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REGULATION OF THE ELECTROGENIC ($Na^+ + K^+$)-PUMP OF EHRLICH CELLS BY INTRACELLULAR CATION LEVELS

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Summary

Ehrlich cells actively accumulate neutral amino acids even if both the Na^+ and K^+ gradients are inverted. The seeming contradiction of this observation to the gradient hypothesis is, however, explained by the presence of a powerful electrogenic Na^+ pump, which stongly raises the electrochemical potential gradient of Na^+ under these conditions. Since the evidence of this pump has so far been found only during abnormal concentrations of alkali ions (low K^+, high Na^+) in these cells, the question arises whether the pump is equally powerful with completely normal cells, when the pump is not 'needed' for amino acid transport. Using the initial rate of uptake of the test amino acid (2-aminoisobutyrate) as a sensitive monitor of the electrical potential at constant cation distribution between cell and medium, a procedure has been devised to split the overall electrical potential into the diffusional and the pump component. With this procedure it could be shown that the electrogenic pump per se is most powerful in K^+-depleted and Na^+-rich cells but declines to a lower 'resting' value according as the electrolyte content of the cell approaches normality. A strong positive correlation between cellular Na^+ content and the electrogenic pumping activity suggests that the intracellular activity of this ion regulates the rate of the electrogenic pump. The low activity of the pump under normal conditions may explain why the existance of this pump has rarely come to attention previously.

Introduction

The gradient hypothesis of active solute transport postulates that the electrochemical potential gradient of an electrolyte ion is the immediate and exclusive driving force for the active transport of the solute concerned. Whereas there cannot be any doubt that the electrochemical potential gradient of Na^+ in

Ehrlich cells drives the active accumulation of neutral amino acids, some observations, however, seem to indicate, that active transport is also possible under inverted electrochemical potential gradients of Na^+, thus suggesting that an energy source other than this gradient be available for this transport system [1]. In the meantime, however, evidence could be produced that under such conditions an electrogenic pump of Na^+ is activated which substantially contributes to the electrical membrane potential thus providing for an adequate electrochemical potential Na^+ gradient, even if the concentration gradients of both Na^+ and K^+ are inverted. This electrogenic pump was shown to be stimulatable by raising the extracellular K^+ [2] and inhibitable by ouabain. It manifests itself by a vigorous extrusion of Na^+ and uptake of K^+, by a rapid rise in electrical potential difference, outward positive, as demonstrated by the distribution of tetraphenylphosphonium [3] and cyanine dye fluorescence [4,5], and by a concomitant rise of the initial rate of 2-aminoisobutyrate uptake. Accordingly, it could be shown that at almost constant concentrations of Na^+ and K^+ in medium and cell the transport of neutral amino acids could be stimulated directly by raising the transmembrane electrical potential, no matter whether this was done by valinomycin, which changes the membrane diffusion component only, or by stimulating the electrogenic pump. The good correlation between membrane potential and initial 2-aminoisobutyrate influx made the latter a sensitive and fast monitor as compared to tetraphenylphosphonium of the pump-induced changes in electrical potential [6].

So far the electrogenic pump was demonstrated only under abnormal conditions of the cellular ion contents, namely with high intracellular Na^+ and low intracellular K^+. The question therefore arises, whether this pump is equally active under normal conditions or whether it is specifically activated by the above mentioned conditions, high intracellular Na^+ and low intracellular K^+. This question is the topic of these investigations.

Methods and Materials

The media employed throughout were Krebs-Ringer phosphate buffers of the following basic composition: 145 mM Na^+, 8 mM K^+, 1.9 mM Ca^{2+}, 1.3 mM Mg^{2+}, 138 mM Cl^-, 1.3 mM SO_4^{2-}, 10 mM P_i, 1% albumin, adjusted to pH 7.4 with 0.31 M HCl. The Na^+ and K^+ concentration was varied as needed by replacing these cations by each other.

