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STEADY-STATE CURRENT-VOLTAGE CHARACTERISTICS OF AMINO ACID TRANSPORT IN RHIZOID CELLS OF *RICCIA FLUITANS*

IS THE CARRIER NEGATIVELY CHARGED?

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The amino acid transport across the plasmalemma of *Riccia fluitans* rhizoid cells has been further characterized by means of current-voltage (*I-V*) analysis. On the basis of two cyclic transport models which include six different carrier states, the question is raised, whether the electrochemical pH-gradient drives a negatively charged carrier or a positively charged alanine-proton-carrier complex across the membrane. *I-V* analysis shows that (1) the typical *I-V* characteristic of L-alanine transport follows a sigmoid curve, (2) maximal accumulation of L-alanine within the cytoplasm is reached after about 1 hour, (3) the electrically accessible cytoplasmic L-alanine concentration is limited to about 20 mM, and (4) the steady-state saturation current depends directly on external L-alanine concentration. It is concluded that (a) these results are consistent with the predictions of the models for a negatively charged carrier, and (b) that the rate-limiting step involves the translocation of the ternary complex.

Introduction

In many plant cells the electrical potential difference across the plasmalemma plays a dominant role in carrier-mediated substrate transport [1]. Although the membrane potential per se may not be important for initial uptake rates, it is an integral part of the allover driving force for substrate accumulation within the cytoplasm. Theoretically, the charge transfer across a membrane can proceed in two ways: Either the (hypothetical) carrier itself is electrically negative, or the carrier is neutral, which means that the co-ion lends its positive charge to the (likewise hypothetical) ternary complex. Although both mechanisms are thermodynamically equivalent, and hence lead to the same substrate accumulation, there are some interesting differences in electrical steady-state behaviour. Heinz and Geck [2] investigated the same question on Na^+/α-aminoisobutyric acid cotransport in Ehrlich cells and came to a best fit with a neutral carrier on the basis of radioisotope measurements. Since cotransport systems in animal cells seem mainly driven by sodium gradients, whereas such systems in plant cells favour the electrical portion of the proton gradient across the membrane, it may well be that there are also differences in the type of charge transfer. A decision on this question can be found by the method of steady-state current-voltage analysis. Recently it has been reported that the total *I-V* characteristic of α-aminoisobutyric acid transport in *Riccia fluitans* indeed follows a sigmoid curve [3], which is to be expected from a carrier-mediated transport, as suggested by Slayman et al. [4], and Hansen and Slayman [5]. For sugar cotransport in *Neurospora* such S-shaped curves have not been found yet, and it has been discussed, whether internal pH-shifts

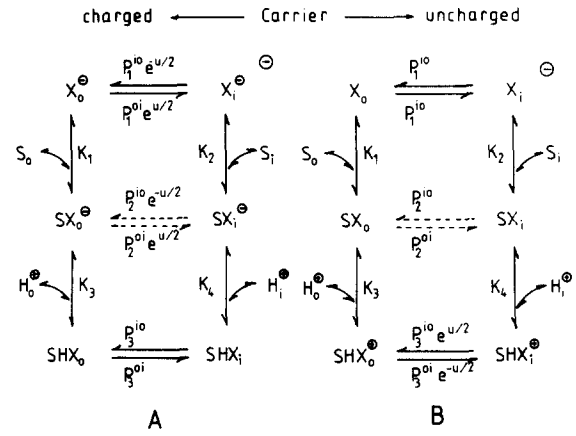

Fig. 1. Two hypothetical carrier schemes for cotransport across a membrane. In scheme A the carrier is assumed electrically negative, whereas in scheme B the carrier is neutral and the ternary complex is positively charged. The K's are the dissociation constants, the P's are the translocation constants, and u is the reduced voltage, given by $zF\psi_m/RT$; i and o denote inside and outside. Note that the translocation of the binary complex is taken into consideration, but is not discussed.

during sugar accumulation and subsequent changes in pump activity could *I-V* recordings, or whether accumulated sugar itself could act as transport inhibitor [5]. In *Riccia* this ceased to be a measuring problem, since the initial external amino acid concentrations have been chosen low enough to prevent imbalancing of other transport processes.

