The human esophagus is lined by a moist stratified squamous epithelium that through eating, drinking, and gastroesophageal reflux is frequently exposed to liquid media whose physicochemical properties are potentially noxious. Among the noxious environments to which these epithelial cells are exposed is that of hyposmolality, i.e., severe hyposmolality generated by the simple act of drinking tap water being capable of inducing cell swelling and lysis. Yet, the esophageal epithelium in most people remains intact and healthy despite exposure to hyposmolal solutions because of protection afforded by its surface cell layers. The surface cell layers are composed of individual pancake-shaped cells whose apical cell membranes and intercellular junctional complexes combine to produce an effective permeability barrier against the influx of luminal content. In particular, the barrier created by these structures limits exposure of the surface cells’ basolateral cell membranes and entire membrane of cells of the deeper layers to the wide swings in osmolality occurring regularly within the esophageal lumen. This capacity of the surface cell layers for protecting deeper cell layers has been previously observed by Goldstein et al. (4) when they reported no effect of luminal hyposmolality on the short-circuit current of Ussing-chambered rabbit esophageal epithelium, whereas short circuit current, a reflection of net transepithelial ion transport, was significantly increased by serosal hyposmolality.

Gastroesophageal reflux disease results from repeated contact of the esophageal epithelium with refluxed gastric acid and pepsin. Based on the prevalence of its characteristic symptom, heartburn, it represents one of the most common modern disorders of adult Americans. Moreover, there is electrical and morphologic evidence that patients with heartburn, even with nonerosive disease, have a breakdown in barrier function of the esophageal epithelium (9–11, 15) and that in a significant percentage of these individuals, breakdown of the epithelial barrier can progress to erosions and ulceration that are grossly visible on upper endoscopy. Indeed, although acid and pepsin may, in fact, have been the initiators of this injury, destruction of the (permeability) barrier, both microscopically and macroscopically, provide luminal content greater access to cells of the lower layers, access that is maximized in the damaged esophagus by the lack of a protective surface mucous layer as exists in stomach and duodenum (1, 2). The consequence of this is that the cells of the lower layers are now exposed and so vulnerable to damage from exposure to luminal envi-

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environments, including hyposmolality from ingestion of tap water.

In this study, we utilized HET-1A cells, a noncancer-derived esophageal epithelial cell line, to examine the effects of hyposmolar stress on human esophageal epithelial cells and to establish the nature of the membrane transport mechanisms responsible for regulatory volume decrease (RVD). Our results indicate that HET-1A cells behave like osmometers under hyposmolar stress and exhibit RVD as a means for protection against hyposmolar-induced cell death. Furthermore, RVD in HET-1A cells appears to occur via two distinct mechanisms: 1) rapidly activated parallel K+ and Cl− conductance pathways and 2) a slowly activated KCl cotransporter. The need for such a combination of mechanisms may indicate the importance of RVD for cell survival in this epithelial cell type.

**MATERIALS AND METHODS**

**HET-1A cell culture.** HET-1A cells, a SV-40 immortalized human esophageal epithelial cell line, were a generous gift from Dr. Gary Stoner (Medicine and Public Health Administration, Ohio State University, Columbus, OH). Given the fact that these cells have the capacity to replicate, they are more representative of cells within the basal layers of esophageal epithelial cells (stratum basalis) than the more mature nonreplicating cells of the upper strata (stratum spinosum and stratum corneum). The cells were grown in 250-ml flasks in DMEM with high glucose (4.5 g/l), supplemented with 2% FCS, 1 mM corneum). The cells were grown in 250-ml flasks in DMEM with high glucose (4.5 g/l), supplemented with 2% FCS, 1 mM corneum) for determination of mean cell volume from the sample in the Coulter Counter, the population was exposed to a hyposmolar stress by adding to the isotonic HEPES buffer, varying amounts of a Na-free HEPES buffer whose osmolality was 40 mosmol/kg H2O and whose composition was (in mM/l) 5 K+, 1 Ca2+, 1 Mg2+, 13.5 Cl−, 10 HEPES, 5 D-glucose, and 4.5 NMDG, pH 7.4 after being adjusted with NMDG. The hyposmolar stress created by this means ranged from 118 to 280 mosmol/kg H2O. After hyposmolar stress, mean cell volume was serially recorded over a 30-min period. At each time point, mean cell volume was determined in triplicate (differing from each by only a few seconds in time) and the mean cell volume plotted for any given time point is the average of the triplicate runs.

