Luminal L-alanine stimulates exocytosis at the K+-conductive apical membrane of Aplysia enterocytes

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Luminal L-alanine stimulates exocytosis at the K+-conductive apical membrane of Aplysia enterocytes. Am. J. Physiol. 275 (Cell Physiol. 44): C1300–C1312, 1998.—In Aplysia intestine, stimulation of Na+ absorption with luminal alanine increases apical membrane K+ conductance (GK,a), which presumably regulates enterocyte volume during stimulated Na+ absorption. However, the mechanism responsible for the sustained increase in plasma membrane K+ conductance is not known for any nutrient-absorbing epithelium. In the present study, we have begun to test the hypothesis that the alanine-induced increase in GK,a in Aplysia enterocytes results from exocytic insertion of K+ channels into the apical membrane. We used the fluid-phase marker horseradish peroxidase to assess the effect of alanine on apical membrane exocytosis and conventional microelectrode techniques to assess the effect of alanine on fractional capacitance of the apical membrane (fCm). Luminal alanine significantly increased apical membrane exocytosis from 1.04 ± 0.30 to 1.39 ± 0.38 ng·min⁻¹·cm⁻². To measure fCm, we modeled the Aplysia enterocyte as a double resistance-capacitance (RC) electric circuit arranged in series. Several criteria were tested to confirm application of the model to the enterocytes, and all satisfied the model. When added to the luminal surface, alanine significantly increased fCm from 0.27 ± 0.02 to 0.33 ± 0.04 (n = 10) after 4 min. There are two possible explanations for our findings: 1) the increase in exocytosis, which adds membrane to the apical plasma membrane, prevents plasma membrane fracture, and 2) the increase in exocytosis delivers K+ channels to the apical membrane by exocytic insertion. After the alanine-induced depolarization of apical membrane potential (Vm), there is a strong correlation (r = 0.96) between repolarization of Vm, which reflects the increase in GK,a, and increase in fCm. This correlation supports the exocytic insertion hypothesis for activation of GK,a.

Membrane capacitance; potassium channels; nutrient absorption; sea hare

IN VIRTUALLY ALL nutrient- and Na+-absorbing epithelia, increases in the rate of Na+ absorption are paralleled by increases in plasma membrane K+ conductance (GK) (2, 5, 11, 13). Upregulation of plasma membrane GK increases diffusional K+ efflux from the cells and restores cell volume after nutrient-induced cell swelling (5, 18, 31, 32). After a meal, regulation of enterocyte volume ensures continuous nutrient absorption by the enterocytes.

Several mechanisms have been examined as possible mediators of the increase in plasma membrane GK. For example, changes in intracellular pH (1, 17), increases in cytosolic Ca²⁺ activity (18), activation of ATP-sensitive K+ channels (KATP channels) by decrease in intracellular ATP concentration (1, 24), and activation of stretch-activated K+ channels (5) have been explored as mechanisms that increase plasma membrane GK.

Although these mechanisms provide a possible link between rate of Na+ absorption and activation of plasma membrane GK, they do not account entirely for sustained modulation of membrane GK during nutrient-induced increases in Na+ absorption. For instance, during nutrient-stimulated Na+ absorption in rabbit proximal convoluted tubule, both intracellular alkalization (1) and acidification have been observed in isolated rabbit proximal convoluted tubule, switching the luminal perfusate to nutrient-free Ringer has little effect on intracellular Ca²⁺ activity (18). Intracellular ATP concentration declines during nutrient absorption (1), but in some cases these decreases in nucleotide concentration may not be sufficient to activate KATP channels and produce macroscopic increases in plasma membrane GK (24). In nutrient-absorbing frog proximal tubule cells, basolateral membrane K+ channels are activated by membrane stretch (29) and nutrient-induced cell swelling (5). However, nutrient-absorbing epithelial cells regulate their volume after nutrient entry (21, 22, 23). Thus the stretch-activated K+ channels do not account for sustained increases in plasma membrane GK during prolonged periods of nutrient absorption.

Taken together, these studies do not reveal the mechanism responsible for the sustained increase in plasma membrane GK in stimulated Na+-absorbing epithelial cells (28). However, exocytic insertion of K+ channels into plasma membranes could increase plasma membrane GK. Today, nine different transport proteins are known to undergo regulated exocytic insertion into plasma membranes as a means of increasing the number of functional transporters in plasma membranes (3). Therefore, we have assessed the role of exocytic insertion of K+ channels as a mechanism to increase plasma membrane GK in Aplysia enterocytes.

Aplysia intestine is an excellent model system for investigation of the role of exocytic insertion as a means of increasing plasma membrane GK. First, GK is located in the apical membrane of Aplysia enterocytes, and, second, luminal alanine increases apical membrane GK (GK,a) (11). In contrast, GK is located in the basolateral membrane of vertebrate enterocytes (13). Hence, in vertebrate intestine, identification of membrane trafficking events associated with K+ channels is complicated by the possibility of parallel trafficking of Na+ pumps to the basolateral membrane.

In this study, we have begun to test the hypothesis that the alanine-induced increase in GK,a in Aplysia enterocytes is due to exocytic insertion of K+ channels into the apical membrane. Accordingly, we assessed the effect of alanine on apical membrane exocytosis and fractional capacitance of the apical membrane (fCm). We report that luminal alanine significantly increases both...
apical membrane exocytosis and $f_{Ca}$. There are two possible explanations, which are not mutually exclusive, for the increases in exocytosis and $f_{Ca}$: 1) the increases reflect exocytic events that prevent plasma membrane fracture as the enterocyte swells and 2) the increases result from exocytic insertion of K$^+$ channels into the apical plasma membrane, as hypothesized. The second explanation is supported by the strong correlation between repolarization of the apical membrane potential ($V_m$), which largely reflects the increase in $G_{K_{ap}}$ and increase in $f_{Ca}$. Hence, the increase in $G_{K_{ap}}$ is tightly correlated with the increase in $f_{Ca}$.

