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DRIVING FORCES AND CURRENT-VOLTAGE CHARACTERISTICS OF AMINO ACID TRANSPORT IN *RICCIA FLUITANS*

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The complete steady-state *I-V* relationship of α-aminoisobutyric acid transport across the plasmalemma of rhizoid cells from *Riccia fluitans* has been measured and analysed with special emphasis on α-aminoisobutyric acid equilibrium and saturation conditions. (A) The electrical data show that: (1) the amino acid-induced electrical current saturates after the addition of the amino acid, regardless of the concentration; (2) a steady state is reached 1–2 h after incubation in α-aminoisobutyric acid, but after less that 5 min in the presence of 1 mM CN^-; (3) the steady-state *I-V* characteristic of α-aminoisobutyric acid transport is a sigmoid curve and fairly symmetric in current with respect to the voltage axis; and (4) the equilibrium potential is clearly a function of the amino acid accumulation ratio. It is suggested that the sigmoid curve represents the characteristic of carrier-mediated α-aminoisobutyric acid transport with a voltage-insensitive step, possibly the translocation of the unloaded carrier, rate-limiting. Since under normal conditions the voltage-sensitive rate constant k_{oi} is much greater than k_{io}, it is further suggested that the energy to drive this system is put into the transfer of positive charge from outside to the cytoplasm. (B) Accumulation ratios have been determined by inspection of current-voltage data, and additionally by compartmental analysis on green thalli from *Riccia fluitans*. Both methods give ratios far too low compared with the thermodynamically possible accumulation of about 10^4. It is suggested that substantial leakages via different non-electrical pathways prevent equilibrium at steady state, and it is concluded that in such leaky systems the thermodynamic equilibrium condition is not suitable for estimating stoichiometries.

Introduction

Analysis of electrogenic transport requires continuous measurements of membrane potential, membrane resistance, and electrical current during the translocation of the substrate of interest. Although many sugar and amino acid transport systems that have been investigated are clearly electrogenic [1–7] and have been addressed as co-transport, such tests have rarely been carried out simultaneously on one object [8,9]. The reason for this is that most studies on co-transport have been performed on either very small cells, such as microorganisms [10], on red blood cells [11], or on tissues of both animals [7] and plants [3–6]. But, even when the required measurements had been carried out successfully, the interpretation of such data had to remain somewhat unclear as long as it could not be demonstrated that the observed effects are not merely voltage-dependent phenomena.

Current-voltage measurements can provide more complete information and inspection of data obtained from rhizoid cells of the aquatic liverwort *Riccia fluitans* (Fig. 1) or from *Neurospora* hyphae [8] indeed proves that very little of the observed resistance changes, for example, at maximal depolarization, can directly be attributed to the

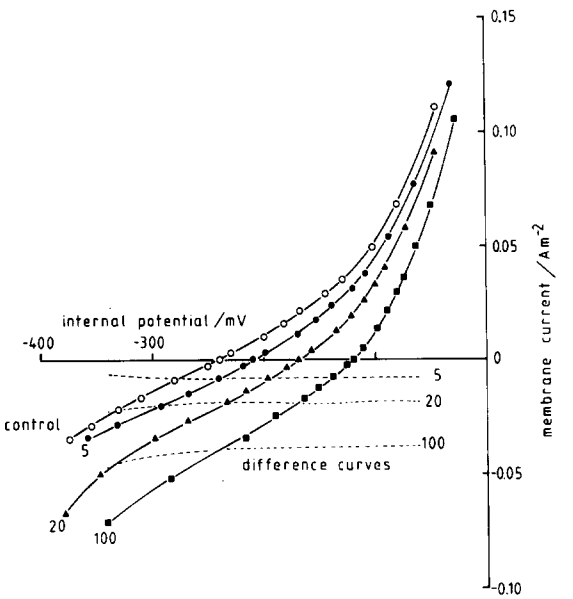

Fig. 1. Current-voltage characteristics, measured on a rhizoid cell of *R. fluitans* before (control) and at peak depolarization after the addition of 5, 20, 100 μM L-alanine to the test medium ($[K^+]_o = 1$ mM). The dashed lines represent the electrical current induced by the L-alanine added at the indicated concentrations. These curves were obtained by graphical subtraction of the control from the corresponding curves.

transport of hexoses [8] or amino acids [9] across membranes.

