Membrane mechanisms for electrogenic Na\(^+\)-independent L-alanine transport in the lizard duodenal mucosa

MARIO DÍAZ, VIRTUDES MEDINA, TOMÁS GÓMEZ, AND ANTONIO LORENZO
Laboratorio de Fisiología Animal, Departamento de Biología Animal,
Universidad de La Laguna, 38206 Tenerife, Spain

Received 27 December 1999; accepted in final form 28 March 2000

Díaz, Mario, Virtudes Medina, Tomás Gómez, and Antonio Lorenzo. Membrane mechanisms for electrogenic Na\(^+\)-independent L-alanine transport in the lizard duodenal mucosa. Am J Physiol Regulatory Integrative Comp Physiol 279: R925–R935, 2000.—The active Na\(^+\)-independent transport of L-alanine across the duodenal mucosa of the lizard Gallotia galloti was studied in Ussing-type chambers using a computer-controlled voltage clamp. Addition of L-alanine to the Na\(^+\)-free bathing solutions resulted in a significant L-alanine absorption (\(J_{\text{net}}\)) that was paralleled by an increase in transepithelial short-circuit current (\(I_{\text{sc}}\)) and potential difference (PD) without apparent changes in the tissue conductance. The concentration dependence of \(J_{\text{net}},\) PD, and \(I_{\text{sc}}\) displayed Michaelis-Menten kinetics. L-alanine-induced electrical changes were completely inhibited by external alkaline pH or by the H\(^+\)-ionophore carbonyl cyanide m-chlorophenylhydrazone in the bathing solution. The alanine-induced electrogenicity was dependent on the presence of extracellular K\(^+\) and could be blocked by serosal Ba\(^{2+}\) or mucosal orthovanadate. These results suggest the existence of an H\(^+\)-coupled L-alanine cotransport at the apical membrane of enterocytes. The favorable H\(^+\) driving force is likely to be maintained by an apical vanadate-sensitive H\(^+\)-K\(^+\)-ATPase, allowing the extrusion of H\(^+\) in an exchange with K\(^+\). Potassium exit through a basolateral barium-sensitive conductance provides the key step for the electrogenicity of L-alanine absorption.

H\(^+\)-L-alanine cotransport; neutral amino acid absorption; intestinal epithelium; H\(^+\)-K\(^+\)-ATPase; Gallotia galloti

THE ACTIVE TRANSPORT OF NEUTRAL amino acids has been widely studied in a variety of tissues and cells from different organisms, and several kinetic models have been postulated to explain the transport mechanisms (4, 11, 16, 18, 20). It is accepted that coupling of amino acid transfer occurs secondarily to metabolic energy stored in the transmembrane Na\(^+\) electrochemical gradient, and it is well established that Na\(^+\)-coupled amino acid cotransport across the apical membranes of small intestine and renal proximal tubules from a variety of species is both rheogenic and conductive (11, 20, 23). Thus the addition of amino acids to the solution bathing the apical surface depolarizes the electrical potential difference (PD) across the apical membrane in association with the net sodium transfer into the intracellular compartment. In polarized epithelia, the Na\(^+\) gradient is maintained by the activity of the Na\(^+\)-K\(^+\)-ATPase that extrudes the Na\(^+\) ions through the basolateral membranes to the serosal compartment; this movement occurs with a concomitant change in the short-circuit current (\(I_{\text{sc}}\)) that can be assessed with short-circuit techniques.

In recent years, increasing evidence has accumulated indicating that active amino acid transport can also take place in the absence of Na\(^+\). The concentrative Na\(^+\)-independent transport of several amino acids, such as L-proline, L-alanine, and β-alanine, amino acid derivatives, such as α-aminoisobutyrate (AIB), and dipeptides, such as glycylsarcosine has been demonstrated in renal epithelia tissues and cultured cell lines (14, 15, 26–29). Some of these studies pointed out the key role of the transmembrane H\(^+\) electrochemical gradient as the major driving force for the Na\(^+\)-independent amino acid transport, and from the results of direct intracellular pH measurements using fluorescent dyes, it has been demonstrated that amino acid absorption occurs along with a concomitant intracellular acidification (26, 27). Recently, we observed the presence of an active absorption of L-alanine transport in the upper small intestine of the lizard Gallotia galloti. The intestinal physiology of this animal model has been studied in our laboratory over the last two decades, and many of the intestinal transport systems at different intestinal segments have been characterized (2, 3, 8, 12).

In the present study, using isotope tracers and voltage clamp techniques, we attempted to determine the characteristics of the active Na\(^+\)-independent L-alanine transport in the isolated lizard duodenum, to analyze the electrical characteristics of the L-alanine transport, and, finally, to examine the possible mechanisms involved in the electrogenicity of the Na\(^+\)-independent amino acid absorption. To our knowledge, the present study represents the first electrophysiological investigation of electrogenic Na\(^+\)-independent L-alanine transport across the in vitro small intestine.
and encourages more detailed studies of the H⁺-coupled transport of organic solutes in the duodenum.