**MATERIALS AND METHODS**

Animals and solutions. Aplysia californica weighing 150–500 g were obtained from Alacrity Marine Biological (Redondo Beach, CA) or the University of Miami Aplysia Resource Growth Facility (UMARGF; Miami, FL). Snails were stored at 14°C in artificial seawater (ASW; Instant Ocean), usually for no more than 2 wk, and fed Gracilaria obtained from UMARGF or romaine lettuce three times each week. Before removal of the intestine, snails were anesthetized by injecting them with a volume of 0.33 M MgCl$_2$ equal to one-half the animal's body weight. They were opened dorsally with stainless steel scissors and forceps, and the entire digestive gland and intestine were removed and placed in standard ASW at room temperature, 20–24°C. ASW contained (in mM) 450 Na$^+$, 530 Cl$^-$, 10 K$^+$, 10 Ca$^{2+}$, and 25 Mg$^{2+}$, and either 10 mannitol or 10 L-alanine (mannitol-ASW and Ala-ASW, respectively). All solutions were buffered at pH 7.5 with 10 mM HEPES. The first 1–4 cm of proximal intestine was removed from the digestive gland, cut open, and pinned, luminal side up, over a nylon mesh support. Next, the luminal surface was rinsed vigorously with mannitol-ASW to remove fecal material. The tissue and mesh support were secured in a vertical position. Next, the tissue was mounted on an O-ring assembly, which was mounted in either the exocytosis chamber or the microelectrode (ME) chamber, described below (Measurement of exocytosis and Electrophysiology). Both chambers allowed superfusion of apical and basolateral surfaces of the tissue with ASW.

Horseradish peroxidase as a marker for exocytosis. All solutions used in the exocytosis studies contained 5 mM $\beta$-(+)-mannose (Sigma) and 1% BSA (Sigma) (Table 1). Because horseradish peroxidase (HRP; type II, Sigma) is rich in mannose residues, mannose was added to ASW to prevent HRP binding to mannose receptors on the cell surface (4); BSA was added to reduce nonspecific binding of HRP to the apical membrane (6). Mannitol (Sigma), a nontransported osmolyte, for the increases in exocytosis and $f_{Ca}$, was used instead of mannose in some experiments when possible, for the increases in exocytosis and $f_{Ca}$, was used instead of mannose. All solutions had the same ionic composition and pH (see MATERIALS AND METHODS). Solute concentrations are in mM except for BSA (%) and Grace's vitamin solution, which is given as final concentration of a stock concentrated solution (1,000×). Ala, alanine; ASW, artificial seawater; s, supplemented.

### Table 1. Composition of ASW solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Mannitol</th>
<th>L-Alanine</th>
<th>Mannose</th>
<th>BSA</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Pyruvate</th>
<th>Grace's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol-mannose-BSA-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala-mannose-BSA-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-mannitol-mannose-BSA-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1×</td>
</tr>
<tr>
<td>s-Ala-mannose-BSA-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1×</td>
</tr>
<tr>
<td>s-mannitol-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-Ala-ASW</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All solutions had same ionic composition and pH (see MATERIALS AND METHODS).
ments was minimized. Immediately after the HRP loading period, the tissue was rinsed with mannitol-mannose-BSA-ASW from a plastic squirt bottle at room temperature (total rinse volume ~250 ml) and dipped in 10 consecutive changes of ice-cold mannitol-mannose-BSA-ASW for 2 min each. Between each 2-min wash, the tissue was rinsed extensively with ice-cold mannitol-mannose-BSA-ASW from a squirt bottle (total rinse volume ~20 ml each). Ice-cold ASW was used to reduce endosomal processing of endocytosed HRP and to reduce exocytosis and endocytosis processes during the rinse protocol. After the last cold wash and rinse, the tissue was dipped in two consecutive 10-ml vials of mannitol-mannose-BSA-ASW at room temperature for 2 min each. The tissue was rinsed extensively with mannitol-mannose-BSA-ASW at room temperature after each 2-min wash (total rinse volume ~250 ml).

Measurement of exocytosis. Exocytosis was quantified by measuring release of HRP into the apical reservoir of a modified Ussing chamber (Jim’s Instruments, Iowa City, IA) designed to measure exocytosis in Aplysia intestine. The tissue was mounted to maximize exposed tissue surface area (0.63 cm²) and to minimize the volume of apical bathing solution (500 µl). Each half chamber contained two vertical ports (diameter 0.38 cm) that allowed access to the apical and basolateral bathing solutions for mixing and sampling. After mounting of the tissue in the exocytosis chamber, the apical and basolateral reservoirs were filled with mannitol-mannose-BSA-ASW at room temperature. The luminal solution was mixed and exchanged five times with fresh mannitol-mannose-BSA-ASW to continue removal of surface-bound HRP. Mixing was accomplished by inserting a Pipetman micropipette tip into the apical solution through the vertical port farthest from the tissue, withdrawing 250 µl of solution, delivering it back into the reservoir, and repeating this action four times. In a dye experiment, five plunger strokes performed in this manner produced a uniform distribution of phenol red in the luminal reservoir. Therefore, to ensure consistent sampling of HRP released into the luminal reservoir during exocytosis periods, the apical bathing solution was mixed with five plunger strokes before removal of 250-µl samples (see below).

Time course of HRP release. HRP was released from the apical membrane, a sampling “window” was established during which release of HRP into the luminal reservoir was constant with time. After loading HRP into enterocytes and rinsing HRP from the intestine, the sampling window was established in the following manner. The apical reservoir was filled with mannitol-mannose-BSA-ASW and mixed, and a 250-µl time zero sample was removed. After replacement of the 250 µl of mannitol-mannose-BSA-ASW removed at time zero, the tissue was allowed to release HRP for 10 min. At the end of the 10-min efflux period, the apical reservoir was mixed and a second 250-µl sample was removed (10-min sample). The remaining apical solution was removed and discarded and replaced with 500 µl of fresh mannitol-mannose-BSA-ASW. The difference between the concentration of HRP present in the time zero sample and in each 10-min sample represented the amount of HRP released into the apical reservoir over that 10-min period. This protocol (called HRP washout) was continued for eight consecutive 10-min HRP release periods.

Effect of low temperature on release of HRP. We evaluated the effect of reduced temperature on HRP release as follows. After HRP washout and the removal and discarding of three consecutive 10-min HRP release samples, a 10-min efflux period was measured, first in the presence of apical s-mannitol-mannose-BSA-ASW and then in the presence of apical s-Ala-mannose-BSA-ASW. Time zero samples were removed and the chamber port was cut off. After loading HRP into enterocytes and rinsing HRP from the intestine, the sampling window was established in the following manner. The apical reservoir was filled with mannitol-mannose-BSA-ASW and mixed, and a 250-µl time zero sample was removed. After replacement of the 250 µl of mannitol-mannose-BSA-ASW removed at time zero, the tissue was allowed to release HRP for 10 min. At the end of the 10-min efflux period, the apical reservoir was mixed and a second 250-µl sample was removed (10-min sample). The remaining apical solution was removed and discarded and replaced with 500 µl of fresh mannitol-mannose-BSA-ASW. The difference between the concentration of HRP present in the time zero sample and in each 10-min sample represented the amount of HRP released into the apical reservoir over that 10-min period. This protocol (called HRP washout) was continued for eight consecutive 10-min HRP release periods.