A complete current-voltage characterisation of a carrier-mediated process describes the transport of the substrate in both directions. However, the measurement of such curves may be complicated. For example, an electrogenic proton pump at the plasmalemma extrudes protons into the external aqueous phase, presumably with ATP as the energy source. If the stoichiometry of H^+/ATP happens to be 1 [13], the reversal potential will be at voltages where the membrane becomes irreversibly damaged upon experimental hyperpolarization. Therefore, only the export of positive charge can be measured in such systems. For a stoichiometry of 2, a complete curve can be measured, as demonstrated for *Acetabularia* [14]. With co-transport of, say, hexoses or amino acids it is the other way around. These are, a priori, uptake systems with equilibrium potentials far more positive than the membrane potential, which is one reason why non-steady-state curves usually are detected incomplete, and therefore yield only import of positive charge [8,9]. Since such substrates are metabolized under normal conditions, the driving gradient always remains inwardly directed.

In this paper, the complete current-voltage characteristic for the transport of the non-metabolizable α-aminoisobutyric acid across the plasmalemma of *Riccia* rhizoid cells has been measured and has been analysed with respect to equilibrium potential, saturation current(s) and voltage-dependence.

Material and Methods

Culture conditions

Green thalli of the aquatic liverwort *Riccia fluitans* were cultured under semi-sterile conditions in twin Erlenmeyer flasks according to Hüsemann and Barz [15] as developed for suspension cultures of *Chenopodium rubrum*. The growth medium contained 1% of the medium of Murashige and Skoog [16], and 99% of the standard test medium. If not noted otherwise, this medium was composed of 0.1 mM KCl, 0.1 mM $CaCl_2$ and 2 mM sodiumphosphate buffer; external pH was 6.5. All other conditions were identical to those described previously [9].

Electrical experiments

In order to control better the horizontal approach of the cells by the microelectrodes, a Leitz and a Narishige micromanipulator were connected to each other in series. The microelectrodes were pulled from glass tubing containing glass fiber (Hilgenberg) on a Getra-vertical or a Camden-horizontal instrument. Typical tip diameters were around 0.4 μm. The electrodes were backfilled with 3 M KCl and had typical resistances between 30 and 60 MΩ. The Ag/AgCl-wires were connected to a high-impedance amplifier (WPI: M707 or KS700); signals were recorded on a pen chart (Kontron, W + W-314). For current-voltage measurements, trains of rectangular pulses of constant current from a stimulator (WPI: series 1800) were fed into the cells and monitored by one or two electrodes at different distances from the injecting electrode, which was usually placed at the midpoint of the rhizoid. The electrical space constant was typically around 0.4 mm. As described previ-

ously [17], the input data were subjected to Cole's theorem [18].

Tracer experiments

Uptake data were obtained from green thalli as described recently [9]. Measurement of release of ^{14}C-labeled compounds was carried out after preloading the tissues for 48 h with the radioactive substrate. The sample was rinsed and then transferred into a Plexiglass efflux chamber, which allowed either unstirred sampling or continuous flow by the non-radioactive but otherwise identical incubation medium. The aqueous samples were mixed with Unisolve-1 (Zinsser) and assayed in a Berthold-Frieseke scintillation counter (BF-5000). The sample with the residual radioactivity was processed like the uptake samples.

Accumulation of amino acids within the cytoplasm was determined either by electrically measuring the equilibrium potential and estimating the pH gradient or by comparing data from uptake measurements and from standard compartmental analysis [19].

Estimation of the cytoplasmic volume

Since *Riccia* thalli are only a few cell-layers thick, the surface/volume ratio is satisfactory. For the average thallus cell, a vacuole-to-cytoplasma ratio of about 5 : 1 ≙ 2–3 μl/μg dry wt. has been obtained from cross-sections.

