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Sodium-coupled glycine uptake by Ehrlich ascites tumor cells results in an increase in cell volume and plasma membrane channel activities

(Cl⁻ channels/cell volume regulation)

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ABSTRACT **The addition of 10 mM glycine to a physiological saline bathing Ehrlich ascites tumor cells is followed by a slow increase in cell volume that plateaus between 15 and 30 min at a level ≈17% greater than the control volume; this increase is not observed when glycine is added to cells suspended in a Na^+-free saline. The results of studies using the patch-clamp technique in the cell-attached mode indicate that, 0.5–3 min after the addition of glycine to the bathing solution, there is a marked increase in the activity of single channels, which in almost all instances were previously present and operant in the plasma membrane. Successfully excised patches of membrane that contained a channel stimulated by glycine fell into two categories. Some became inactive within 15 sec in spite of the fact that the GΩ seal remained intact. Others persisted for the lifetime of the seal. All of the persistent channels had an 11-fold selectivity for Cl^- over K^+ and a conductance of 23 pS when bathed by symmetrical 150 mM KCl solutions. Although the ionic specificities of the other channels have not been identified, there is reason to suspect that they might be K^+ channels whose activities are dependent on factors lost when the patch is excised. Swelling induced by exposing these cells to a 50% hypotonic perfusate stimulated the activities of Cl^- channels whose properties closely resemble those stimulated by the addition of glycine to the perfusate, strongly suggesting that the glycine-induced stimulation of Cl^- channel activity is part of a volume-regulatory response to cell swelling. If the increase in channel activity induced by the addition of glycine to the perfusate is indeed a response to cell swelling, then this volume-regulatory response must be extremely sensitive inasmuch as it appears to be "triggered" by an average increase in cell volume that does not exceed 5%.**

The results of previous studies reported by this laboratory indicate that the addition of sugars or amino acids to the solution bathing the mucosal surface of *Necturus* small intestine results in an initial, rapid depolarization of the electrical potential difference across the apical membrane (ψ^{mc}) and a decrease in the ratio of the resistance of the apical membrane (r^m) to that of the basolateral membrane (r^s)—i.e., (r^m/r^s). These responses are followed by a slower, partial repolarization of ψ^{mc} that is paralleled by an increase in (r^m/r^s) to levels that exceed those observed in the absence of the sugar or amino acid (1). The initial responses can be attributed to the activation of rheogenic and conductive carrier-mediated pathways for the coupled entry of Na^+ and sugars or amino acids into the cell across the apical membrane (1, 2). The secondary responses appear to be, at least in part, due to an increase in the conductance of the basolateral membranes to K^+, (g^s_K), which is blocked by the presence of Ba^{2+} in the serosal solution and by exposure of the tissue to metabolic inhibitors (1, 3, 4).

In addition, circumstantial evidence has been reported suggesting that the increase in g^s_K may be part of a "volume-regulatory response" to cell swelling resulting from the intracellular accumulation of sugars and amino acids in osmotically active forms (4, 5). Thus, exposure of *Necturus* small intestine to a 12% hypotonic solution results in a hyperpolarization of ψ^{mc} and an increase in (r^m/r^s) that can be blocked by Ba^{2+} and metabolic inhibitors (4). Further, the delayed repolarization of ψ^{mc} and increase in (r^m/r^s) observed after the addition of galactose to the mucosal solution under isotonic conditions can be prevented by rendering the galactose-containing perfusate 20% hypertonic, presumably preventing or reducing cell swelling (6).

These findings suggested the unitary hypothesis that the same mechanisms may be responsible for the homocellular regulation of intracellular ion (primarily Na^+ and K^+) activities in the face of increased rates of Na^+-coupled solute entry across the apical membrane and, in turn, increased Na^+–K^+ pump activity in the basolateral membrane and the regulation of cell volume in the face of cell swelling (4, 7, 8).

To explore this hypothesis and possible underlying mechanisms further, we initiated a series of studies on the effects of Na^+-coupled amino acid uptake on the membrane transport properties of Ehrlich ascites tumor cells using the "patch-clamp" technique. The rationale behind choosing this well-established cell line is 4-fold.