Ehrlich ascites tumor cells were propagated and collected as described previously [1]. K^+-depleted cells were obtained by preincubations in half-isotonic K^+-free Krebs-Ringer phosphate buffer for 15 min at 0°C, and in isotonic K^+-free Krebs-Ringer phosphate buffer for 10 min at 0°C, and again for 10 min at 37°C. After this procedure the cells contained approx. 15 mM K^+ and 200 mM Na^+ as compared to 185 mM K^+ and 30 mM Na^+ after the normal preincubation in K^+-containing media for 30 min at 37°C.

To a suspension of K^+-depleted cells in K^+-free buffer at 37°C K^+-containing Krebs-Ringer phosphate buffer was added to give a final K^+ concentration of 15 mM and to start thus K^+ uptake. At different time intervals after the K^+ addition buffer, which contained 2-aminoisobutyric acid (0.1 mM) and as needed $^{42}K^+$, the inhibitor ouabain (3 mM) and the ionophore valinomycin (30 μM),

was added for the final 1-min incubation. The final concentration of valinomycin had to be high enough to be effective in the albumin containing medium. Since in the buffer valinomycin is not soluble to the required extent, a well-mixed suspension of valinomycin in buffer was used for the addition.

After the 1 min incubation with 2-aminoisobutyrate the uptake was stopped by cooling the sample in an ice-bath (—4°C). After centrifugation in a cooled centrifuge (0°C, 10 min, 4000 × *g*) the supernatant was decanted for further analysis; the cellular mass was freeze-dried and extracted with water and the protein-free extract, after addition of 3% trichloroacetic acid, used for further analysis as described elsewhere [1].

Na^+ and K^+ were determined by flame photometry (Eppendorf Gerätebau Netheler und Hinz GmbH), radioactivity by liquid scintillation spectrometry (TriCarb, Packard Instrument Company). According to repeated previous determinations 16% of the wet pellet weight was used to correct for the extracellular space of the pellet.

Valinomycin was obtained from Boehringer Mannheim GmbH and dissolved in ethanol (2.5 mg/ml), strophantin g (ouabain) from E. Merck, Darmstadt, bovine albumin (Cohn fraction V) from Carl Roth KG, Karlsruhe, 2-aminoisobutyric acid from Serva Feinbiochemica, Heidelberg, 2-[1-^{14}C]aminoisobutyrate from NEN Chemicals, Dreieichenchain, and ^{42}K from Amersham Buchler, Braunschweig, G.F.R.

Results and Discussion

The electrical potential of the cell membrane presumably consists of several components: a Gibbs-Donnan potential, a membrane-diffusion potential, due to unequal distribution of Na^+ and K^+ and unequal mobility of these ions, and the pump potential, due to the activity of an electrogenic pump. Thus, an active ion pump, provided it is electrogenic, does not only influence the membrane potential indirectly by creating a disequilibrium of ions, but contributes to the potential directly by its own activity.

In order to study the electrogenicity of the pump it is necessary to separate the electrogenic component from the other components of the electrical membrane potential. This separation is possible due to the fact that this electrogenic component is specifically and almost instantaneously inhibitable by ouabain, whereas the membrane diffusion component will persist as long as the unequal distribution of Na^+ and K^+ remain. The Na^+-dependent amino acid uptake can be used as a monitor of the membrane potential under the condition that the ion distribution is the same in test and control. Differences in amino acid uptake are then the result of changes in membrane potential [6].

Accordingly, the ($Na^+ + K^+$)-pump of Ehrlich cells depleted of K^+ was triggered by addition of extracellular K^+ and allowed to pump for certain periods of time to obtain different ion distributions. Then in one series 2-aminoisobutyrate was added and its initial uptake was measured in the presence of a functioning ion pump, while in a second series with 2-aminoisobutyrate ouabain was added to measure the initial 2-aminoisobutyrate uptake at similar ion distributions but without a functioning ion pump. Thus, a comparison between the initial uptakes of the amino acid with and without

