na^+] and a_{Na}. In terms of percentage decrease, however, the change in a_{Na} is more striking, decreasing about 55% while cellular [Na^+] decreases only by 30%. Thus, at the two lowest environmental [Na^+] tested γ_{app} falls to 0.11. V_m also depolarizes in conjunction with the alteration in Na^+, but the magnitude of the change supports the relative insensitivity of V_m to variations in environmental ion contents [18].

TABLE I

EFFECT OF VARYING EXTRACELLULAR Na^+ ON INTRACELLULAR [Na^+], ACTIVITY AND MEMBRANE POTENTIAL IN THE PRESENCE OF 2-AMINOISOBUTYRIC ACID

Ehrlich ascites tumor cells were incubated in physiological saline or in saline with extracellular Na^+ ([Na^+]$_e$) reduced by equivalent K^+ replacement. 2-Aminoisobutyric acid (final concentration = 0.25 mM) was added and incubation continued for 60 min to establish steady-state accumulation of 2-aminoisobutyric acid. The average intracellular concentration ([Na^+]$_c$) was determined by spectrophotometry, while the intracellular activity (a_{Na}) was measured using Na^+-selective glass microelectrodes. The apparent activity coefficients (γ_{app}) were calculated as the ratio of a_{Na} to [Na^+]$_c$. Data present the mean and S.E. for activity and [Na^+]$_c$ determinations. The number of observations is given in parentheses.

[Na^+]$_e$ (mM)	[Na^+]$_c$ (mM)	a_{Na} (mM)	γ_{app}	V_m (mV)
154	25.3 ± 0.5	4.4 ± 0.6 (30)	0.17 ± 0.02	-20.8 ± 2.1
100	21.0 ± 0.2	3.4 ± 0.4 (22)	0.16 ± 0.02	-19.3 ± 1.5
75	20.9 ± 0.8	3.1 ± 0.4 (6)	0.15 ± 0.02	-17.8 ± 1.6
50	18.7 ± 1.1	2.1 ± 0.3 (21)	0.11 ± 0.03	-15.3 ± 2.3
25	17.6 ± 0.8	2.0 ± 0.2 (15)	0.11 ± 0.02	-14.2 ± 0.8

The molar free energy available from the Na^+ electrochemical potential gradient can be calculated from these results:

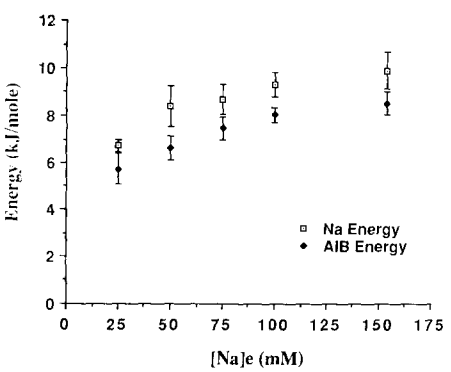

Fig. 5. Comparison of Na^+ and 2-aminoisobutyric acid (AIB) energy gradients in media of varying [Na^+]. Ehrlich ascites tumor cells were incubated in media of varying [Na^+] (range: 25–154 mM) in the presence of 2-aminoisobutyric acid (initial concentration = 0.25 mM) for 60 min to establish steady-state conditions with respect to 2-aminoisobutyric acid accumulation and ion contents. V_m, intracellular a_{Na}, [Na^+] and [2-aminoisobutyric acid] were determined on parallel samples. $\Delta\mu_{AIB}$ and $\Delta\tilde{\mu}_{Na}$ were calculated from equations Eqns. 1 and 2 in the text. Error bars show the most probable errors.

$$\Delta\tilde{\mu}_{Na} = RT \ln\{\gamma_{Na}[Na^+]_e/\gamma_{app}[Na^+]_c\} - FV_m \quad (2)$$

where γ_{Na} and γ_{app} are the Na^+ activity coefficients in the environment (e) and cytoplasm (c), respectively, and F is Faraday's constant. Coupled with our concomitant measurements of the accumulation capacity for 2-aminoisobutyric acid, these data permit comparison of the energy available from the Na^+ gradient to that needed for 2-aminoisobutyric acid accumulation. Fig. 5 shows that both $\Delta\tilde{\mu}_{Na}$ and $\Delta\mu_{AIB}$ are sensitive to $[Na^+]_e$, decreasing as Na^+ is replaced by K^+. At all $[Na^+]_e$ tested, however, $\Delta\tilde{\mu}_{Na}$ exceeds $\Delta\mu_{AIB}$.

Discussion

The Na^+ gradient hypothesis is based on two premises: (1) energy stored in the Na^+ electrochemical gradient ($\Delta\tilde{\mu}_{Na}$) is adequate to drive organic solute accumulation; and (2) a mechanism exists for coupling Na^+ and organic solute movements and satisfies the required efficiency for energy transfer. In Ehrlich ascites tumor cells, Na^+ has been shown to alter both the maximum flux and the apparent Michaelis-constant describing the transport of certain amino acids [3,19,20]. These effects provide a basis for the transfer of energy between the Na^+ gradient and amino acid accumulation.

The more serious concern with the hypothesis has been the failure to demonstrate the adequacy of energy from the Na^+ gradient (cf. Ref. 6). Comparisons of the magnitudes of the Na^+ gradient ($\Delta\tilde{\mu}_{Na}$) and amino acid gradient ($\Delta\mu_A$) in steady-state cells have yielded a deficiency of about 1.8 kJ/mol [6–9]. More stringent tests involving metabolic depletion and reversals of the Na^+ gradient have lead to findings of even larger discrepancies [21]. Speculation concerning the energy deficiency has focused on possible underestimates of $\Delta\tilde{\mu}_{Na}$ [5,6]. These possibilities include nuclear sequestration of Na^+, activation of electrogenic cation transport (with membrane hypolarization) and overestimates of the intracellular Na^+ activity coefficient. However, studies using cytoplasts derived from ascites tumor cells, in which the membrane potential was monitored and no nuclear sequestration was present, have shown a persistent Na^+ energy deficiency [8].