Results

Electrical data

1. Membrane potential and conductance. As reported previously [9], an electrogenic and stereospecific amino acid (probably proton co-) transport system with high affinity for neutral amino acids (e.g., apparent K_m for α-aminoisobutyric acid approx. 12 μM) exists within the plasmalemma of *R. fluitans* thallus and rhizoid cells. The kinetics of the changes in membrane potential ($\Delta\psi_m$) and of membrane conductance ($g_m = 1/r_m$) before and after the addition of 50 μM α-aminoisobutyric acid, and in the presence or absence of 1 mM CN^- are shown in Fig. 2. While recording A demonstrates the typical rapid depolarization with sharp increase in g_m at maximal depolarization, recording B, which has been measured in the presence of 1 mM CN^-, only displays a small depolarization and likewise a small increase in g_m. Both repolarization as well as the recovery of the conductance take place spontaneously. It would be an error to conclude that the observed Δg_m were in any way amino acid-specific. Since the *I-V* curves in Figs. 1 and 3 are clearly nonlinear, any depolarization of the membrane positively has to result in a merely voltage-dependent increase in the conductance. Therefore only difference *I-V* curves, as shown in the lower parts of Figs. 1 and 3, can be attributed directly to the amino acid

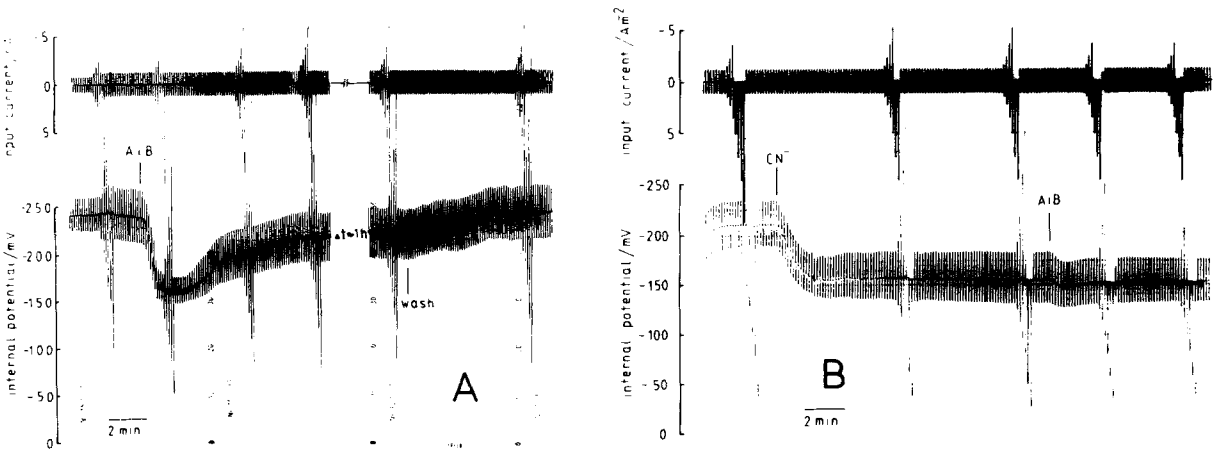

Fig. 2. Original recordings, photographed from the pen chart, of membrane depolarization, repolarization, and change in membrane resistance from *R. fluitans* rhizoid cells after the addition of 50 μM α-aminoisobutyric acid to the test medium, as indicated. A, in the absence of; B, in the presence of: 1 mM CN^-. The measurement of *I-V* curves is indicated by trains of altered electrical current.

