Volume regulatory responses of basolateral membrane vesicles from Necturus enterocytes: Role of the cytoskeleton

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ABSTRACT Previous studies from this laboratory have demonstrated that basolateral membrane vesicles isolated from Necturus maculatus small intestinal epithelial cells possess a K⁺ channel that is inhibited by ATP. In the present studies, we demonstrate that these vesicles, which are essentially devoid of soluble cytoplasmic contaminants, exhibit volume regulatory responses that parallel those of intact epithelial cells. Thus, suspension of these vesicles in a solution that is hypotonic to the intravesicular solution increases channel activity whereas suspension in a solution that is hypertonic to the intravesicular solution decreases, and may abolish, channel activity. These volume regulatory responses appear to be mediated by the same Kₐ₅₆ channel and depend on an intact actin cytoskeletal network. The responses to both hypotonic and hypertonic challenge are abolished by cytochalasin D or by incubating the vesicles under conditions that are known to depolymerize actin. Phalloidin, which is known to stabilize actin filaments, partially prevents the action of cytochalasin D. Thus, the present results indicate that the Kₐ₅₆ channel activity of basolateral membrane vesicles from Necturus basolateral membranes respond to hypo- and hypertonic challenge monotonically around an isotonic "set point" and that these responses depend on an intact actin cytoskeleton.

Exposure of virtually all animal cells to solutions hypotonic to that of the intracellular compartment results in an increase in the conductance of their plasma membranes to K⁺ and Cl⁻ (1–3). In the case of Na⁺-absorbing epithelial cells, this response is restricted to their basolateral membranes (4). The resulting loss of KCl, accompanied with water, serves either to limit cell swelling (5) or to actually restore the initially swollen cell toward its original volume; the latter is referred to as "regulatory volume decrease" (RVD) (1). Further, this response is very sensitive. For example, Lau et al. (6) have reported that an increase in basolateral membrane K⁺ conductance of Necturus small intestinal epithelial cells can be detected after exposure of the tissue to a solution that is only 6% hypotonic to the normal amphibian Ringer's. However, the mechanisms responsible for RVD are, in many, if not most, instances, poorly understood (cf. 3).

Recently, K⁺ channels have been identified in a preparation of purified basolateral membrane vesicles from Necturus enterocytes that are inhibited by ATP and that, according to the current convention, are referred to as Kₐ₅₆ channels (7). The present study was designed to explore whether these channels might be responsible for the increase in basolateral membrane K⁺ conductance in response to cell swelling after "hypotonic shock." The results indicate that the activity of this channel is not only increased by exposure to a hypotonic solution but also is decreased by exposure to a hypertonic solution and that these volume regulatory processes depend on an intact actin cytoskeleton.

METHODS

The method for isolating a basolateral membrane fraction from Necturus enterocytes has been described in detail (8). In brief, a membrane fraction enriched in Na⁺-, K⁺-ATPase activity was isolated from mucosal scrapings of Necturus small intestine by differential centrifugation without the use of enzymes. This method results in a >20-fold enrichment of Na⁺-, K⁺-ATPase activity over that in the crude homogenate with minimal contamination by enzyme markers for membranes other than the basolateral membranes. The membranes were frozen, were stored in liquid N₂, and were thawed immediately before use.