First, these cells have served as a model system for the study of Na^+-coupled amino acid uptake for many years. It is well established that they accumulate amino acids such as glycine by the Na^+-dependent "A system" to levels much greater than that in the suspension medium (9, 10) and that this accumulation is accompanied by cell swelling (10, 11). Thus, these symmetrical cells are confronted by the same problems that confront small intestinal cells when Na^+-coupled amino acid uptake across the apical membrane is activated by the addition of amino acids to the mucosal bathing solution. Further, Dawson and Smith (12) have reported that the addition of the amino acid analogue 2-aminoisobutyric acid to the incubation medium brings about a depolarization of the electrical potential difference across the plasma membrane of these cells, which is followed by a slower spontaneous repolarization; this biphasic response closely resembles that observed in *Necturus* small intestine (1).†

Second, the volume-regulatory responses of these cells to challenge by exposure to anisotonic media have been extensively studied by Hoffmann and her collaborators (13). These

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†Hacking and Eddy (11) also have provided evidence for a biphasic response in the transmembrane electrical potential difference, estimated by using a fluorescent carbocyanine dye after the addition of amino acids to the incubation medium.

investigators have provided substantial evidence that the "regulatory volume decrease" after exposure to a hypotonic solution is due, at least in part, to increases in the permeabilities of the plasma membrane to K^+ and Cl^-, which permit the loss of KCl accompanied by water.

Third, average volume changes resulting from amino acid accumulation can be monitored in "living cells" so that one need not determine wet weights, dry weights, extracellular spaces, etc., to ascertain increases in cell water content.

Finally, these cells proved to be amenable to the patch-clamp technique without the need to resort to enzymatic treatments, which appear to be necessary to obtain GΩ sealing to the basolateral membranes of epithelial cells (see refs. 14 and 15).

MATERIALS AND METHODS

Ehrlich ascites tumor cells (Lettre strain; hyperdiploid) were maintained by weekly intraperitoneal transplantation in Ha/ICR male mice (Harlan, Houston). After 8–10 days, the cells were harvested by aspiration and washed free of ascitic fluid by suspending 1 ml of the aspirated fluid in 50 ml of physiological saline (150 mM Na^+ and Cl^-/5 mM K^+/1 mM Ca^{2+}/4 mM HPO_4^{2-}/1 mM $H_2PO_4^-$/1 mM Mg^{2+} and SO_4^{2-}/10 mM 4-morpholinepropanesulfonic acid/10 mM mannitol) and centrifuging at 100 × *g* for 2 min. The pellet was resuspended in 50 ml of saline, and this washing procedure was repeated two more times. Cells were kept at room temperature (23–25°C) throughout all procedures.

For physiological saline solutions containing glycine, 10 mM glycine was substituted for mannitol. Hypotonic solutions were prepared by diluting the physiological saline with an equal volume of distilled water. The pH of all solutions when equilibrated with air was 7.4.

Patch-clamp studies followed the general procedures outlined by Hamill *et al.* (16) and Sakmann and Neher (17). Patch pipettes were fabricated from 1.0-mm OD square-bore borosilicate glass (Glass Company of America, Bargaintown, NJ) by utilizing a two-stage puller (Narishige, Japan). The pipette was coated with Silgard 184 (Dow Corning, Midland, MI) and cured with a heat gun. The pipette tip was heat-polished with a homemade apparatus. By using the "bubble number technique" described by Sakmann and Neher (17) to give a relative index of tip diameter, the most successful seals were obtained with pipettes that were heat-polished from a starting value of 5 to a final value of 2–2.5; this corresponds to a tip diameter of ≈1 μm and a resistance of 5–10 MΩ. Each pipette was constructed and filled with a filtered (0.2-μm Millipore syringe filter) solution immediately before use. The filling solution for cell-attached and isolated inside-out patches contained 150 mM KCl and 106 nM $CaCl_2$.

Experiments were conducted at room temperature in a perfusion chamber (2 mm × 5 mm) mounted on the stage of a Nikon Diaphot microscope, which allowed a complete solution change in 10 sec. The cells were visualized with Hoffmann modulation optics and cell-attached and inside-out patches were obtained as described by Hamill *et al.* (16). Single-channel currents were amplified by a List EPC-7 voltage-clamp and recorded on either a Gould strip chart recorder or a video data recording system (PCM-1; Medical Systems, Greenvale, NY). Records were filtered by using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and observed with a digital oscilloscope (Nicolet, model 3091). An "outward current" is defined as a cationic flow out of the cell or an anionic flow into the cell as per the usual convention.