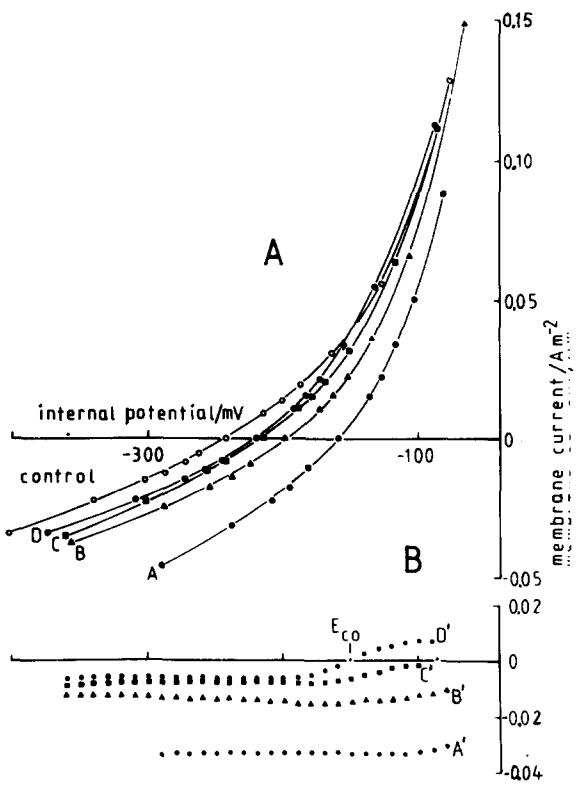

Fig. 3. Part A. Current-voltage characteristics of *R. fluitans* rhizoid cells, measured before (control) and at different times after the addition of 50 μM α-aminoisobutyric acid to the test medium. A, approx. 1 min; B, approx. 6 min; C, approx. 10 min, D approx. 70 min. Part B. A', B', C', D', are difference curves (compare Fig. 1) derived by subtraction of the control from the A, B, C, D curves. For better accuracy, the subtraction has not been carried out graphically, but directly from the Cole-correction data.

transport, provided no other electrogenic membrane element or even metabolism has been interfered with. But since α-aminoisobutyric acid has been shown not be to metabolized [20], such apprehensions are not stressed.

The amino acid-induced electrical current. The difference *I-V* curves in Figs. 1, 3 and 4 represent the net current induced by the addition of the cited amino acids under the indicated conditions. Fig. 1 shows that this current depends upon external L-alanine concentration, and Fig. 3B additionally states that this current also changes time-dependently during depolarization and repolarization, respectively. The net currents, measured at peak depolarization (Fig. 1; Fig. 3B, curve A') are independent of voltage, and thus describe characteristics of ideal current sinks. Since at this initial phase of transport the internal amino acid concentration should still be too low for any equilibration potential E_{co} ($i_{co} = 0$) to be measured, naturally no net movement of 'amino acid export current' can take place over the voltage ranges tested. After about 1 h of incubation in α-aminoisobutyric acid, this has changed. Curve D' (Fig. 3B) intersects the voltage axis at approx. −150 mV, which represents the equilibrium potential under the given conditions. Furthermore, the curve displays the net saturation current for α-aminoisobutyric acid import (i_s^-) as well as for α-aminoisobutyric acid export (i_s^+). Since after another 1 h of incubation in this amino acid the parameters of the *I-V* curve have not changed at all, it is assumed that a steady state has been reached. Curves B' and C', measured during the repolarization phase, have intermediate character and become interesting as soon as the equilibrium potential is within measuring range. For an assumed proton co-transport, no net current flows at

$$E_{co} = RT/F \ln([\mathrm{AiB}]_o[\mathrm{H}^+]_o/[\mathrm{AiB}]_i[\mathrm{H}^+]_i) \quad (1)$$

which gives the direct relation of already accumulated α-aminoisobutyric acid (AiB) to the measured equilibrium potential (E_{co}). Since the external pH was set at 6.5, the ΔpH across the plasmalemma can be assumed small. The accumulation ratio would then be about 30 after 10 min (C') and roughly 300 after 70 min (D').

When the same experiment is carried out in the presence of 1 mM CN^- (Fig. 2B, Fig. 4), astonishingly, a steady state is reached within a few minutes. Given that the cytoplasmic pH did remain constant, an apparent α-aminoisobutyric acid accumulation of 130 could be calculated. However, since the driving potential was less negative than in the control situation, the cytoplasmic pH probably did change as a consequence of the CN^- treatment.

Tracer experiments

Accumulation ratios. The accumulation of amino acids within the cytoplasm of *R. fluitans* thallus cells is clearly a function of the external amino

