Biochimica et Biophysica Acta 939 (1988) 179–188
Elsevier

BBA 73910

Kinetics of the Na^+/alanine cotransporter in pancreatic acinar cells

P. Jauch and P. Läuger

Department of Biology, University of Konstanz, D-7750 Konstanz, F.R.G.

(Received 5 October 1987)

Key words: Sodium ion/alanine cotransport; Amino acid transport; Secondary active transport; Kinetics; Whole-cell recording; (Mouse pancreas)

Electric currents associated with Na^+-coupled alanine transport in pancreatic acinar cells were investigated by the technique of tight-seal whole-cell recordings. In a previous study the observed concentration dependence of alanine-dependent currents was found to be consistent with a 'simultaneous' transport mechanism with 1:1 stoichiometry. In the present work the sidedness of the cotransporter was investigated by comparing inward (I'') and outward currents (I') measured under mirror-symmetrical conditions. I' and I'' were found to be nearly equal (within a factor of approx. 2) in a wide range of Na^+ and alanine concentrations. The transport model was further tested by 'infinite-cis' experiments with fixed, saturating concentrations of Na^+ and L-alanine on one side of the membrane and variable concentrations on the other. By measuring transmembrane currents as a function of Na^+ and alanine concentrations, numerical values of the equilibrium dissociation constants of both substrates could be estimated.

Introduction

The plasma membrane of many mammalian cells contains cotransport systems capable of promoting uphill transport of amino acids driven by an electrochemical gradient of Na^+ [1–6]. Most sodium/amino-acid cotransporters are electrogenic, i.e., they translocate net charge across the membrane [7,8]; accordingly, these systems can be studied by electrophysiological techniques [9–21]. Recently, the method of whole-cell current recording [22] has been applied to the investigation of the sodium-alanine cotransporter in pancreatic acinar cells [20,21]. In whole-cell recording experiments the cell is attached to the tip of a glass pipette with a high seal-resistance (> 10 GΩ) between glass and membrane. A major advantage of the whole-cell recording technique is the possibility of controlling the intracellular concentration of solutes by internal perfusion of the pipette [23]. Pancreatic acinar cells are particularly suitable for studying Na^+-coupled amino-acid transport, since they have a high rate of protein synthesis and accumulate amino acids with high efficiency [12,13,24–32].

In a previous study [20,21] alanine-dependent inward currents have been measured under 'zero-trans' conditions, i.e., with finite concentrations of Na^+ and L-alanine on one side of the membrane and vanishing concentrations on the other. From the results of these experiments, as well as from measurements of the reversal potential at a given concentration ratio of the amino acid, evidence was obtained that the cotransporter translocates Na^+ and L-alanine with 1 : 1 stoichiometry. The observation that *N*-methylated amino acids may substitute for L-alanine, as well as the pH dependence of currents indicate that the transport system is similar to (or identical with) system 'A' which is widespread in animal cells [2,20,21,23]. The concentration dependence of inward currents was found to be consistent with a

'simultaneous' mechanism involving formation of a ternary complex between cotransporter, amino acid and Na^+.

In this communication the sidedness of the cotransporter is studied in more detail by comparing inward and outward currents under mirror-symmetrical 'zero-trans' conditions. Furthermore, 'infinite-cis' experiments are described in which fixed, saturating concentrations of Na^+ and L-alanine are present on one side of the membrane and variable concentrations on the other. The analysis of these experiments represents an additional test for the validity of the transport model and yields numerical estimates for a number of kinetic parameters.

Materials and methods

Materials

Reagents were obtained from the following sources: Collagenase ('high purity'), tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) and L-alanine from Sigma; Eagle basal medium from Serva. Electrolytes and buffers were analytical grade.

Cell preparation

Single cells and small cell clusters were prepared by enzymatic dissociation of mouse pancreatic tissue, as described previously [20,34]. A small piece of pancreas from a white laboratory mouse was injected with a solution containing 140 mM NaCl, 5 mM KCl, 10 mM Hepes (pH 7.3) and 100 units/ml high-purity collagenase (Sigma) and incubated with 1 ml of the same solution at 37 °C for 30 min. During the incubation period mild shearing forces were applied by repeated gentle pipetting. The resulting suspension of rounded-up cells and small cell clusters was diluted 5-fold with collagenase-free buffer medium and the cellular material was allowed to settle. The supernatant was removed and replaced with buffer medium. The cell suspension could be stored in Eagle basal medium at 3 °C for 24 h without significant change in the electrical properties of the cells.

Current measurements

Whole-cell recordings of membrane currents were carried out as described in detail previously [20]. The cell suspension was transferred to a thermostated perspex chamber mounted on the stage of an inverted microscope. The chamber could be perfused by different electrolyte solutions from a number of reservoirs. By placing the tip of the micropipette with the attached cell close to the inlet of the solution, the solution composition in the vicinity of the cell could be changed withing less than 1 s.

Pipettes were pulled from glass capillaries as described by Hamill et al. [22]. Pipettes had internal tip diameters of 1–2 μm; when filled with a 150 mM NaCl solution they exhibited resistances of 4 to 8 MΩ. The pipette was connected to the amplifier head stage of the L/M-EPC-5 patch-clamp system (List-Electronic, Darmstadt, F.R.G.) mounted on a hydraulic micromanipulator. Current signals were recorded on tape. Silver-silver chloride electrodes were used inside the pipette and in the bath. The lower part of the pipette was filled with a chloride-free sulfate solution and the upper part (in which the electrode was located) with a solution containing 150 mM chloride. The external silver-silver chloride electrode was connected to the bath medium by a salt bridge.