Kₐ₅₆ channel activity of the vesicles was assayed by using ⁸⁶Rb⁺ as a tracer for K⁺, according to the method of Garty et al. (9), as described (10). Vesicles were loaded by addition of 200 µl of membranes (1.5–4 mg protein/ml) to 50 µl of 0.5 M K₂SO₄ and 10 mM K-Hepes (pH 7.0) and other reagents as indicated. The osmolarity of the loading solution was adjusted with sucrose as indicated in the text. The mixture was frozen in liquid N₂ and was thawed; during the freeze–thaw cycle, the intravesicular compartment equilibrated with the loading solution and the cytoplasmic contents retained during the isolation procedure were washed out. Columns were prepared from Dowex 50W-X-8 (Tris form), were poured into glass Pasteur pipettes, and were pretreated with three drops of 30% BSA. The columns were washed with 4 ml of a solution of sucrose and 10 mM Tris-Hepes (pH 7.6) adjusted to the osmolarity of the loading solution. The vesicle suspension (200 µl) was pipetted onto the Dowex column to remove extravesicular K⁺ and was eluted with 2 ml of sucrose and 10 mM Tris-Hepes (pH 7.0) buffer under mild vacuum; the sucrose wash was adjusted to the test osmolarity. Thus, the vesicles were eluted into a buffer that is isotonic, hypertonic, or hypotonic relative to the intravesicular solution. After the vesicles were collected, a 10-µl aliquot of ⁸⁶Rb⁺ (1–4 µCi) was added to initiate uptake. At timed intervals starting immediately (~5 sec) after the addition of tracer (nominally "zero time"), 200-µl aliquots were withdrawn and placed on a second Dowex column to remove all extravesicular tracer. The vesicles were eluted from the column with 2 ml of the sucrose buffer directly into scintillation vials and were assayed for ⁸⁶Rb⁺ content. Intravesicular ⁸⁶Rb⁺ was expressed as the percent of total radioactivity in a 200-µl aliquot of reaction mixture normalized to the protein content of the vesicle suspension. As discussed (10), because the intravesicular compartment is markedly electrically negative with respect to the external compartment, only channels oriented so that the intravesicular compartment corresponds to the intracellular compartment would be active.

Abbreviation: RVD, regulatory volume decrease.
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Depolymerization of the actin cytoskeleton was accomplished, according to the method of Spudich and Watt (11), by incubating the vesicles overnight at 5°C in a markedly hypotonic buffer comprised of 2 mM Tris-HCl (pH 8), 0.5 mM DTT, 0.2 mM ATP, and 0.2 mM CaCl₂. The control for these studies were vesicles that were exposed to this depolymerizing buffer for 5 min before conducting the studies. It should be noted that ATP must be in the Mg²⁺ form to inhibit this channel (7) and that the buffer is Mg²⁺-free.

One-dimensional SDS-polyacrylamide gels were run in a Bio-Rad Mini-Protein II apparatus using the buffer system of Laemmli on a 7.5–20% gradient gel (12). Protein bands were visualized by staining with Coomassie brilliant blue (Bio-Rad). Wet electrophoretic transfers were done after protein separation on SDS/PAGE onto a 0.45-μm nitrocellulose membrane in a transfer buffer consisting of 25 mM Tris, 190 mM glycine, and 20% methanol according to the procedure of Towbin et al. (13). The transfer was performed for 1 h at 100 V in a Bio-Rad Mini Transtblot assembly according to the manufacturer’s instructions. After the transfer, the nitrocellulose membranes were blocked in 5% nonfat dry milk in 0.2% Tween 20 in PBS (1.8 mM KH₂PO₄/10 mM Na₂HPO₄/137 mM NaCl, pH 7.4) overnight. The nitrocellulose was washed three times in the same buffer to remove the blockers and then was exposed to anti-actin IgG (A 5060, Sigma) diluted 1:5,000 in PBS plus Tween 20, the bound antibody then was labeled with donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia) diluted 1:25,000 in PBS for 1 h. The nitrocellulose was washed a final three times in PBS. The antigen–antibody reaction was detected by placing the membrane in the ECL reagent (1:1 mixture) for 1 min according to the manufacturer’s instructions (Amersham Pharmacia). The ECL reagent was drained, and the membrane was placed into a plastic holder in an x-ray cassette and was exposed to Kodak X-Omat AR film.

Osmolarities were determined with a Wescor (Logan, UT) Model 5500 vapor pressure osmometer. Protein was determined by the method of Bradford (14). All experiments were performed in duplicate. Values are expressed as the arithmetic mean plus and minus the standard error of the mean for n = 3 experiments unless otherwise stated.