Changes in cell volume were monitored with a Coulter Counter with a channel analyzer (model C-1000).

Results are reported as the mean ± SEM.

RESULTS

Effect of Na-Coupled Glycine Uptake on Cell Volume. A series of experiments was performed to monitor changes in average cell volume that result from Na-coupled glycine uptake by these cells. Exposure to 10 mM glycine resulted in a slow increase in cell volume that plateaued within 15–30 min at an average value 17 ± 0.01% (n = 3) greater than the control value (Fig. 1). The steady-state increase in average cell volume observed in the presence of 10 mM glycine is in remarkably good agreement with that determined by Hempling and Hare (10) using different techniques. The addition of glycine to a suspension medium rendered Na-free by substitution with choline did not result in a detectable increase in cell volume.

Effects of Glycine on Channel Activity in the "Cell-Attached" Configuration. Spontaneous channel activity was observed in ≈10% (n = 78) of the cells in which patch-clamp seals in the range of 2–10 GΩ were obtained. Fig. 2 *Left* illustrates a typical response of a single channel before and after the addition of 10 mM glycine to the bathing solution when a cell was clamped in the cell-attached mode with the pipette held at −25 mV. In the absence of glycine, the channel was rarely active, and then only briefly. The addition of glycine to the perfusate resulted, after a variable delay of 0.5–3 min, in a dramatic increase in the channel's activity with the channel primarily in the open state; this increase in channel activity could persist for as long as 10 min.

It should be noted that: (*i*) in only one instance did the addition of glycine to the solution bathing attached cells with quiescent patches elicit *de novo* channel activity (Fig. 2 *Right*); (*ii*) in many instances (n = 47), patches displayed channel activity that was not dramatically stimulated by glycine as judged by visible inspection of the recording (a possible explanation for this finding will be discussed below); (*iii*) in every instance where channel activity was markedly stimulated by glycine (n = 31), the patch appeared to contain only a single channel; and (*iv*) in all instances the direction of current flow was consistent with a cationic current leaving the cell and/or an anionic current entering the cell.

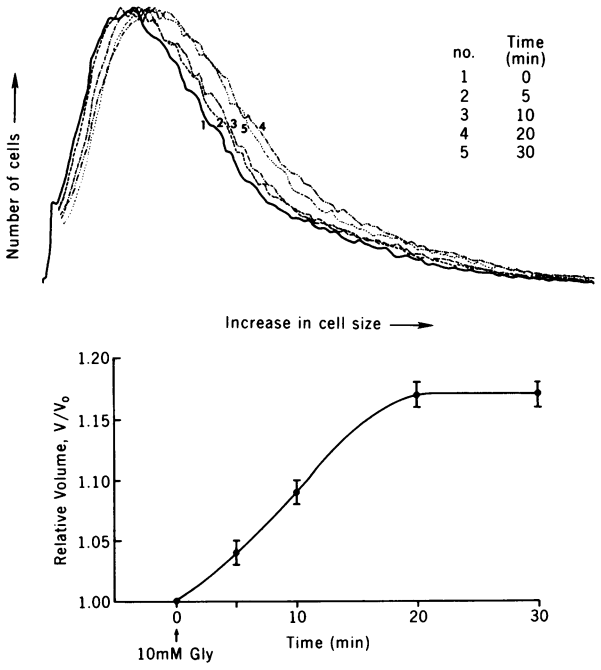

FIG. 1. Changes in volume after the addition of 10 mM glycine to the solution bathing Ehrlich ascites cells. (*Upper*) Volume distribution of a population of cells measured at 5, 10, 15, and 30 min after exposure to glycine. (*Lower*) Relative change in average volume calculated as the ratio of the average volumes at various times after the addition of glycine to the cell suspension to those observed in the glycine-free saline. Average volumes were determined from the weighted mean of the population distributions illustrated in *Upper*.