We have recently utilized ion-selective microelectrodes to estimate intracellular activities in Ehrlich cells [10]. In steady-state cells, in the absence of added amino acids, we found that approx. 82% of intracellular Na^+ is not electrochemically active ($\gamma_{app} = 0.18$). In light of this, for the present investigation we undertook a re-evaluation of the energy balance serving Na^+-dependent 2-aminoisobutyric acid transport.

Mitchell [22] has devised a simple test of the hypothesis in terms of energy expenditure necessary to maintain a steady state, asymmetrical distribution of the amino acid. In essence, the Na^+ gradient energy coupled to amino acid transport is adequate to drive the accumulation if:

$$\nu_{AIB} \cdot \Delta\mu_{AIB} < \nu_{Na} \cdot \Delta\tilde{\mu}_{Na} \quad (3)$$

where ν_{AIB} and ν_{Na} represent the number of molecules of 2-aminoisobutyric acid and Na^+ moved by the coupling process, respectively, and $\Delta\mu_{AIB}$ and $\Delta\tilde{\mu}_{Na}$ are defined by Eqns. 1 and 2. The ratio ν_{AIB}/ν_{Na} defines the transport stoichiometry. Our previous studies, performed under similar conditions, yield a stoichiometry of unity for 2-aminoisobutyric acid co-transport with Na^+ [15]. Consequently, the simplest energetic test only requires that the energy available from $\Delta\tilde{\mu}_{Na}$ exceed that needed for 2-aminoisobutyric acid accumulation.

Our results (Fig. 5) provide a clear answer to the question of energy adequacy. In steady state, metabolically competent cells $\Delta\tilde{\mu}_{Na}$ significantly exceeds $\Delta\mu_{AIB}$ over the entire range of $[Na^+]_e$ tested. This conclusion should be compared to that reached using the usual assumption that the cellular Na^+ activity coefficient equals that in the medium. In this case, cells in physiological saline have a calculated $\Delta\tilde{\mu}_{Na}$ of 6.4 ± 0.3 kJ/mol. Since the measured $\Delta\mu_{AIB} = 8.5 \pm 0.5$ kJ/mol under these same conditions, there would be an apparent energy deficiency of 2.1 kJ/mol. This compares well with the energy imbalance reported under similar conditions by others [6–9]. As $[Na^+]_e$ is reduced, the apparent energy deficiency increases until at the lowest $[Na^+]_e$ tested (25 mM), the energy shortfall is 3.5 kJ/mol. This approximates the deficiency found in cells with reversed Na^+ gradients [3]. Our finding that γ_{app} in the cytoplasm is markedly lower than that in the medium

(Table I) provides a complete account of the apparent deficiency. In physiological saline, the measured $\Delta\tilde{\mu}_{Na}$ exceeds $\Delta\mu_{AIB}$ by 1.4 kJ/mol while in medium containing 25 mM Na^+, $\Delta\tilde{\mu}_{Na}$ exceeds $\Delta\mu_{AIB}$ by 1.0 kJ/mol.

Another area of uncertainty relates to the effect of added amino acids on V_m. Several studies have described depolarization, consistent with stimulation of rheogenic Na^+ entry through the co-transport system. However, it is not clear whether the depolarization is transient, with subsequent repolarization toward resting levels [8,16], or whether it is progressive to new stable levels [7,9]. In an earlier study, we used microelectrodes to determine the average V_m of the cell population in the absence of amino acids (-22.3 ± 0.8 mV) and during 20 min after addition of 10 mM 2-aminoisobutyric acid (-16.7 ± 0.7 mV) [15]. Unfortunately, we did not analyze the time course of the potential change after 2-aminoisobutyric acid addition. Thus, if there is any tendency for repolarization it would not be seen.

In the present study, we followed the response of the cell population during discrete time intervals after 2-aminoisobutyric acid addition (Fig. 2), as well as the continuous responses of individual cells (Fig. 3). Both protocols establish the transient nature of the V_m change. The initial depolarization persists for only about 2 min after addition of 2-aminoisobutyric acid. Repolarization to control levels is complete within 10 min. These findings are in excellent quantitative and qualitative agreement with those of Laris and co-workers using potential sensitive fluorescent dye to monitor V_m [8,16]. The ouabain sensitivity of the repolarization phase (Fig. 4) establishes that electrogenic Na^+ extrusion is at least partially responsible. The studies do not eliminate the possible participation of other rheogenic pathways in either the depolarization or repolarization. For example, a consistent finding associated with amino acid accumulation is an alteration in K^+ transport. While it is clearly expected that ouabain-sensitive K^+ uptake would increase, alterations in conductive K^+ pathways must also be considered [1,23].

Taken together, the results of this investigation are the first based on direct measurements to confirm the energetic adequacy of the Na^+ electrochemical potential gradient for amino acid accumulation. Previous estimates assuming equivalency of Na^+ activity coefficients in the extracellular and cellular compartments always showed energy deficits [6–9]. It should be recognized that these authors did emphasize that the discrepancy probably derived from overestimates of a_{Na}. This indeed proves to be the case, since γ_{app} is only about 25% γ_{Na} in the medium. In addition, membrane repolarization, at least partially driven by electrogenic Na^+/K^+ transport, contributes to the available energy.

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