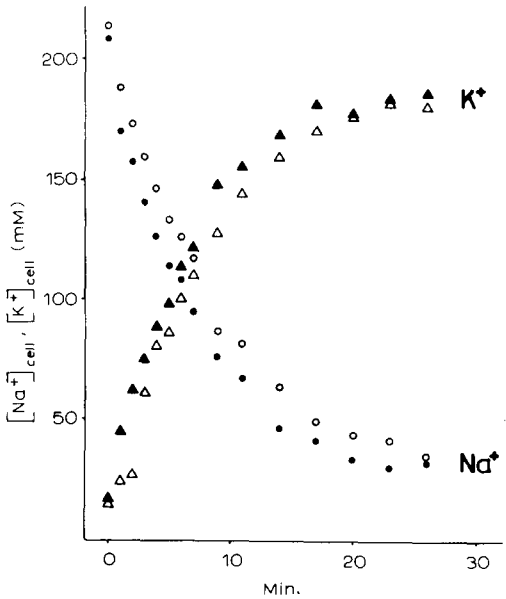

Fig. 1. Na^+ and K^+ content of K^+-depleted Ehrlich ascites tumor cells after the addition of 13 mM K^+ as a function of time. Ordinate: cellular concentration of Na^+ and K^+, respectively, (mM). Abscissa: time interval after the addition of K. ▲, ● = control; △, ○ = in the presence of 3 mM ouabain during the final minute of incubation.

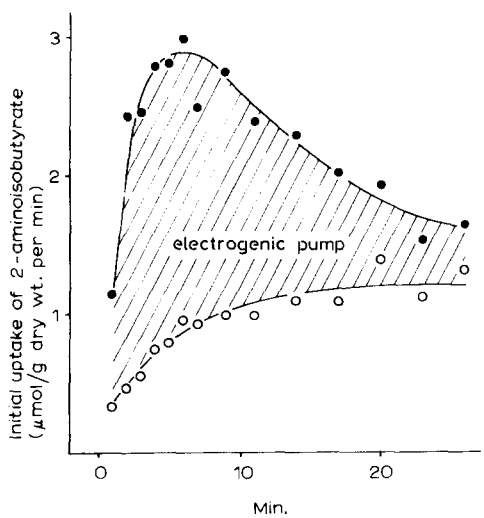

Fig. 2. Initial rate of 2-aminoisobutyrate net uptake under the influence on the electrogenic pump. To K^+-depleted and Na^+-rich cells 13 mM K^+ was added at time 0 to each sample of two series, in order to initiate the electrogenic pump. Subsequently the initial rate of 2-aminoisobutyrate net uptake was measured in the first series by the addition of 0.1 mM 2-aminoisobutyrate to the samples at varying time intervals after K^+ addition (●). In the parallel series the procedure was the same except that 3 mM ouabain was added together with the 2-aminoisobutyrate in order to test the initial 2-aminoisobutyrate uptake after the blocking of the electrogenic pump (○). The upper curve gives the initial 2-aminoisobutyrate uptake under the influence of the total electrical potential difference. The lower curve gives this uptake under the influence of merely the membrane diffusion potential difference remaining after the blockage of the electrogenic pump. The cross-hatched area between the two curves represents the contribution of the electrogenic pump to the initial 2-aminoisobutyrate uptake. Ordinate: initial uptake of 2-aminoisobutyrate (1 min) in μmol/g dry weight per min. Abscissa: time in minutes after the addition of K^+ to the suspensions.