Gigaohm seals between cell membrane and pipette were formed as described by Hamill et al. [22]. In the whole-cell configuration (Fig. 1) seals were usually mechanically stable enough so that the cell (or cell cluster) could be detached from the bottom of the chamber by slowly lifting the pipette. Under the usual experimental conditions, seals maintained their high electrical resistance for times up to 2 h.

The pipette solution always contained 1 mM EGTA and the external medium 2 mM $CaSO_4$. In order to minimize leakage conductance of the cell membrane, SO_4^{2-} was used as anion instead of Cl^- in most experiments. Since the solubility product of $CaSO_4$ is $6 \cdot 10^{-5}$ M^2, the concentration of free Ca^{2+} was about 0.6 mM at the highest sulfate concentration (100 mM). All experiments were carried out at 37 °C and at a pH value of 7.25–7.30 which was stabilized by addition of 10 mM $Tris_2SO_4$.

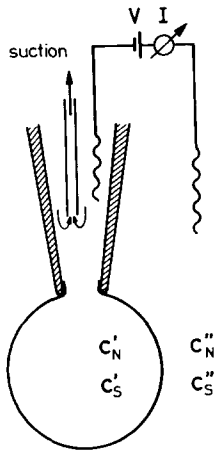

Fig. 1. Arrangement for whole-cell recordings from pancreatic acinar cells. The micropipette is internally perfused by applying suction to the central glass capillary (see text for further explanation). The distance between the tip of the capillary and the tip of the pipette is about 150 μm. c_N and c_S are the concentrations of Na^+ and alanine, respectively.

Internal perfusion of the pipette

In order to control the composition of the intracellular solution, the pipette was internally perfused (Fig. 1) using a slightly modified version of the method of Soejima and Noma [23]. It consists in a double perfusion system in which in a first step the perfusate is brought to a distance of a few mm from the pipette tip via a concentric plastic tube (not shown in the figure). Thereafter suction is applied to the thin glass capillary inside the plastic tube which is brought with its tip to a distance of about 100–200 μm from the tip of the patch pipette. This causes a flow of perfusate toward the tip of the pipette.

Experimental results

Inward and outward currents under 'zero-trans' conditions

For a comparison with theoretical predictions it is advantageous measuring currents under 'zero-trans' conditions, i.e., with zero concentrations of Na^+ and amino acid on one side and finite concentrations on the other side. From the ratio of inward and outward currents recorded from the same cell, information on the sidedness of the cotransporter may be obtained. After establishment of the whole-cell configuration, both the pipette and the bath are perfused with Na^+- and alanine-free Tris solutions. The cell interior equilibrates with the pipette within a few minutes, as indicated by the drop of membrane potential to nearly zero. After equilibration, the current recorded under short-circuit conditions is zero (Fig. 2). Change of the external medium to a solution containing 50 mM Na^+ leads to a small inward current I_o''. This current is likely to result from uncoupled sodium transport by the cotransporter, as well as from transport through other conductive pathways. When the extracellular medium is changed to a solution containing 5 mM L-alanine and 50 mM Na^+, a large inward current I_t'' is observed. The difference $I'' \equiv I_t'' - I_o''$ of the total inward current I_t'' and the alanine-independent inward current I_o'' is thought to represent the alanine-coupled sodium current. A few seconds after the inward current has reached its peak value, the pipette is perfused by a solution containing 50 mM Na^+ and 5 mM L-alanine (as in the extracellular medium); the current then declines to

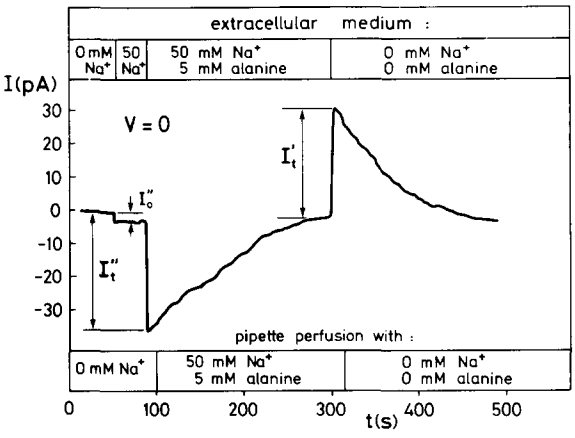

Fig. 2. Inward and outward short-circuit currents measured under 'zero-trans' conditions. Extracellular medium: 100 mM $Tris_2SO_4$ or (75 mM $Tris_2SO_4$ + 25 mM Na_2SO_4), 2 mM $CaSO_4$ (pH 7.3); the concentration of free Ca^{2+} under this condition is about 0.6 mM. Pipette medium: 100 mM $Tris_2SO_4$ or (75 mM $Tris_2SO_4$ + 25 mM Na_2SO_4), 1 mM EGTA (pH 7.3). The currents were measured from a single cell; the temperature was $T = 37\,^\circ$C. The current trace has been redrawn from the original record. As indicated, the current amplitudes were taken a few seconds after the change of the external solution, after the system had reached a quasi-stationary state.