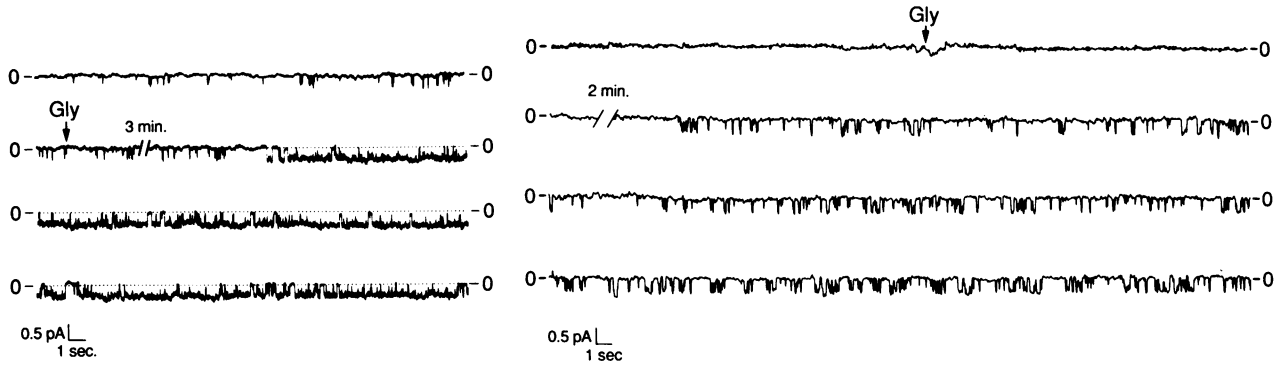

FIG. 2. Cell-attached recordings of single channel currents before and after the addition of 10 mM glycine to the bathing solution. (*Left*) Responses of a channel active prior to the addition of the amino acid ($n = 31$). (*Right*) Tracing of the single instance when a channel was activated in a previously quiescent patch. The pipette contained 150 mM KCl and 106 nM Ca^{2+} (Ca EGTA buffer, pH 7.2); the "holding potential" was −25 mV.

Finally, channel activity could be neither initiated in quiescent patches nor stimulated in patches containing an active channel by varying the pipette potential over a range of ±50 mV.

Results on "Excised Patches" Containing a Channel Stimulated by Glycine. Patches containing a channel whose activity was stimulated by the addition of glycine to the bathing solution were excised, generally within 30 sec after a clear increase in channel activity was observed, by the procedure of Hamill *et al.* (16) for the preparation of "inside-out" patches.

Successfully excised patches ($n = 14$) fell into two categories. Some ($n = 9$) spontaneously inactivated within 15 sec before their ionic selectivity could be ascertained in spite of the fact that the GΩ-seal remained intact. Others ($n = 5$) persisted for the lifetime of the patch, often as long as 10–15 min.

The current–voltage relation of one such persistent channel when both solutions (i.e., micropipette and external bath) contained 150 mM KCl is illustrated in Fig. 3; the conductance of this channel under this condition was 26 pS when the electrical potential difference across the patch, ψ^{oi}, was 0 mV. The rectification shown when the Cl^- current was directed inwardly (i.e., from the bath to the pipette) was observed in all instances and resembles the phenomenon observed by Frizzell *et al.* (18) for some Cl^- channels present in the apical membrane of tracheal epithelial cells. When the outer bathing solution was switched to one containing 30 mM KCl, the conductance declined to ≈50% of the value observed in the presence of symmetrical 150 mM KCl solutions, and the current–voltage relation displayed an extrapolated reversal potential of approximately −30 mV (Fig. 4), indicating that the channel is preferentially selective for Cl^- over K^+. All of the persistent channels excised to date displayed similar properties; the average conductance of these Cl^- channels when bathed by 150 mM KCl at zero potential was 23 ± 2 pS (range, 18–27 pS) ($n = 5$) and the permeability to Cl^- averaged 11.1 ± 0.4 ($n = 5$) times greater than that to K^+. (We were never successful in reversing the direction of current flow across excised patches when the outer bathing solution contained 30 mM KCl; all attempts to do so resulted in a loss of the GΩ-seal. The extrapolated reversal potential probably provides a minimal estimate of the selectivity of the channel to Cl^- over K^+.)

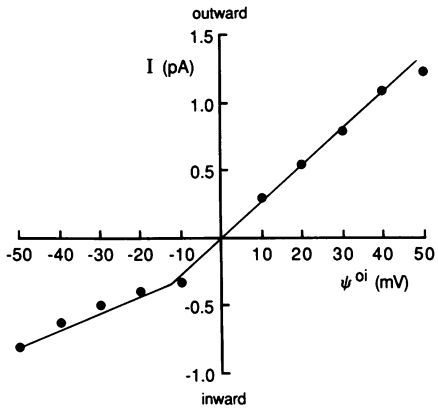

FIG. 3. Current–voltage relation of an excised patch containing a channel that was activated after the addition of glycine to the bathing solution. The patch of membrane was bathed symmetrically with 150 mM KCl and 106 nM Ca^{2+} (Ca EGTA buffer, pH 7.2). ψ^{oi} is the electrical potential difference across the patch with reference to the outer solution. An outward current is defined as a cationic current leaving the cell (entering the pipette).