Effect of low temperature on release of HRP. We evaluated the effect of reduced temperature on HRP release as follows. After HRP washout and the removal and discarding of three consecutive 10-min HRP release samples, a 10-min efflux period at room temperature was measured as described above. Immediately afterward, the apical and basolateral reservoir solutions were removed and replaced with 8–10°C mannitol-mannose-BSA-ASW, and the entire exocytosis chamber was set into crushed ice. After 5 min, the ice was removed and replaced with ice-water with a temperature of 8–10°C. The apical and basolateral solutions reached and maintained a temperature of 8–10°C after 3 min. Chamber temperature was monitored with an infrared thermometer (TempTestr, Cole-Parmer) into the apical reservoir and recording the steady-state temperature achieved after ~10 s. Crushed ice was added periodically to maintain the bath temperature at 8–10°C. Once the chamber temperature reached 9°C, HRP release was determined in a second 10-min efflux period.

Effect of alanine on exocytosis. To assess the effect of luminal alanine on apical membrane exocytosis required modifications of the HRP loading protocol. First, HRP loading time was extended from 70 to 150 min, ASW was replaced with fresh ASW every 30 min, and all ASW were supplemented (called s-ASW) with 1 mM acetate, 1 mM butyrate, 1 mM pyruvate, and Grace’s vitamin solution at 1× final concentration in ASW (all supplements purchased from Sigma). Second, the HRP release time was reduced from 10 to 3 min to minimize contributions from constitutive and nonspecific HRP release that would obscure alanine-stimulated HRP release. We also shifted our sampling window from 40 to 70 min into the HRP washout protocol to reduce nonspecific HRP release. Finally, it was necessary to modify our mixing protocol to release HRP trapped between microvilli of the apical brush border. Accordingly, all mixing was done through the chamber port nearest the tissue, the number of mixing strokes was increased from 5 to 10, and the mixing stream was directed over the luminal surface of the tissue (called alanine HRP flush protocol). This allowed thorough flushing of exocytosed HRP from the luminal surface.

The effect of luminal alanine on exocytosis from the apical membrane was assessed in the following manner. After removal and discarding six consecutive 10-min HRP washout samples, a 3-min HRP efflux period was measured, first in the presence of apical s-mannitol-mannose-BSA-ASW and then in the presence of apical s-Ala-mannose-BSA-ASW. Time zero samples were removed through the chamber port nearest the tissue immediately after filling the luminal reservoir through the chamber port nearest the tissue surface. As described above, the stream of ASW was directed over the tissue surface to facilitate surface removal of HRP. Before addition of alanine to the luminal solution, this reservoir was rinsed quickly with a fresh exchange of s-mannitol-mannose-BSA-ASW. As a measure of tissue viability, the effect of alanine on short-circuit current (Isc) was recorded after exocytosis measurements in an Ussing chamber that exposed 0.63 cm² of tissue surface area. We compared the effects of mannitol and alanine on HRP catalytic rates because the alanine exocytosis experimental protocol required release of HRP into mannitol-ASW and Ala-ASW, respectively. HRP catalytic rates were assessed in 20% mannitol-ASW (2 mM mannitol) and 20% Ala-ASW (2 mM alanine) with HRP concentrations of 4, 8, and 16 ng/ml; percentages represent final ASW concentrations.

Electrophysiology. MEs were prepared as previously described (10). However, agar bridges and calomel electrodes were modified to reduce access resistances. Agar bridges connected the ME chamber to calomel electrodes and were prepared from PE-90 tubing filled with 3% bacteriological agar (Sigma) in 0.45-µm-filtered 3 M KCl and were kept short (11 cm). The transepithelial potential difference (Vte) was recorded with calomel electrodes from which the ceramic junctions had been cut off.
After the tissue was mounted in the ME chamber, the tissue was short-circuited with a voltage clamp (model VCC600, Physiologic Instruments) that automatically compensates for fluid resistance. \( V_a \) was recorded with a high-impedance electrometer (model FD 223, World Precision Instruments). Both \( V_a \) and the \( I_a \) were sampled at 1,500 samples/s with a Biopac analog-to-digital converter connected to a 160-MHz Motorola MacOs microcomputer equipped with a high-resolution color monitor. Data were saved on 100-megabyte Zip disks with an Epson Zip drive for later analysis.

Determination of \( f_C_a \) with MEs. \( f_C_a \) was measured to evaluate changes in apical membrane surface area (SAa) that occur during alanine-stimulated Na\(^+\) absorption. The major assumption in the ME technique to measure membrane fractional capacitance is that the transcellular pathway can be represented by two linear circuits arranged in series (Fig. 1). To determine \( f_C_a \), a transepithelial current pulse clamps \( V_t \) from zero to a new value \((\Delta V_t = -40 \text{ mV})\) instantaneously \((<1 \text{ ms})\). In response to the current pulse, \( V_a \) will change with a time course that depends on the resistances and capacitances of the apical and basolateral membranes \((R_a, C_a, R_b, C_b\), respectively\). The increment in \( V_a \) as a function of time is described by the following relation \((12, 19)\):

\[
\Delta V_a(t) = \Delta V_t(f_{R_a}) + \Delta V_t(f_{C_a} - f_{R_a}) e^{-\frac{t}{\tau}}
\]

where \(\Delta V_a(t)\) is the change in \(V_a\) at a point in time \((t)\) after a step change in \(V_t\), \(f_{C_a}\) is the fractional capacitance of the basolateral membrane \((f_{C_a} = C_b/(C_b + C_a))\), \(f_{R_a}\) is the fractional resistance of the apical membrane \((f_{R_a} = R_a/(R_a + R_b))\), and \(\tau\) is the time constant for interaction between the two RC circuits arranged in series. This is the time required for \(\sim 63\%\) dissipation of the capacitive transient; \(\tau\) is given by \((12)\):

\[
\tau = \left(\frac{C_a + C_b}{R_a/R_b + (R_a + R_b)}\right)
\]

When \( V_t \) is clamped to a new value, no current flows through \( R_a \) and \( R_b \) at time zero. Thus \(\Delta V_a(t) = \Delta V_t f_{C_a}\) and \(\Delta V_a/\Delta V_t = f_{C_a} \). At \(t = \tau\), \(\Delta V_a/\Delta V_t = f_{R_a}\); at this time, no current flows through the capacitance of the circuit. Depending on the magnitude of \( f_{R_a} \) and \( f_{C_b} \), \( V_a \) at time zero \((V_a(0))\) may either overshoot during a transepithelial current pulse, undershoot, or equal the final steady-state value of \( V_a \) \((V_a(\infty))\) \((19)\). When \( f_{R_a} > f_{C_b} \), \(\Delta V_a\) is characterized by an "overshoot." Finally, when \( f_{R_a} = f_{C_b} \), \(\Delta V_a\) is "square" because Eq. 2 reduces to

\[
\Delta V_a = \Delta V_t (f_{R_a})
\]

In Aplysia enterocytes, we have observed mostly overshoots \((\sim 90\%)\) and a few square waves. Thus on average \( f_{C_b} > f_{R_a} \). However, in this study we only observed overshoots.