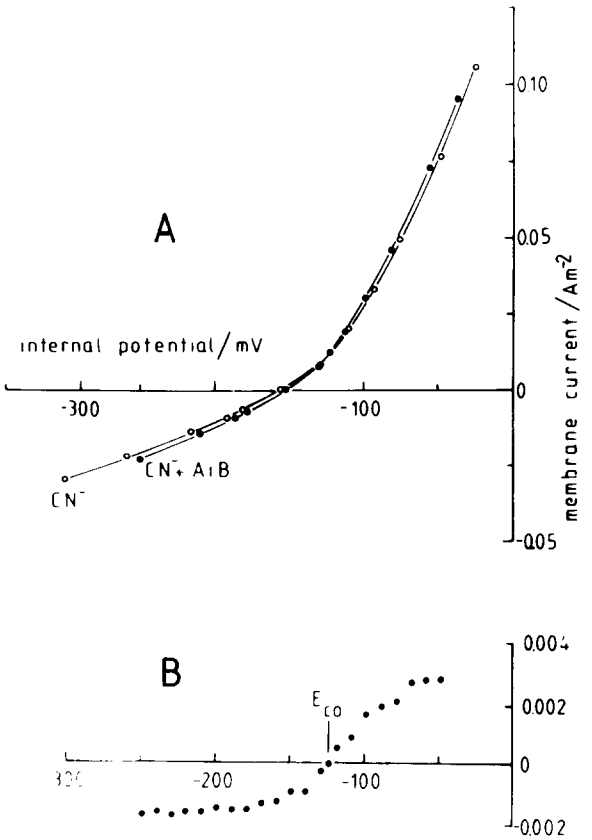

Fig. 4. Part A. Current-voltage characteristics of *R. fluitans* rhizoid cells, measured in the presence of 1 mM CN^- and 3 min after the addition of 0.1 mM α-aminoisobutyric acid to the test medium. Part B. *I-V* difference curve, derived as described in the legend of Fig 3.

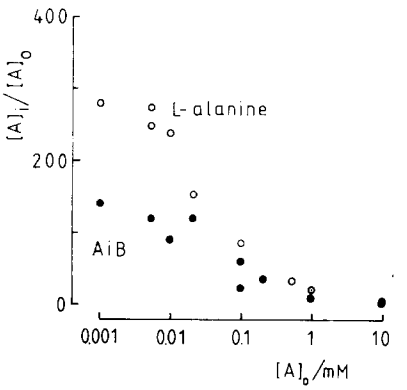

Fig. 5. Ratio of internal and external amino acid concentration ($[A]_i/[A]_o$) vs. external amino acid concentration after 2 h of α-amino[^{14}C]isobutyric acid and L-[^{14}C]alanine uptake into green thallus cells of *R. fluitans*.

acid concentration (Fig. 5). For example, after 2 h uptake, the ratio $[A]_i/[A]_o$ is not even unity with $[A]_o = 10$ mM, but about 150 for α-aminoisobutyric acid or about 300 for L-alanine at 1 μM external alanine. The higher alanine ratio may simply be the result of alanine being metabolized and therefore taken out of the transport cycle. But compared with the ratios of about 1600 or 2500 reported for the hexose transport in *Chlorella* [2] and *Neurospora* [8], respectively, the accumulation of amino acids in *Riccia* appears to be very low. Even given an error of 2 or 3 in estimating the cytoplasmic volume, the corrected data would still be far below the possible accumulation of about 10^4 under the experimental conditions reported for Fig. 5.

Inhibition and stimulation of the amino acid transport. Since it is assumed that the amino acid transport is linked to the electrochemical proton gradient produced and maintained by the electrogenic pump at the plasmalemma, inhibition or stimulation of this pump resulting in a change in this gradient could likewise influence the transport of amino acids across that membrane. Therefore, the following agents have been tested at different concentrations: ammonium, which is electrogenically transported across the plasmalemma of *R. fluitans* by a specific ammonium/methylammonium uptake system [21], electrically short-circuits the pump; cyanide (CN^-), which is known to inhibit ATP synthesis and therefore decreases the pump activity due to lack of ATP; and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which as a powerful uncoupler also prevents ATP synthesis, but additionally induces a proton leak in the plasmalemma [22]. The results are given in Fig. 6. It is remarkable that, although ammonium and CN^- at 10^{-4} M each depolarize the plasmalemma to essentially the same potential, the inhibition of α-amino[^{14}C]isobutyric acid uptake is some 50% by NH_4^+, but over 80% by CN^-. Since CCCP as a protonophore dissipates a good deal of the protonmotive force ($\Delta\psi_m$ and ΔpH), the observed stronger inhibition of about 90% appears reasonable.

