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Effect of extracellular glucose and K^+ on intracellular osmolytes and volume in a human kidney cell line

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TABLE OF CONTENTS

1. ABSTRACT

The goal of this study was to assess the effect of extracellular glucose and K^+ ($[K]_o$) on the intracellular osmolyte content and cell volume maintenance and regulation in a human embryonic kidney cell line (tsA201a). Cell volume maintenance was studied by isotonic (313 ± 5 mOsm) replacement of culture media by a glucose-free Ringer solution containing (in mM) 0, 3, 6, or 10 K^+. Cell volume regulation was studied by exposing cells to hypotonic (250 ± 5 mOsm) glucose-free Ringer solution containing the various $[K]_o$. The results showed that: 1) intracellular osomlyte content (i.e. Na^+, Cl^-, Urea and free amino acids (FAA)) and cell volume increased when culture media was replaced with isotonic Ringer at all $[K]_o$; 2) osmolyte content decreased with continuous exposure to isotonic Ringer at all $[K]_o$ but cell volume changes depended on $[K]_o$. Volume recovery occurred at 6 and 10 mM K^+; 3) exposure to hypotonic Ringer induced swelling at all $[K]_o$ followed by a reduction in measured intracellular osmolytes. Regulatory volume decrease occurred in 6 or 10 mM K^+ but swelling continued in 0 or 3 mM K^+; and 4) addition of ouabain produced swelling without recovery under iso- and hypotonic conditions. These results indicate that the removal of extracellular glucose produced a transient inhibition of the Na^+/K^+ ATPase resulting in a transient increase in the intracellular content of Urea, FAA and cell volume and $[K]_o$ regulated an as yet unidentified intracellular osmolyte.

2. INTRODUCTION

In animal cells, water is in thermodynamic equilibrium across the plasma membrane. Cell volume is largely determined by the intracellular osmolyte concentration. Thus changes in cell volume under iso- or hypotonic conditions result from net gain or loss of intracellular osmolytes (1-4).

The extracellular solution surrounding mammalian cells may change in composition in response to dynamic environmental forces. Homeostasis and volume are maintained by ion and osmolyte transport across the plasma membrane. Regulation of cell volume, therefore, is a dynamic process in order to maintain constant volume and cell viability. Unregulated volume changes are known to disrupt intracellular metabolic processes such as mRNA processing and protein synthesis and can ultimately result in cell lysis (5, 6).

The contribution of various transport proteins and ion channels to iso- and hypotonic volume regulation varies according to cell type. Many cells have a tendency to swell under isotonic conditions in the presence of impermeant solutes (27, 7). High resting K^+ conductance found in many cells, including epithelial cells (8), may also contribute to isotonic volume regulation. Ouabain produces a net increase in intracellular Na^+, resulting in the swelling of human epithelial T84 cells (9). Removal of K^+ or the addition of ouabain inhibits the Na^+/K^+ ATPase but does not produce cell swelling in rat and rabbit renal cortex tissue or rat adipocytes (10, 11).

Most cells respond to a hypotonic environment by swelling followed by the recovery of cell volume. Volume recovery under these conditions is known as regulatory volume decrease (RVD). This process requires the movement of osmolytes, followed by water from the intracellular to extracellular milieu. The ultimate movement of water allows the cell to regain a normal volume. The cellular mechanisms responsible for RVD appear to be dependent on cell type.

Separate K^+ and Cl^- pathways are responsible for volume recovery in some epithelial cell lines, Ehrlich ascites tumor cells and lymphocytes (12). In human epithelial cells, RVD is associated with activation of volume-regulated anion channels. As anions leave the cell through open volume regulated anion channels, the membrane becomes depolarized. Membrane depolarization results in activation of voltage-gated K^+ channels (1, 13, 14) and K^+ moves out of the cell. The net efflux of KCl, osmolytes and water allow the cell to recover its original volume. Volume recovery usually occurs within 20 minutes (1, 2).

Inorganic ions and FAA are used as osmolytes in most mammalian cells. Kidney epithelial cells also use polyhydric alcohols (e.g., sorbitol, inositol), methylamines (e.g., glycerophosphorylcholine, betaine) and urea, as osmoregulators (for review, see ref. 15). These osmolytes are used to counteract the physiological exposure of kidney cells to large extracellular tonicities, without compromising their intracellular ionic strength.