TABLE I

RATIO I'/I'' OF OUTWARD (I') AND INWARD (I'') SHORT-CIRCUIT CURRENTS MEASURED UNDER MIRROR-SYMMETRICAL ZERO-TRANS CONDITIONS

I' and I'' are defined by $I' \equiv I(c'_N = c_N,\ c'_S = c_S,\ c''_N = c''_S = 0)$ and $I'' \equiv -I(c'_N = c'_S = 0,\ c''_N = c_N,\ c''_S = c_S)$; c'_N, c''_N, c'_S and c''_S are the concentrations of sodium and alanine, respectively in the intracellular ($'$) and extracellular ($''$) medium (Fig. 1). Each pair of I' and I'' has been measured on the same cell. The observed total currents I'_t and I''_t have been corrected for the leakage current I_o according to $I' = I'_t - I''_o$ and $I'' = I''_t - I''_o$ (Fig. 2).

c_N (mM)	I'/I'' ratio			
c_S (mM):	2	4	8	20
10	2.2	1.4	–	0.8
24	1.3	1.2	1.5	2.4
80	0.8	0.75	–	–
200	0.8	0.9	–	1.0

nearly zero within 200 s. This decline is likely to reflect the disappearance of Na^+- and alanine-concentration gradients across the cell membrane. When thereafter the extracellular medium is changed to a solution free of Na^+ and alanine, the direction of the Na^+ and alanine gradients is reversed and a large outward current I'_t is observed. I'_t again declines to zero when the pipette is perfused with a Na^+- and alanine-free medium.

In the example represented in Fig. 2 the inward and outward currents measured under mirror-symmetrical conditions are nearly equal. Analogous zero-trans experiments with symmetrical gradients have been carried out for a number of other concentrations. In Table I the ratio I'/I'' of outward (I') to inward (I'') short-circuit currents is given for different sodium and alanine concentrations; I' and I'' have been corrected for leakage currents according to $I' = I'_t - I''_o$ and $I'' = I''_t - I''_o$ (Fig. 2). It is seen from Table I that inward and outward currents are equal within a factor of about two. Within the limits of experimental error no systematic variations of I'/I'' with concentration can be detected. This indicates that the cotransporter is functionally nearly symmetric.

Effect of extracellular sodium and alanine on outward currents ('infinite-cis' experiments)

In the following we describe experiments under 'infinite-cis' conditions [35] in which the cell is loaded with sodium and alanine at saturating concentrations. In this case a large outward current into a sodium- and alanine-free extracellular solution is observed. In the 'infinite-cis' experiment the reduction of this outward current by addition of Na^+ and alanine to the extracellular medium is measured.

In the first part of the experiment represented in Fig. 3, uncoupled Na^+ currents in the absence of alanine were determined. At the beginning, 200 mM Na^+ were present on both sides of the cell membrane. Replacement of the extracellular medium by a Tris solution containing 0 mM or 100 mM Na^+ resulted in small outward currents (I_1^o and I_2^o, respectively) through leakage pathways. Thereafter, both sides were equilibrated with solutions containing high concentrations of Na^+ (200 mM) and L-alanine (20 mM). Subsequent removal of Na^+ and alanine in the extracellular

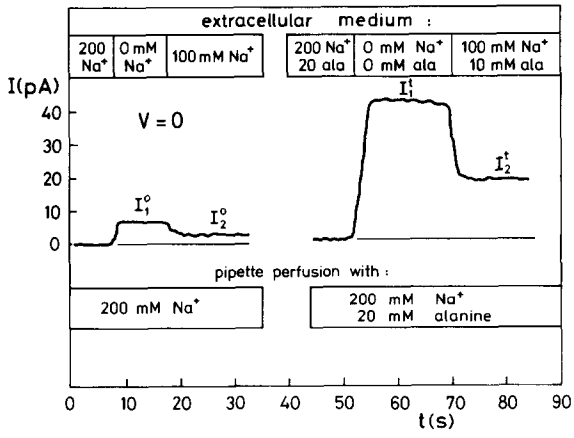

Fig. 3. 'Infinite-cis' experiments at zero voltage with fixed, saturating concentrations of sodium (200 mM) and L-alanine (20 mM) on the cytoplasmic side and variable concentrations on the extracellular side. I_1^o and I_2^o are alanine-independent leakage currents observed in the presence of sodium gradients alone, I_1^t and I_2^t are alanine-dependent outward currents in the presence and in the absence of extracellular substrates. Extracellular medium: 100 mM ($Tris_2SO_4 + Na_2SO_4$), 2 mM $CaSO_4$ (pH = 7.3); pipette medium: 100 mM Na_2SO_4, 10 mM $Tris_2SO_4$, 1 mM EGTA, (pH 7.3). The currents were measured from a single cell; $T = 37°C$. The current traces have been redrawn from the original record.

medium leads to a large outward current I_1^t. This current is reduced to a smaller outward current I_2^t when finite concentrations of Na^+ (100 mM) and L-alanine (10 mM) are present in the extracellular medium.