ouabain, respectively, should give an indication of the pump potential at different ion distributions. Such an experiment is demonstrated in the first figures. Fig. 1 shows the changes in ion content of K^+-depleted Ehrlich cells upon the addition of K^+ to the external medium as a function of time after K^+ addition. The addition of ouabain at the final minute of incubation has only a small effect on the ion content (closed symbols without ouabain, open symbols with ouabain). The 2-aminoisobutyrate uptake during the final minute of incubation, however, is remarkably different between the two batches as seen in Fig. 2. The continuously rising curve of the batches to which ouabain was added for the final incubation with 2-aminoisobutyrate (open symbols) shows the effect of changing internal ion content on amino acid transport in absence of pump activity. The increasing 2-aminoisobutyrate uptake is a result of the increasing chemical potential gradient of Na^+ and the increasing membrane diffusion potential due to the increasing disequilibrium of cations. In the absence of ouabain, i.e. with operating pump (solid symbols) transport activity is maximal at early times after addition of K^+, and declines as the time interval between K^+ and 2-aminoisobutyrate addition increases. The difference between the two series with and without ouabain can be attributed to the electrogenicity of the ion pump and shows the response of the pump potential to the variation in cellular ion content. The pump potential is seen to decline as the intracellular ion composition approaches its normal state with increasing incubation time in K^+ buffer. (No explanation can be given for the somewhat reduced 2-aminoisobutyrate uptake of inhibited cells right after addition of K^+ as compared to the following time intervals. As the electrolyte content (Fig. 1) shows, there is no delay in K^+ uptake, i.e the pump is immediately active.)

The question arises whether this decline of the pump potential is predictable from the equation under constant pumping rate without postulating a regulatory reduction of pump activity. To answer this question we shall first see what the behavior of the electrogenic pump potential is to be expected under the condition that the pump activity is constant thoughout the whole experiment. An equation has to be derived showing the behavior of the potential in the presence of the electrogenic pump in the transient state, i.e. before reaching its static head. The equation derived by Mullins and Noda [7] for electrogenic pumps, only applies to the steady state and cannot be used here. On the other hand, an equation given by us in a previous paper [2] applies to a transient state though, giving the electrogenic contribution by an additional term in the Goldman equation. This term, however, is only of qualitative significance, since it is itself a function of the electrical potential which it helps to generate. A more precise evaluation of this relationship is given by the following equation, which is derived in the Appendix:

$$\zeta_{tot} = \zeta_{diff} + (j^2/2) + j\sqrt{\zeta_{diff} + j^2/4}$$

$$\zeta_{tot} = \exp(-F\Delta\Psi/RT)$$

$$\zeta_{diff} = (p_n \cdot n'' + p_k \cdot k'')/(p_n \cdot n' + p_k \cdot k')$$

where n', n'' = extra- and intracellular concentrations of Na^+; k', k'' = extra- and intracellular concentrations of K^+; and p_n, p_k = permeation probabilities for

Na^+ and K^+.

$$j = (\nu_n - \nu_k) \cdot J_r / (p_n \cdot n' + p_k \cdot k')$$

where J_r = rate of the ($Na^+ + K^+$)-pump; and ν_n, ν_k = stoichiometric coefficients of the ($Na^+ + K^+$)-pump for Na^+ and K^+.

It clearly differentiates between the diffusional component and the contribution of the electrogenic pump to the total membrane potential. Provided that the pump term j remains constant, i.e. the stoichiometry and the rate of the pump as well as the permeabilities for Na^+ and K^+ are constant at any time of the experimental procedure, the equation predicts that the total membrane potential increases continuously with increasing diffusion potential after an initial jump when the pump starts operating. Since this predicted behavior of the membrane potential fundamentally differs from the experimental observations shown in Fig. 2, the assumption of a constant pump term j is clearly incompatible with the experimental findings. This forces us to assume that the electrogenic pump is strongly activated by the unphysiological composition of cellular electrolytes (high Na^+, low K^+), but declines drastically as the physiological electrolyte composition of the cell interior is restored by the activity of the pump. In Fig. 3 the data of the experiment are replotted to show the dependence of amino acid transport on cellular Na^+, from which the dependence of the pump potential on cellular Na^+ was derived (difference curve). This dependence is well comparable to the dependence of the ($Na^+ + K^+$)-ATPase activity of plasma membranes of Ehrlich cells on Na^+ [8,9], which was half-maximally activated at 16 mM Na^+, maximally at 60 mM Na^+.