Effects of Hypotonic Challenge on Membrane Channel Activity. The effects on channel activity after abrupt exposure of cells clamped in the cell-attached configuration to a 50% hypotonic solution are illustrated in Fig. 5. Clearly, there was a dramatic increase in channel activity with the channels going from a predominantly closed state to a predominantly open state within a few seconds; except for the difference in response time, these effects closely resemble those elicited after the addition of glycine to an isotonic perfusate (Fig. 2 *Left*).

The current–voltage relation of an excised "inside-out" patch containing a channel stimulated by exposure to a 50% hypotonic saline solution when the inner ("cytoplasmic") solution contained 30 mM KCl and the pipette contained 150 mM KCl is shown in Fig. 6. The extrapolated reversal potential is consistent with an 11-fold selectivity for Cl^- over

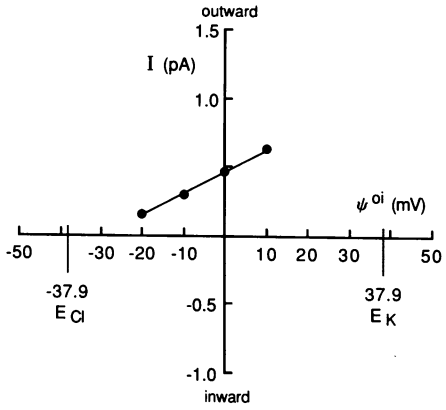

FIG. 4. Current–voltage relation of the glycine-stimulated channel described in Fig. 3 exposed to 30 mM KCl and 106 nM Ca^{2+} (Ca EGTA buffer, pH 7.2) at the inner surface. E_{Cl} and E_K are the predicted reversal potentials for a channel that is either ideally selective for Cl^- or K^+, respectively.

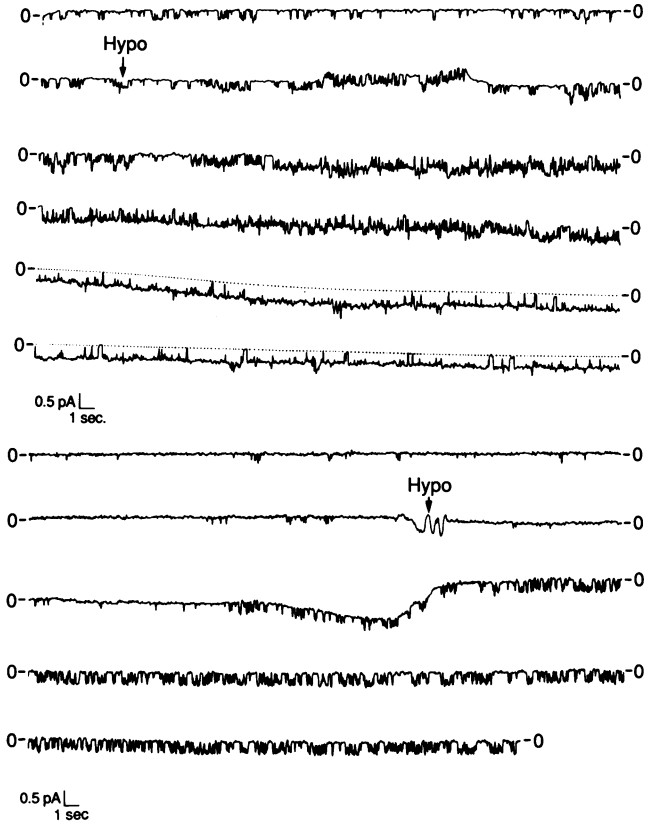

FIG. 5. Stimulation of membrane channel activity after exposure of cells, patch-clamped in the cell-attached configuration, to a 50% hypotonic solution.

K^+; the conductance of this channel under these conditions is ≈17 pS. A second channel successfully excised under these conditions also exhibited an 11-fold selectivity for Cl^- over K^+ and a conductance of 18 pS.