From the experiment represented in Fig. 3, the ratio I_1/I_2 of the alanine-dependent currents in the absence and in the presence of extracellular substrates may be obtained:

$$\frac{I_1}{I_2} = \frac{I_1^t - I_1^o}{I_2^t - I_2^o} \tag{1}$$

The leakage currents I_1^o and I_2^o varied considerably from cell to cell. When I_1^o and I_2^o become large, the quantities $I_1 = I_1^t - I_1^o$ and $I_2 = I_2^t - I_2^o$ are subjected to large errors. For this reason only those experiments have been used for the determination of I_1/I_2 in which the contribution of leakage current to the total current was less than 25%. As will be shown later, for the evaluation of kinetic parameters it is convenient plotting the quantity

$$p \equiv \frac{I_1}{I_2}\left(1 - \frac{c_N'' c_S''}{c_N' c_S'}\right) \tag{2}$$

as a function of the extracellular concentrations c_N'' and c_S'' of Na^+ and alanine; c_N' and c_S' are the intracellular concentrations of Na^+ and alanine, respectively. Experimental values of p are represented in Fig. 4 for different values of c_N'' and c_S''. The straight lines have been drawn according to the relation

$$p = 1 + \alpha c_N'' + \beta c_S'' + \gamma c_N'' c_S'' \tag{3}$$

which follows from the analysis of the transport model (see below); α, β and γ are c_N''- and c_S''-independent combinations of kinetic constants.

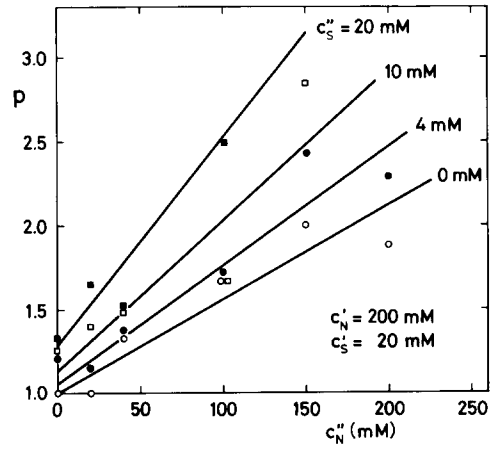

Fig. 4. Results of 'infinite-cis' experiments at different extracellular concentrations of sodium (c_N'') and L-alanine (c_S'') and fixed intracellular concentrations ($c_N' = 200$ mM, $c_S' = 20$ mM). The quantity p has been determined according to Eqns. 1 and 2 using the experimental protocol indicated in Fig. 3. Current measurements were carried out at 37 °C with single cells or small aggregates of electrically coupled cells. Each pair of current values (I_1 and I_2) was determined from the same cell (or cell aggregate). Extracellular medium: 100 mM ($Tris_2SO_4 + Na_2SO_4$), 2 mM $CaSO_4$, pH 7.3; pipette medium: 100 mM Na_2SO_4, 10 mM $Tris_2SO_4$, 1 mM EGTA, pH 7.3. Each experimental point represents the average from measurements with several individual cells. The straight lines have been drawn according to Eqn. 3 using the following parameter values determined by least-square fitting: $\alpha = 5.67 \cdot 10^{-3}$ mM^{-1}, $\beta = 1.44 \cdot 10^{-2}$ mM^{-1}, $\gamma = 3.34 \cdot 10^{-4}$ mM^{-2}.

Kinetic analysis

Previously it has been shown that the results of current measurements under 'zero-trans' conditions are consistent with a 'simultaneous' mechanism involving the formation of a ternary complex NCS between cotransporter C, driving ion N and substrate S [21]. In the following, kinetic parameters of the cotransporter are evaluated on the basis of the 'simultaneous' model, using data from the experiments described in this study.

Evaluation of kinetic parameters of the 'simultaneous' mechanism

The 'simultaneous' mechanism (Fig. 5) is based on the assumption that the binding sites for N and S are alternately accessible from the cytoplasmic side (states C′, NC′, C′S, NC′S) and from the extracellular side (states C″, NC″, SC″, NC″S). Conformational transitions moving the binding sites from an inward-facing to an outward-facing configuration are assumed to occur only in the empty (C′ ↔ C″) and in the fully occupied states (NC′S ↔ NC″S). This assumption which implies complete coupling of the fluxes of N and S is based on the finding that the alanine-independent Na^+-current is only a small fraction of the total current measured at saturating alanine concentra-

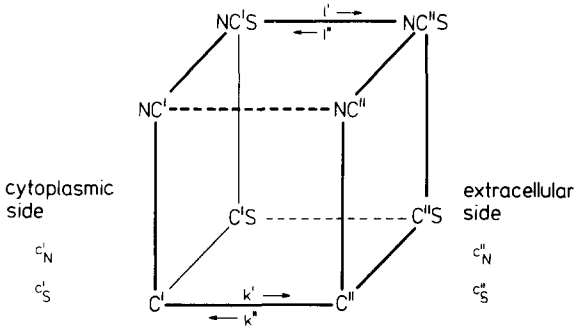

Fig. 5. 'Simultaneous' mechanism for the transport of ion N and substrate S by the cotransporter C. The binding sites for N and S are alternately accessible from the cytoplasmic side (states C′, NC′, CS′, NC′S) and from the extracellular side (states C″, NC″, C″S, NC″S). It is assumed that conformational transitions switching the binding sites from the inward-facing to the outward-facing configuration are possible only in the empty and fully occupied states (C and NCS). c'_N, c''_N, c'_S and c''_S are cytoplasmic and extracellular concentrations of N and S.

tions [20]. We further assume that the rate constants for binding and release of N and S are large so that the association-dissociation reactions in the membrane/solution interfaces are always in equilibrium [36].