Fusicoccin (FC), which hyperpolarizes the plasmalemma of *Riccia* by 30 to 35 mV, is supposed to stimulate the electrogenic proton-extru-

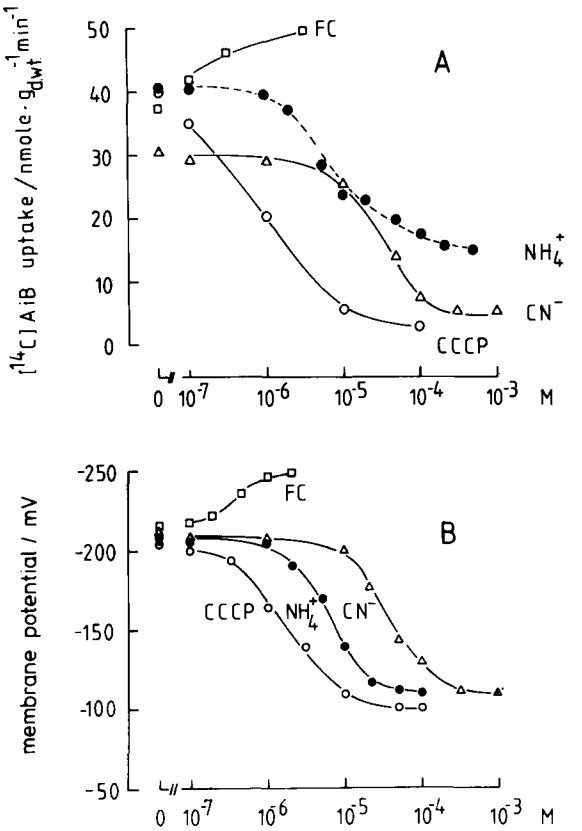

Fig. 6. Action of different substances at various external concentrations on α-amino[^{14}C]isobutyric acid uptake (1 min) into green cells of *R. fluitans* (A), and on the membrane potential (B).

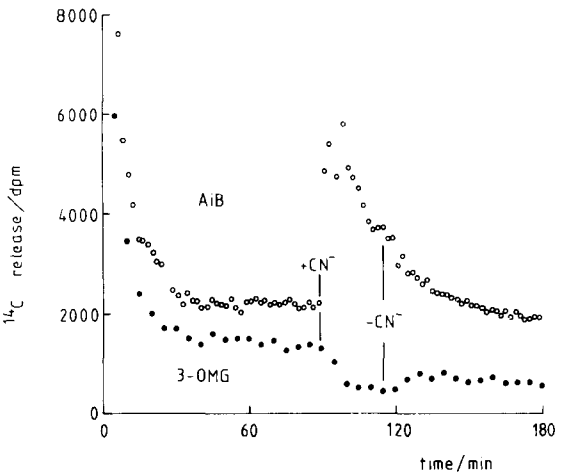

Fig. 7. Effect of 1 mM CN^- on the release of α-amino[^{14}C]isobutyric acid and 3-*O*-methyl[^{14}C]glucose from green thallus cells of *R. fluitans*. External concentrations were 0.1 mM α-aminoisobutyric acid and 3-*O*-methylglucose each; incubation time was 48 h.

sion pump of a wide variety of plant cells [23,24], and therefore should increase the protonmotive force across the plasmalemma. As Fig. 6 demonstrates, FC indeed stimulates α-amino[^{14}C]isobutyric acid uptake by about 20% and thus seems to confirm the above notion. The effect of CN^- on the release of α-amino[^{14}C]isobutyric acid is demonstrated in Fig. 7. After about 1.5 h of efflux, 1 mM CN^- is added and a very rapid but transient release of label is observed. This appears trivial, since the degradation of the inwardly directed driving force should indeed favor export of positive charge. However, the same experiment carried out with ^{14}C-labeled 3-*O*-methylglucose, where export is inhibited by 1 mM cyanide (Fig. 7), indicates that this interpretation may be too simple, and that import and export of such substrates need not be mediated energetically in the same way.

Discussion

The rapid depolarization and spontaneous repolarization, as observed upon addition of amino acids to cells of *R. fluitans*, proves this transport to be clearly electrogenic. The speed and the extent of such potential changes depend upon time-dependent interaction of different factors. These are (1) external/internal amino acid concentration, as indicated by the 'migration' of the equilibrium potential on the voltage axis (Fig. 3B, Eqn. 1), (2) the permeability of the counterion (probably K^+; unpublished results), and (3) the activity of the electrogenic pump, which may be stimulated by an acidification of the cytoplasm due to proton import along with the amino acid. In this context it is interesting to find that the spontaneous repolarization also takes place in the presence of 1 mM CN^- (Fig. 2B).