**MATERIALS AND METHODS**

Adult male and female *Gallotia galloti* specimens weighing 25–40 g were killed by spinal transection. The duodenum was then removed, opened along the mesenteric border, and rinsed several times in ice-cold bathing solution gassed with 5% CO₂ and 95% O₂. The intestinal segment was mounted in water-jacketed Ussing chambers with an exposed area of 0.21 cm². The tissue was bathed on both sides with 4 ml of Ringer solution continuously gassed with 95% O₂, and the temperature was maintained at 27°C. The standard bathing solution contained (in mM) 107 NaCl, 4.5 KCl, 25 NaHCO₃, 1.8 Na₂HPO₄, 0.2 NaH₂PO₄, 1.25 CaCl₂, 1.0 MgCl₂ with a final pH 7.3. Na⁺-free solutions were prepared by omitting KCl from the nominal Na⁺ solution. The solution pH was adjusted with HCl. K⁺, Na⁺-free solutions were prepared by omitting KCl from the nominal Na⁺-free solution.

Unidirectional amino acid fluxes were measured under short-circuit conditions using the procedure described in detail by Bolaños et al. (3). Briefly, 20 min after the tissue was properly mounted in the chamber, 5.0 μCi [1-14C]alanine was added to the serosal or the mucosal side of the tissue. After an additional period of 20 min in which the isotope reached the steady state, duplicate 200-μl aliquots were taken from the unlabeled side of the tissue at regular 20-min intervals for 1 h. The sample volume was replaced by an equal volume of cold Ringer solution to maintain the compartment volume constant. Isotope radioactivity was measured in a liquid scintillation spectrometer (LKB-1208, Rackbeta), and the unidirectional and net fluxes were determined using a computer program written in our laboratory (7), which also provided the statistical tools required for data analysis.

The electrical measurements were made using calomel (for voltage sensing) and Ag-AgCl electrodes (for current passage) connected to the bathing solutions through 4% vol/wt agar bridges. Electrical measurements were continuously monitored with an automatic computer-controlled voltage-clamp device (AC-microclamp, Aachen, Germany). The electrode offset potential and the solution resistance were determined at the beginning of every experiment and were automatically corrected for. The tissues were first incubated under open-circuit conditions for 20 min and then short-circuited, with the PD and the Iₑ determined every minute. Every 5 s, the tissues were pulsed with ±10-μA pulses of 1-s duration, and from the displacement of the PD, the tissue conductance (Gₛ) was derived. All three parameters were recorded using a dot matrix printer.

Results are expressed as means ± SE. Statistical comparison of mean values were made using one-way ANOVA and two-tailed Student’s t-test where appropriate. Curve fitting of experimental data was performed by nonlinear regression analysis using a computer iteration procedure. Experimental PD and Iₑ data were fitted to the following equations:

\[
PD = PD_0 + PD_{max} \times \frac{[\text{L-alanine}]}{(K_m + [\text{L-alanine}])}
\]

\[
I_{sc} = I_{sc,0} + I_{sc,max} \times \frac{[\text{L-alanine}]}{(K_m + [\text{L-alanine}])}
\]

where \(K_m\) is the apparent Michaelis-Menten constant, \(PD_{max}\) and \(I_{sc,max}\) are the PD and \(I_{sc}\) estimates at saturation, and \(PD_0\) and \(I_{sc,0}\) are the background PD and \(I_{sc}\) values displayed at 0 mM L-alanine, respectively.

**Results**

### Transepithelial L-alanine fluxes

The unidirectional and net fluxes of L-alanine (1 mM) across the isolated lizard duodenum in the presence and in the absence of sodium ions in the bathing solution are given in Fig. 1. Under control conditions, using Na⁺-containing Ringer solutions, the mucosal-to-serosal flux \(J_{m,s}\) was 50.9 ± 4.9 nmol/cm²·h and the flux in the opposite direction \(J_{s,m}\) was 35.8 ± 5.8 nmol/cm²·h, resulting in a statistically significant difference from zero net absorptive flux \(J_{net}\) of 15.2 ± 1.7 nmol/cm²·h \((P < 0.01)\). Once the bathing solutions were replaced with Na⁺-free solutions, both the unidirectional and net fluxes were considerably decreased compared with the fluxes in the presence of Na⁺. Thus \(J_{m,s}\) was reduced to 37.1 ± 3.2 nmol/cm²·h \((P < 0.01)\), and the calculated \(J_{net}\) was decreased by 48% to 7.8 ± 0.1 nmol/cm²·h. From these data, it can be stated that the reduction of \(J_{m,s}\) observed in the ab-

---

**Fig. 1.** Unidirectional and net L-alanine fluxes across the duodenal mucosa of the lizard under short-circuit conditions. Net fluxes were determined as the difference between mucosal-to-serosal flux \(J_{m,s}\) and serosal-to-mucosal flux \(J_{s,m}\) in Na⁺-containing (Ringer solution) and Na⁺-free solutions. The concentration of L-alanine was 1 mM throughout the experiment when added to both sides of the tissue. Results are means ± SE for 12 determinations. **Significantly different from zero with a probability value \(P < 0.01\). ”Statistically different from zero with a probability value \(P < 0.01\).
sence of Na⁺ accounts for the decrease in the $J_{\text{net}}$, because there was no significant difference in $J_{\text{e-m}}$ between the presence and absence of sodium. The $J_{\text{net}}$ observed under Na⁺-free conditions was significantly different from zero ($P < 0.05$), indicating that the lizard duodenum displays different Na⁺-dependent and Na⁺-independent L-alanine active transport pathways.