Denoting the fraction of transporter molecules which are in state A by x[A], the association-dissociation reactions at the cytoplasmic interface are described by equilibrium constants K'_N, K'_S, $K_N^{S'}$ and $K_S^{N'}$:

$$K'_N = \frac{x[C']c'_N}{x[NC']}; \quad K_N^{S'} = \frac{x[C'S]c'_N}{x[NC'S]} \tag{4}$$

$$K'_S = \frac{x[C']c'_S}{x[C'S]}; \quad K_S^{N'} = \frac{x[NC']c'_S}{x[NC'S]} \tag{5}$$

c'_N and c'_S are the cytoplasmic concentrations of Na^+ and S; $K_N^{S'}$ and $K_S^{N'}$ are the equilibrium constants for the dissociation of Na^+ and S, respectively, from the ternary complex NC′S. Analogous relations hold for the extracellular interface. The equilibrium constants K'_N, $K_N^{S'}$, etc., as well as the translocation rate constants k', k'', l' and l'' (Fig. 4) depend, in general, on membrane potential $V \equiv \psi' - \psi''$. According to the principle of microscopic reversibility (or detailed balance), the kinetic constants are connected by

$$\frac{K'_N}{K_N^{S'}} = \frac{K'_S}{K_S^{N'}}; \quad \frac{K''_N}{K_N^{S''}} = \frac{K''_S}{K_S^{N''}} \tag{6}$$

$$\frac{K''_S K_N^{S''}}{K'_S K_N^{S'}} \cdot \frac{k''l'}{k'l''} = \exp(u) \tag{7}$$

$$u \equiv \frac{\psi' - \psi''}{RT/F} = \frac{V}{RT/F} \tag{8}$$

R is the gas constant, T the absolute temperature, and F the Faraday constant. Eqn. 6 follows directly from Eqns. 4 and 5; for a derivation of Eqn. 7, see Ref. 36.

If the membrane contains N transporter molecules per unit area, the electric current density I associated with sodium-coupled substrate transport is given by

$$I = e_0 N(l'x[NC'S] - l''x[NC''S]) \tag{9}$$

where e_0 is the elementary charge. Introducing the abbreviations

$$n \equiv \frac{c_N}{K_N}; \quad s \equiv \frac{c_S}{K_S} \tag{10}$$

$$Q \equiv \frac{K_N}{K_N^S} = \frac{K_S}{K_S^N} \tag{11}$$

$$P \equiv 1 + n + s + nsQ \tag{12}$$

$$H \equiv k + \ln sQ \tag{13}$$

and using the notation $n' \equiv c'_N/K'_N$, $Q' \equiv K'_N/K_N^{S'}$, etc., the following relation is obtained for the current density I in the stationary state [37]:

$$I = \frac{e_0 N}{\chi} \frac{k'l''}{K''_N K_S^{N''}} [c'_N c'_S \exp(u) - c''_N c''_S] \tag{14}$$

$$\chi \equiv P'H'' + P''H' \tag{15}$$

Inward and outward currents measured at $V = 0$ under mirror-symmetrical zero-trans conditions have been found to be approximately equal in the whole concentration range of Na^+ and alanine

(Table I):

$$I(c'_N = c_N,\ c'_S = c_S,\ c''_N = c''_S = 0)$$
$$\approx I(c'_N = c'_S = 0,\ c''_N = c_N,\ c''_S = c_S) \quad (16)$$

This nearly symmetrical behaviour is only possible if the following relations hold which are obtained from Eqn. 14:

$$\frac{K''_N}{K'_N} \approx \frac{K''_S}{K'_S} \approx \frac{k'}{k''} \equiv \kappa \quad (17)$$

$$\frac{1+(l'/k'')}{1+(l''/k')} \approx \frac{K_N^{S'}}{K_N^{S''}} \approx \frac{K_S^{N'}}{K_S^{N''}} \approx \frac{l'}{l''} \equiv \lambda \quad (18)$$

The simplest condition by which Eqns. 17 and 18 may be satisfied consists in the assumption that the cotransporter is approximately symmetric with respect to its kinetic parameters, meaning that κ and λ are close to unity; this assumption is used for the following analysis of the experimental results. It should be emphasised, however, that an independent test of the condition $\kappa \approx \lambda \approx 1$ is not possible on the basis of stationary short-circuit current measurements alone, but requires additional experiments such as current-voltage measurements or relaxation studies. Introducing the relations $k' = k'' = k$, $K'_S = K''_S = K_S$, etc., into Eqn. 14 yields:

$$I = I_\infty \frac{c'_N c'_S - c''_N c''_S}{w} \quad (19)$$

$$w \equiv B_1 + B_2(c'_N + c''_N) + B_3(c'_S + c''_S) + c'_N c'_S(1 + B_4 c''_N + B_5 c''_S) + c''_N c''_S(1 + B_4 c'_N + B_5 c'_S) + B_6 c'_N c'_S c''_N c''_S \quad (20)$$

$$I_\infty = e_0 N(1/k + 1/l)^{-1} \quad (21)$$

$$B_1 \equiv \frac{2\mu K_S K_N^S}{1+\mu};\quad \mu \equiv k/l \quad (22)$$

$$B_2 \equiv \frac{\mu K_S K_N^S}{K_N(1+\mu)};\quad B_3 \equiv \frac{\mu K_N^S}{1+\mu} \quad (23)$$

