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# Electrophysiological Investigation of the Amino Acid Carrier Selectivity in Epithelial Cells from *Xenopus* Embryo

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**Summary.** The electrical responses induced by external applications of neutral amino acids were used to determine whether different carriers are expressed in the membrane of embryonic epithelial cells of *Xenopus laevis*. Competition experiments were performed under voltage-clamp conditions at constant membrane potential.

Gly, L-Ala, L-Pro, L-Ser, L-Asn and L-Gln generate electrical responses with similar apparent kinetic constants and compete for the same carrier. They are $[Na]_o$ and voltage-dependent, insensitive to variations in $[Cl]_o$ and $[HCO_3]_o$, inhibited by $pH_o$ changes, by amiloride and, for a large fraction of the current, by MeAIB. The increase in $[K]_o$ at constant and negative membrane potential reduces the response, whereas lowering $[K]_o$ augments it.

L-Leu, L-Phe and L-Pro appear to compete for another carrier. They generate electrogenic responses insensitive to amiloride and MeAIB, as well as to alterations of membrane potential, $[Na]_o$ and $[K]_o$. Lowering $[Cl]_o$ decreases their size, whereas increasing $[HCO_3]_o$ at neutral $pH_o$ increases it.

It is concluded that at least two and possibly three transport systems (A, ASC and L) are expressed in the membrane of the embryonic cells studied. An unexpected electrogenic character of the L system is revealed by the present study and seems to be indirectly linked to the transport function.

L-Pro seems to be transported by system A or ASC in the presence of Na and by system L in the absence of Na. MeAIB induces an inward current.

**Key Words** Na-gradient coupled transports · amino acid transports · electrogenic transports · epithelial cells · *Xenopus laevis* embryo

## Introduction

In the past ten years, a large body of information has confirmed the idea that in mammalian and avian epithelia the amino acid uptake is mediated by a set of membrane proteins showing substrate specificity. However, important common properties of these carriers have also been recognized, so that a simplified classification has been proposed in which neutral amino acids can be accepted by three major classes of transporters.

For polar and linear neutral amino acids including small ones (Oxender & Christensen, 1963; Schultz & Curran, 1970; Thomas & Christensen, 1971; Collarini & Oxender, 1987) the process has proved to be achieved by two similar transport mechanisms ("system A" and "ASC"), energized by the sodium (Na) electrochemical gradient built up by the Na/K ATPase. The membrane carriers are assumed to be intrinsic proteins capable, under physiological conditions, of binding one (or two) Na ions and one amino acid molecule on the extracellular side of the membrane and releasing them intracellularly. The binding of Na increases the carrier affinity for the amino acid (AA) so that the asymmetrical distribution of Na on either side of the membrane determines an uphill influx of the solute (Curran et al., 1967; Christensen et al., 1973). Moreover, as Na and AA are simultaneously translocated, presumably with a constant stoichiometry, the rate of solute uptake is proportional to the net influx of Na. Accordingly, the symport is electrogenic and the inward current (or the membrane depolarization) induced by external application of neutral amino acids can be regarded as an index of the transport efficiency (Kehoe, 1976; Bergman & Bergman, 1981, 1985; Gunter-Smith, Grasset & Schultz, 1982; Jung, Schwartz & Passow, 1984; Jauch, Petersen & Läuger, 1986; Barbour, Brew & Attwell, 1988).

Branched chains and aromatic neutral amino acids, on the other hand, are recognized by another class of membrane carriers ("system L"). This transport system appears to be unaffected by changes in membrane potential and sodium concentration (Kilberg & Christensen, 1980; Collarini & Oxender, 1987). Energization of the transport of these amino acids by the proton gradient has been questioned (Kilberg & Christensen, 1980). The transport could possibly be electroneutral.

Several amino acids can compete for both sys-

tems (A and ASC), thereby revealing overlapping properties of the transporters. However, since the ASC system is unaffected by 2(methylamino) isobutyrate (MeAIB) which inhibits the A system, it seems likely that each of these two transport systems corresponds to a particular type of membrane protein.

In this paper, we present the results of an electrophysiological investigation of the neutral amino acid transport in embryonic epithelial cells from *Xenopus*. The aim of this work was to determine whether distinct transport systems are already expressed in these (endoderm) cells at an early stage of development. By means of the voltage-clamp technique, competition experiments at constant membrane potential were designed in which voltage related interactions between two transport systems were prevented. We have studied the selectivity of the Na-dependent carrier system (system A or ASC); and we have shown that an electrogenic Na- and voltage-independent system (presumably system L) is already present in the embryonic cells. Our experiments also suggest that L-proline is recognized and taken up by both the A (or ASC) and the L-systems.

## Materials and Methods

### Endoderm Cells

*Xenopus laevis* embryos were obtained from standard breeding procedures. Neurula embryos staged 16–24 (Nieuwkopf & Faber, 1975) were used according to a dissection procedure already described (Bergman & Bergman, 1985). The ventral halves of the embryos were cut into thin slices, which were continuously superfused in a small (150 $\mu$l) Perspex chamber circulated with Ringer's or test solutions at a constant rate (15 ml/min). A three-way stopcock (Kilb & Stämpfli, 1955) permitted us to exchange the superfusion solutions within less than 12 sec.

At the beginning of each experiment, the slices were superfused with a Ca-free Ringer solution and mechanically dissociated into small clusters of 10 to 30 cells. The experiments were undertaken after a complete reaggregation of the cells which generally occurred 15 min after readmission of the normal Ringer's solution. All experiments were made at room temperature.

### Electrical Measurements

The membrane potential was measured with glass micropipettes filled with a mixture of $K_2SO_4$ (0.5 M) and KCl (3 M) solutions. Taking advantage of the low electrical resistance of the gap junctions between cells belonging to the same cluster, the membrane potential was measured by impaling one cell of the aggregate. An additional micropipette served to inject the clamp current through a neighboring cell. Similar results were obtained when impaling only one cell with a double-barrelled microelectrode.