DISCUSSION

The results of the present study indicate that exposure of Ehrlich ascites tumor cells to glycine (10 mM) is followed by a gradual increase in cell volume and a dramatic increase in the activities of channels which, with one exception (Fig. 2 *Right*), were previously present and operant in the plasma membrane. The increase in cell volume can be attributed to the Na^+-coupled uptake of the amino acid, which is accumulated within the cells in an osmotically active form (10, 11).

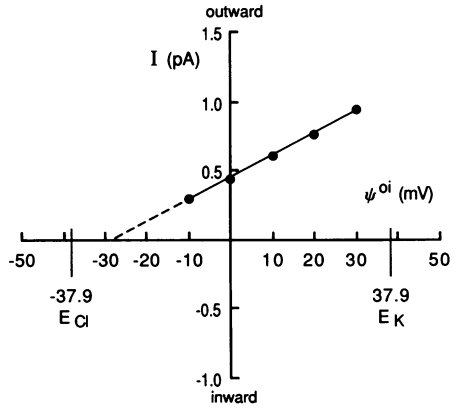

FIG. 6. Current–voltage relation of an excised inside-out patch containing a channel stimulated by hypotonic challenge. The inner surface was bathed with a solution containing 30 mM KCl, while the pipette contained 150 mM KCl.

These observations do not definitively establish a causal relation between the increase in cell volume resulting from the intracellular accumulation of glycine and the increase in channel activity. However, the findings that cell swelling induced by hypotonic challenge stimulates the activities of Cl^- channels whose properties closely resemble those stimulated by glycine strongly suggest that these phenomena are not merely coincidental but that the glycine-induced increase in channel activity is part of a volume-regulatory response triggered by cell swelling.

It is of interest to consider these findings in the light of previously published studies on the responses of cells to swelling resulting either from exposure to hypotonic media or an increase in intracellular osmolyte concentration.

Hoffmann and her collaborators (13) have shown that swelling induced by exposure of Ehrlich cells to hypotonic media brings about an increase in the permeabilities of the plasma membrane to K^+ and Cl^-, and similar findings have been reported by Grinstein *et al.* (19) for hypotonic challenge of lymphocytes. Ussing (20) has reported that cell swelling increases the permeability of the inner-facing membranes of isolated frog skin to Cl^- (and perhaps K^+), and evidence has been presented that swelling increases the conductance of the basolateral membranes of *Necturus* small intestine (4) and toad (21) and frog (22) urinary bladders to K^+.

Hempling and Hare (10) found that the bidirectional fluxes of $^{42}K^+$ across the plasma membranes of Ehrlich ascites tumor cells are significantly increased during the accumulation of glycine and after a steady-state level of intracellular glycine was achieved. This finding together with the results of the electrophysiological studies of Dawson and Smith (12), cited above, are consistent with the notion that Na^+-coupled amino acid accumulation by these cells brings about an increase in the conductance of the plasma membrane to K^+. Finally, Hacking and Eddy (11) have shown that glycine accumulation by these cells is accompanied by cell swelling and a decrease in intracellular K^+ content, which are abolished when the incubation medium is rendered hypertonic.

Na^+-coupled amino acid uptake by isolated rabbit jejunal enterocytes (23) and isolated rat hepatocytes (24) is also accompanied by an increase in membrane K^+ permeability, and evidence has been presented for the case of hepatocytes that this increase is a response to cell swelling (25, 26).

It should be emphasized that, in the present studies, the only "persistent" channels stimulated by glycine that we have successfully excised from cell-attached patches to date are Cl^- channels with a unitary conductance of ≈23 pS in the presence of 150 mM KCl. At the same time, in a majority of instances, channels activated by the addition of glycine to the perfusate in the cell-attached mode spontaneously and rapidly inactivated after the patch was excised, in spite of the fact that the GΩ seal remained intact. It is tempting to speculate on the basis of the findings cited above that these may be K^+ channels whose activity is dependent upon intracellular factors and/or factors loosely attached to the membrane that are lost shortly after excision; but further studies are needed to examine this possibility.‡ In this respect it is of interest that Grinstein *et al.* (27) reported that the increase in K^+ permeability of rabbit thymocyte membranes in response to exposure to a hypotonic bathing solution could