The two transport schemes used as theoretical basis throughout this study (Fig. 1) are in principal the most used ones in cotransport studies, and their basic validity and applicability to in vivo systems will be tested. These models implicate that a (mobile) carrier exists within the membrane in at least six (arbitrarily chosen) states, from which the transmembrane transport of four states will be taken into account.

Materials and Methods

Growth conditions. Green thalli of the aquatic liverwort *Riccia fluitans* were grown under sterile conditions in large Petri-dishes according to Hüsemann and Barz [6]. The growth medium contained 1% of the medium of Murashige and Skoog [7], and 99% of a standard medium, which was composed of 0.1 mM KCl, 0.1 mM $CaCl_2$, and 2 mM sodium phosphate buffer. The tissues with the 2–4 mm long rhizoid cells were then transferred into a test medium which typically contained 0.1 mM KCl, 0.1 mM $CaCl_2$, 1 mM NaCl and 2 mM Tris-HCl at pH 7.6. All other conditions were identical to those described previously [3].

Electrical experiments. These were carried out as described repeatedly [3,8]. In order to control better the horizontal approach of the cells by microelectrodes, a Leitz- and a Narishige micromanipulator (MM33) were mounted and connected to each other in series allowing a controlled drive of less than 0.5 μm. The microelectrodes were pulled from glass tubing containing solid glass fiber (Hilgenberg) on a Getra-vertical or a Camden-horizontal instrument. The electrodes were backfilled by syringe with 0.5 or 3 M KCl and had typical resistances around 50 mΩ. The Ag/AgCl electrodes were connected to a high-impedance amplifier (WPI: KS 700 or S 7071). Signals were recorded on a pen chart (Kontron W + W 314) or storage oscilloscope (Gould, OS 4020). 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 more electrodes at defined distances from the injecting electrode, which was placed either at the midpoint or the tip of the rhizoid cell. The electrical space constant was typically around 0.4 mm. As described [3,8], the input data were subjected to the correction method according the Cole's theorem [9].

Extrapolation of the I-V curves. The *I-V* carrier curves have been obtained by the following method: The control curve was measured repeatedly in the minute prior to adding the alanine. This was necessary to guarantee that leaks around the electrode did not open especially in the extreme parts of the curves, and that the membrane was not damaged in any way. After adding the alanine, *I-V* curves were measured in intervals and at different external alanine concentrations, as indicated in the figures shown below. Under the assumption that during the incubation with alanine neither pump nor passive pathway changed considerably, it is allowed to extrapolate the electrical portion of the amino acid pathway by subtracting the control *I-V* curves from the test *I-V* curves [3–5]. In an attempt to avoid possible changes, the

initial alanine concentration has been kept low (10 μM) which resulted in only minor and transient depolarizations. It has been demonstrated recently [3] that the spontaneous repolarization following peak depolarization takes place in spite of the presence of 1 mM CN^-, from which it is assumed that mainly decreased driving forces (due to increased internal amino acid) partly restore the membrane potential. Under the given experimental conditions a changed pump activity while approaching a steady state is therefore considered small. As to the possibility of changed leaks, two more points should be stressed: Firstly, internal acidification in the presence of alanine proved less than 0.1 pH units; secondly, the strongest electrical signal during alanine incubation is surely the peak-depolarization, but since the current-source type curves [3–5] usually obtained at this point do not indicate any conductance change, there is little reason to believe that this should occur during repolarization.

Results and Discussion

Basic properties

In Fig. 2A the well-known membrane potential changes after adding different L-alanine concentrations are shown. Since the L-alanine accumulates very fast [3], each concentration indicated is measured on a different cell preparation from the same batch, in order to guarantee identical experimental conditions. Fig. 2B gives the lapse of the membrane potential after the addition of 10 μM L-alanine which is the basis for the *I-V* curves of Fig. 3. It is noteworthy to point out that the steady-state membrane potential never completely reaches the original resting potential. Fig. 2C then demonstrates the steady-state situation (basis for Fig. 4) with only minor changes in membrane potential after the addition of 20 to 500 μM alanine. This latter experiment proves that alanine is not metabolized as quickly as it is taken up, and it clearly states that cytoplasmic alanine has reached a value which prevents further increase; the system is completely saturated. The test with 100 μM methylamine is a control which tells us that other transport systems [10] still work quite well beside the saturated amino acid system.