The reference electrode was made of a large Ag-AgCl bar connected by a NaCl-Agar bridge with the solution bathing the preparation. The cell aggregates were voltage clamped by means of a high-impedance voltage follower (WPI M707), together with a clamp amplifier (BIOLOGIC C.A. 100) equipped with a 1-$\mu$A current-injecting head stage (HS 170). Membrane current and membrane potential were continuously recorded by means of a dual channel pen recorder.

### Solutions

The normal Ringer's solution (control) had the following composition (in mM): 115, NaCl; 2.5, KCl; 2, $CaCl_2$; 2, $MgCl_2$; 3, $Na_2HPO_4$; 0.5, $NaH_2PO_4$. The pH of the control solution was fixed to 7.2. Modifications of the solution are mentioned in the corresponding sections. The various amino acids, as well as the transport inhibitors (amiloride and MeAIB, Sigma) were added to the control solutions immediately before use.

## Results

### Membrane Depolarizations in Response to Different Amino Acids

In the present series of experiments, the endoderm cells were not voltage clamped. They were exposed to various neutral amino acids: glycine (Gly), L-alanine (L-Ala), L-serine (L-Ser), L-proline (L-Pro), L-asparagine (L-Asn), L-glutamine (L-Gln), L-leucine (L-Leu), and L-phenylalanine (L-Phe) either at constant or variable concentrations. The spontaneous membrane potential was continuously recorded, especially during the transitory (pulses) applications of amino acids.

At a constant and high (10 mM) concentrations, all the neutral amino acids induced a membrane depolarization with a time course very similar to that already described for L-Asn (Bergman & Bergman, 1985). The responses to L-Leu and L-Phe, however, were always smaller than those to other amino acids. All the amino acid-induced responses increased with increasing concentrations in the Ringer's solution. The dose-response relationships reached on asymptote at concentration between 10 and 20 mM and could be satisfactorily described by first-order Michaelis-Menten kinetics. Double reciprocal plots of the experimental data are presented in Fig. 1 for different AA.

From those plots, the apparent kinetic constants $K_m$ (dissociation equilibrium constant) and $\Delta V_{max}$ (maximal membrane depolarization) were determined graphically, as was an apparent index of the transport capability of the membrane ($\Delta V_{max}/K_m$). The results of these measurements are presented in Table 1, which reveals that the apparent kinetic constants are of the same order of magnitude for all amino acids. It should be pointed out

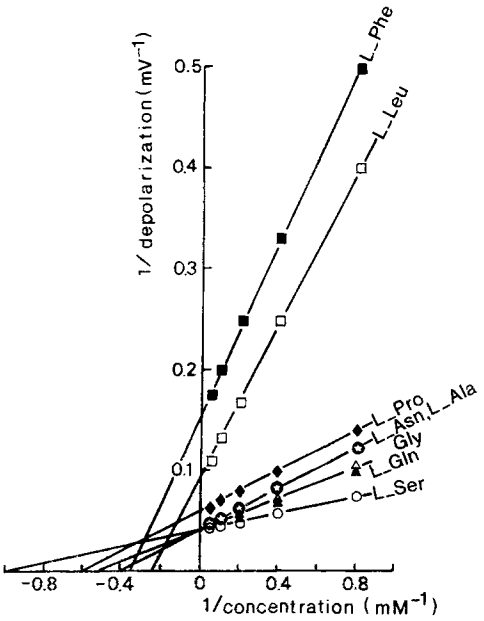


**Fig. 1.** Lineweaver-Burk representation of the steady-state membrane depolarization as a function of the external concentration in different amino acids. Most of these curves were obtained from different cell aggregates at their spontaneous membrane potentials varying between −65 and −80 mV

that D-isomeric forms of the amino acids did not produce significant responses.

## Several Amino Acids Compete for the Same Carrier

The following experiments were undertaken to determine whether different types of membrane carriers are implied in the transport of the various AA tested in the preceding section.

The endoderm cells were voltage clamped to their resting level (zero current potential). Under this condition, an external application of a transported amino acid induces an inward current (Bergman, Bergman & Mouttapa, 1986) which is assumed to reflect the Na influx coupled to the amino acid uptake (Jung et al., 1984; Jauch et al., 1986).

In the experiments reported here, two different amino acids at saturating concentrations (10 to 20 mM) were applied to the cells, first successively, then simultaneously, in order to establish whether the corresponding current responses are additive. To simplify the procedure, the first amino acid was generally L-Asn whereas the second one was chosen among the various amino acids already tested (*see* Table 1).

**Table 1.**

| Amino acid | $K_m$ (mM/liter) | $\Delta V_{max}$ (mV) | $\Delta V_{max}/K_m$ |
|---|---|---|---|
| Glycine | 1.9 | 25 | 14.2 |
| L-alanine | 2.5 | 26 | 11.1 |
| L-serine | 1.3 | 25 | 20 |
| L-proline | 1.6 | 16.5 | 11.1 |
| L-asparagine | 2.5 | 25 | 10 |
| L-glutamine | 1.8 | 22 | 12.5 |
| L-leucine | 4.2 | 11.1 | 2.7 |
| L-phenylalanine | 2.8 | 6.5 | 2.32 |

Apparent kinetic constants determined graphically from depolarization-concentration relationships obtained in the presence of various neutral amino acids at the resting potential (mean values for two to four experiments performed on different cell aggregates).