The \( f_{C_a} \) is determined from the capacitive transient in \( V_a \). As described above, the increment in \( V_a \) at time zero \((\Delta V_a(0))\) divided by \( \Delta V_a \) is equal to \( f_{C_a} \). The magnitude of \( V_a(0) \) was determined with Biopac's Acknowledge software by measuring the difference between the mean \( V_a \) immediately preceding the upstroke of the capacitive transient and peak value of \( V_a(0) \). \( f_{C_a} \) was then calculated from the following relation:

\[
f_{C_a} = 1 - f_{C_b}
\]

Usually \( V_a(0) \) is estimated from a graph of \( \ln(V_a(t) - V_a(\infty)) \) vs. time \((19)\). However, in preliminary studies, \( V_a(0) \) was the same whether estimated as described above or by the semilogarithmic plot. Therefore, to facilitate and simplify determination of \( f_{C_a} \), we used the method described above.

Experimental protocol. After mounting the tissue luminal side up in the ME chamber, the apical and basolateral surfaces were superfused with aerated s-mannitol-ASW in which room air was filtered with a gas-wash bottle filled with deionized, distilled water. The tissue was short-circuited and, after \( I_a \) stabilized \((\sim 15 \text{ min})\), an enterocyte was impaled with an ME. Once \( V_a \) stabilized, current pulses sufficient to drive \(\Delta V_a \) to \(-10, -20, -30, \text{ and } -40 \text{ mV}\) were passed across the tissue every 10 s for a duration of 0.7 s to assess the effect of \(\Delta V_a \) on \( f_{C_a} \) and \( f_{R_a} \). After a control period consisting of \(-10 \text{ 40-mV pulses, the apical superfusate was switched to s-Ala-ASW and the tissue was continuously pulsed with 40-mV pulses every 10 s. After } V_a \text{ repolarized (\sim 4 min), the apical superfusate was switched back to s-mannitol-ASW.}\)

Statistical analysis. All data are reported as means \(\pm\) SE (except for Fig. 2). Statistical analyses were performed by use of either the paired Student's \(t\)-test or ANOVA with \( P < 0.05 \) as the level of significance. With ANOVA, statistical difference between treatments was established with the Fisher potential least significant difference test and the Scheffé \(F\)-test. Treatment were considered significantly different if both Fisher and Scheffé tests agreed.

**RESULTS**

HRP activity is influenced by ASW ionic concentration. HRP was used to monitor apical membrane exocytosis in A. californica proximal intestine. Because HRP is extracted from plants and normally exists in a relatively low-ionic strength intracellular environment \((\sim 8 \text{ mM NaCl}; \text{ Ref. } 30)\), we suspected that the high ionic strength of our ASW \((450 \text{ mM NaCl})\) would "salt out" the enzyme and reduce its catalytic activity. Accordingly, ASW was diluted 1:3 and 1:10 with distilled water, and HRP activity in the diluted ASW was compared with that in full-strength ASW. Diluting ASW by 1:3 and 1:10 increased HRP activity significantly (ANOVA) by factors of three and five, respectively (Fig. 2). To increase HRP assay sensitivity, all exocytosis samples were diluted to a final concentration of 20% ASW before HRP activity was measured. Because HRP standard curve samples were produced in 8% ASW, exocytosis HRP activities (AU/min) were
doubled before HRP concentrations were determined from standard curves.

Time course of HRP release. To evaluate the effect of alanine absorption on apical membrane exocytosis, a sampling window was established during which release of HRP into the apical reservoir was constant with time. Release of HRP during the first 10-min efflux period was significantly higher (ANOVA) than subsequent efflux periods (Fig. 3). This suggests that a small amount of HRP remained adsorbed onto the tissue surface and was released into the luminal reservoir. HRP release dropped during the second efflux period and by the third efflux period assumed a rate that remained constant during the next five 10-min efflux periods. Therefore, all exocytosis experiments were performed between 40 and 80 min after the start of HRP washout.

Low temperature inhibits HRP release. We measured the effect of low temperature on HRP release to confirm that release of HRP from the intestine into the luminal reservoir represented release of HRP by exocytosis and not release of adsorbed HRP. HRP release from the apical surface at 9°C was significantly reduced by 72% compared with the control (24°C; Fig. 4). These data are consistent with HRP release by exocytosis from the apical surface of Aplysia enterocytes.

Luminal alanine stimulates exocytosis. If the alanine-induced increase in $G_{K,a}$ results from fusion of K$^+$ channel-containing cytoplasmic vesicles with the apical membrane, luminal addition of alanine should stimulate apical membrane exocytosis. Addition of alanine to the intestine's apical surface significantly increased release of HRP by 37% above the control (paired t-test; Fig. 5). This increase in HRP release cannot be attributed to alanine stimulation of HRP catalytic activity. The slopes of the relation between HRP catalytic activity (AU/min) and HRP concentration (4, 8, and 16 ng/ml; 2–4 replicates for each HRP concentration) were virtually identical for 20% mannitol-ASW (2 mM mannitol; 0.021 AU⋅ml⁻¹⋅min⁻¹⋅ng⁻¹) and 20% Ala-ASW (2 mM alanine; 0.020 AU⋅ml⁻¹⋅min⁻¹⋅ng⁻¹). As a measure of tissue viability, we assessed the effect of alanine on $I_{sc}$ after completion of the exocytosis studies. Because alanine increased $I_{sc}$ by 6.3 µA/cm² (Fig. 5), A. californica proximal enterocytes retain their nutrient-absorbing capacity even after extended exposure (2.5 h) to HRP.