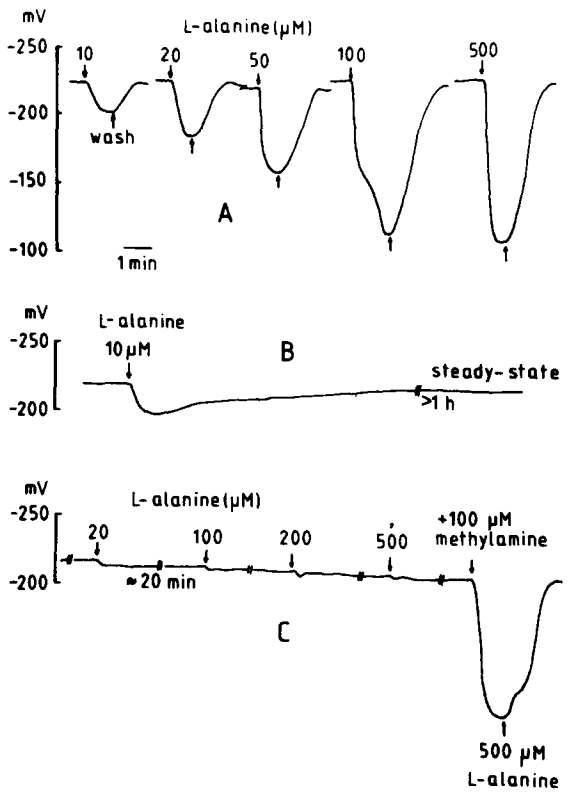

Fig. 2. Changes in membrane potential (ψ_m), recorded in rhizoid cells of *R. fluitans* under different conditions. (A) Depolarizations of ψ_m in the presence of various external L-alanine concentrations; each measurement in a different cell. (B) Time-course of ψ_m in the presence of 10 μM L-alanine (no wash). (C) Reaction of ψ_m to the addition of the indicated L-alanine concentrations after incubating for about 1 h with 10 μM L-alanine. The double bars mark 20 min of continuous recording each.

Carrier schemes

The two models in Fig. 1 predict a sigmoid current-voltage behaviour for the electrically driven alanine transport across the plasmalemma. Four parameters are experimentally accessible: Firstly, the equilibrium potential (E_{co}), the point on the voltage axis marking zero net current through the system; secondly, the saturation currents (i_s+, i_s-) at extreme voltages of opposite signs with respect to the equilibrium potential. Two other parameters, the short-circuit current (at $V=0$) and the slope conductance at limiting steepness will not be of any importance in this study, and are therefore

not discussed. Solving for the negatively charged carrier, the net current at steady state is

$$i_n = zFX_t \frac{P_1^{io}K_2K_4A - P_1^{oi}K_1K_3B}{AC \cdot \exp(zu/2) + BD \cdot \exp(-zu/2)} \quad (1)$$

and consequently for the positively charged ternary complex

$$i_n = zFX_t \left(P_3^{oi}[S]_o[H^+]_o \cdot \exp(-zu/2)B - P_3^{io}[S]_i[H^+]_i \cdot \exp(zu/2)A \right) / (AC + BD) \quad (2)$$

where X_t denotes the total amount of carrier molecules within the membrane, z is the stoichiometric coefficient, F is the Faraday, and u, the reduced voltage is $zF\Delta\psi/RT$ [11,12].

$$A = K_1K_3P_1^{oi} + K_3P_2^{oi}[S]_o + P_3^{oi}[S]_o[H^+]_o \cdot \exp(-zu/2)$$

$$B = K_2K_4P_1^{io} + K_4P_2^{io}[S]_i + P_3^{io}[S]_i[H^+]_i \cdot \exp(zu/2)$$

$$C = K_2K_4 + K_4[S]_i + [S]_i[H^+]_i$$

$$D = K_1K_3 + K_3[S]_o + [S]_o[H^+]_o$$

Since these equations are rather unhandy, some simplifications can be done: The current through the system gets voltage insensitive at extreme voltages with respect to the equilibrium potential. For very negative voltages and P_2 assumed negligible (P_2 is reinserted, if necessary), Eqn. 1 yields the saturation current

$$i_{s^-} = zFX_tP_3^{oi} \frac{[S]_o[H^+]_o}{K_1K_3 + K_3[S]_o + [S]_o[H^+]_o} \quad (1a)$$

and Eqn. 2

$$i_{s^-} = zFX_tP_1^{io} \frac{K_2K_4}{K_2K_4 + K_4[S]_i + [S]_i[H^+]_i} \quad (2a)$$