MATERIALS

_Necturus maculosus_ were obtained from Nasco (Fort Atkinson, WI) and Kons Scientific (Germantown, WI). Radioisotopes were obtained from NEN, and Universal scintillation fluid was from ICN. All other chemicals and reagents were obtained from Sigma.

RESULTS

The results of a series of experiments in which the vesicles were preloaded with a solution having an osmolarity of 410 mOsm and ⁸⁶Rb⁺ uptake was determined during exposure to solutions having osmolarities of 350 mOsm (16% hypotonic), 410 mOsm (isotonic), or 480 mOsm (18% hypertonic) are shown in Fig. 1. Clearly, exposure to the hypotonic solution stimulated ⁸⁶Rb⁺ uptake by the vesicles whereas exposure to the hypertonic solution essentially abolished uptake.

To exclude the possibility that the results shown in Fig. 1 were attributable to the absolute osmolarities of the solutions, a series of experiments was performed in which the vesicles were preloaded with a solution having an osmolarity of 460 mOsm. As shown in Fig. 2, exposure of these vesicles to a solution having an osmolarity of 400 mOsm (18% hypotonic) resulted in an increase in ⁸⁶Rb⁺ uptake compared with control whereas exposure to a solution having an osmolarity of 520 mOsm (13% hypertonic) once more abolished uptake. Thus, these results are attributable to the osmotic gradient, not to the absolute osmolarities.

The relation between ⁸⁶Rb⁺ uptake and extravesicular osmolarity in vesicles preloaded with a solution having an osmolarity of 410 mOsm is shown in Fig. 3. There is a graded increase in uptake as the osmolarity of the extravesicular solution is decreased from 430 mOsm to 375 mOsm; the decline in uptake in the presence of 325 mOsm is likely caused by bursting of vesicles. Clearly, the volume regulatory responses of these vesicles are very sensitive to changes in the osmotic gradient.

Previous studies using these vesicles (10) have shown that the K⁺ (Rb⁺) permeability is mediated by an ATP-inhibitable K<sub>ATP</sub> channel that has been characterized after incorporation...
into planar phospholipid bilayers (7). Inasmuch as this channel also is inhibited by glibenclamide (7, 15), studies were carried out to determine whether these channels are responsible for the volume regulatory response to hypotonic shock or whether the response is attributable to the activation of a different type of channel that was previously quiescent. As shown in Fig. 4, 50 mM glibenclamide markedly inhibited $^{86}$Rb$^{+}$ uptake by vesicles exposed to a solution isotonic to the intravesicular compartment as well as vesicles exposed to a hypotonic solution. These findings strongly suggest that it is the activity of the native K$_{ATP}$ channel that is enhanced by hypotonic shock.

There is a convincing body of evidence implicating the F-actin cytoskeleton in RVD, some of it based on the action of cytochalasin(s) (see below). This fungal toxin, which is often, mistakenly, referred to as a “depolymerizing agent,” actually reversibly binds to the barbed (“growing”) end of the F-actin filament and prevents further elongation. Depending on the dynamics of the G-actin-to-F-actin transition, it may result in depolymerization and shortening of the filament at the nonbarbed end of the filament. Cytochalasin also may prevent attachment of the barbed end of the filament to membrane-binding proteins and appears to disrupt F-actin networking: i.e., cross-linking of individual filaments (16–18). As shown in Fig. 5, exposure of vesicles to 10 mM cytochalasin D completely eliminated the response to both hypertonic and hypotonic shock but had no effect on vesicles exposed to an isotonic solution.