$$B_4 \equiv \frac{1}{K_N(1+\mu)};\quad B_5 \equiv \frac{1}{K_S(1+\mu)} \quad (24)$$

$$B_6 \equiv \frac{2}{K_S K_N^S(1+\mu)} \quad (25)$$

I_∞ is the limiting current which is observed under the condition $c''_S = c''_N = 0$, c'_N, $c'_S \to \infty$. From 'zero-trans' experiments the following numerical estimates have been obtained: $K''_N(1 + k''/k') \approx 77$ mM; $K''_N/K''_S \approx 4.1$; $K_N^{S''}/(1 + l''/k') \approx 16$ mM [21]. Using the symmetry condition introduced above, this yields: $K_N \approx 39$ mM, $K_S \approx 9.4$ mM, $B_1 \approx 304$ mM^2, $B_2 \approx 3.9$ mM, $B_3 \approx 16$ mM.

In the 'infinite-cis' experiments described in this study, the outward current I_1 measured at high cytoplasmic concentrations c'_N and c'_S and zero extracellular concentrations c''_N and c''_S is compared with the current I_2 measured with the same values of c'_N and c'_S, but finite values of c''_N and c''_S:

$$I_1 \equiv I(c'_N,\ c'_S > 0,\ c''_N = c''_S = 0,\ u = 0) \quad (26)$$

$$I_2 \equiv I(c'_N,\ c'_S,\ c''_N,\ c''_S > 0,\ u = 0) \quad (27)$$

From Eqns. 19 and 20 one obtains

$$\frac{I_1}{I_2} = \frac{c'_N c'_S}{c'_N c'_S - c''_N c''_S} \cdot p \quad (28)$$

$$p = 1 + q[c''_N(B_2 + B_4 c'_N c'_S) + c''_S(B_3 + B_5 c'_N c'_S) + c''_N c''_S(1 + B_4 c'_N + B_5 c'_S + B_6 c'_N c'_S)] \quad (29)$$

$$= 1 + \alpha c''_N + \beta c''_S + \gamma c''_N c''_S \quad (29a)$$

$$1/q \equiv B_1 + B_2 c'_N + B_3 c'_S + c'_N c'_S \quad (30)$$

The quantities p, α, β and γ have been originally introduced in Eqns. 2 and 3 as phenomenological parameters.

The results of the "infinite-cis" experiments are represented in Fig. 4 in which the quantity p (Eqn. 2) is plotted as a function of c''_S and c''_N. From the experimental values of p, numerical estimates for the quantities B_4, B_5 and B_6 may be obtained from Eqn. 29 using a least-square fitting procedure. The straight lines in Fig. 4 have been drawn according to Eqn. 29a with the following parameter values:

$$\alpha \equiv q(B_2 + B_4 c'_N c'_S) = 5.67 \cdot 10^{-3}\ \text{mM}^{-1} \quad (31)$$

$$\beta \equiv q(B_3 + B_5 c'_N c'_S) = 1.44 \cdot 10^{-2}\ \text{mM}^{-1} \quad (32)$$

$$\gamma \equiv q(1 + B_4 c'_N + B_5 c'_S + B_6 c'_N c'_S) = 3.34 \cdot 10^{-4}\ \text{mM}^{-2} \quad (33)$$

Using the results from the previous 'zero-trans' experiments ($B_1 \approx 304$ mM^2, $B_2 \approx 3.9$ mM, $B_3 = 16$ mM), the following estimates are obtained: $1/B_4 = K_N(1+\mu) \approx 149$ mM, $1/B_5 = K_S(1+\mu) \approx 65$ mM. The coefficient B_6 which is rather sensitive to experimental errors in the determination of p could not be evaluated with sufficient reliability. From B_4 and B_5 one obtains $K_N/K_S \approx 2.3$, which is somewhat smaller than the previous estimate, $K_N/K_S \approx 4.1$. By averaging the parameter values determined from 'zero-trans' and 'infinite-cis' experiments, the following set of numerical estimates for the kinetic constants is obtained:

$K_N \approx 33$ mM; $K_S \approx 11$ mM
$K_N^S \approx 20$ mM; $K_S^N \approx 6.4$ mM
$k/l \approx 4.4$

These values should be considered as tentative in view of the symmetry assumption introduced above ($\kappa \approx \lambda \approx 1$) which requires further experimental tests.

Alternative transport mechanisms; four-state model with ordered binding of Na$^+$ and S

Besides the 'simultaneous' reaction mechanism (Fig. 5), alternative cotransport models have been discussed in the literature [38–40]. Previously it has been shown that a 'consecutive' mechanism (which does not require formation of a ternary complex SCN) is inconsistent with the observed properties of the Na$^+$/alanine-cotransporter [21]. A third transport model is depicted in Fig. 6. It is based on the assumption that Na$^+$ has to bind from the cytoplasmic side before a substrate S can bind from the same side and, vice versa, that S has to bind from the extracellular side before an extracellular Na$^+$ can bind. This model is basically different from the simultaneous mechanism of Fig. 5 since it does not involve transitions between two conformations of the cotransporter. A similar mechanism has been discussed previously by Hopfer and Groseclose [38]. The model of Fig. 6 corresponds in enzyme kinetics to an 'ordered' bisubstrate reaction with 'glide' symmetry [41,42]; with only four states (C, CN, SC, SCN) it is formally much simpler than both the 'simultaneous' and the 'consecutive' model.