‡The results of preliminary studies employing the "whole-cell" recording technique indicate that when the cells are bathed with the physiological saline solution (containing 150 mM NaCl) and the pipette contained 150 mM KCl the addition of 10 mM glycine to the perfusate results in a rapid depolarization of ψ^{oi} followed, within minutes, by a spontaneous repolarization. The most probable explanation for this finding is an increase in the K^+ conductance of the plasma membrane but more extensive studies are needed to explore this possibility.

not be reproduced in isolated membrane vesicles and concluded that ". . . components essential for the volume- and Ca^{2+}-induced changes in K^+ permeability are lost or inactivated during membrane isolation." [Yatani *et al.* (28) have reported that the spontaneous activity of atrial muscarinic K^+ channels in the cell-attached mode disappears after patch excision but can be reactivated by the addition of a guanine nucleotide protein (G_K) to the "inner" solution.] Further, the persistent activity of the Cl^- channels in the excised patch suggests that the mechanism responsible for inactivating this channel in the intact cell is lost. The identification of these components would provide important insight into the regulation of these conductive pathways.

Finally, as indicated above, increases in plasma membrane channel activities were observed within 3 min after the addition of glycine to the bathing medium. During this period the *average* increase in cell volume does not exceed 5% over the control volume (Fig. 1). However, caution must be exercised when comparing average responses of a large population of cells with results obtained with the patch-clamp technique, which reflect the responses of individual cells. Tupper *et al.* (29) have demonstrated, using synchronized cultures of Ehrlich cells, that amino acid transport by the A system is minimal during the M and early S phases of the growth cycle and is maximal during the late S and early G_2 phases; their analysis (see figure 4 of ref. 29) suggests that in an asynchronously growing population, approximately half of the population would exhibit moderate to maximal rates of glycine transport, and the other half would exhibit moderate to minimal rates. Thus, it is quite possible (*i*) that our experimental approach selects cells with rapid rates of amino acid accumulation (and swelling) and discriminates against cells with slower rates, and (*ii*) that spontaneously active channels that were not stimulated after the addition of glycine to the perfusate were possessed by cells in stages of their growth cycle when the rate of glycine transport is minimal.

Nonetheless, if these glycine-stimulated increases in channel activities are, in fact, regulatory responses to cell swelling, our findings suggest that the underlying mechanism(s) must be very sensitive to changes in cell volume. In this respect Lau *et al.* (4) found that an increase in basolateral membrane K^+ conductance could be detected after exposure of *Necturus* small intestine to a perfusate that was only 6% hypotonic compared to the control, and Lohr and Grantham (30) have reported that the volume of renal proximal tubule cells remained constant (within 3%) when gradually exposed to an increasingly hypotonic perfusate at the rate of 1.5 milliosmolar per minute down to an osmolarity almost half that of the isotonic control. Sackin (33) has recently identified stretch-activated K^+ channels in the basolateral membranes of *Necturus* proximal tubule cells and has argued, on the basis of a simple model, that the membrane tension necessary to stimulate the activity of these channels could result from a 1% increase in cell volume. Finally, the finding that gradual isotonic cell swelling resulting from intracellular glycine accumulation stimulates Cl^- channels whose properties are virtually identical to those stimulated by rapid swelling after exposure to a hypotonic solution strongly suggests that the "immediate signal" for these responses are mechanical forces. Such forces could be tension developed in the membrane itself or in cytoskeletal elements anchored to the membrane (see refs. 31 and 32).§ In any event, the possibility that membrane transport properties may be dramatically altered by small changes in cell volume must be seriously considered when interpreting the results of experiments where the experimental manipulation itself could result in cell volume changes.

§As discussed by Sachs (32), it is highly unlikely that channel activity can be stimulated simply by enlargement of the channel diameter due to tension developed in the surrounding membrane. In the present studies, where cell swelling activated channels residing within membrane patches clamped in the "cell attached" configuration, the possibility of a direct effect of membrane tension on those channels can be virtually dismissed. Thus, assuming that the cell interior is isobaric, Laplace's law dictates that the tension developed in the quasi-hemispherical patch of membrane contained within the pipet would be minute compared to the tension of the remaining cell membrane. In short, the cell is likely to burst before any significant tension could be developed in the "clamped patch." Thus, activation of these channels must be the result of mechanical or mechanochemical events resulting from stretching of membrane external to the "patch" or cytoskeletal elements.

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