Cultured cells are commonly used to study volume regulatory mechanisms, yet there have been no systematic studies assessing the result of isosmotic replacement of culture media by Ringer solution and its effect on intracellular osmolyte content and cell volume. Furthermore, mechanisms responsible for isotonic and anisotonic volume regulatory mechanisms in human embryonic epithelial renal cells have not been studied. The present work was undertaken to fill the aforementioned gaps. Intracellular osmolytes (i.e., Na^+, K^+, Cl^- Urea and FAA) and cell volume were measured in a human embryonic epithelial cell line (tsA201a) under iso- and hypotonic conditions. The results reveal that, under isotonic conditions, removal of glucose produces changes in intracellular osmolyte content and cell volume as well as an unexpected effect of K_o removal leading to an increase of an as yet unidentified intracellular osmolyte. These effects could have important implications for researchers interpreting results in which cultured cells are used for studying cell volume regulation.

3. MEASUREMENT OF CELL VOLUME AND INTRACELLULAR CONTENT

3.1. Cell Culture

Human epithelial tsA201a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 U/ml) in 5% CO_2 maintained at 37°C. Cells were split with 0.25% trypsin-EDTA every 2-3 days. All cell culture reagents were purchased from Fisher Scientific, Pittsburgh, PA.

3.2. Calcein-AM Loading

A stock solution (1 mM) of Calcein-AM was reconstituted in dimethylsulfoxide (DMSO) and stored at -20° C in a sealed dessicator. Cells were plated on poly-L-lysine coated coverslips and incubated in DMEM containing 2.5 µM calcein-AM (Molecular Probes, Eugene, OR) for 30 minutes at room temperature. The dye-loading solution was replaced with DMEM supplemented with 10% FBS for 30 minutes at 37° C in 5% CO_2. Cells were washed once with isotonic Ringer's solution (313 mOsm) and placed in an isotonic Ringer's bath.

3.3. Solutions

The composition of Ringer solution was (in mM): 100 NaCl, 0, 3, 6 or 10 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, pH 7.2 (NaOH). The nominal concentration of K^+ in 0 mM K^+ Ringer solution was measured to be < 0.3 mM. For comparison, the composition of culture media (DMEM) for tsA201a cells included (in mM): 5.3 K^+, 154 Na^+, 118 Cl^-, 1.8 Ca^{2+}, 1 Mg^{2+}, 0.9 NaH_2PO_4, 44 $NaHCO_3$, 1 Na pyruvate, 25 glucose and 6.8 FAA. Osmolality of Ringer solutions was adjusted to 313 ± 5 (isotonic) or 250 ± 5 (hypotonic) mOsm using a 1 M stock solution of sucrose while keeping the ionic concentration constant. The presentation of the experimental results will use the term "isotonic" to refer to solutions with an osmolarity of 313 ± 5 mOsm and "hypotonic" to refer to solutions of 250 ±

5mOsm. Osmolality was confirmed prior to each experiment using a vapor pressure osmometer (Wescor, Logan, UT). 30 mM ouabain was added to isotonic solutions as indicated.

3.4. Cell volume measurements

Calcein-AM loaded cells were placed in a recording dish and bathed in isotonic 3 mM K^+ Ringer's solution to establish a baseline volume. Cells exhibiting uniform fluorescence were chosen for each experiment. These cells were then exposed to either isotonic or hypotonic test solutions containing the various K^+ concentrations by lowering a capillary tube filled with isotonic Ringer's solution into the bath, positioned with the opening toward the cell(s) to be superfused, in a "sewer-pipe" fashion. Once the capillary tube was positioned using a SF-77 Perfusion Fast-Step system (Warner Instrument Corporation, Hamden, CT), a leuer valve was manually opened to begin superfusion within 5 seconds, changing the solution at the cell within 10 seconds. Cells were superfused for 30 - 60 minutes at a rate of 0.5 ml/min.

Fluorescent images were taken using phase contrast microscopy with an inverted, epi-fluorescence microscope (Nikon Diaphot, Tokyo, Japan) and a Hamamatsu C5985 CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). An HMX-3 mercury lamp (Nikon Instrument Group, Melville, NY) was used as the excitation light source. Exposure time and intensity was limited by a computer-controlled automatic high speed shutter (Uniblitz, Vincent Associates, Rochester, NY) and neutral density filters (Omega Optical, Brattleboro, VT).

Images were taken at regular intervals throughout superfusion. Mean pixel intensity (MPI) of a cell at each time point was determined from within a small box of fixed size placed in the cell interior using Scion Image 1.57c software. MPI measurements taken during superfusion with experimental test solution (F_t = fluorescence in response to experimental test solution) were normalized with respect to MPI measurements during superfusion with isotonic Ringer's solution (F_i = fluorescence in response to isotonic solution). The change in fluorescence was calculated from the normalized fluorescence ratio $F_i/F_t = [((F_i/F_t) - 1)/(F_i/F_t)]*100$ and was interpreted to represent the percent change in cell volume (16).