For some studies on RVD, cell populations were preincubated in the following pharmacologic agents for 15 min before measurement of mean cell volume: barium chloride (BaCl2), H2DIDS, R−-butylindazone (DIOA), indanyloxyacetic acid-94 (IAA), N-phenylanthranilic acid (DPC), and bumetanide (BUM). All chemicals were from Sigma except DPC, which was purchased from Fluka (Milwaukee, WI), BaCl2, which was purchased from Specialties Chemicals Division (Morristown, NJ), and DIOA, which was purchased from Research Biochemicals (Natick, MA).

Statistical significance was determined using Student’s  \( t \)-test for paired samples unless otherwise indicated.

**RESULTS**

The mean cell volume for HET-1A cells in isotonic HEPES buffer at room temperature averaged 1,702 ± 28 μm³ (n = 160). After exposure to osmolalities ranging from 280 to 118 mosmol/kg H2O, the cells swelled in direct proportion to the magnitude of the hyposmolar stress, behaving effectively like osmometers (Fig. 1). Notably, at the lowest osmolality, 118 mosmol/kg H2O, there was greater variability in mean cell volume, a reflection of cell lysis as evidenced by the appearance of smaller particles with the much larger swollen cells. Because cell fragility was clearly in evidence at 280 mosmol/kg H2O, but not at 160 mosmol/kg H2O, additional experiments were carried out using exposures at the latter osmolality.

![Fig. 1. The % change in mean cell volume for HET-1A cells is plotted against bathing solution osmolality. Δ % change in mean cell volume in μm³ from cells in isosmolal solution.](http://ajpgi.physiology.org/)
Notably, HET-1A cells exhibited RVD in response to hyposmolal stress. As illustrated in Fig. 2, hyposmolal stress (160 mosmol/kg H2O) initially resulted in a period of rapid cell swelling that peaked 15–20% above baseline ∼3–5 min postexposure. After the rapid increase in cell volume and despite continued exposure to hyposmolal stress, HET-1A cells exhibited a gradual and progressive decline until cell volume returned to baseline, which could require ≥25–30 min postexposure. After reaching baseline, cell volume continued to decline modestly (∼5%) over the ensuing 5–10 min before increasing and stabilizing at the initial baseline value (data not shown).

After establishing the presence of RVD after hyposmolal stress in HET-1A cells, the transmembrane pathways responsible for the process were investigated by monitoring RVD in the presence of pharmacologic agents affecting known mechanisms for RVD in other cell types (5, 13). Specifically, the presence of a K⁺ conductance pathway was assessed by monitoring RVD in HET-1A cells exposed to hyposmolal stress in the presence of the K⁺ channel inhibitor BaCl₂ (5 mM). As depicted in Fig. 3, BaCl₂ in isosmotic solution had no significant effect on resting cell volume, whereas exposure to BaCl₂ during hyposmolal stress resulted in both greater swelling and reduced capacity for RVD than untreated controls. Furthermore, and supporting a role for the outward movement of K⁺ in RVD in HET-1A cells, RVD under hyposmolal stress was monitored during exposure to high (20 mM) K⁺ buffer. As shown in Fig. 4, high K⁺ in isosmotic solution had no effect on resting cell volume, whereas high K⁺ during hyposmolal stress resulted in both greater swelling and inhibition of RVD compared with (control) cells in the standard buffer with 5 mM K⁺.