As a further test of the actin-dependence of the volume regulatory responses, vesicles were incubated under F-actin depolymerizing conditions for 20 h (see Methods). As shown in Fig. 6, the volume regulatory responses to anisotonic challenge were abolished by the treatment (Fig. 6A). Incubation of vesicles in the depolymerizing buffer for only 5 min had no effect on the responses to anisotonic challenge (data not shown). In parallel experiments, vesicles were analyzed for their actin content by immunoblots. Incubation of the vesicles for 20 h under depolymerizing conditions completely stripped the membranes of bound actin (Fig. 6B). A control 5-min incubation showed actin retention by the vesicles. For comparison, fresh membrane vesicles were treated under other conditions known to disrupt the cytoskeleton. Both low salt washing and incubation in 1 mM EDTA (pH 9) (19) caused a significant depletion of actin. In contrast, extraction of the vesicles with TX-100 to remove membrane and soluble components (20) resulted in retention of actin in the stripped cytoskeleton. These results demonstrate that the vesicles retain an intact cytoskeleton and that disruption of the cytoskeleton, as indicated by actin depletion, is associated with the loss of the volume regulatory responses to anisotonic solutions. Finally, as shown in Fig. 7, preloading the vesicles with 10 mM phalloidin, a fungal toxin that binds to, and stabilizes, F-actin (16), partially prevents (compare Fig. 7 with Figs. 1 and 5) the ability of 10 mM cytochalasin D to abolish volume regulatory responses.

**Fig. 3.** $^{86}$Rb$^{+}$ uptake in 2 min under anisotonic conditions. Vesicles were equilibrated with 410 mOsm solution and were assayed for $^{86}$Rb$^{+}$ in solutions as indicated in the figure.

**Fig. 4.** Inhibition of $^{86}$Rb$^{+}$ uptake by glibenclamide. Glibenclamide (50 μM) was added to basolateral membrane vesicles that were equilibrated with a 410 mOsm solution and were incubated for 1 min at room temperature. The vesicles then were assayed for $^{86}$Rb$^{+}$ uptake as described in Fig. 1.

**Fig. 5.** Blocking the volume regulatory response by Cytochalasin D. Vesicles were equilibrated with a 410 mOsm solution and were incubated with 10 μM Cytochalasin D for 1 min at room temperature. The treated vesicles then were assayed for $^{86}$Rb$^{+}$ uptake as described in Fig. 1.
DISCUSSION

The results of the present investigation clearly indicate that exposure of vesicles derived from the basolateral membrane of *Necturus* enterocytes to solutions that are hypotonic to the intravesicular solution increases the activity of glibenclamide-sensitive K<sub>ATP</sub> channels whereas exposure to solutions that are hypertonic to the intravesicular solution decreases and can abolish channel activity. Although vesicle size was not determined in these studies, it would be difficult to explain these findings other than by invoking swelling and shrinking in response to the imposed osmotic gradients. Further, disruption of the actin cytoskeletal network either with cytochalasin D or, nonpharmacologically, by preincubating these vesicles under F-actin-depolymerizing conditions abolishes these responses.

As mentioned above, it is well established that exposure of epithelial cells to a hypotonic solution (hypotonic shock) results in an increase in the K<sup>+</sup> and the Cl<sup>-</sup> conductances of their basolateral membranes (4). This permits the efflux of KCl and water, which either limits the degree of swelling (5) or actually restores volume toward the control value (so-called regulatory volume decrease, or RVD) (1, 4). Further, several groups have demonstrated that exposure of epithelia to a hypertonic solution reduces the K<sup>+</sup> and Cl<sup>-</sup> conductances of their basolateral membranes (21–24); indeed, Macri et al. (24) have reported that hypertonic shock sufficient to shrink rabbit proximal tubule cells by only 20% decreases the K<sup>+</sup> conductance of their basolateral membranes to "nonmeasurable values." Further, Lau et al. (25) have demonstrated that the increase in the K<sup>+</sup> conductance of the basolateral membrane of *Necturus* small intestinal epithelial cells after the addition of galactose to the luminal perfusate can be blocked by rendering that solution 20% hypertonic. The responses of the K<sup>+</sup> channel in *Necturus* basolateral membrane vesicles to hypo- and hypertonic shock parallel these whole-cell responses; the complete closure of this channel after hypertonic shock mimics the findings of Macri et al. (24). Although the activation of new, previously quiescent K<sup>+</sup> channels could explain the response to hypotonic shock, the decrease in basolateral membrane K<sup>+</sup> conductance in response to hypertonic shock, however, clearly must be attributable to a decrease in the activity of the channels responsible for that conductance under control (isotonic) conditions. Our results indicate that, in this preparation, the same channel appears to be responsible for the basal conductance as well as the responses to hypo- and hypertonic challenge. There is also a convincing body of evidence implicating the actin cytoskeletal network in RVD. Several groups have shown that treatment with cytochalasin blunts or abolishes RVD (1, 3, 26, 27), and it is also well established that hypotonic shock results in reversible changes in the organization of the cortical cytoskeleton (27–31). The role of the cytoskeleton in the responses of cells after exposure to a hypertonic solution has been less well studied. Foskett and Spring (32) found that, although cytochalasin blocked RVD in *Necturus* gallbladder epithelial cells, it did not block the regulatory volume increase.