Volume changes in cells first exposed to culture media and then to Ringer's solution were measured visually because the culture media contains 0.04 mM phenol red and the coloration interferes with Calcein fluorescence signal. In these experiments, cell volume was calculated using a visual measurement of cell diameter and assuming that the cells are spheres and applying $V=4/3\pi r^3$. This is a good qualitative estimation of volume. This method, however, is inherently less quantitative than the fluorescent assay because it is based on a 2-dimentional measurement and volume changes in the z-dimension are not represented.

3.5. Intracellular ionic content measurements

Each run consisted of $5.1 \times 10^7 \pm 0.2$ cells. Cells were removed from the culture media and incubated for 5 or 20 min in isotonic Ringer's containing 0, 3, 6, or 10 mM K^+. Cells were immediately placed on ice (4°C), and washed 3 x in cold isotonic sucrose. Cells were pelleted and then freeze-dried. Intracellular water content was determined from the difference between wet and dried cell weight. Dry cell pellets were dissolved in 90% formic acid. Cl^- content was assessed using a chloridometer (Corning, Chloride Analyzer, Model 925). Total intracellular K^+ and Na^+, content was assessed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) at the Zentrallabor Chemische Analytik, Technsche Universität Hamburg-Harberg, Germany (Dipl. Ing. J. Kunze, director). Weights were measured in pre-tared eppendorf tubes. Values are expressed as µmol/g (dry wt.).

3.6. Measurement of intracellular FAA and Urea content

Freeze-dried cells were homogenized in 70% ethanol. Homogenate was boiled to precipitate protein and centrifuged (20,000 x g for 20 min.). Residue was taken up in 300 µl 0.2 N lithium citrate buffer (pH 2.2) and the FAA and Urea contents were measured using high pressure liquid chromatography (HPLC, Beckman, System Gold; see ref. 17). Values are expressed as µmol/g (dry wt.).

3.7. Data analysis

Student's t-test was used to analyze data for statistically significant differences with $P > 0.05$. Statistical comparisons between experimental groups are indicated in the figure legends.

4. CELL VOLUME AND INTRACELLULAR CONTENT CHANGES UNDER VARIED CONDITIONS

4.1 Fluorescence signal is linear with changes in osmolality

The ability of tsA201a cells to act as osmometers in response to changes in external osmolality was tested in cells loaded with calcein-AM. Cells were challenged with iso-, hypo- or hyper-tonic NaCl Ringer's solution for 45 - 60 minutes and the ratio, F_t/F_i, was determined. Figure 1 shows that the average peak change (± SEM) in relative fluorescence (F_t/F_i) during superfusion was linear in solutions with osmolality ranging from 250 to 410 ± 5 mOsm. Peak change in cell volume occurred within 20 min. in all superfusion experiments.

Linear regression analysis through the data points in Figure 1 yields a slope of 0.59 with a y-intercept of 0.33. A cell that is an ideal osmometer should yield a line with a slope of 1.0 and a y-intercept of 0.0. The difference between measurements made in tsA201a cells and that of an ideal osmometer is most likely due to compartmentalization of intracellular water that cannot fully equilibrate with calcein during volume regulation. Similar deviations from ideality have been observed in other preparations using similar fluorescence assays (e.g. ref. 18) and represent an underestimation of actual cellular volume changes. There was no evidence of dye compartmentalization or photobleaching (data not shown) that may also result in deviations from ideality.

Table 1. Change in intracellular ionic and amino acid content

	Media µMol/g(dry wt)	**6K** µMol/g(dry wt)	**%Δ**
Cl^-	103 ± 9	332 ± 43	322[1]
K^+	511 ± 31	381 ± 55	75
Na^+	36 ± 4	107 ± 12	299[1]
FAA (total)	55 ± 8	86 ± 16	156[1]
Glu	9 ± 1	27 ± 4	314[1]
Urea	5.4 ± 0.7	9 ± 1	167[1]
NH_3^+	1.6 ± 0.5	2.5 ± 0.5	156

Intracellular ionic and amino acid content measured in cells grown in DMEM and then exposed to glucose-free isotonic 6mM K^+ Ringer for 5 min. Values are expressed in µMol/g(dry wt) to avoid the effect of net plasmalemmal water fluxes on the osmolyte values. ([1]) indicates significant change ($p \leq 0.05$, Student's t-test) in ion or amino acid content between the two groups.