If the loss of K⁺ was through K⁺ channels during RVD, these channels would require for maintenance of electrochemical equilibrium a parallel conductive loss of anions, and specifically, conductive Cl⁻ loss. Therefore, RVD in HET-1A cells during hyposmolal stress (160 mosmol/kg H2O) was monitored in the presence of three known inhibitors of Cl⁻ conductance pathways: H₂DIDS, IAA, and DPC. H₂DIDS (0.2 mM), IAA (50 μM), and DPC (50 μM) had no effect on resting cell volume in HET-1A cells in isosmolal solution (data not shown). However, H₂DIDS and IAA, but not DPC, significantly inhibited RVD, and the lack of inhibition of RVD by DPC was not a result of the absence of membrane charge, because repeat experiments in a low (1 mM) K⁺ buffer produced the same outcome (Fig. 5, A and B). Also, whereas both H₂DIDS and IAA produced significantly greater increases in cell volume,
to further establish that the response to DIOA was dependent on the activity of a KCl cotransporter. To volume (data not shown).

The above experiments clearly suggest that two different mechanisms are operative for RVD under hyposmolar stress in HET-1A cells, parallel K+ and Cl− channels, and a KCl cotransporter. Support for this concept can also be generated by a comparison of the

Fig. 5. Effects of chloride channel blockers, H2DIDS (0.2 mM), indanyloxyacetic acid-94 (IAA; 50 μM), and N-phenylanthranilic acid (DPC; 50 μM), the latter in both normal (5 mM) and low K DPC (1 mM DPC-LK) solutions, on mean cell volume in HET-1A cells after hyposmolal stress (160 mosmol/kgH2O) is shown for the early maximal change in cell volume above baseline at 2–5 min (A) and for the change in cell volume at 30 min (B). *Δ %change in mean cell volume from initial volume in isosmolal solution; *P < 0.05 compared with untreated hyposmolal stressed controls; n = 4–7 cells/group. Mean cell volume for HET-1A cells in isosmolal solution modestly declined during the experiment, and neither H2DIDS, IAA, nor DPC in isosmolal solution had any significant effect on resting mean cell volume (data not shown).

The above experiments clearly suggest that two different mechanisms are operative for RVD under hyposmolar stress in HET-1A cells, parallel K+ and Cl− channels, and a KCl cotransporter. Support for this concept can also be generated by a comparison of the

Fig. 6. Effect of 30 μM DIOA on the regulatory volume decrease exhibited by HET-1A cells during exposure to 160 mosmol/kgH2O. Δ %change in mean cell volume from initial volume in isosmolal solution; *P < 0.05 compared with untreated hyposmolal controls; n = 8 cells/group. Mean cell volume for HET-1A cells in isosmolal solution modestly declined during the experiment, and R'1-butylindazone (DIOA) in isosmolal solution had no significant effect on resting mean cell volume.

Fig. 7. Effect of 0.1 mM bumetanide on the regulatory volume decrease exhibited by HET-1A cells during exposure to 160 mosmol/kgH2O. Δ %change in mean cell volume from initial volume in isosmolal solution; *P < 0.05 compared with untreated hyposmolal controls; n = 3 cells/group. Mean cell volume for HET-1A cells in isosmolal solution modestly declined during the experiment, and bumetanide produced a small but statistically insignificant increase in resting cell volume after 20 min in isosmolal solution.

Fig. 7. Effect of 0.1 mM bumetanide on the regulatory volume decrease exhibited by HET-1A cells during exposure to 160 mosmol/kgH2O. Δ %change in mean cell volume from initial volume in isosmolal solution; *P < 0.05 compared with untreated hyposmolal controls; n = 3 cells/group. Mean cell volume for HET-1A cells in isosmolal solution modestly declined during the experiment, and bumetanide produced a small but statistically insignificant increase in resting cell volume after 20 min in isosmolal solution.

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by the noncancer-derived basal cell type HET-1A cell line have the capacity for RVD when challenged by hyposmolal stress and that this response appears to be mediated by loss of cytosolic K⁺ and Cl⁻ through two mechanisms: 1) a rapid loss via parallel K⁺ and Cl⁻ channels and 2) slow onset loss via a KCl cotransporter. The presence of RVD in esophageal epithelial cells was initially documented by Snow et al. (12) using a freshly isolated basal cell-enriched population from the rabbit esophagus. In these experiments, the investigators showed that RVD was inhibited by barium and high extracellular K⁺, supporting loss of K⁺ through a K⁺ channel, and by depletion of cell Cl⁻, H₂DIDS, and DPC, supporting loss of Cl⁻ through a DPC-sensitive Cl⁻ channel. In our experiments, RVD in HET-1A cells was blocked by barium, high K⁺ solution, H₂DIDS, and IAA, all supporting the loss of KCl through parallel K⁺ and Cl⁻ channels. In HET-1A cells, unlike rabbit basal cells, the chloride channel blocker IAA but not DPC was effective in blocking RVD. DPC was ineffective even in low K⁺ solution, which maximizes membrane charge. Nonetheless, although the nature of the Cl⁻ channels involved with RVD appear to differ, these results indicate that both human and rabbit esophageal epithelial cells exhibit RVD and have at least one mechanism, parallel operation of K⁺ and Cl⁻ channels, in common.