**FIG. 6.** (A) Blocking the volume regulatory response by depolymerization of actin. Vesicles were incubated overnight under conditions that promote the depolymerization of actin (see Methods). The vesicles then were equilibrated with a 410 mOsm solution and were assayed for <sup>86</sup>Rb<sup>+</sup> uptake as described in Fig. 1. Note that the higher specific channel activity, compared with that shown in previous figures, is likely attributable to loss of protein (e.g., cytoskeletal proteins) in the depolymerization procedure. (B) Immunoblot of actin in the basolateral membrane vesicles after depolymerization. Vesicles were treated as described in Methods under various conditions that are known to disrupt the cytoskeleton. The depolymerized vesicles were run in a parallel experiment to the transport assay shown in A.

**FIG. 7.** Protection of volume regulatory response from the action of cytochalasin D. Vesicles were treated with 5 μM phalloidin for 30 min at room temperature. The vesicles then were exposed to cytochalasin D and were assayed for <sup>86</sup>Rb<sup>+</sup> uptake as described in Fig. 5.
in response to hypertonic shock, but their studies did not specifically examine the effects of this agent on the basolateral K\(^+\) conductance. The present findings are consistent with earlier findings regarding RVD and also suggest that an intact actin cytoskeletal arrangement is required for the decrease in basolateral membrane K\(^+\) conductance after hypertonic shock.

In a recent, exhaustive review, Lang et al. (3) cite a plethora of possible cell signals and mechanisms that have been implicated in the responses of numerous cells to anisotonic shock. In as much as the vesicles used in the present studies are essentially devoid of soluble cytoplasmic components, many of the signals cited by Lang et al., such as "molecular crowding" or signals dependent on soluble cytoplasmic molecules (e.g., cAMP), soluble proteins, or changes in bulk pH or Ca\(^{2+}\) activities, can be excluded. Although we cannot exclude the involvement of cytoplasmic factors in the volume regulatory responses of the intact cell, the conclusion that K\(_{\text{ATP}}\) channel activity in our basolateral membrane vesicle preparation is responsive to deformation of the membrane and underlying cytoskeleton, alone, being increased by an increase in membrane tension and decreased by a decrease in tension, seems inescapable. In this regard, Schütt and Sackin (33) found that mechanosensitive cation channels present in vesicles isolated from plasma membranes of Xenopus oocytes exhibited an increase in open probability in response to swelling.