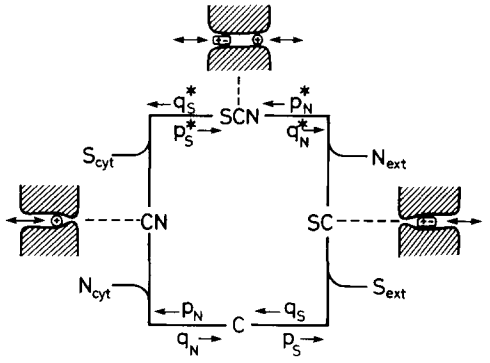

Fig. 6. Four-state model of the cotransporter. Cytoplasmic alanine (S) is assumed to bind only after cytoplasmic sodium (N) has been bound; at the extracellular side the binding order is reversed. In state SCN alanine exchanges only with the cytoplasm, sodium only with extracellular medium. p_N, p_N^*, p_S, p_S^* are bimolecular association rate constants; q_N, q_N^*, q_S, q_S^* are dissociation rate constants.

For the reaction cycle of Fig. 6, the electric current density associated with coupled transport of Na$^+$ and S may be evaluated using standard methods [43]. The result reads:

$$I = \frac{e_o N}{\rho} p_N^* p_S q_N q_S^* [c_N' c_S' \exp(u) - c_N'' c_S''] \tag{34}$$

$$\begin{aligned}\rho \equiv\ & q_N q_S (q_N^* + q_S^*) + p_N q_S (q_N^* + q_S^*) c_N' \\ & + p_S^* q_N^* (q_S + p_S c_S'') c_S' + p_N p_S^* (q_N^* + q_S + p_N^* c_N'') c_N' c_S' \\ & + p_N^* q_S^* (q_N + p_N c_N') c_N'' + p_S q_N (q_N^* + q_S^*) c_S'' \\ & + p_N^* p_S (q_S^* + q_N + p_S^* c_S') c_N'' c_S''\end{aligned} \tag{35}$$

$$\frac{p_N p_S^* q_N^* q_S}{p_N^* p_S q_N q_S^*} = \exp(u) \tag{36}$$

An analogous result is obtained for the mirror-symmetrical version of the four-state model of Fig. 6 in which CN is formed by binding of Na$^+$ from the extracellular side and SC by binding of S from the cytoplasm. The equations describing the mirror-symmetrical version are obtained from Eqns. 34–36 by the substitutions $I \leftrightarrow -I$, $u \leftrightarrow -u$, $c_N' \leftrightarrow c_N''$, $c_S' \leftrightarrow c_S''$.

Comparison of Eqns. 35 and 36 with the result from the 'simultaneous' model (Eqns. 14 and 15) shows that the concentration dependence of the current under 'zero-trans' conditions ($c_N' = c_S' = 0$ or $c_N'' = c_S'' = 0$) is identical for both models. A

similar statement applies to measurements of I_1/I_2 (Eqns. 26 and 27), i.e., the same dependence of I_1/I_2 on c''_N and c''_S at fixed c'_N and c'_S is predicted for both mechanisms. This means that the 'simultaneous' model and the 'ordered' four-state model cannot be strictly distinguished on the basis of the available experimental data. However, the 'ordered' mechanism is unlikely in view of the observed functional symmetry of the cotransporter. It may be shown from Eqn. 34 that for the model of Fig. 6 a symmetrical behaviour of the cotransporter (Eqn. 16) is only possible when the relations $q_N \approx q_S$, $q^*_N \approx q^*_S$, $p^*_N \approx 2p_N$ and $p^*_S \approx 2p_S$ hold. The existence of such a strong correlation between the different rate constants is unlikely, however.

Discussion

The results presented in this study are consistent with a 'simultaneous' model of the sodium, alanine cotransporter in which both substrates are translocated together in a single step. Information on the kinetic parameters of the model may be obtained from the concentration dependence of alanine-dependent inward and outward currents. Inward and outward currents measured under mirror-symmetrical conditions were found to be nearly equal (within a factor of approx. 2) in a wide range of Na^+ and alanine concentrations. According to this observation, the analysis was based on the assumption that the cotransporter is approximately symmetrical with respect to its kinetic constants. Furthermore, it was assumed that binding and release steps of Na^+ and alanine are not rate limiting. It is pertinent to emphasize the tentative nature of these assumptions. In particular, inward (I'') and outward currents (I') can be approximately equal even when individual kinetic constants are asymmetric, since the ratio I'/I'' may be insensitive to the values of certain kinetic parameters. The symmetry of the reaction scheme has been introduced here as the simplest assumption by which the experimental data can be explained; the validity of this assumption should be further checked by independent experiments, such as current measurements under nonstationary conditions. It is pertinent to mention that other sodium-coupled cotransport systems exhibit a distinct asymmetry in their kinetic properties. A well-studied example is the Na^+/D-glucose cotransporter from small intestine which exhibits influx and efflux rates differing by up to a factor of ten at mirror-symmetrical conditions [44].

The equilibrium dissociation constants of Na^+ and alanine which have been evaluated using the symmetry assumption are $K_N \approx 33$ mM and $K_S \approx 11$ mM, respectively. These values mean that at normal extracellular concentrations the transport system operates near saturation with respect to Na^+, but below saturation with respect to alanine.