As a direct measure of pump activity the ouabain-inhibitable unidirectional K^+ influx may serve. Since only a minor fraction of the unidirectional K^+ influx

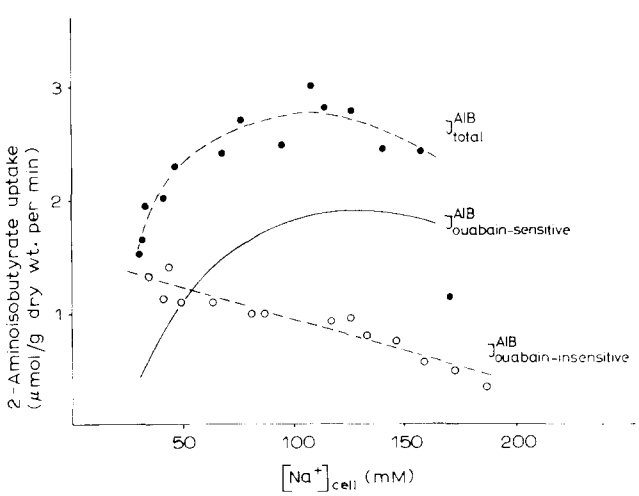

Fig. 3. The dependence of the initial rate of 2-aminoisobutyrate uptake on the intracellular Na^+ concentration. The same data as in Fig. 2 are plotted against the cellular Na^+ concentration at the end of the corresponding 1-min 2-aminoisobutyrate uptake. The symbols have the same meaning as in Fig. 2. The solid line in the middle represents the difference between the ordinate values of the two curves. Ordinate: initial uptake of 2-aminoisobutyrate in μmol/g as dry weight per min. Abscissa: intracellular Na^+ concentration in mM. J^{AIB} = 2-aminoisobutyrate uptake.

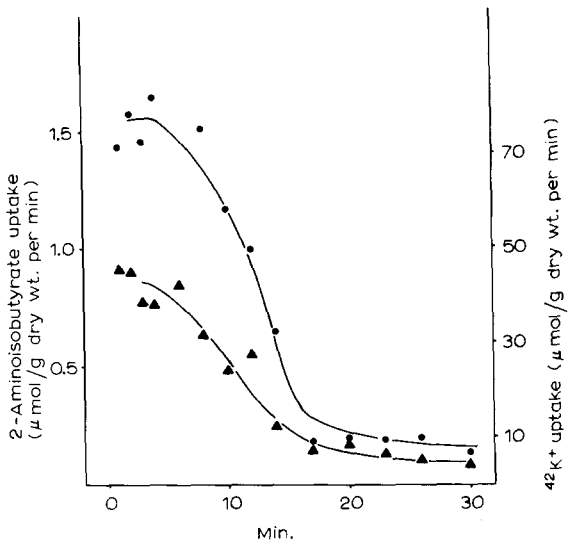

Fig. 4. The ouabain sensitive 2-aminoisobutyrate and $^{42}K^+$ uptake. In an experiment similar to that of Fig. 2 and 3 the electrogenic pump was initiated by the addition of 15 mM K^+ at time 0. Subsequently the initial rates of the ouabain inhibitable 2-aminoisobutyrate and ^{42}K, uptake, respectively, were measured at the time intervals indicated on the abscissa. Ordinates: initial rate of 2-aminoisobutyrate uptake in μmol/g dry weight per min after subtraction of the ouabain insensitive part (●), and the initial rate of isotopic K^+ uptake after subtraction of the ouabain-insensitive part in μmol/g dry weight per min (▲). Abscissa: time in minutes after the addition of K^+.

is diffusional influx [10], the indirect influence of ouabain on it due to the lowering of the membrane potential is insignificant. An experiment was conducted in the same manner as the one described before except that besides 2-[^{14}C]aminoisobutyrate also $^{42}K^+$ was added for the final 1 min incubation. Fig. 4 shows the ouabain-inhibitable unidirectional uptakes of 2-aminoisobutyrate and K^+ at the different times after starting the pump by adding K^+. Obviously, the time course of both parameters is similar, as indicates that indeed changes in the pump rate are responsible for the changes in pump potential monitored by the ouabain inhibitable 2-aminoisobutyrate uptake.