Electrophysiology: testing the double RC circuit model. In our measurements of $f_{C_o}$, we assumed that the enterocyte transcellular pathway can be represented by a series combination of two linear RC circuits (Fig. 1). To confirm that this model accurately predicts the electrical behavior of A. californica proximal enterocytes, at least three criteria must be met. First, because two linear RC circuits arranged in series will charge and discharge through the same circuit, the on and off responses in a capacitive transient should be identical (12). Figure 6 illustrates a representative capacitive transient in $V_a$ produced from a step change in $V_a$ from 0 to −40 mV. The on and off responses are equal. Thus charging and discharging of the double RC circuit
occurs through the same pathway. Second, fC_b and fR_a, and hence τ, should be independent of the magnitude of \( \Delta V_t \). Accordingly, fC_b and fR_a were determined with \( \Delta V_t = -10, -20, -30, \) and \(-40 \text{ mV} \). This experimental protocol shows that fC_b and fR_a are independent of \( \Delta V_t \) (Fig. 7), and therefore τ (see Eq. 2) is independent of \( \Delta V_t \). Third, capacitance is an inherent property of the membrane surface area and the ability of the membrane to store electrical charge. Therefore, it should be possible to change fR_a without changing fC_b. Superfusion of the apical surface with alanine reduced fR_a before changes in fC_b occurred (see Fig. 9), as predicted by the double RC circuit model.

fR_a is not voltage dependent. To apply the double RC electric circuit model to Aplysia enterocytes, G_K,a and therefore fR_a must be voltage independent at least over the time course of the 700-ms voltage pulse (19). fR_a is not influenced by a 30-mV depolarizing voltage clamp applied to \( V_a \) (Table 2). Thus G_K,a is voltage independent, and these data provide additional support for application of the double RC circuit model to Aplysia enterocytes.

G_K,a is not activated by depolarization of \( V_a \). We assessed the role of voltage-gated K_1 channels in the alanine-induced increase in G_K,a. In a previous study, luminal alanine depolarized \( V_a \) by an average of 12 mV (11). Accordingly, \( V_a \) and fR_a were measured before and during a 12-mV depolarizing voltage clamp applied to \( V_a \). If depolarization-sensitive voltage-gated K_1 channels reside in the apical membrane, we predicted that a 12-mV voltage clamp would reduce fR_a and cause gradual hyperpolarization of \( V_a \) as these channels open. However, \( V_a \) remained constant, and fR_a increased slightly over the 3-min voltage clamp period (Fig. 8). Thus G_K,a is not activated by physiological changes in \( V_a \).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>30-mV Clamp</th>
</tr>
</thead>
<tbody>
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<td>s-Ala-ASW 0.39</td>
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<td>Snail 2</td>
<td>s-Mannitol-ASW 0.49</td>
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<td></td>
<td>s-Ala-ASW 0.43</td>
<td>0.43</td>
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Values for fractional resistance of apical membrane (fR_a) were obtained from 2 different snail intestines. To determine fR_a, transepithelial potential was pulsed to –10 mV for 700 ms under control conditions [no voltage clamp applied to apical membrane potential (\( V_a \))] and when a 30-mV depolarizing voltage clamp was applied to \( V_a \).
Effect of alanine on electrical properties of Aplysia enterocytes. Within the first 1 min after superfusion of the apical membrane with s-Ala-ASW, $V_a$ depolarized by 8 mV, $I_{sc}$ increased by 9 µA/cm², and $fR_a$ dropped slightly by 0.03 (Fig. 9). $fC_a$ remained fairly constant during this time but increased slightly 50 s after superfusion of alanine onto the luminal surface. Over the next 3.5 min, $V_a$ repolarized by 8 mV, $I_{sc}$ gradually increased by 3.5 µA/cm² before decreasing by 1 µA/cm², $fR_a$ increased slightly by 0.01, and $fC_a$ climbed gradually until reaching a peak value 0.06 above prealanine $fC_a$. Fifty seconds before alanine was superfused onto the apical surface, $fC_a$ was 0.27 ± 0.02. After 4 min of exposure to alanine, $fC_a$ increased significantly (paired t-test) to 0.33 ± 0.04 (Fig. 9). Thus alanine increased the SAa.

After the alanine-induced depolarization of $V_a$, there was a time-dependent repolarization of $V_a$ that was paralleled by a time-dependent increase in $fC_a$, suggesting a relationship between the two variables (Fig. 10). Because increases in $G_{K,a}$ will drive $V_a$ toward the Nernst equilibrium potential for $K^+$ ($E_{K,a}$), the correlation between the time-dependent changes in $V_a$ and $fC_a$ ($r = 0.96$) suggests that the increase in $G_{K,a}$ depends on the increase in $fC_a$.

In several experiments, the MEs remained in the enterocyte for several minutes after the luminal superfusate was switched to alanine. In these extended time course impalements, there was a decrease in $fC_a$ after the increase stimulated by alanine (Fig. 11). These data suggest that after extended periods of exposure to alanine, the rate of endocytosis at the apical membrane

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Fig. 8. A 12-mV voltage clamp to $V_a$ has no effect on $fR_a$ and $V_a$ in Aplysia enterocytes. Error bars, ±SE (n = 5).

Fig. 9. Effect of luminal alanine (10 mM) on time course of $V_a$, $I_{sc}$, $fR_a$, and fractional capacitance of the apical membrane ($fC_a$) in Aplysia intestine; man, supplemented mannitol-ASW; ala, supplemented Ala-ASW. Increase in $fC_a$ at peak (~4 min after alanine) is significantly different from prealanine value, $P < 0.05$ (paired t-test). Error bars, ±SE (n = 10).
Luminal alanine reduces \( f_{Ca} \). The alanine-induced decrease in \( f_{Ra} \) is consistent with conductive alanine transport across the apical membrane. Equation 2 shows that addition of a conductive pathway to the apical membrane should decrease \( R_a \) and hence reduce \( \tau \). At the peak alanine-induced depolarization of \( V_a \), \( \tau \) was reduced significantly (paired t-test) by 20% (Fig. 12). This provides additional support for application of the double RC circuit model to Aplysia enterocytes.

**DISCUSSION**

Fluid-phase markers, including HRP, have been used extensively to investigate endocytic and exocytic events associated with trafficking of ion channels and transport proteins in numerous cell types and tissues (4, 6, 33, 34). Importantly, HRP follows traffic of proteins, such as the antidiuretic hormone-sensitive water channels, to the apical membrane (6), and HRP is internalized by epithelial cells into the endosomal compartment.

![Graph](image1)

**Fig. 10.** Correlation between time-dependent increase in \( f_{Ca} \) and time-dependent repolarization of \( V_a \) after alanine-induced depolarization of \( V_a \) in Aplysia intestine. Data are average values for \( f_{Ca} \) and \( V_a \) from Fig. 9 between 30 and 150 s after addition of alanine.