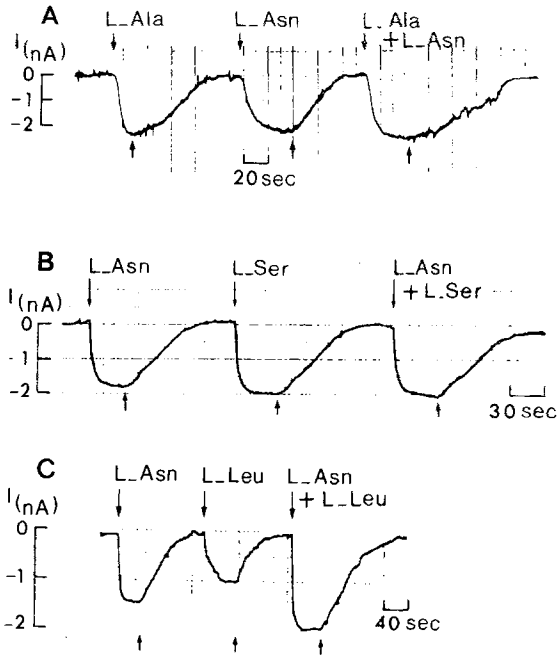


**Fig. 2.** Competition experiments under voltage-clamp conditions. Membrane current recordings in response to external applications of L-amino acids at saturating concentrations (20 mM) either separately or simultaneously. The experiments were performed on three different aggregates voltage clamped to their spontaneous resting level; −84 mV (*A*), −70 mV (*B*), −86 mV (*C*). Negative currents are inward

Figure 2 shows the result of such experiments when either L-Ala (*A*) or L-Ser (*B*) was added to L-Asn. It can be observed that the current response obtained when the two amino acids were applied simultaneously was not larger than either of the separate responses, thereby suggesting that the pairs of amino acids compete for the same membrane carrier.

**Table 2.**

| Substrate 1 | Substrate 2 | $\alpha$ |
|---|---|---|
| L-Asn | Gly | 1.04 ± 0.03 ($n$ = 10) |
| L-Asn | L-Ala | 1.03 ± 0.05 ($n$ = 5) |
| L-Asn | L-Ser | 1.04 ± 0.03 ($n$ = 4) |
| L-Asn | L-Pro | 1.14 ± 0.04 ($n$ = 5) |
| L-Asn | L-Leu | 1.47 ± 0.15 ($n$ = 6) |
| L-Asn | L-Phe | 1.62 ± 0.26 ($n$ = 12) |

Mean value ± SE for the competition coefficient $\alpha$ calculated from Eq. (1). Substrates 1 and 2 are the two amino acids generating the current responses $\Delta I_1$ and $\Delta I_2$, respectively; $n$ reflects the number of experiments performed on different cells.

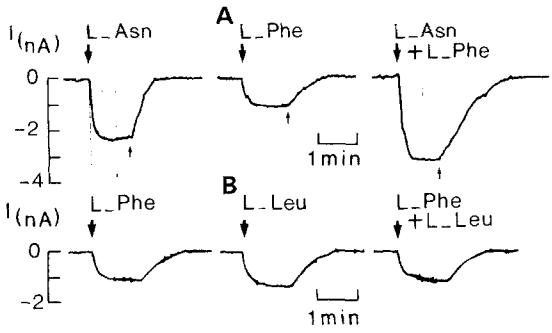


**Fig. 3.** Competition experiments under voltage-clamp conditions similar to those presented in Fig. 2. (*A*) Between L-Asn and L-Phe (20 mM). (*B*) Between L-Phe and L-Leu (20 mM). These results suggest that L-Phe and L-Leu compete for a common route different from that used by L-Asn and other amino acids (*see* Table 2)

In contrast, Fig. 2*C* shows that the simultaneous application of L-Leu and L-Asn induces a compound response significantly larger than either the L-Asn or the L-Leu applied alone.

This result supports the view that L-Asn and L-Leu are transported by two distinct carriers. It should be noted, however, that the compound response is not as large as the sum of the two separate responses.

In an attempt to quantify the additive character of the current responses, the results of these competition experiments were expressed in the form of a dimensionless index $\alpha$ (Samarzija, Hinton & Frömter, 1982)

$$\alpha = \frac{\Delta I}{1/2(\Delta I_1 + \Delta I_2)} \quad (1)$$

where $\Delta I$ is the total current response, $\Delta I_1$ and $\Delta I_2$ are the current responses to amino acids 1 and 2, respectively. Clearly, fully competitive responses should give values of $\alpha = 1$, whereas fully independent responses should rise to values of $\alpha = 2$.

Table 2 summarizes the results obtained from different experiments. Two categories of responses emerge: for L-Ala, L-Asn, Gly, L-Pro, L-Ser, $\alpha$ is close to 1, whereas for L-Leu and L-Phe, $\alpha$ is significantly higher than 1 but smaller than 2.

These data confirm the idea that L-Ala, L-Asn, Gly, L-Ser and likely L-Pro are translocated by the same type of carrier, whereas L-Leu and L-Phe use another route. In fact, a few competition experiments (Fig. 3) between L-Leu and L-Phe lead to the conclusion that these amino acids share a common carrier.

The observation that $\alpha$ is slightly higher than 1 in the first group could be due to the fact that, at the concentrations used (20 mM), the separate responses were not fully saturating. The fact that $\alpha$ is smaller than 2 could be due to space clamp defects (*see* Discussion) or could reveal the existence of interactions, either direct (incomplete selectivity of the carrier) or indirect ($[Na]_i$ increase coupled with AA uptake).

## The Voltage Dependence of the Amino Acid Responses

Most electrogenic dissipative processes are expected to be voltage dependent. In the particular case of glucose and small neutral amino acids, the transmembrane electrical gradient is assumed to provide a substantial fraction of the energy required for taking up the substrates against their concentration gradients (Geck & Heinz, 1976; Carter-Su & Kimmich, 1980). It follows that any membrane depolarization that reduces the potential difference ($E_m$-$E_{Na}$) between the membrane potential and the Na-equilibrium potentials should reduce the turnover rate of the carrier (Gibb & Eddy, 1972; Geck & Heinz, 1976). This has been confirmed in several preparations. In the particular case of the L-Asn transport in *Xenopus* embryo, it has been proposed (Bergman & Bergman, 1985) that the membrane potential affects the maximum translocation velocity but not the apparent affinity of the carrier for the substrate.