In Fig. 5 a third series of assays is shown in which besides 2-aminoisobutyrate and ouabain valinomycin was added for the final 1 min incubation period. Due to the increase in K^+ permeability effected by valinomycin the membrane diffusion potential is predominantly determined by the K^+ distribution. As compared to the corresponding potential difference without valinomycin the membrane diffusion potential and the 2-aminoisobutyrate uptake is little effected by the addition of valinomycin at low internal K^+ concentrations in the beginning of the experiment. With rising internal K^+ concentration the effect of valinomycin increases reaching its maximum at the end of the experiment at high cellular K^+ concentrations as can be predicted from the Goldman equation. At the end of the experiment, when the pumping cells have reached steady-state, the 2-aminoisobutyrate uptake and hence the membrane potential of the nonpumping cells in the presence of valinomycin is higher than that of the pumping cells.

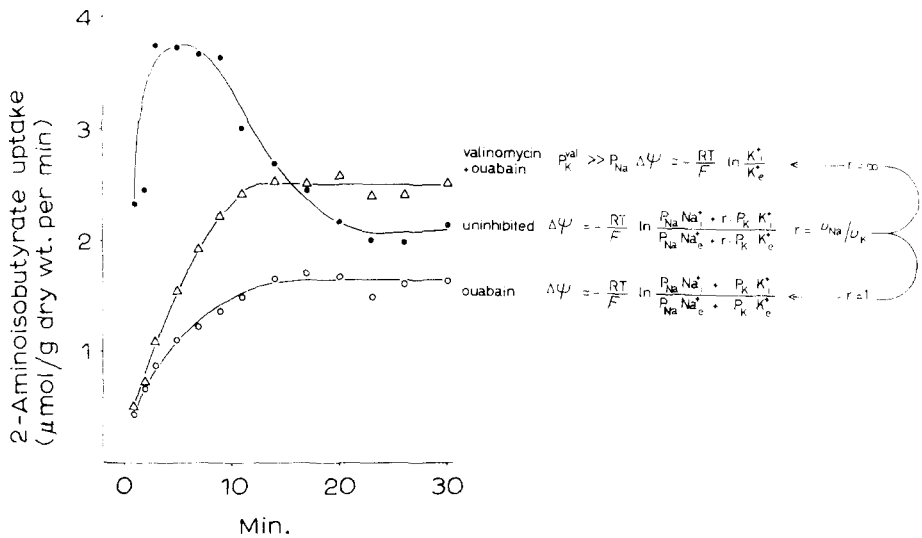

Fig. 5. The initial rate of 2-aminoisobutyrate uptake. The effect of ouabain and ouabain + valinomycin, respectively. The experiments were carried out as those described before. At time 0, 13 mM K^+ was added to K^+-depleted Ehrlich ascites tumor cells. As in the experiment of Fig. 2 the initial rate of 2-aminoisobutyrate net uptake was tested at various time intervals after K^+ addition as indicated on the abscissa. The symbols have the same meaning as in Fig. 2, representing the total 2-aminoisobutyrate uptake (●) and the ouabain-insensitive 2-aminoisobutyrate uptake (○), respectively. △, ouabain-insensitive 2-aminoisobutyrate uptake in the presence of 30 μM valinomycin. Ordinate: initial rate of 2-aminoisobutyrate uptake in μmol/g dry weight per min. Abscissa: time in minutes after addition of K^+. The equations on the right side are to predict the membrane potential at the final steady state for each condition: From bottom to top: 1. Equation applying in the presence of ouabain, i.e. in absence of the electrogenic pump. 2. the membrane potential in steady state, in the presence of an electrogenic pump, according to Mullins and Noda [7], where r is the ratio of the stoichiometric coefficients of Na^+ to K^+ transport by the pump. 3. the membrane potential in absence of the electrogenic pump, but in presence of valinomycin, where the permeability of Na^+ is negligible as compared to that of K^+. If r is either 1 or ∞ the equation of Mullins and Noda is reduced as indicated.