![Graph](image2)

**Fig. 11.** Left: decreases in \( f_{Ca} \) observed after extended luminal superfusion with alanine in 3 snail intestines. Right: \( V_a \) and \( f_{Ra} \) for each tissue over same period.
(4, 6). In absorptive epithelial cells of rat small intestine, HRP enters endosome-like vesicles and in some instances even enters Golgi cisternae (9). Hence, HRP is a suitable fluid-phase marker with which to follow exocytic events in solute- and water-transporting epithelial cells. However, the high ionic concentration of our ASW, compared with the cytoplasmic ionic concentration of the plant from which HRP is extracted, may influence HRP activity. Indeed, HRP catalytic activity increased significantly with ASW dilution (Fig. 2). Thus HRP activity is strongly dependent on either the osmolality or ionic concentrations of ASW. By diluting HRP samples in full-strength ASW (i.e., 100%) to 20% ASW before quantifying HRP activity, we increased the sensitivity of our HRP assay by a factor of approximately four.

The higher rates of HRP release during the first and second 10-min efflux periods of HRP washout probably resulted from both exocytosis and surface release of HRP (Fig. 3). Between 40 and 80 min of the washout, HRP release results largely from exocytosis alone. If the amount of HRP released from the tissue surface is proportional to the amount of surface-bound HRP, it follows that as the amount of surface-bound HRP declines, the rate of HRP surface release will decrease with time. Therefore, if HRP release into the luminal reservoir results from surface release only, the rate of release should decline continually as a function of time. However, HRP release from the luminal surface remained nearly constant between 40 and 80 min. Therefore, HRP release between 40 and 80 min after initiation of HRP washout results largely from exocytosis and not surface release.

To confirm that HRP release from the luminal surface of Aplysia intestine results from exocytosis, we assessed the effect of low temperature on HRP release, because exocytosis is sensitive to reduced temperature (4, 6, 16, 34). Compared with room temperature, cold reduced HRP release by 73%. Although low temperature will decrease release of surface-bound HRP, the large effect of low temperature on HRP release and the constant rate of HRP release between 40 and 80 min in the washout study suggest that low temperature reduced primarily exocytosis. Therefore, we conclude that HRP release from 40 to 80 min after initiation of HRP washout provides a reliable estimate of the basal rate of apical membrane exocytosis in Aplysia intestine.

Rates of HRP release measured during the low-temperature studies were ~20 times higher than rates measured during the HRP washout and the alanine studies. The higher rates observed during the low-temperature studies may be associated with seasonal changes in constitutive exocytosis, because the temperature experiment was performed several weeks after the HRP washout studies and several months before the alanine studies. However, because there are no published studies on exocytosis in Aplysia intestine, we can only speculate as to the cause of these differences in rates of exocytosis.

Alanine significantly increased exocytosis at the apical membrane (Fig. 5), which supports the exocytic insertion hypothesis for activation of G\(_{\text{K,A}}\). Alanine, however, could conceivably displace HRP from the apical surface, or alanine could stimulate HRP catalytic activity. If so, the increase in HRP release would not reflect stimulated exocytosis. Two observations show that alanine stimulates exocytosis and not surface release of HRP, and a third shows that alanine does not stimulate HRP activity. 1) In many preliminary experiments, i.e., before the final alanine exocytosis protocol was worked out, alanine failed to consistently stimulate HRP release. In most cases, there was no stimulation or even a slight decrease. If alanine displaced HRP bound to the apical surface, we would have observed consistent stimulated HRP release. 2) In three of the five tissues used in the alanine exocytosis experiments, we repeatedly (twice) tested the effect of alanine on HRP release (data not shown) after the stimulated release illustrated in Fig. 5. In two tissues, alanine repeatedly stimulated HRP release, but, in the third tissue, alanine failed to repeatedly stimulate release, even though basal rates of HRP release were similar in the three tissues. Again, if alanine simply displaced HRP from the apical surface, HRP release should have been stimulated repeatedly in all three tissues. 3) Furthermore, alanine had no effect on HRP catalytic activity. Thus the alanine-stimulated increase in HRP release cannot be attributed to alanine stimulation of HRP catalytic activity.
Detection of the alanine-induced increase in apical membrane exocytosis required thorough flushing (the alanine HRP flush protocol) of the luminal surface before removal of HRP exocytosis samples. The apical surface of Aplysia intestine is covered with closely packed microvilli (25) that may trap HRP released by exocytosis at the base of the microvilli. Microvilli of intestinal brush borders are packed with cytoskeletal proteins from their tips to their bases (10). This arrangement of proteins and the narrow width of microvilli prevent the movement of vesicles into the microvilli. Indeed, electron micrographs of intestinal and renal proximal tubule brush borders show that vesicles fuse with the apical membrane at the base of microvilli (7, 10).Therefore, it is necessary to flush the apical surface thoroughly to remove HRP released by alanine-induced exocytosis and trapped between microvilli. The necessity for the alanine HRP flush protocol also supports the above arguments that alanine-stimulated HRP release is due to exocytosis and not surface release.

Peak values for alanine-stimulated $I_{sc}$ in the exocytosis studies (Fig. 5) were $\sim 47\%$ lower than peak values for alanine-stimulated $I_{sc}$ recorded in the ME studies (Fig. 9). The difference may reflect tissue "rundown" and a reduced capacity for alanine absorption by intestine used in the exocytosis studies. The extended duration of the alanine exocytosis protocol ( $\sim 4$ h) vs. the length of the ME protocols (<2 h) may account for the difference in magnitudes of alanine-stimulated $I_{sc}$ in the two experimental protocols. However, in a previous study, we found alanine-stimulated $I_{sc}$ to vary from 8 to 12 $\mu$A/cm$^2$ (11). Thus the lower stimulated $I_{sc}$ in the alanine exocytosis experiment may result from both rundown and tissues that exhibit somewhat lower alanine-stimulated $I_{sc}$. We did not measure alanine-stimulated $I_{sc}$ at the beginning of the experiment and compare this to the stimulated $I_{sc}$ at the end because this would have extended the experimental protocol, which was already 4 h long. This would have necessitated additional tissue handling and possibly increased edge damage, which would have reduced the tissue area that released HRP.

Our electrophysiological studies show that the alanine-induced changes in $V_a$, $R_a$, and $I_{sc}$ recorded in this study are similar to those reported previously (11) and that Aplysia enterocytes can be modeled as two RC circuits arranged in series. Furthermore, alanine activation of the apical membrane Na$^+$-alanine cotransporter reduces $r$, as expected for a conductive cotransporter whose activation reduces $R_a$ (see Eq. 2). This observation supports application of the double series RC circuit to Aplysia enterocytes. In addition, values for $r$ recorded in Aplysia enterocytes fall within the range reported by Garcia-Diaz and Esgis (12) for Necturus gallbladder (30–130 ms) and agree favorably with values reported for Necturus small intestine before and after luminal addition of galactose: 59 ms and 46 ms, respectively (19).