For reasons of symmetry, similar equations (not given) can be obtained for extreme voltages of opposite sign, again with respect to the equilibrium potential. As X_t, P, K, F (and also ΔpH) are assumed constant during the experiment, the saturation current i_{s^-} solely depends on the substrate (alanine) concentration. According to Eqn. 1a, which describes the charge transport of an empty and negatively charged carrier, i_{s^-} should be a direct function of external alanine concentration. But if the carrier is electrically neutral (which for a proton cotransport is equivalent with a positively charged ternary complex), according to Eqn. 2a the steady-state saturation current i_{s^-} should not depend on external alanine directly. So, in order to decide on this issue, the steady-state I-V saturation currents have been measured and are compared with above predictions of Eqns. 1a and 2a.

I-V curves

In Fig. 3 the time-dependent development of the carrier I-V difference curves is shown from the moment of the alanine addition up to the almost steady-state accumulation in the cytoplasm. In order to fully understand the different curves, one has to recall that with a given constant external alanine concentration (here 10 μM) the internal alanine level will increase according to the actual driving force until either a true equilibrium or a steady state is reached. Since during this time the equilibrium potential and the net current are subject to changes, naturally the I-V curves will change too. The fact that L-alanine is metabolized may prevent the achievement of a thermodynamic equilibrium, but is irrelevant for I-V analysis which measures only the transport of the electrically accessible internal alanine anyway. As demonstrated by the curves in Fig. 3, a steady-state is approximated within about one hour after which the I-V characteristics do not change any more, neither in shape nor in location in the I-V coordinates. The near steady-state curve in Fig. 3 (45 min) is S-shaped and thus confirms one of the predictions from the models in Fig. 1. The other curves in Fig. 3 (0.5 to 32 min) have intermediate character, but can easily be understood by taking the location of the equilibrium potential on the voltage axis into account. This equilibrium potential (at $i_n = 0$) is a function of internal alanine vs. external alanine at all times and is given for both models by

$$E_{co} = \frac{RT}{zF} \ln \left(\frac{[S]_o[H^+]_o}{[S]_i[H^+]_i} \cdot \frac{K_2K_4P_1^{io}P_3^{oi}}{K_1K_3P_1^{oi}P_3^{io}} \right) \quad (3)$$

Since at steady-state it can be assumed that $K_2K_4P_1^{io}P_3^{oi} = K_1K_3P_1^{oi}P_3^{io}$, Eqn. 3 reduces to the often used [3–5]

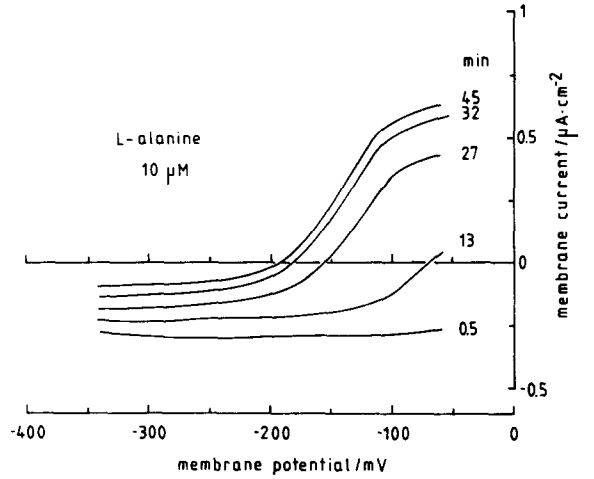

Fig. 3. Current-voltage characteristics (difference curves) measured in a rhizoid cell of *R. fluitans* in the presence of 10 µM L-alanine. The curves were extrapolated as described in Methods by subtraction of the control curve (without alanine) from the curves measured at the indicated times (after adding alanine). The characteristics represent the voltage-dependent electrical current carried by the investigated transport system under the given conditions.