In the following voltage-clamp experiments, the membrane potential was varied and the amplitude of the membrane currents in response to amino acid pulses at saturating concentrations were compared. Figure 4 reports the results of such experiments performed on different cell aggregates.

Figure 5 shows the effect of membrane potential on the responses induced by several amino acids. Two types of current-voltage relationships

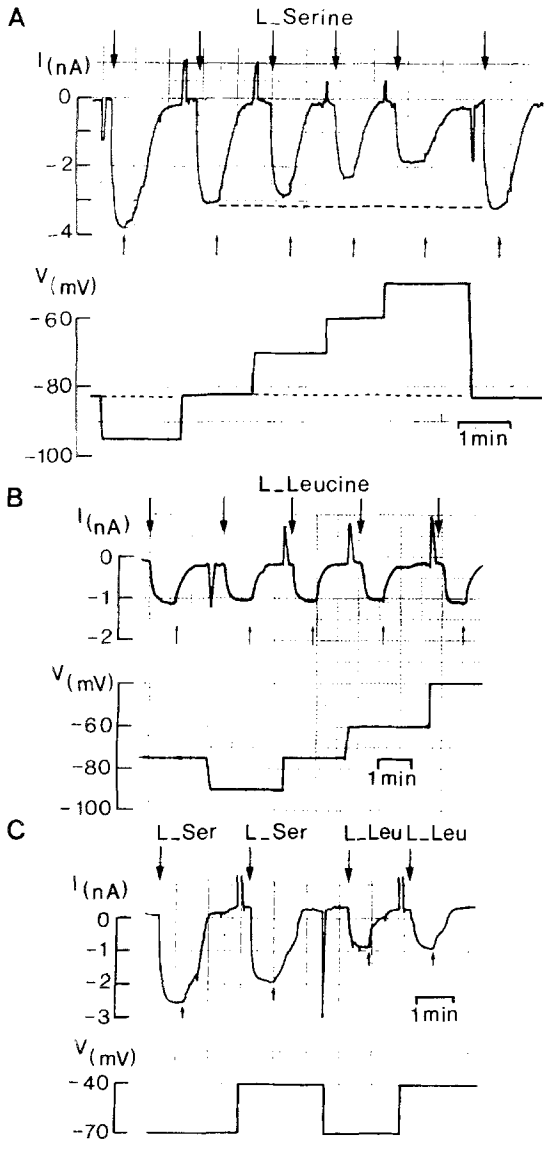


**Fig. 4.** Voltage sensitivity of the extra currents elicited by L-Ser and L-Leu in two different preparations (*A* and *B*) or in the same preparation (*C*). The cells were voltage clamped to various levels from their resting polarization (−80 mV in *A*, −76 mV in *B*, −70 mV in *C*). To facilitate the comparison, the background current induced by the changes in membrane potential was mechanically compensated (peaks) on the pen recorder before application of the amino acid. The size of these peaks reflects the change in holding current arising from the change in membrane potential. All amino acid pulses were saturating (20 mM)

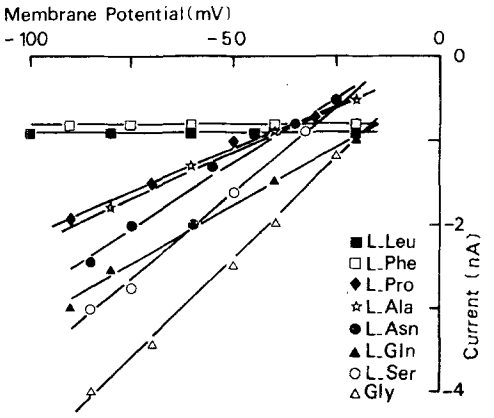


**Fig. 5.** Amino acid-induced extra currents as a function of the membrane potential (abscissa) under voltage-clamp conditions. The current values (ordinate) were obtained from experiments similar to those reported in Fig. 4. They reflect the difference between the total membrane current measured during exposure to saturating amino acid concentrations (20 mM) and the background current measured at the same potential in the absence of amino acid. Negative currents are inward. In most cases the different straight lines (drawn by eye) correspond to different cell aggregates

can be distinguished. For all amino acids other than L-Leu and L-Phe, the amino acid-induced current decreased with membrane depolarization, disappearing completely in the vicinity of the zero membrane potential. In contrast, the responses to L-Leu and L-Phe appeared to be totally insensitive to membrane potential variations.

## Amino Acid Responses and Ionic Environment

The preceding observations prompted us to ascertain whether all the recorded amino acid responses are Na dependent. The cells were voltage clamped to their natural resting level (near −70 mV), and the current responses to the application of a 20 mM pulse of amino acid were first recorded in normal Ringer's, then in a modified solution in which most of the Na content was replaced by an equimolar amount of choline chloride or Tris chloride. The complete elimination of Na rendered the membrane irreversibly leaky. Therefore, 10% of the normal NaCl content was maintained in all experiments.

As already shown for L-Asn (Bergman & Bergman, 1985) the current responses to Gly, L-Ala, L-Ser, L-Pro, L-Gln were always markedly reduced in low Na Ringer's. However, this was not the case for L-Leu and L-Phe (Fig. 6*A* and *B*).