Luminal addition of alanine increased $fC_a$ by 22% after $\sim 3$ min (Fig. 9). This is consistent with an alanine-induced increase in $SA_{a}$. Although an increase in $fC_a$ can result from an increased rate of endocytosis at the basolateral membrane, which, in turn, will decrease $C_{ib}$, this is not likely for four reasons. First, increased endocytosis at the basolateral membrane may reduce the number of Na$^+$ pumps and thereby limit the ability of enterocytes to match basolateral Na$^+$ extrusion to nutrient-induced apical Na$^+$ entry. Second, alanine absorption activates a conductive pathway in the basolateral membrane of Aplysia enterocytes (11). Increases in the rate of basolateral membrane endocytosis would be counterproductive to addition of a conductive pathway at this barrier. Third, increases in membrane area that exceed 3% cause membrane rupture (20). Because the alanine-induced increase in $fC_a$ exceeds 3% and is not accounted for by increases in cell volume and accompanying membrane stretch (see APPENDIX), membrane area must increase by addition of membrane to the apical membrane. Fourth, reduction in surface area of the basolateral membrane ( $SA_{a}$; decrease in $C_{ib}$) during increases in the rate of nutrient absorption would likely lead to rupture of the basolateral membrane as the enterocyte began to swell. Furthermore, alanine-stimulated apical membrane exocytosis confirms that the alanine-induced increase in $fC_a$ reflects increased apical membrane exocytosis.

During nutrient absorption, two phenomena can increase $SA_{a}$: 1) membrane stretch associated with osmotic cell swelling (5) and 2) addition of membrane by stimulated exocytosis (33). Membrane stretch, however, cannot account entirely for the alanine-induced increase in $fC_a$ (see APPENDIX). Thus the alanine-induced increase in $fC_a$ must result, at least in part, from increased exocytosis at the apical membrane, as confirmed by the alanine-induced increase in apical membrane exocytosis.

After the alanine-induced depolarization of $V_a$, there is a repolarization over the next $\sim 3$ min. Five mechanisms may contribute to the repolarization of $V_a$: 1) increase in $G_{K,a}$, 2) increase in basolateral membrane electrogenic Na$^+$ pump activity, 3) stimulation of Na$^+$-3HCO$^-$ cotransporter, 4) increase in basolateral membrane $G_K$ ($G_{K,b}$), and 5) change in reversal potential for the Na$^+$-alanine cotransporter ($E_{AlaNa,a}$), which is given by

$$E_{AlaNa,a} = 2.3 (RT/2F) \log (\text{[Na]}_i/\text{[Na]}_o)$$

where o and i denote extracellular and intracellular concentrations, respectively, R is the gas constant, T is absolute temperature, z is valence, and F is Faraday's constant.

In Aplysia enterocytes, luminal alanine increases $G_{K,a}$ (11). This increase contributes to repolarization of $V_a$ because the increase in $G_{K,a}$ will drive $V_a$ toward $E_{K,a}$, which is approximately $\sim 75$ to $\sim 100$ mV in vertebrate and invertebrate cells (8). In Aplysia intestine, the alanine-stimulated increase in Na$^+$ pump activity has no effect on repolarization of $V_a$, since ouabain, a specific blocker of the Na$^+$ pump (15), has no effect on repolarization but completely blocks the in-
crease in \( I_C \) (11). A role for \( Na^+\)-HCO\(_3^-\) inward-directed cotransport in repolarization of \( V_a \) (17) can be ruled out because we used nominally HCO\(_3^-\)-free ASW gassed with room air. During alanine absorption, accumulation of alanine in the cytosol of enterocytes will reduce \( E_{\text{Ala-Na}},a \) and hyperpolarize \( V_a \). However, the change in \( E_{\text{Ala-Na}},a \) probably contributes little to repolarization of \( V_a \). In the presence of alanine, the apical membrane becomes predominantly \( K^+ \) conductive even in the presence of the \( Na^+-\)alanine cotransporter conductive pathway (11). This conclusion is supported by the effect of alanine washout on \( V_a \). In eight snail intestines, we washed alanine off the luminal surface after recording the effects of alanine on the various electrical parameters. In these eight tissues, addition of alanine to the luminal surface depolarized \( V_a \) by \( \sim 10 \) mV \( \pm 1.39 \), whereas removal of alanine after the increase in \( G_{K,a} \) caused a \( < 1 \) \( \pm 0.62 \) mV change in \( V_a \) (these data are significantly different, paired t-test). Virtually the same effect of alanine washout on \( V_a \) was found in a previous study (11). Thus, if \( G_{K,a} \) is the predominant conductive pathway in the apical membrane in the presence of alanine, then alanine washout should have little effect on \( V_a \), as observed. We can rule out voltage-dependent \( K^+ \) channels as the basis for the increase in \( G_{K,a} \) because a 12-mV depolarizing voltage clamp applied to \( V_a \) for 3 min had virtually no effect on \( V_a \) and \( R_a \). An increase in \( G_{K,b} \) can also contribute to the repolarization of \( V_a \) under short-circuit conditions (\( V_{a,sc} \)) is given by

\[
V_{a,sc} = (E_a R_b + E_b R_a)/(R_a + R_b)
\]

where \( E_a \) and \( E_b \) are the electromotive forces of the apical and basolateral membranes, respectively. The prealanine \( R_a \) is 0.30, and \( R_b \) is 2.3 times larger than \( R_a \). Therefore, \( V_a \) is dominated by \( E_a \). With alanine, \( R_a \) falls to \( \sim 0.27 \), \( R_b \) becomes 2.7 times larger than \( R_a \), and \( V_a \) shifts even closer to \( E_a \). After the alanine-induced decrease in \( R_a \), \( R_b/R_a \) remains constant for the next \( \sim 3.5 \) min. During this time \( G_{K,a} \) increases (11), and there must be a parallel increase in \( G_b \) because the \( R_a \) constant. The ionic nature of the increase in \( G_{K,b} \) is not known. If it represents an increase in \( G_{K,b,p} \), this could contribute to repolarization of \( V_a \), as mentioned above. However, the much larger \( R_b \) means \( V_a \) is dominated by \( E_a \). Although we cannot rule out a contribution of an increase in \( G_{K,b} \) to repolarization of \( V_a \), this analysis suggests that the repolarization of \( V_a \) reflects mostly the change in \( E_a \). Taken together, these data show that the alanine-induced increase in \( G_{K,a} \) is the predominant factor responsible for repolarization of \( V_a \). Hence, we conclude that the repolarization of \( V_a \) after the alanine-induced depolarization is an indirect but good measure of the increase in \( G_{K,a} \).