**Electrical measurements.** The determination of the bioelectrical parameters associated with the transport of L-alanine fluxes was performed under short-circuit conditions, and the results are illustrated in Fig. 2. The tissues were maintained for 20–40 min under open-circuit conditions and then short circuited. As has been reported several times for this same preparation (8, 12), no differences were observed between the transmural PD, $I_{\text{sc}}$, and $G_t$ between stable open-circuit and short-circuit measurements. As can be seen in Fig. 2, the initial 40 min provide steady-state measurements for PD, $I_{\text{sc}}$, and $G_t$ both in the presence or in the absence of sodium.

In the presence of sodium (Fig. 2A), the serosal side of the tissue was found to be electrically positive compared with the luminal side. The average transepithelial PD was 1.09 mV, and the mean $I_{\text{sc}}$ was 10.1 μA/cm². These values were slightly smaller than in other studies in this same preparation (12). The transepithelial conductance remained unaltered throughout the experiment ~7.5 mS/cm².

In the absence of sodium (Fig. 2B), both PD and $I_{\text{sc}}$ were considerably smaller than in the presence of sodium ($P < 0.01$ for both parameters), reflecting the suppression of the basally active electrogenic sodium transport (12). The $G_t$ was 6.7 ± 0.84 mS/cm² and therefore was not significantly affected by the removal of extracellular sodium. The lack of change in conductance when sodium was replaced by choline suggests that the paracellular pathways are equally permeable for both cations.

The addition of L-alanine (1 mM) to the mucosal and serosal compartments brought about considerable changes in both PD and $I_{\text{sc}}$. As can be seen in Fig. 2A, the addition of the amino acid produced a statistically significant increase of PD and $I_{\text{sc}}$, which was not followed by any change in the $G_t$. PD and $I_{\text{sc}}$ were progressively elevated in the presence of the L-alanine and reached a steady state 20 min after the addition of the amino acid.

Figure 2B shows the results of the addition of L-alanine to the bathing solution in the complete absence of sodium. Interestingly, PD and $I_{\text{sc}}$ significantly increased ($P < 0.01$), without apparent changes on $G_t$. Again, the electrical parameters reached a steady state 20–30 min after the presence of amino acid in the bathing solution.

The effects of L-alanine on the duodenal electrical characteristics were also observed when L-alanine was added only to the luminal solution (data not shown), either in the presence or in the absence of sodium, suggesting that these effects might be triggered at the luminal membrane of the cells.

**Kinetic analysis of L-alanine fluxes.** The existence of active L-alanine fluxes in the absence of sodium was further explored by determining the transport kinetic under short-circuit conditions. Figure 3 summarizes the results of the experiments designed to determine the effects of different concentrations of L-alanine (50 μM to 10 mM) on the transepithelial $J_{\text{net}}$. These experiments were carried out in the presence of identical L-alanine concentrations on the mucosal and serosal reservoirs under short-circuit conditions. Under these conditions, the changes on the net transmural fluxes...
are the consequence of active transport process activity, because the diffusive components are reasonably identical in the two opposite directions (mucosa-to-serosa and serosa-to-mucosa). As can be seen in Fig. 3, the increase on the external L-alanine concentration was followed by a hyperbolic rise of the calculated net L-alanine fluxes. The kinetic constants describing this saturable transport were computed from the Lineweaver-Burk plot and were $K_m = 0.168 \pm 0.03$ mM and a maximal net flux of $14.88 \pm 1.37$ nmol/cm$^2$ h. These results strongly suggest that the presence of a carrier-mediated system is responsible for the net Na$^+$-independent transport.

Kinetic analysis of L-alanine-induced electrical responses. As was observed above, the addition of 1 mM L-alanine to the external solution induced significant changes on the transepithelial PD and $I_{sc}$ both in the presence and absence of sodium. Under short-circuit conditions and in the absence of transepithelial gradients, the presence of an Na$^+$-independent L-alanine saturable transport indicates the presence of some secondary active transport system driving the net movement of L-alanine to the serosal side. In an attempt to determine the possible relationship between the concentration of L-alanine in the bathing solutions and the bioelectrical parameters, we assessed the variations on the PD and $I_{sc}$ in response to changes in the external L-alanine concentration under Na$^+$-free conditions. The substrate-dependence curves for the PD and $I_{sc}$ elicited in the presence of different concentrations of L-alanine are shown in Fig. 4. Clearly, both PD and $I_{sc}$ displayed saturable functions of amino acid concentration. The kinetic parameters were derived from nonlinear regression analysis of the experimental data fitted to the saturation equations (see MATERIALS AND METHODS). The computed parameters showed apparent $K_m$ values of $0.82 \pm 0.26$ and $0.65 \pm 0.21$ mM for PD and $I_{sc}$, respectively, which were not statisticallcally different. The $PD_{max}$ and $I_{sc_{max}}$ estimates, representing the L-alanine-induced increase on PD and $I_{sc}$ values at saturation, were $0.86 \pm 0.08$ mV and $4.53 \pm 0.39$ $\mu$A/cm$^2$. The offset values, corresponding to the PD$_o$ and $I_{sc_{o}}$ obtained in the absence of L-alanine, were fairly similar to the values obtained above and have also been published elsewhere (8, 12).