It should be emphasized that the results of the present studies cannot distinguish between changes in the activity of single K\(_{\text{ATP}}\) channels and changes in the number of active channels in response to anisotonic challenge. Indeed, our earlier findings that these channels reconstituted into planar phospholipid bilayers are fully active in the absence of ATP (7) suggests that the only way activity can be changed in the absence of ATP, as in the present studies, is by changing the number of active channels. But, an alternative, and more attractive, explanation is that the changes in \(^{86}\text{Rb}^+\) uptake are attributable to changes in the activity of single channels that are regulated by stretch on the cytoskeletal elements anchored to the membrane and that this is determined by the radius of curvature of the membrane and is maximal when the membrane is forced into a planar configuration. In this respect, it is of interest that Sackin (34, 35) and Filipovic and Sackin (36) have presented evidence for the presence of stretch-activated K\(^+\) channels in the basolateral membranes of Necturus renal proximal tubule. These channels are activated by cell swelling in response to hypotonic shock as well as by stretching an excised patch of membrane. In studies by Cemerek and Sackin (37) on frog proximal tubule, basolateral membrane K\(^+\) channel activity was increased by membrane stretch as well as by swelling associated with activation of Na\(^+\)-coupled sugar or amino acid transport under isotonic conditions and was insensitive to ATP. Kawahara (38) also has identified stretch-activated K\(^+\) channels in the basolateral membranes of frog proximal tubule, but the effect of ATP, apparently, was not explored in those studies. And, Hunter (39) has identified stretch-activated cation channels in the basolateral membrane of frog proximal tubule that do not distinguish between Na\(^+\) and K\(^+\). In the light of these findings, it is not unreasonable to assume, pending further investigation, that the K\(^+\) channel in the Necturus basolateral membrane vesicle is stretch-activated. On the other hand, Mauzer et al. (40, 41) have reported that, in Ambystoma, a close relative of Necturus, the predominant channel in the basolateral membrane of isolated proximal tubule cells is a K\(_{\text{ATP}}\) channel that is insensitive to cell swelling (in the cell-attached patch mode) or stretch (in the excised patch mode); the activity of this channel is, however, regulated by the cytoskeleton. It is of interest that, in the studies by Mauzer et al. (41), cell swelling resulted in hyperpolarization of the membrane potential but did not affect the activity of the channels in the cell-attached patch. These findings led the authors to suggest that hypotonic shock leads "to the opening of a swelling-activated K\(^+\) exit pathway" (p. 167). Another possible explanation for these results is that, as pointed out by Hamill and McBride (42, 43), "patch-clamping" may disrupt membrane-cytoskeletal interactions and, as a consequence, an "...ion channel may show either a decrease or an increase in its native mechanosensitivity or even acquire mechanosensitivity" (ref. 43, p. 621). Thus, failure to discern mechanosensitivity of a patch-clamped channel need not necessarily exclude the possibility that the channel is stretch-activated. Our channel, examined in basolateral membrane vesicles, is not subject to this potential artifact of patch-clamping.

We are unaware of any previous studies examining the responses of epithelial stretch-activated K\(^+\) channels to both hypo- and hypertonic shock, but mechanosensitive channels whose activity is decreased by cell swelling and increased by cell shrinking have been identified in the osmoreceptors in the hypothalamus responsible for the regulation of antidiuretic hormone secretion (44). Likewise, Ji et al. (45) have recently reported that the activity of the amiloride sensitive epithelial Na\(^+\) channel expressed in Xenopus oocytes is decreased by osmotic swelling and is increased by osmotic shrinking of the eggs.

Finally, it is of interest that the K\(_{\text{ATP}}\) channel in atrial myocytes, which, unlike the present channel, falls into the Type 1 category proposed by Ashcroft and Ashcroft (46), appears to be mechanosensitive (47), and its sensitivity to ATP is reduced by cytchalasin (48). Preliminary studies on the present channel reconstituted into planar phospholipid bilayers also indicate that cytchalasin D reactivates channels that were inactivated by MgATP (data not shown). Clarification of the relations among ATP, the actin cytoskeleton, and these K\(_{\text{ATP}}\) channels awaits further studies. In summary, the present results indicate that the K\(^+\) channel activity of basolateral membrane vesicles from Necturus basolateral membranes respond to hypo- and hypertonic challenge monotonically around an isotonic "set point" and that these responses depend on an intact actin cytoskeleton.

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