(4) One would expect that the electrical potential difference across the plasmalemma should likewise influence the speed of depolarization and the extent thereof, but the voltage-insensitivity of the amino acid-induced current (Figs. 1 and 3)

makes this questionable, at least for certain voltage ranges.

Driving forces

The assumption of a thermodynamic equilibrium between solute gradient and the driving force has been fundamental to recent studies of solute transport [25]. For a proton-linked system, which electrogenically transports α-aminoisobutyric acid(AiB), this can be expressed as follows:

$$RT/F \ln([\mathrm{AiB}]_i/[\mathrm{AiB}]_o)$$

$$= n(\Delta\psi_m - (RT/F)\ln([H^+]_i/[H^+]_o)) \quad (2)$$

with the driving force

$$\Delta\mu_{\mathrm{AiB}} - n(\Delta\psi_m - (RT/F)\ln\Delta\mathrm{pH}) \quad (2a)$$

and the stoichiometry

$$\mathrm{AiB}/\mathrm{H}^+ = \Delta\mu_{\mathrm{AiB}}/\Delta\tilde{\mu}_{\mathrm{H}^+} = n \quad (2b)$$

R, T, F have their usual meanings and $\Delta\psi_m$ is the altered membrane potential (inside negative).

Fig. 5 gives two important results. Firstly, the measured α-aminoisobutyric acid accumulation is far below the thermodynamically possible ratio of about 10^4. Secondly, $[\mathrm{AiB}]_i/[\mathrm{AiB}]_o$ varies strongly with $[\mathrm{AiB}]_o$, which means that in order to satisfy Eqn. 2, $\Delta\psi_m$, ΔpH and/or the stoichiometry must also depend upon $[\mathrm{AiB}]_o$. Whereas there is no doubt that this is true for $\Delta\psi_m$, a change in ΔpH can only be assumed. But since the changes in internal pH during α-aminoisobutyric acid uptake are probably small, the change in protonmotive force can be taken as essentially identical with $\Delta\psi_m$, and therefore cannot explain the massive discrepancy of $\Delta\mu_{\mathrm{AiB}}$ vs. $\Delta\tilde{\mu}_{\mathrm{H}^+}$! Is, therefore, a change in stoichiometry the only alternative? Certainly not! Several reasons can be given for low accumulation ratios.

(a) Leakages in transport. Since electrogenic amino acid transport is assumed to occur by a ternary carrier-substrate-proton complex, only a tightly coupled system will yield the theoretical equilibrium. Amino acid which has been translocated electrically may leave the cytoplasm again as binary carrier-substrate complex which would be electrically neutral and therefore not subject to electrical driving forces [26]. Since these driving forces can be very high, such an arrangement protects the cell from overcharging the pools in case of higher external substrate concentrations.

(b) Poor carrier-substrate efficiency, which includes the possibility of non-carrier-mediated leakage of amino acid through the membrane. From the accumulation data and the considerations above, it can be concluded that stoichiometries, as obtained from such leaky systems, are apparent and cannot have any meaning as to the 'real' coupling between carrier, substrate, and co-ion.

(c) Even the possibility of a cotransport other than one linked to the proton should be considered. A potassium co-transport has been discussed by Van Bel and Van Erven for the transport of glutamic acid in tomato xylem vessels [27]. On the basis of several potassium effects, e.g., on the uptake of ^{14}C-labeled amino acids [28] or on the location of the equilibrium potential (data not shown), such a mechanism has been considered in our working group, too. But in the light of arguments (a) and (b) proton co-transport would not have to be immediately excluded. Also, there is always some uncertainty as to the real pH gradient across the plasmalemma, because protons may circulate within the membrane without ever measurably influencing external or internal pH.

Voltage dependence

Whereas a driving force includes electrical as well as chemical forces, and describes the theoretical power to transport a substrate, e.g., against its concentration gradient, the voltage dependence only refers to electrical transport parameters, which can be seen under a merely kinetic or thermodynamic aspect. A voltage change will alter the driving force, but not necessarily the electrical current, as shown by the current-voltage difference curves in Figs. 1, 3, and 4. As will be shown below, this occurs because mainly voltage-insensitive processes rule the current-voltage relationships within that voltage range.