Given the hyperbolic relationship between the electrical parameters and the L-alanine concentration, we performed regression analyses to test whether the magnitude of the unidirectional or net fluxes could provide a prediction of the change on the $\delta$PD or $\delta I_{sc}$. These analyses were performed in those experiments where the electrical PD, the $I_{sc}$ values, and the amino acid fluxes were measured simultaneously. The results of these analyses are summarized in Fig. 5. The data could be significantly fitted to linear equations with $r^2$ above 0.8 in all cases, indicating that the magnitude of net L-alanine fluxes may be adequately inferred from

---

Fig. 3. Concentration dependence for net L-alanine flux ($J_{net}$) under short-circuit conditions in the presence of Na$^+$-free solutions. L-alanine was added to the mucosal and serosal solutions in small volumes. The rectangular hyperbola represents the Michaelis-Menten ($K_m$) fit for the data by nonlinear regression analysis. Each measurement represents the mean $\pm$ SE of 5 different experiments.

Fig. 4. Kinetic analyses of PD (A) and $I_{sc}$ (B) at increasing L-alanine concentrations in Na$^+$-free media added to both sides of the epithelia. Data are fitted with Michaelis-Menten equation (for details, see MATERIALS AND METHODS). Each point corresponds to the mean of 10 determinations.
Effects of variations on the pH gradient. The absence of sodium in the bathing solution raises the question of what sort of electrochemical gradient could be responsible for the net charge carried during the amino acid transport across the duodenal mucosa. The possibility of an electrochemical gradient for $H^+$ driving the uphill movement of organic solutes such as amino acids, lactate, and small peptides has been shown several times in the literature (14, 15, 25–30).

To assess the possible involvement of $H^+$ on the net transport of L-alanine, we carried out different experiments to determine the effects of changes of pH gradients on the L-alanine-induced PD and $I_{sc}$. In the first set of experiments, the pH of the external bathing solution, deprived of sodium, was shifted from the standard value of 7.2 to either more alkaline (8.2) or more acidic (5.6), whereas the transmural $I_{sc}$ was monitored. As can be seen in Fig. 6A, when the external pH was buffered at 8.2, the addition of L-alanine to the mucosal bathing solution clearly failed to increase $\delta I_{sc}$. Instead, it seems that alkalinization of the bathing solution causes a reduction in electrical parameters. On the contrary, in the presence of more acidic bathing solution (Fig. 6B), mucosal application of L-alanine readily augmented normalized $\delta I_{sc}$, indicating that the effects of L-alanine were pH dependent. A more detailed analysis of the time course for the L-alanine PD activation indicated that the activation was faster at pH 5.6 than at pH 7.2, suggesting that the rate of electrogenicity was influenced by the pH (data not shown).

Fig. 5. Regression analyses for the L-alanine fluxes and normalized ($\delta$) PD (A) or $\delta I_{sc}$ (B) simultaneously determined in the same tissues in the absence of Na$^+$ in the bathing solution. Linear equations and correlation coefficients are indicated. Each data point represents the average of 6 determinations.

Fig. 6. pH dependence of Na$^+$-independent L-alanine induced electrogenicity. Effect of an alkaline (A) and an acidic (B) media on the epithelial response to L-alanine. C: effects of the application of the $H^+$-ionophore carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) to the mucosal solutions. L-alanine (2 mM) was added only to the mucosal solutions. The results illustrate the average of 3 experiments.
In the second set of experiments, the pH of the bathing solutions was maintained at 7.2, and the effects of the H⁺-ionophore (CCCP) on the response to L-alanine were assessed. The results of these experiments are depicted in Fig. 6C. As expected, the presence of L-alanine causes a significant rise of $I_{sc}$ which reached a plateau 20 min after the addition of the amino acid. Clearly, the addition of the H⁺-ionophore to the mucosal solution produced a dramatic reduction of $I_{sc}$ that eventually abolished the amino acid-induced effects.

**Effects of NEM and orthovanadate on L-alanine-induced electrogenicity.** If the electrochemical gradient for H⁺ is the energy source for L-alanine entry across the apical membrane of the lizard duodenum, the question arises, what is the mechanism(s) extruding H⁺ out from the intestinal cells, thereby keeping the transmembrane proton gradient?