An important parameter for the understanding of the coupling mechanism is the quantity $Q \equiv K_N/K^S_N = K_S/K^N_S$. Under the assumptions mentioned above, Q is estimated to be about 1.7, meaning that Na^+ and alanine bind nearly independently of each other. This further means that the contribution of binding affinities to the coupling of the fluxes of Na^+ and substrate is small. Coupling is likely to be of kinetic origin, resulting from the low rate of conformational transitions in the partially occupied states CN and SC of the cotransporter.

References

1 Johnstone, R.M. (1979) Can. J. Physiol. Pharmacol. 57, 1–15.
2 Christensen, H.N. (1984) Biochim. Biophys. Acta 779, 255–269.
3 Stevens, B.R. Kaunitz, J.D. and Wright, E.M. (1984) Annu. Rev. Physiol. 46, 417–433.
4 Kilberg, M.S. (1985) Curr. Top. Cell. Regulation 25, 133–163.
5 Englesberg, E. and Moffett, J. (1986) J. Membr. Biol. 91, 199–212.
6 Schultz, S.G. (1986) in Physiology of Membrane Disorders (Andreoli, T.E., Hoffman, J.F., Fanestil, D.D. and Schultz, S.G., eds.), pp. 283–294, Plenum Press, New York.
7 Heinz, E., Geck, P. and Pietrzyk, C. (1975) Ann. N.Y. Acad. Sci. 264, 428–441.
8 Philo, R.D. and Eddy, A.A. (1978) Biochem. J. 174, 811–817.
9 Hoshi, T., Sudo, K. and Suzuki, Y. (1976) Biochim. Biophys. Acta 448, 492–504.
10 Kehoe, J.S. (1976) Cold Spring Harbour Symp. Quant. Biol. 40, 145–155.
11 Okada, Y., Tsuchiya, W., Irimajiri, A. and Inouye, A. (1977) J. Membr. Biol. 31, 205–219.
12 Iwatsuki, N. and Petersen, O.H. (1980) Nature 283, 492–494.
13 Iwatsuki, N. and Petersen, O.H. (1980) Pflügers Arch. 386, 153–159.

14 Bergman, C. and Bergman, J. (1981) J. Physiol. (London) 318, 259–278.
15 Bergman, C. and Bergman, J. (1985) J. Physiol. (London) 366, 197–220.
16 Frömter, E. (1982) Pflügers Arch. 393, 179–189.
17 Gunter-Smith, P.J., Grasset, E. and Schultz, St.G. (1982) J. Membr. Biol. 66, 25–39.
18 Jung, D.W., Schwarz, W. and Passow, H. (1984) J. Membr. Biol. 78, 29–34.
19 Singh, J. (1984) Pflügers Arch. 402, 176–184.
20 Jauch, P., Petersen, O.H. and Läuger, P. (1986) J. Membr. Biol. 94, 99–115.
21 Jauch, P. and Läuger, P. (1986) J. Membr. Biol. 94, 117–127.
22 Hamill, O.P., Marti, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
23 Soejima, M. and Noma, A. (1984) Pflügers Arch. 400, 424–431.
24 Bégin, N. and Scholefield, P.G. (1965) J. Biol. Chem. 240, 332–337.
25 Cheneval, J.P. and Johnstone, R.M. (1976) Biochim. Biophys. Acta 433, 630–637.
26 Petersen, O.H. (1976) Physiol. Rev. 56, 535–577.
27 Tyrakowski, T., Milutinovic, S. and Schulz, I. (1978) J. Membr. Biol. 38, 333–346.
28 Schulz, I. and Ullrich, K.J. (1979) in Membrane Transport in Biology (Giebisch, G., ed.), Vol. IVB, pp. 811–852, Springer, Berlin-Heidelberg-New York.
29 Iwamoto, Y. and Williams, J.A. (1980) Am. J. Physiol. 238, G440–G444.
30 Laugier, R. and Petersen, O.H. (1981) Biochim. Biophys. Acta 641, 216–221.
31 Mann, G.E. and Peran, S. (1986) Biochim. Biophys. Acta 858, 263–274.
32 Norman, P.S.R. and Mann, G.E. (1986) Biochim. Biophys. Acta 861, 389–394.
33 Kilberg, M.S. (1982) J. Membr. Biol. 69, 1–12.
34 Iwatsuki, N. and Petersen, O.H. (1985) J. Membr. Biol. 86, 139–144.
35 Stein, W.D. (1986) Transport and Diffusion across Cell Membranes, Chapter 4, Academic Press, Orlando.
36 Läuger, P. (1984) Biochim. Biophys. Acta 779, 307–341.
37 Turner, R.J. and Silverman, M. (1980) Biochim. Biophys. Acta 596, 272–291.
38 Hopfer, U. and Groseclose, R. (1980) J. Biol. Chem. 255, 4453–4462.
39 Kessler, M. and Semenza, G. (1983) J. Membr. Biol. 76, 27–56.
40 Restrepo, D. and Kimmich, G.A. (1985) Am. J. Physiol. 248, C498–C509.
41 Segel, I.H. (1975) Enzyme Kinetics, John Wiley, New York.
42 Turner, R.J. (1981) Biochim. Biophys. Acta 649, 269–280.
43 Hill, T.L. (1966) J. Theor. Biol. 10, 442–459.
44 Semenza, G., Kessler, M., Schmidt, U., Venter, J.C. and Fraser, C.M. (1985) Ann. New York Acad. Sci. 456, 83–96.