The contribution of K ions to the Na-coupled uptake of amino acids has been questioned several times (Eddy, 1968; Eddy & Hogg, 1969; Burckhardt et al., 1980; Grasset, Gunter-Smith & Schultz, 1983; Bergman & Bergman, 1985; Bussolati et al., 1986). The interpretation is still uncertain because in most cases the membrane potential could not be controlled or was associated with alterations of the K gradient. We present (Fig. 6*C* and *D*) an example of experiments in which the membrane was voltage clamped to its normal resting level (−70 mV). 20

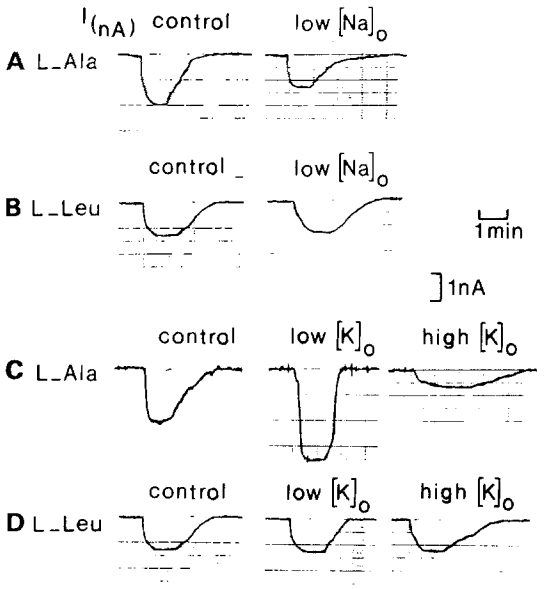


**Fig. 6.** Effect of changes in the cationic environment on the extra currents elicited by 20 mM pulses of L-Ala or L-Leu. The preparations were voltage clamped to their resting level in normal (115 mM) and low (11.5 mM) $[Na]_o$ (rows *A* and *B*) or in normal (2.5 mM), low (0 mM) and high (30 mM) $[K]_o$ (rows *C* and *D*). In row *C*, the changes in time course of the amino acid responses are typical and were regularly observed in response to alterations of $[K]_o$. In all solutions the changes in the background current before amino acid applications were compensated mechanically on the pen recorder. The corresponding variations of the holding current were: 0.6 nA (outward) in *A* and *B*, 1.3 nA (outward) in low $[K]_o$ and 2.6 nA (inward) in high $[K]_o$; they were immediately reversed on returning to the control solution.

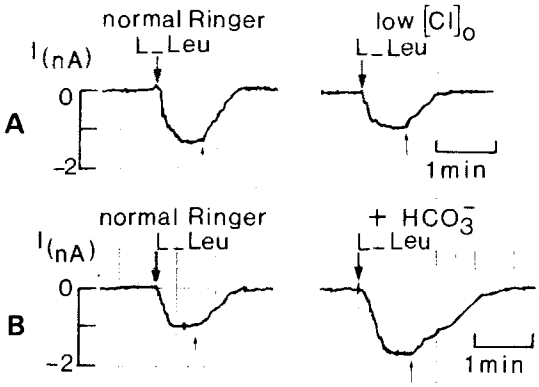


**Fig. 7.** Effect of alterations of the anionic content of the external medium on L-Leu-induced currents at saturating (20 mM) concentration. Row *A*: change in $[Cl]_o$ (replaced by methyl sulfate). Row *B*: increase in $[HCO_3]_o$ at constant and normal pH (7.2)

mM pulses of L-Ala or L-Leu were added to either a normal Ringer's solution ($[K]_o$ = 2.5 mM) or a Ringer's in which the K concentration was altered ($[K]_o$ = 0 or 30 mM). The Na-electrochemical gradient was kept constant in these experiments, which show that increasing $[K]_o$ reduces the membrane current elicited by L-Ala whereas lowering $[K]_o$ increases it. This result is in agreement with a previous and similar observation made with L-Asn (Bergman et al., 1986) or L-Glu (Barbour et al., 1988). In contrast, the L-Leu response appears to be insensitive to changes in $[K]_o$. We conclude that, at constant and negative membrane potentials, external K exerts an inhibitory effect that is selective for the Na-coupled and voltage-sensitive transports. Moreover, K ions are not implicated in the electrical responses associated with external applications of L-Leu.

Owing to the observation that external application of both L-Leu and L-Phe induces an inward current that remains unaltered in the presence of a low Na- or K-modified Ringer's solution, we tested whether an efflux of chloride ions could account for the membrane current associated with the amino acid uptake. We have substituted Na-methylsulfate (115 mM) for an equimolar amount of NaCl and compared the response to L-Ala, L-Ser and L-Leu in this medium to those obtained in normal Ringer's. We have observed that the alterations of the Cl electrochemical gradient, at constant membrane potential, never modify the size of the current responses to L-Ala (four experiments) or L-Ser (three experiments). In one experiment, the L-Leu response was found unchanged by low Cl solutions, in six other experiments (Fig. 7*A*) the Leu-induced current was reduced from 0.61 ± 0.21 to 0.45 ± 0.19 nA (mean values and SEM). These results reveal that chloride ions are not directly implicated in the electrical response elicited by external applications of L-Leu in endoderm cells. One possibility is that changes in $[Cl]_o$ might induce alterations of pH inside the cells via the balance of a (hypothetical) $Cl/HCO_3$ exchange. Consequently, we replaced a fraction (20 mM) of the external NaCl by an equimolar amount of $NaHCO_3$ in the external solutions, $pH_o$ being readjusted to 7.2 by adding a small amount of HCl to Ringer's. We have observed (Fig. 7*B*) that the L-Leu response was always reversibly augmented by a factor of about 30% in this medium. This result is exactly the opposite of what was expected and does not support the view that the modifications in the anionic content of the external solution reported here induce changes in the L-Leu response that can be ascribed to the activation of a membrane $Cl/HCO_3$ antiporter.