RVD was also inhibited in HET-1A cells by DIOA, but not by BUM, suggesting that in addition to parallel K⁺ and Cl⁻ channels for RVD, there is a second mechanism compatible with a KCl cotransporter. The presence of a KCl cotransporter was also supported by an analysis of the kinetics of RVD in the presence of various inhibitors. Specifically, channel blockade with Ba²⁺ or H₂DIDS, but not with the KCl cotransport inhibitor DIOA, resulted in an early (2–5 min) inhibition in RVD, whereas inhibition of KCl cotransport by DIOA exerted a significant inhibitory effect on RVD later (at 30 min). These data indicate there are two mechanisms for RVD in hyposmolal-stressed HET-1A cells—an early response due to parallel operations of K⁺ and Cl⁻ channels—and joining them, a later response due to the activation of a KCl cotransporter.
Dual mechanisms for RVD have been reported previously (5, 14, 6) in such diverse cell types as Necturus gallbladder cells, Ehrlich ascites tumor cells, and human retinal pigmented epithelial cells. It is presently unknown whether rabbit esophageal epithelial cells also possess a KCl cotransporter. It is of interest, however, that the basal cell population of rabbit esophageal epithelial cells responded rapidly to the hyposmolar challenge with swelling, and RVD was completed within 5 min (12). A fast response such as this, according to Spring and Hoffman (13) suggests the dominance of parallel K⁺ and Cl⁻ channels for KCl loss during RVD. In contrast, the HET-1A cell population swelled rapidly but then could require ≥30 min before completion of RVD. Although this difference in RVD between rabbit basal cells and HET-1A cells may represent differences in species, phenotype, or methodology, this difference could also reflect a fundamental difference in the mechanism(s) for RVD. For example the slow response of RVD in HET-1A cells reflects the predominance of the KCl cotransporter, as is the case in duck red blood cells in which the KCl cotransporter is the exclusive means of RVD in response to hyposmolar stress and which requires ~90 min for completion (8, 7).

The importance of RVD as a mechanism for defense in human esophageal epithelial cells is worth emphasizing. For instance, in the pathologic setting of GERD when there is a breakdown in epithelial barrier function, the cells of the lower layers may be exposed to luminal contents (e.g., tap water) whose osmolality is low enough for cell destruction by volume-induced lysis. RVD, therefore, represents in this setting, a potential protective defense against cell death by enabling cells to tolerate the hyposmolar environments for considerable periods long enough to permit clearance of the noxious environment from the lumen by swallow-induced peristalsis. Yet, protection against hyposmolar stress by RVD under the complex pathologic conditions produced by GERD should not be assumed, because RVD is also dependent on environmental pH. For instance, Snow et al. (12) have shown that the rapid RVD in rabbit basal cells is inhibited under mildly acidic conditions (pH 6.8). This inhibitory effect, however, may be limited or transient in cells with dual mechanisms, such as the HET-1A cells, because it has been shown in Ehrlich ascites tumor cells that acidic pH may shift the operation of RVD from parallel K⁺ and Cl⁻ channels to the KCl cotransporter (5, 7). In this respect, it was notable in our studies that RVD was inhibited but not abolished in HET-1A cells exposed to extracellular acidity (pH 6.6). This suggests that the apparent redundancy in mechanisms for RVD, and in particular, the presence of a KCl cotransporter, may serve as an additional protective role in human esophageal epithelial cells under hyposmolar stress in an acidic environment.

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