The possible involvement of an active mechanism allowing the recirculation of H⁺ across the mucosal membrane was assessed. Because an NEM-sensitive H⁺-ATPase was shown to be present in microsomal fractions of rabbit colonic mucosa (10), the effect of NEM on the development of L-alanine-induced electrical changes was also tested. We performed a series of experiments where NEM (1 mM) was added to the mucosal bath 30 min before the addition of L-alanine while the pH of the bathing solutions was maintained at 5.6. Our experiments showed that the addition of NEM to the mucosal or serosal solutions did not prevent the increase on $I_{sc}$ induced by L-alanine (Fig. 7A), indicating that an H⁺ pump was not involved in the maintenance of the H⁺ gradient across the mucosal membrane of the enterocytes.

With the use of apical membrane vesicles isolated from rabbit colon (10) and Caco-2 cells (1), the presence of a vanadate-sensitive potassium-dependent proton pump that is involved in the regulation of intracellular pH has been demonstrated. We have tested for the presence of a similar ATPase activity involved in the recirculation of H⁺ through the enterocyte cell membrane in another series of experiments where the effects of vanadate on the Na⁺-independent L-alanine-induced rise of $I_{sc}$ were determined. The results depicted in Fig. 7B clearly show that preincubation with mucosal vanadate (100 μM) for 60 min or more completely prevents the increase of $I_{sc}$ induced by mucosal L-alanine, despite extracellular pH being maintained at an electrochemical gradient for protons that is very favorable for H⁺-coupled L-alanine entry. Although the effect of vanadate cannot exclude other possibilities, these experiments clearly indicate that L-alanine electrogenicity is vanadate sensitive.

**Effects of barium chloride.** The experiments shown above suggest the possibility that a vanadate-sensitive H⁺/K⁺ active mechanism could mediate the exchange of H⁺ by K⁺ at the apical membrane and that the basolateral exit of potassium ions could be responsible for the net charge accumulation accounting for the serosa-positive PD. If this were the case, we could suppose that the blocking of basolateral pathways for potassium exit would alter the transepithelial potential. Thus we have designed experiments using barium chloride, a well-known inhibitor of several types of K⁺ channels in a number of tissues including epithelia (9) to determine whether a potassium conductance was secondarily activated by L-alanine. The results presented in Fig. 8 illustrate the effects of the addition of 5 mM BaCl₂ to the serosal solution on the L-alanine-induced activation of $I_{sc}$. As can be seen, the application of barium chloride completely suppressed the effect of L-alanine and readily returned the $I_{sc}$ to control values, suggesting that L-alanine generated an electrogenic current flow carried by K⁺ ions. Parallel experiments in the absence of K⁺ and Na⁺ in the bathing solutions revealed that the electrical effects of the Na⁺-independent L-alanine transport were dependent on the presence of K⁺ ions in the extracellular fluids.

**Effects of CL, BCH, and L-serine.** The amino acid analogs BCH and CL have been shown to competitively inhibit the Na⁺-independent L-alanine transport in a number of tissues and cell lines (4, 6, 11). To address the substrate specificity for the Na⁺-independent L-alanine transport demonstrated here, we have performed a series of experiments determining the effects of several of the well-known inhibitors on the lizard duodenum L-alanine transport. Figure 9 shows the results of the incubation with the analogs BCH and CL.
at concentrations reported in the literature to inhibit alanine transport on the unidirectional and net L-alanine fluxes across the isolated lizard duodenal mucosa. The effects of the amino acid analogs were assayed in separate experiments after the steady state of L-alanine transport had been reached and were added to both the mucosal and serosal compartments. As can be seen in Fig. 9, the addition of BCH (20 mM) or CL (20 mM) completely abolished the net L-alanine fluxes by significantly reducing the mucosa-to-serosa fluxes ($P < 0.001$) without altering the fluxes in the opposite direction. Interestingly, despite the dramatic reduction of $J_{\text{net}}$, the addition of the amino acid analogs did not alter any of the electrical parameters measured (Table 1). The use of L-serine (20 mM) instead of CL or BCH did not cause any change on the transepithelial transport (Fig. 9C) or on the electrical parameters (Table 1), suggesting that the amino acid analogs CL and BCH might compete with L-alanine for the transporter. In another series of experiments, the effects of the addition of CL to the mucosal bathing solution on the transepithelial electrical activity was assessed. To avoid transmural osmotic side effects, the same concentration of D-mannitol was added to the serosal compartment. The results shown in Fig. 9D clearly indicate that the mucosal addition of CL induced an increase on $I_{\text{sc}}$. Subsequent addition of 5 mM L-alanine did not change the transepithelial $I_{\text{sc}}$. The effects of CL were reversible on removal of the amino acid from the bathing solutions.

**DISCUSSION**

A number of studies have demonstrated the active transport of amino acids across the small intestine in different species of vertebrates from fish to mammals, and, although several kinetic models have been postulated, it is generally accepted that active amino acid absorption involves an Na$^+$-coupled cotransport across the apical membranes of small intestine and renal proximal tubules.