## The Particular Case of L-Proline

A close examination of Fig. 1 (*see also* Table 2) suggests that among the amino-acid induced responses sensitive to alterations of both the membrane potential and the external Na concentration, L-Pro can be regarded as a special case. Therefore,

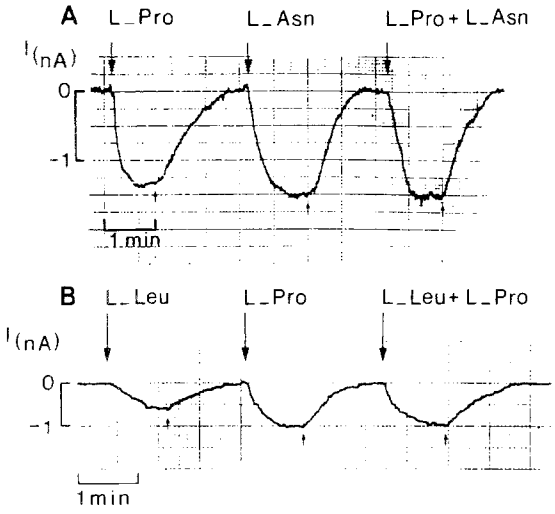


**Fig. 8.** Competition experiments similar to those presented in Figs. 2 and 3. (*A*) Between L-Pro and L-Asn in $[Na]_o$ 115 mM; (*B*) between L-Pro and L-Leu in $[Na]_o$ 11.5 mM. The holding potential was fixed to −80 mV. Experiments were performed on two different cell aggregates

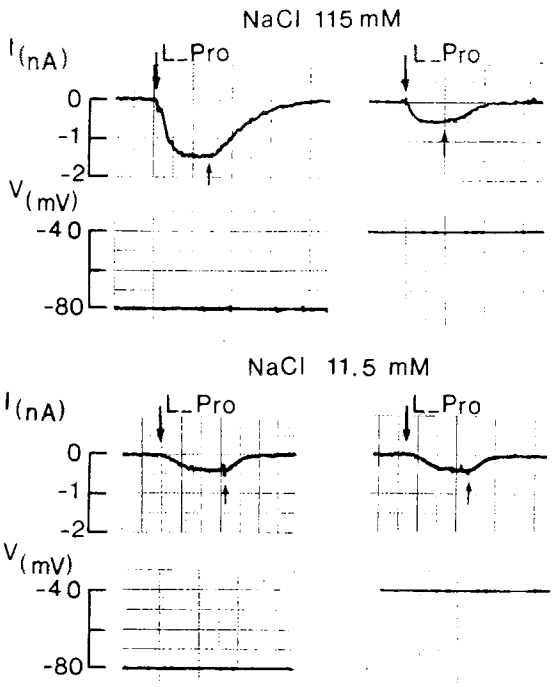


**Fig. 9.** Differential voltage sensitivity of the proline response in normal and low $[Na]_o$. The cell were voltage clamped to −80 mV (left) and −40 mV (right) in 115 mM Na (upper row) and 115 mM Na (lower row). It is assumed that in low $[Na]_o$ most of L-Pro is transported by the Leu/Phe carrier

we have developed a specific set of competition experiments with either L-Asn or L-Leu to evaluate the origin of the typical characteristics of the response to this amino acid. As shown in Fig. 8, the responses to L-Pro do not appear additive with either L-Asn or L-Leu. To confirm this view, we have examined the voltage sensitivity of the L-Pro induced current, either in the presence of the normal Na content (115 mM) or at low $[Na]_o$ (11.5 mM). In the first case, we assume that L-Pro is transported predominantly by the Na- and voltage-dependent carrier. In the second case, the Na gradient is markedly reduced so that one would expect that L-Pro is transported mainly by the carrier that usually recognizes L-Leu and L-Phe. Figure 9 supports this view showing that, in the presence of Na, the L-proline response is markedly reduced when the membrane is depolarized to −40 mV, whereas it remains unaffected by the same voltage drop in the absence of Na. These results, together with those of the competition experiments against L-Asn and L-Leu, suggest that L-Pro can be transported by two distinct membrane carriers.

### Amino Acid Responses and Transport Inhibitors

Amiloride has been shown to inhibit several dissipative Na pathways in epithelia membranes, including the Na-coupled amino acid transports (Fehlman et al., 1981; Renner et al., 1988). We have exposed the endoderm cells to amiloride (0.5 mM) and found that the Na-dependent AA responses described above were markedly depressed, but never abolished, in the presence of this inhibitor. In contrast, both the L-Leu and the L-Phe responses in voltage-clamped cells were found to be insensitive to amiloride, thereby indirectly confirming that the corresponding carrier does not recognize Na as a cationic activator (Fig. 10*A* and *B*).

MeAIB (*see* Introduction) is assumed to compete selectively with neutral amino acids for the transport system A (Bass et al., 1981; Boerner & Saier, 1982). On the other hand, any Na-coupled transport that persists in a high concentration in this inhibitor, should be mediated by a membrane carrier different from system A, with the ASC system being the most likely candidate (Christensen, Liang & Archer, 1967; Thomas & Christensen, 1971; Englesberg & Moffett, 1986; Saier et al., 1988). To test for such a MeAIB-resistant transport, the membrane of the endoderm cells was voltage clamped to the resting level (near −70 mV) in control Ringer's, and 20 mM pulse of L-Ser (presumably activating both systems A and ASC) was applied and elicited a rather large inward current regarded as a control response (Fig. 10*C*). Then a 20 mM pulse of MeAIB was given. This induced a small and fully reversible inward current. Finally, a third modified Ringer's solution containing 20 mM MeAIB and 20 mM L-Ser was superfused. It can be shown that the compound response to MeAIB and L-Ser is significantly smaller than the response to L-Ser alone, thereby