On the other hand, changes in membrane potential or proton motive force do indeed alter radioactive amino acid uptake, e.g., hyperpolarization stimulates (fusicoccin) and depolarization of the plasmalemma (ammonium) inhibits ^{14}C-labeled

amino acid uptake (Fig. 6). In the presence of metabolic inhibitors (CN^-, CCCP) this uptake is decreased even more, although the membrane potential is at about the same level as in the presence of 0.1 mM ammonium. This could mean that either the cross-membrane pH difference has been changed by massively decreasing the activity of pH-regulating mechanisms, or the transport of amino acids is closely linked to metabolic energy. Whereas little evidence has been brought forward for the latter suggestion so far [29], there is some experimental proof for a strong acidification of the cytoplasm after the addition of CN^- (see below).

The parameters of the current-voltage relationships

The typical sigmoid carrier curves (Figs. 3 and 4) have several parameters of interest. (1) The saturation current i_s^- and i_s^+, which represent the voltage-insensitive portions of the curves far from equilibrium; (2) the equilibrium potential the conditions of which are given in Eqns. 1 and 5, and (3) the voltage-dependent slope at maximal steepness of the curve (which will not be dealt with in this work).

The current saturation, regardless of external amino acid concentration, is probably one of the most puzzling results, and on first sight one will find it difficult to find a connection with the classical Michaelis saturation. But it simply indicates that some reaction step other than the charge transfer limits the electrical 'amino acid current' across the plasmalemma. And since association/dissociation of carrier and transported substrates can be assumed to be very fast reactions, it is more likely that the translocation of either the unloaded carrier or of the uncharged carrier/amino acid complex are rate-limiting.

If the membrane is considered to be a single and symmetric energy barrier to the translocation of charge [30], the ratio of voltage-sensitive rate constants can be written as

$$k_{io}/k_{oi} = k_{io}^0/k_{oi}^0 \cdot \exp(zF\Delta\psi_m/RT) \quad (3)$$

where the subcripts i and o mean inside and outside, the superscript zero designating the translocation rate constants at zero membrane potential. Recently, Hansen et al. [12] have demonstrated that all those cyclic models of transport systems with a single charge transfer step can be reduced to a two-state model. Only two steps are considered: Besides the charge-carrying step (k_{io}, k_{oi}), all accessible and inaccessible voltage-insensitive steps are lumped together and given the pseudo-rate constants x_{io} and x_{oi}. Several equations were derived which enable one to obtain ratios of such constants, once the equilibrium potential and the saturation currents at steady state are known:

For the saturation currents

$$i_s^-/i_s^+ = -x_{io}/x_{oi} \quad (4)$$

for the equilibrium potential

$$E_{co} = RT/zF \ln(k_{oi}^0 x_{io}/k_{io}^0 x_{oi}) \quad (5)$$

and for the driving force

$$\Delta\psi_m - E_{co} = RT/zF \ln(k_{io}x_{oi}/k_{oi}x_{io}) \quad (6)$$

According to the data from Figs. 3 and 4, $x_{oi}/x_{io} = 1.17$ without CN^-, and 1.54 in the presence of 1 mM CN^-. So, one basic result is that CN^- has not much influence on the ratio x_{oi}/x_{io} (the voltage-independent rate constants), which makes k_{io}/k_{oi} clearly a function of the driving force (Eqn. 6). The second important result is that k_{oi} is much larger than k_{io} in the absence of CN^-. This is not surprising, since it states that the energy to drive this system is seemingly put into the transfer of positive charge from the outside to the cytoplasmic side of the membrane. The third result is that in the presence of CN^- the driving force ($\Delta\psi_m - E_{co}$) has been reduced considerably in a way that already after a few minutes of exposure to 1 mM CN^- very little power to accumulate is left, although the substantial membrane potential (−150 mV in Fig. 4) could still do so. But preliminary [^{14}C]DMO (5,5-dimethyloxazolidine-4-dione) distribution tests (data not shown) strongly indicate a rapid acidification of the cytoplasm with CN^- added.

Subsequently, this causes a break-down of the driving force, and also explains the negative equilibrium potential (−120 mV) observed already a few minutes after the addition of α-aminoisobutyric acid. If this pH shift can be confirmed, e.g., by direct measurements with pH-sensitive micro-

electrodes, it could give strong evidence for this system to be a proton co-transport.

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