The data reported here demonstrate the presence of net L-alanine absorption across the isolated lizard duodenum. Net alanine transport is observed under controlled short-circuit conditions and in the virtual absence of electrochemical gradients between the luminal and serosal sides of the tissue and both in the presence and in the absence of sodium ions in the bathing solution. Although the magnitude of net L-alanine fluxes was considerably higher in the presence of sodium, a significant fraction of the total L-alanine absorption occurs under Na$^+$-free conditions, which indicates that the isolated lizard duodenum develops active processes for both Na$^+$-dependent and Na$^+$-independent L-alanine transport.

The results of the bioelectrical determinations measured under Na$^+$-free conditions show that, together with the development of a net L-alanine absorption, an increase in transepithelial PD and $I_{\text{sc}}$ are induced by the presence of L-alanine in the bathing solution; these effects are due to the presence of L-alanine in the luminal side of the tissues, because the replacement of L-alanine with D-mannitol in the serosal compartment does not affect the temporal evolution of the bioelectrical parameters. These data indicate that L-alanine triggers the activation of some electrogenic processes other than the transepithelial movement of sodium toward the serosal compartment that lead to the development of a serosally positive PD.

The kinetic analyses of both $J_{\text{net}}$ and electrical parameters are consistent with the existence of a carrier-mediated mechanism responsible for the net alanine transport and the development of a serosally positive PD. More interestingly, from the magnitude of the developing PD and $I_{\text{sc}}$, an indirect estimation of the net alanine flux can be achieved. However, these analyses revealed that the apparent $K_m$ constants for the net alanine flux (0.168 mM) are slightly smaller than the constants computed over the PD or $I_{\text{sc}}$ analyses (0.82 and 0.65 mM, respectively). The reason for this discrepancy is unknown, but because the development of the L-alanine-induced electrogenicity involves several membrane transport activities coupled in a sequential manner, it can reasonably be supposed that the kinetic constants for the electrical parameters are conditioned by the transport mechanism exhibiting the smaller
affinity and the stoichiometric ratio of the different substrates.

It is thought that amino acid transport by the small intestinal brush-border membranes is mediated by a number of systems, including an $\text{Na}^+$-independent system identical to system L for neutral amino acids, the $\text{Na}^+$-independent $\text{y}^+$ system for cationic amino acids, and, more recently, the $\text{b}^0,\text{H}^+$ for neutral and cationic amino acids together with at least four $\text{Na}^+$-dependent systems (11, 18, 20). The $\text{Na}^+$-independent system described here in the apical membrane of duodenal cells is novel, mainly because it is electrogenic and exhibits an unusual dependence on the pH gradient.

The results shown in Fig. 6 provide direct evidence for coupling between L-alanine and $\text{H}^+$ movements across the luminal membrane of duodenal enterocytes. The strongest evidence supporting $\text{H}^+$-alanine cotransport are 1) the ability of a pH gradient (alkaline inside) to drive net L-alanine-induced electrogenicity in the absence of other energy sources and 2) the inhibitory effects of the proton ionophore CCCP. Although direct intracellular pH measurements will be necessary to confirm this hypothesis, it is evident that the most straightforward explanation of these findings is that the transporter catalyzes a cotransport driven by the favorable electrochemical gradient for protons. In agreement with our findings, several reports have unambiguously demonstrated $\text{H}^+$-coupled cotransport.

![Fig. 9. Effects of cycloleucine (A; CL), 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, B), and l-serine (C) on Na$^+$-independent L-alanine unidirectional and net fluxes under short-circuit conditions. The amino acid and analogs were added to both sides of the epithelia at the concentrations specified in the figures. L-alanine (1 mM) was present throughout the experiment and was added to the bathing solutions 20 min before the control values were obtained. D: time course for the change of transepithelial $I_m$ before and after the addition of CL to the mucosal bathing solution. D-mannitol (20 mM) was added to the serosal media. Results are means ± SE for 6 (A, B, and C) and 3 experiments (D). " and **Statistically different from the controls with probability values $P < 0.05$ and $P < 0.01$, respectively.](http://ajpregu.physiology.org/)

Table 1. Electrical parameters associated to the Na$^+$-independent L-alanine transport under control conditions and in the presence of different amino acids and analogues

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>PD, mV</th>
<th>$I_m$, $\mu A/cm^2$</th>
<th>$G_t$, $mS/cm^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.50 ± 0.70</td>
<td>4.44 ± 2.04</td>
<td>9.95 ± 0.40</td>
</tr>
<tr>
<td>BCH (20 mM)</td>
<td>1.60 ± 0.70</td>
<td>5.36 ± 2.14</td>
<td>10.40 ± 0.60</td>
</tr>
<tr>
<td>Control</td>
<td>1.30 ± 0.68</td>
<td>5.75 ± 2.14</td>
<td>10.45 ± 0.50</td>
</tr>
<tr>
<td>CL (20 mM)</td>
<td>1.06 ± 0.70</td>
<td>5.36 ± 2.14</td>
<td>10.60 ± 0.60</td>
</tr>
<tr>
<td>Control</td>
<td>0.86 ± 0.39</td>
<td>5.89 ± 3.15</td>
<td>9.40 ± 0.45</td>
</tr>
<tr>
<td>L-Ser (20 mM)</td>
<td>0.58 ± 0.21</td>
<td>5.02 ± 2.17</td>
<td>10.30 ± 0.55</td>
</tr>
</tbody>
</table>