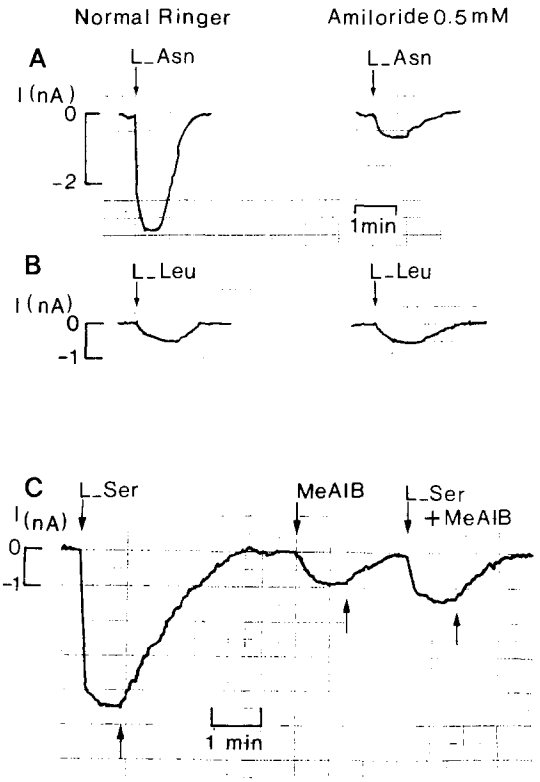


**Fig. 10.** Inhibitory effects of amiloride and MeAIB on membrane currents induced by different amino acids. Amiloride (0.5 mM) inhibits the (20 mM) Asn response (*A*) and leaves the (20 mM) Leu response unaffected (*B*). MeAIB (20 mM) induces a relatively small inward current, which is inhibitory for a large fraction of the L-Ser response (*C*). Experiments *A* and *B* were performed on the same preparation, voltage clamped to its resting level (−76 mV). Experiment *C* was done on another aggregate, voltage clamped to −70 mV

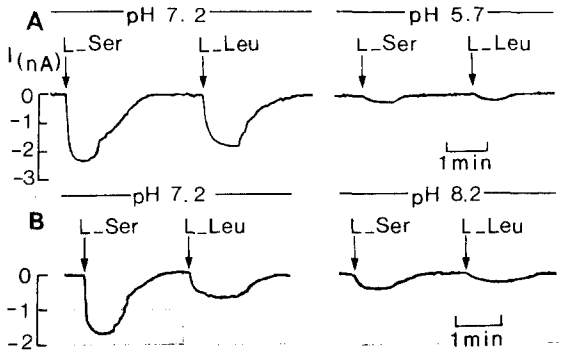


**Fig. 11.** The effect of external pH alterations of L-Ser and L-Leu responses. (*A*) Acidification of the Ringer solution from 7.2 to 5.7. The two experiments were made on the same preparation successively (holding potential: −68 mV). The change in pH induced an inward current of 2.5 nA (*not shown*). (*B*) Alkalinization of the Ringer's solution from 7.2 to 8.2. The two experiments were made on another preparation voltage clamped to −72 mV. The change in pH induced an outward current of 1.2 nA (*not shown*). In all cases the amino acid concentration was 20 mM/liter. The ionic conductance changes induced by external pH alterations are fully reversible

revealing that a large fraction of the serine-induced current has been inhibited by MeAIB. The results presented in Fig. 10*A* and *C* confirm the view that amiloride and MeAIB inhibit the amino acid transport in two different ways. Amiloride alone does not induce any significant current (12 experiments, *not shown*); it is, however, capable of inhibiting a current elicited by an amino acid presumably cotransported with Na. This agent bears a guanidinium residue; it inhibits several Na cotransports and likely interferes with the Na binding sites of the amino acid carrier. In contrast, MeAIB, which is known to compete with AA, is recognized and apparently preferred to the amino acid at a molecular site where it plays the role of carrier activator since it induces an inward current like natural ligands. Moreover, it may also be cotransported, apparently at a rate slower than the amino acids. Eventually, assuming that a 20 mM MeAIB concentration saturates all the carriers in the presence of 20 mM L-Ser, it might be speculated that the (small) difference current between the compound response and the MeAIB-induced response in Fig. 10*C* reveals the existence of the transport System ASC in the endoderm cells of *Xenopus* embryo.

Alterations of external pH were recognized a long time ago as being a particularly efficient inhibitor of amino acid transport (Oxender & Christensen, 1963). This effect can be attributed to several factors (Makowske & Christensen, 1982; White, 1985). Moreover, the proton electrochemical gradient has been considered as a possible source of energy for system L (Kilberg & Christensen, 1980). Consequently, we investigated the effect of alterations of the Ringer's pH from 7.2 (normal) to either 5.7 or 8.2 on the current responses to L-Ala, L-Ser and L-Leu under voltage-clamp conditions. We found (Fig. 11*A* and *B*) that all the responses were diminished by these manipulations. The responses to L-Ala and L-Ser were reduced by a factor of seven at pH 5.7 and six at pH 8.2, whereas the L-Leu responses were diminished to about half their normal size when pH was varied in either direction. These results are in qualitative agreement with the conclusions of Christensen and his group (*see* above) based on amino acid flux measurements in different tissues.

## Discussion

The present study is based upon the assumption that the membrane currents elicited by external applications of neutral amino acids reflect the uptake

of these solutes rather than the activation of ionic channels. This view is supported by results from earlier electrophysiological investigations (*see* Introduction), which corroborate previous kinetic analyses of amino acid flux measurements. A few additional observations reported here reinforce this view: (i) the apparent $K_m$ values summarized in Table 1 satisfactorily agree with those usually proposed for amino acid carriers at high Na concentration (*see* Thomas & Christensen, 1971) and are several orders of magnitude higher than those of specific ligands activating ionic channels; (ii) no desensitization of the electrical responses is observed even during long lasting (several tens of seconds) applications of amino acids; (iii) little selectivity is observed for amino acids known to be taken up but not yet identified as agonists of ligand-gated channels; (iv) finally, inhibitors like MeAIB or amiloride markedly (and for different reasons) reduce the responses. All of these observations leave little room for an alternative interpretation of the electrical signals in terms of ion movement tightly coupled with the uptake of amino acids.

Our results suggest that at an early stage of development the embryonic cells of *Xenopus laevis* already possess at least two and possibly three different electrically identifiable carrier systems resembling those of adult superior vertebrates.

This conclusion is based largely upon the results of competition experiments. Concerning the technical aspect of the work, it should be emphasized that, in nonvoltage-clamped cells, the uptake of one neutral solute can interfere in a competitive-like manner with that of another one if the transports are both voltage sensitive and electrogenic. In the particular case of Na-coupled transports, an apparent competition is expected even if two solutes are transported by two independent carriers since the membrane depolarization associated with one solute transport reduces the Na-electrochemical gradient available for the other one. In the experiment presented here, this source of error was in principle eliminated since the cell membranes were voltage clamped.