For non-pumping cells the Goldman equation can be used to estimate the membrane potential, while for the pumping cells in steady-state an analogous equation derived by Mullins and Noda [7] is applicable. Comparing the three equations and the experimental data for the 2-aminoisobutyrate uptake at high cellular K^+ concentrations allows some conclusions about the stoichiometry of the ($Na^+ + K^+$)-pump. Since the potential of pumping cells is higher than for non-pumping cells the pump must be electrogenic ($r > 1$) also in steady-state when its rate is reduced. On the other hand, the pump cannot be a pure Na^+ pump with K^+ distributing passively following the membrane potential ($r = \infty$). In this case in steady-state the K^+ distribution would be at electrochemical equilibrium and therefore the addition of valinomycin would not lead to the increase in membrane potential as the experimental results indicate.

The investigations show that the membrane potential of Ehrlich cells is not only a membrane diffusion potential but that there is an additional component due to the activity of the ($Na^+ + K^+$)-pump. The activity of the pump is

regulated by the internal ion composition. It is stimulated under high cellular Na^+ and low K^+. Its rate is reduced at low Na^+ and high K^+. It is electrogenic under all conditions. It is an $(Na^+ + K^+)$-exchange pump, which tightly links outward Na^+ movement with inward K^+ movement. As in erythrocytes, electrogenicity is brought about by an unequal stoichiometry of Na^+ and K^+ [11].

Appendix

J_{Na}, J_K Netfluxes of Na^+ and K^+, uptake positive;
J_r rate of the $(Na^+ + K^+)$-pump;
k', k'' extra- and intracellular concentrations of K^+;
n', n'' extra- and intracellular concentrations of Na^+;
p_n, p_k permeation probabilities for Na^+ and K^+;
t' $p_n \cdot n' + p_k \cdot k'$;
t'' $p_n \cdot n'' + p_k \cdot k''$;
ν_n, ν_k stoichiometric coefficients of the $(Na^+ + K^+)$-pump for Na^+ and K^+;
j $(\nu_n - \nu_k) \cdot J_r/t'$;
ζ electrochemical activity coefficient, $\exp(-F\Delta\Psi/RT)$;
ζ_{diff} t''/t'.

Na^+ and K^+ are transported through a symmetrical barrier membrane [12] via two routes, a passive electrodiffusional one and a electrogenic $(Na^+ + K^+)$-pump. The fluxes of the two ions are described by the following equations:

$$J_{Na} = p_n \cdot (n' \cdot \zeta^{1/2} - n'' \cdot \zeta^{-1/2}) - \nu_n \cdot J_r$$
$$J_K = p_k \cdot (k' \cdot \zeta^{1/2} - k'' \cdot \zeta^{-1/2}) + \nu_k \cdot J_r \quad (1)$$

Neglecting the fluxes of other ions one gets:

$$J_{Na} + J_k = t' \cdot \zeta^{1/2} - t'' \cdot \zeta^{-1/2} - \Delta\nu \cdot J_r = 0 \quad (2)$$

$$\zeta^{1/2} - \zeta_{diff} \cdot \zeta^{-1/2} - j = 0 \quad (3)$$

This equation holds also if the permeation of other ions is taken into consideration; for this case t' and t'' would have additional terms of the form $p_x \cdot x$. Solving Eqn. 3 one gets:

$$\zeta = \zeta_{diff} + j^2 \cdot \frac{1 + \sqrt{1 + 4 \cdot \zeta_{diff}/j^2}}{2} \quad (4)$$

One sees that $(\delta\zeta/\delta\zeta_{diff})_j$ and $(\delta\zeta/\delta j)_{\zeta_{diff}}$ are positive. This is true also if the barrier is not symmetrical.

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