Each value corresponds to the mean ± SE of at least 4 different experiments. The control values were obtained 20 min after the addition of L-alanine. The concentration of L-alanine was 1 mM throughout the experiment and was added to both the serosal and mucosal solutions. The compounds 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), cycloleucine (CL), and l-serine (L-Ser) were added in 100-μl aliquots to both sides of the tissues. PD, potential difference; $I_m$, short-circuit current; $G_t$, tissue conductance. No significant differences were observed.
systems for amino acids in rabbit kidney, including the H\textsuperscript{+}-\textit{d}-alanine cotransport in the pars convoluta and pars recta (14), the H\textsuperscript{+}-AIB cotransport in the proximal tubule (15), and the H\textsuperscript{+}/L-alanine symport in the proximal tubules (13, 30). Recently, Thwaites et al. (26) identified an H\textsuperscript{+}-coupled \textit{b}-alanine transporter in an intestinal derived cell line (Caco-2). Direct intracellular pH measurements in Caco-2 cells showed that cells undergo a rapid intracellular acidification when exposed to \textit{b}-alanine. Interestingly, the concentrative uptake of \textit{b}-alanine in Caco-2 cells monolayers displays similarities with the L-alanine transport described here; inasmuch as it takes place under voltage clamp conditions, in the absence of sodium, and exhibits a concentration-dependent increase of an inward $I_{sc,i}$ it is clearly electrogenic (26).

The Na\textsuperscript{+}-independent L-alanine transport system reported here is dramatically inhibited by the analog BCH and by CL, a nonhydrolyzable amino acid analog, but not by L-serine. There is general agreement that this substrate specificity defines the activity of system L, which, on the other hand, has also been demonstrated in isolated guinea pig enterocytes by Del Castillo and Muñiz (6). It is generally assumed that system L mediates the uniport of amino acids with bulky side chains and has been postulated to serve in many circumstances to mediate efflux from the cells (20); however, several pieces of evidence indicate that system L functions as an amino acid-proton cotransport. For instance, Mitsumoto et al. (17) showed that an inward H\textsuperscript{+} gradient stimulated the Na\textsuperscript{+}-dependent L-leucine uptake by system L in membrane vesicles from Chang liver cells. These same authors reported that the L-leucine uptake was inhibited by BCH. Our observation that BCH and CL inhibit L-alanine transport without altering the electrical parameters and the finding that mucosal CL stimulated an electrogenic process that is not additive to the L-alanine-induced electrical effects suggest that the Na\textsuperscript{+}-independent L-alanine transport system present in the duodenal mucosa might well correspond to the activity of system L. To our knowledge, this work provides the first demonstration of the presence of active Na\textsuperscript{+}-independent H\textsuperscript{+}-coupled neutral amino acid absorption in the small intestine of vertebrates. The existence of a transapical pH gradient in the form of an acidic microclimate in the small intestine emphasizes the potential relevance of such a transport system in driving amino acid absorption (29). It should be pointed out that because L-alanine-induced electrogenicity is readily achieved at pH 7.2, the apical membrane potential likely provides a major contribution to the driving forces for H\textsuperscript{+} movement, a large chemical gradient is not necessary to achieve the uphill movement of L-alanine.

In addition to the characterization of the Na\textsuperscript{+}-independent L-alanine transport, we have worked on the identification of the mechanisms involved in the recirculation of H\textsuperscript{+} across the luminal plasma membrane that allow the maintenance of the H\textsuperscript{+} electrochemical gradient capable of driving the transport of L-alanine against its concentration gradient.

Previous studies on guinea pig distal colon (21, 22), rabbit colon (10, 24), and \textit{Amphiuma} jejunum (32) have demonstrated the existence of an H\textsuperscript{+}-dependent K\textsuperscript{+}-ATPase in the intestine. A novel K\textsuperscript{+}-ATPase, distinct from both Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and gastric H\textsuperscript{+}-K\textsuperscript{+}-ATPase, has been identified in the epithelial cells of guinea pig distal colon (22, 31), and this ATPase activity has been ascribed to the H\textsuperscript{+}-K\textsuperscript{+} exchange in the apical membrane of colonocytes that mediates the K\textsuperscript{+} uptake process for transepithelial K\textsuperscript{+} absorption (22, 31). On the other hand, Kaunitz and Sachs (10) demonstrated two different proton-stimulated ATPases in membrane vesicles isolated from rabbit distal colon; one is NEM sensitive but vanadate insensitive and the other is NEM insensitive but vanadate sensitive. The finding that the electrogenicity of L-alanine transport in the lizard duodenum can be prevented by incubation with orthovanadate strongly suggests that vanadate-sensi-
ELECTROGENIC Na\(^+\)-INDEPENDENT ALANINE TRANSPORT

tive ATPase activity is present in the apical membrane of the lizard duodenal enterocytes, particularly in view of the resistance to NEM, but this idea should be confirmed using biochemical and molecular approaches. However, if this were the case, it would be expected that the ATPase activity requires the presence of K\(^+\) ions in the extracellular solution. This hypothesis was confirmed by the experiments showing that in the absence of K\(^+\), the addition of L-alanine failed to induce any increase in the transmural \(I_{sc}\).