In fact, due to electrical coupling between the several cells constituting the aggregate under investigation, it is difficult to ascertain whether the membrane potential was uniformly fixed in all cells and circumstances. It may thus be argued that space clamp defects could account for the observation that coefficient $\alpha$ as calculated from Eq. (1) was always found to be smaller than 2 when two distinct amino acid pathways were simultaneously activated. This interpretation cannot be discarded, but we regard it as unsufficient. Different control experiment performed with a third micropipette measuring the voltage at different distances from the main voltage electrode showed indeed that in small aggregates the membrane potential could be easily controlled between $-100$ and $-30$ mV, even in the presence of saturating concentrations of amino acids or external K concentrations lower than 30 mM. Clamp defects frequently occurred at positive potentials in normal medium and at negative potentials in K-rich solutions. We attribute these failures to membrane breakdown in the cell impaled by the current electrode when too much current had to be passed. This was never the case, however, in the experiments described in this paper, since in most cases the holding potential was fixed at the spontaneous resting level. In the particular case of the experiments reported in Fig. 5, the current-voltage relationship is restricted to the negative voltage range and only qualitative conclusions are drawn from this figure. One could still anticipate alterations of the internal ionic content in response to changes in either the membrane potential or the bath composition. Previous observations (Bergman & Bergman, 1985) showed that the endoderm cell are predominantly K-permeable under physiological conditions and (*unpublished observations*) develop a chloride conductance when the internal Ca concentration increases (usually in leaky cells). The membrane permeability of these embryonic cells is also reversibly modified by external pH alterations (*see* Fig. 11). We postulate that the internal composition of the cells was regulated and not significantly altered in all the short-lasting experiments presented in this paper.

The Na-dependent carrier system is inhibited at negative and constant membrane potential by an excess in K in the *cis* compartment, as well as variations of external pH on either side of neutrality. It is insensitive to the external Cl concentration, markedly voltage dependent, poorly selective for linear neutral amino acids and finally, inhibited by amiloride and the specific inhibitor MeAIB. These findings permit the conclusion that the transport system A is present in the cell membrane studied here.

The sensitivity of these amino acid responses to variations of external K concentrations is not expected for a classical Na/solute symport at a constant membrane potential. In recent years, however, it has been shown that a K efflux appears to be linked to the (Na-dependent) amino acid uptake in several cell types (Eddy, 1968; Grasset et al., 1983; Brown & Sepúlveda, 1985; Kristensen, 1986). This K efflux seems to arise from a $[Ca]_i$-induced increase in the K conductance (Bear & Petersen, 1987; Sheppard, Giraldez & Sepúlveda, 1988), the meaning of which remains hypothetical (Schultz, Hudson & Lapointe, 1985). If a similar mechanism existed in our preparation, an inward K current could be expected to take place at high $[K]_o$ when

the membrane is voltage clamped to a negative level. If this were the case, however, the amino acid-induced current should be larger in K-rich solutions than in Ringer's (since an inward K current should be superimposed on the Na current). The opposite finding is observed, thereby reinforcing the view (Bergman & Bergman, 1985; Barbour et al., 1988) that the reversal of the K electrochemical gradient or the increase in $[K]_o$ *per se* exerts an inhibitory effect (Eddy & Hogg, 1969) on the Na-coupled amino acid uptake, possibly directly on the carrier mechanism (Bergman et al., 1986; Barbour et al., 1988), or perhaps indirectly by, for example, opposing a potassium efflux which might be required as an exchange for the Na entry and (or) the amino acid accumulation (*see* Schultz et al., 1985; Kristensen, 1986; Bear & Petersen, 1987).

The fact that serine induces a small current even in the presence of MeAIB suggests that another Na-dependent system is also present. It is tempting to conclude that the membrane pathway for that amino acid corresponds to system ASC, since that carrier is most efficient for serine transport and is widespread. Among the Na-dependent transport systems, a specific pathway for proline, termed "system P," has been identified in mammalian cell lines (Englesberg & Moffett, 1986). Moreover, a transport system specific for L-Asn and L-Gln has been also discovered in hepatocytes and hepatoma cell lines (Kilberg & Christensen, 1980; Kilberg Handlogten & Christensen, 1980). There is no evidence in our results for such specific transporters in the endoderm cells of *Xenopus*. L-Pro, L-Asn and L-Gln apparently compete with all other polar amino acids at the level of the same Na- and voltage-dependent systems. L-Pro, however, also appears to compete with L-Leu and L-Phe at the level of a carrier present in the endoderm cell membranes.

The latter carrier is Na-, K- and voltage-independent. Accordingly, it resembles "system L" in many respects. The surprise is that it is electrogenic but insensitive both to membrane potential changes and to alterations of most electrochemical gradients.

If the proton distribution provides the energy for this transport, a membrane depolarization would be expected to be inhibitory, and a lowering of external pH, facilitory. This is not the case, although an increase in pH has been found to be inhibitory. If an outward Cl antiport were implied, decreasing $[Cl]_o$ should facilitate the transport, whereas a membrane depolarization should reduce it. This has not been observed. Moreover, a decrease in $[Cl]_o$ reduces the response, whereas a rise in $[HCO_3]_o$ increases it. These results remain to be explained on the basis of further investigations.

We conclude that, in view of the data presented here, none of the electrochemical gradients we have manipulated directly contributes to the L-Leu (or L-Phe) transport, or more precisely, to generate the membrane currents elicited by these two neutral amino acids. The electrogenic responses associated with external applications of these substrates are likely related to the transport activity since saturable current-concentration relationships can be obtained that are very similar to those obtained with Na- and voltage-dependent responses. We suggest that the electrogenic character is indirect and involves a voltage-independent rate-limiting step that has to be taken into account for a complete interpretation of the L-Leu and L-Phe transport process.

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