One of the hallmarks of plasma membrane ion channel activation by exocytic insertion of channels is a parallel increase in plasma membrane capacitance and plasma membrane conductance to the particular ion (20). For example, in cultured, \( Cl^- \)-secreting airway epithelia, increases in intracellular cAMP concentra-

\[ \text{tion cause parallel increases in whole cell membrane capacitance and plasma membrane Cl}^- \text{conductance (33). In toad urinary bladder, antidiuretic hormone increases membrane water permeability and membrane capacitance simultaneously during the first 10 min of stimulation (26). Therefore, the correlation, illustrated in Fig. 10, between the time-dependent repolarization of \( V_a \) and the time-dependent increase in \( f_{Ca} \) during alanine absorption in Aplysia enterocytes suggests that \( K^+ \) channels are delivered to the apical membrane by exocytic insertion. An alternative explanation for our findings is that the alanine-induced increases in exocytosis and \( f_{Ca} \) may reflect an attempt to prevent membrane fracture as the enterocyte swells osmotically. This explanation, however, does not account for the correlation between \( V_a \) and \( f_{Ca} \). The alanine-stimulated increase in \( f_{Ca} \) in Aplysia enterocytes is similar to that reported by Lapointe et al. (19) for Necturus small intestine: 15 mM luminal galactose increased \( f_{Ca} \) significantly by 19%. The increase in \( f_{Ca} \) may result from exocytic insertion of \( K^+ \) channels into the basolateral membrane, because the \( G_{K} \) in Necturus intestine resides in the basolateral membrane and increases during galactose absorption (13, 14).

In three ME experiments, the MEs remained in the cell for several minutes after recording the effect of alanine on the various electrophysiological parameters (Fig. 11). In all three tissues, there was a decrease in \( f_{Ca} \) after the alanine-stimulated increase. The decrease could result from either a decrease in \( C_{Ca} \) or increase in \( C_{bp} \), or both. Careful inspection of the data, however, shows a parallel increase in \( R_a \) with no change or a small hyperpolarization of \( V_a \). If the increase in \( R_a \) is due to an increase in basolateral membrane conductance, the decrease in \( f_{Ca} \) may result from an increase in exocytosis at this barrier. Therefore, the drop in \( f_{Ca} \) may be due to an increase in \( C_{bp} \).

In summary, alanine stimulates exocytosis at the \( K^+ \)-conductive apical membrane of Aplysia enterocytes. There are two possible explanations for addition of membrane to the apical plasma membrane: 1) this prevents plasma membrane fracture and 2) it increases \( G_{K,a} \) by exocytic insertion of \( K^+ \) channels. The tight correlation between repolarization of \( V_a \) and increase in \( f_{Ca} \) supports the second explanation. In any case, future studies should be directed at distinguishing between these two possibilities.

**APPENDIX**

Because of osmotic enterocyte swelling, the increase in \( f_{Ca} \) we observed with luminal alanine could result from an increase in \( S_A \) due to membrane stretch (5). However, the calculation below will show that this is not likely. For this calculation, we have to estimate the percent increase in cell volume that occurs when Aplysia enterocytes are exposed to alanine. The large cell size of Aplysia (\( \sim 80 \) \( \mu \)m in length; Ref. 25), low alanine-stimulated \( I_{sc} \) (\( \sim 13 \) \( \mu A/cm^2 \)), and high intracellular osmolarity (\( \sim 1,000 \) mosmol/l) suggest that alanine does not increase cell volume by more than 10\%. This value is supported by the following observations. In renal proximal tubule cells, 10–40 mM nutrients increase cell volume by 18–25\% (5, 18), and 20–25 mM glucose or alanine increases

cell volume by 5–10% in isolated guinea pig enterocytes (21). The intracellular osmolarity of these cells is ~300 mosm/l, or one-third that of Aplysia enterocytes. If the vertebrate and mammalian cells were the same size as Aplysia and had the same low alanine-stimulated \( I_C \), and the same osmolarity, they would increase their volumes by no more than approximately one-third of that exhibited, ~3–8%. Aplysia enterocytes are ~80 µm in length and ~6 µm on a side (25). Without microvilli, enterocyte volume is 2,880 µm\(^3\), or ~3,000 µm\(^3\), and the surface area is 1,992 µm\(^2\), or ~2,000 µm\(^2\). If cell swelling increased cell volume by 10%, this would increase volume to 3,300 µm\(^3\). In the intact tissue, cell swelling is going to occur predominantly by increases in cell length, and we assume that the microvilli do not accommodate any of the increase in cell volume. Thus a 10% increase in volume requires an increase of 8 µm in length to 88 µm.

Our average prealanine \( f_C \) is 0.27. With an enterocyte surface area of 2,000 µm\(^2\), the \( S_A \) is 540 µm\(^2\). If we account for the ~20-fold increase in \( S_A \) associated with apical microvilli (35), the \( S_A \) is 10,800 µm\(^2\). Given that 1 cm\(^2\) of plasma membrane has a capacitance of 1 µF, or 100 µm\(^2\) = 1 pF (5, 33), we use surface area and capacitance interchangeably. This is justified because capacitance is proportional to the surface area of the capacitor. Thus the \( S_A \) is \( f_C \) (prealanine) = \( C_f/(C_a + C_b) = 0.27 = 10,800 \mu m^2/(10,800 \mu m^2 + C_b) \). Solving for \( C_b \), we have \( S_A = C_a = 29,200 \mu m^2 \). We assume that the length of the apical membrane from tight junctions to the base of the microvilli is ~21 µm and that one-half of the increase in cell length (4 µm) occurs in the apical membrane. This gives an alanine \( S_A \) of 12,720 µm\(^2\) and an alanine \( S_A \) of 29,300 µm\(^2\). The increase in \( S_A \) is probably an overestimate because of the long length of the lateral membranes in epithelial cells and the significant surface area associated with the apical brush border, which may accommodate some of the volume change. Indeed, scanning electron micrographs of the extensively microvillus-covered surface of lymphocytes show that as lymphocytes swell the number of microvilli decreases and the diameter of the cell increases (27). The microvilli provide a reservoir of membrane that allows cells to double in size (27). Before enterocyte swelling, \( C_f/C_b = 10,800 \mu m^2/29,200 \mu m^2 = 0.37 \), which corresponds to \( f_C \) of 0.27. After enterocyte swelling, \( C_f/C_b = 12,720 \mu m^2/29,300 \mu m^2 = 0.43 \), or \( f_C \) of 0.30. This represents a 16% increase in \( C_f/C_b \). Our results show an average increase in \( C_f \) from 0.27 (\( C_f/C_b \) = 0.37) to 0.33 (\( C_f/C_b \) = 0.49), a 32% increase in \( C_f/C_b \). Therefore, alanine-induced increases in enterocyte swelling and the accompanying increase in surface area do not account completely for the increase in \( C_f/C_b \).

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