The barium sensitivity of the L-alanine-stimulated positive \(I_{sc}\) and PD demonstrated here is consistent with electrogenic K\(^+\) absorption during L-alanine transport. Ba\(^{2+}\) was effective when applied to the serosal media after PD and \(I_{sc}\) had reached steady-state values, suggesting that the amino acid had activated a basolateral conductance for potassium. The fact that the electrical parameters returned to control levels after barium indicates that the L-alanine-induced electrogenic is totally carried by K\(^+\) ions moving through the epithelial barrier from the mucosal to the serosal solutions. If intracellular K\(^+\) is above electrochemical equilibrium, this conductance would favor a transcellular K\(^+\) transport from the mucosal to the serosal side. Therefore, a barium-sensitive K\(^+\) conductance at the basolateral membrane activated by the Na\(^+\)-independent L-alanine transport seems likely.

In summary, inhibition by orthovanadate indicates the presence of an ATPase, whereas inhibition by serosal BaCl\(_2\) suggests the existence of a conductive K\(^+\) exit. The simplest model that would accommodate both of these requirements is a luminal exchange pump with a conductive K\(^+\) exit.

The mechanism of L-alanine-coupled electrogenic K\(^+\) absorption postulated here seems to be very similar to models proposed for active potassium absorption for other preparations, including rat colon (19), turtle colon (9), and guinea pig colonocytes (5). Interestingly, proton secretion in the guinea pig distal colon is dependent on the presence of K\(^+\) and is sensitive to vanadate (21). It is clear that the cellular mechanisms for the Na\(^+\)-independent H\(^+\)-dependent L-alanine absorption overlap some of the pathways involved in the regulation of intracellular pH, acid secretion, and potassium absorption observed in other preparations, which, to our knowledge, points out the energetic versatility of the membrane electrochemical gradients.

A synthesis of the effects of inhibitors, pH gradients, kinetic analyses, and electrical measurements into a cellular model is suggested by the available information and is depicted in Fig. 10. According to this hypothetical model, the luminal absorption of L-alanine is mediated by the activity of an \(H^+\)-L-alanine cotransport that uses the favorable electrochemical gradient for H\(^+\) to energize the uphill entry of L-alanine into the cell. H\(^+\) recirculates through the apical membrane in an electroneutral exchange process with K\(^+\), hence maintaining a low intracellular H\(^+\) concentration. The exchange mechanism with K\(^+\) and the extrusion of protons are dependent on cellular energy and are carried out by the functioning of an \(H^+\)-K\(^+\) pump located at the apical membrane of duodenal enterocytes. Potassium ions entering the cell would predominantly leave the cell by the basolateral membrane through a barium-sensitive K\(^+\) conductance, which is responsible for the electrogenicity of the L-alanine transport. The dissipation of the transcellular H\(^+\) electrochemical gradient or the blockade of the ATPase activity eventually inhibits L-alanine absorption. Finally, to enable transepithelial absorptive transport, L-alanine must exit the cell via a basolateral pathway, which is likely to be a facilitated diffusion process that might be the ubiquitous system L for neutral amino acids described in other preparations. Further speculation about the nature of the individual transport systems, driving forces, and ionic conductances is unwarranted in the absence of direct measurements of intracellular pH, apical membrane potential, and studies of L-alanine transport across apical and basolateral membrane vesicles.

**Perspectives**

At present we are investigating the mechanisms involved in the active Na\(^+\)-dependent L-alanine transport. In light of the data reported here, the electrogenic Na\(^+\)-independent L-alanine transport might represent a significant contribution to neutral amino acid absorption in the duodenum. Therefore, it was important to assess the possibility of Na\(^+\)-dependent and Na\(^+\)-independent processes coexisting under physiological circumstances, which, in turn, could provide a wider range of physiological conditions under which amino acids may be absorbed. Presumably, the integrated action of the different transport systems is regulated to satisfy the nutritional requirements of the organism.

We express our gratitude to Lupe Acosta for cooperation and extremely efficient lizard hunting and for maintaining the animals in the “five-star” terrarium. Also, we are very grateful to Miguel A. Valverde and Miquel Moretó for helpful comments and critical review of the manuscript. We are indebted to Neil Abrey, who kindly revised the English usage, syntax, and spelling in the manuscript.

**REFERENCES**


5. Del Castillo J, Súbaran-Carrasaco MC, and Burguillos L. K\(^+\) transport in isolated guinea pig colonocytes: evidence for