$$E_{co} = \frac{RT}{zF} \ln\left(\frac{[S]_o[H^+]_o}{[S]_i[H^+]_i}\right) \qquad (3a)$$

which then permits a direct estimate of $[S]_i$, if E_{co} is measured (as in Fig. 3), or the location of E_{co}, if $[S]_i$ is known (e.g. radioisotope experiments). Although Eqn. 3a describes the thermodynamical equilibrium, it nevertheless gives valuable hints at nonequilibrium also. For example, insertion of low cytoplasmic alanine concentrations, which is the experimental situation shortly after the addition of alanine to the test medium, into Eqn. 3a immediately tells that the *I-V* curves must be incomplete which is exactly what the 'early' *I-V* curves in Fig. 3 state. The crucial experiment for the decision negative carrier or positive ternary complex is given in Fig. 4. After a steady-state was reached (Fig. 3), the external L-alanine concentration was increased in the indicated steps from 10 µM to 200 µM in roughly 20-min intervals, in order to give the system enough time to reach a new steady state. Characteristically, each concentration change altered the saturation current i_s- and also shifted the equilibrium potential along the voltage axis. According to the models of Fig. 1, this would be in

TABLE I

Cytoplasmic L-alanine concentration $[S]_i$ and the accumulation ratio $[S]_i/[S]_o$ calculated on the basis of the equilibrium potentials (E_{co}) from Fig. 4, an internal pH of 7.2, and the given external L-alanine concentrations as indicated.

E_{co}(mV)	$[S]_o$(µM)	$[S]_i$(mM)	$[S]_i/[S]_o$
−205	10	11.8	1187
−200	20	19.5	977
−183	50	25.0	503
−156	100	17.5	175
−140	200	18.8	94

agreement with Eqn. 1a which predicts such a concentration-dependent saturation current for a cotransport system with a negatively charged carrier. But basically the same result should be found looking at the export saturation current i_{s+} which subsequently should likewise change with internal alanine. However, as Fig. 4 states, the i_{s+} levels do not differ in the expected manner and even seem to be convergent. Closer inspection of the data clears up this interesting result. From the equilibrium potentials in Fig. 4, the given external L-alanine concentrations, and a cytoplasmic pH of 7.2 (measured by means of ion-exchanger filled glass electrodes; Bertl and Felle, in preparation),

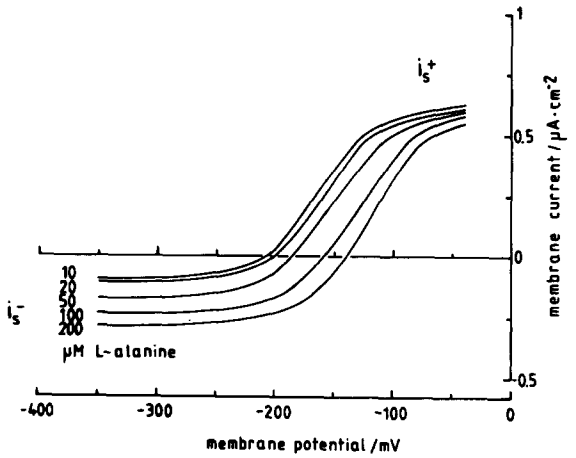

Fig. 4. Steady-state current-voltage (difference) curves measured in a *R. fluitans* rhizoid cell in the presence of increasing external L-alanine concentration. There was a 20–25-min interval between each concentration change. Evaluation and extrapolation of the curves between −50 and −350 mV as described in Methods and in Ref. 3. Comparison with Fig. 2C points out that although there is only little change in ψ_m, there can be a substantial shift in E_{co}. See text.

the respective cytoplasmic alanine concentrations have been calculated according to Eqn. 3a. Table I gives the results, the most important of which is that accumulation of free L-alanine within the cytoplasm seems limited to about 20 mM. Since this holds for the investigated interval of 20 to 200 μM external alanine, the insensitivity of the observed saturation current i_{s+} to (wrongly) assumed changes in internal alanine concentration is cleared up. The idea that the amino acid carrier in the plasmalemma of *Riccia fluitans* may be electrically negative rather than neutral, is still favoured. If this should turn out to be true, then a further important consequence follows: Figs. 3 and 4 demonstrated a strong current saturation of the system at voltages not too far from the respective equilibrium potential, which means nothing else as that the charge carrying process cannot be the rate-limiting step. And since the binding of L-alanine to the carrier may be too fast to be rate-limiting, the transport of the ternary complex probably is. This idea that the transport step which actually carries the substrate should be rate-limiting may sound like a paradoxon, but, as long as the substrate transport takes place reasonably fast, the search for the rate-limiting step is probably more of experimental than of physiological relevance.

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