4.2. Effect of isosmotic replacement of culture media by isotonic glucose-free ringer on intracellular osmolyte content and cell volume

Table 1 shows the total intracellular content of Na^+, K^+, Urea, NH_3^+, Glutamate (Glu) and FAA in cells incubated in the culture media and subsequently exposed for 5 min to glucose-free Ringer solutions containing either 0, 3, 6 or 10 mM K^+. Individual amino acid composition was also assayed but is not shown. Glu was found to be in the highest concentration of any individual amino acid, representing 16% of FAA and showed the greatest changes under experimental conditions.

Replacement of culture media by isotonic, glucose-free 6 mM K^+ Ringer produced a significant increase in Na^+, Cl^-, Urea and FAA while K^+ content decreased. The increase in FAA content was mainly attributed to an increase in Glu. The measured increase in the intracellular osmolyte content suggested that cells should swell when initially exposed to isotonic Ringer. Figure 2 shows that this was the case, with cells swelling slowly to 105-110% of their original volume over 30 min after immediate exposure to isosmotic Ringer containing 6 mM K^+. Volume measurements of the effect of immediate replacement of culture media by Ringer solution were based on visual measurements of cell diameter (see Methods) because the coloration of DMEM media compromised fluorescent measurements.

4.3. Osmolyte content recovered over time in isotonic ringer

Cl^-, Na^+, Glu and total FAA content decreased, approaching that measured in culture media with continuous exposure to isotonic Ringer for 20 min. (Table 2). The recovery was greater in low (i.e., 0 and 3 mM) than in higher (i.e., 6 and 10 mM) K^+ Ringer's solutions. Exposure to 0 mM K^+ was accompanied by cell swelling (Table 2), a condition known to activate the Na^+/K^+ ATPase (19). The intracellular K^+ content, however, remained high in all conditions. Urea and NH_3^+ content did not change significantly from 5 to 20 min. with the exception that it increased in 6 mM K^+ isotonic Ringer. A return toward original levels of intracellular osmolytes is expected to lead to cell volume recovery from the observed swelling (Figure 2). Cells exposed to 6 or 10 mM K^+ isotonic Ringer did recover from swelling (Figure 3). Cells exposed to 3 mM K^+ Ringer remained swollen whereas those exposed to 0 mM K^+ Ringer continued to swell (Figure 3).

4.4. Effect of K_o on hypotonic-induced changes in cell volume and intracellular osmolyte content

The ability of tsA201a cells to regulate their volume with exposure to 0, 3, 6, or 10 mM K^+-containing hypotonic, glucose-free, Ringer's solutions was tested. Table 3 shows the intracellular osmolyte content of cells exposed for 5 and 20 min to hypotonicity at the various K_o. A comparison of Tables II and III shows that the Cl, total FAA , Glu and Urea content at 5 min under hypotonic conditions is similar to that measured at 20 min of exposure to isotonic Ringer with the corresponding $[K^+]_o$ although NH_3^+ content tended to increase. Also, the K^+ content was greater and the Na^+ content was smaller after 5 min in hypotonic Ringer than that measured after 20 min of isotonic exposure. The K^+ content increased further and the content of Na^+ decreased further with continued exposure to hypotonic Ringer. This continued exposure also resulted in a net loss of FAA.

Cl^-, FAA , Glu and Urea content after 5 min in hypotonicity were similar to that measured after 20 min of exposure to isotonic Ringers. Thus the rate of recovery under hypotonic conditions is greater than the recovery under isotonic conditions. The observed increase in K^+ and decrease in Na^+ can be explained by activation of the Na^+/K^+ ATPase induced by cell swelling. Similar effects have been demonstrated in other cell types including cardiac myocytes (19).

Figure 4 shows the change in cell volume with exposure to hypotonic Ringers containing 0, 3, 6 or 10 mM K^+. Hypotonicity induced a significant increase in cell volume within 5 min, under all conditions. The concentration of the measured intracellular osmolytes generally decreased over time at all $[K]_o$, yet recovery of cell volume was only observed in cells exposed to Ringer's containing 6 or 10 mM K^+. In the presence of 0 and 3 mM K^+, the cells responded to hypotonicity by swelling continuously reaching 20-25% of their original volume over a 60 min period.

A comparison between Tables II and III indicates that during the first 5 min of exposure to Ringer's there are relatively lower contents of intracellular Cl^- and Na^+ but higher content of K^+ in hypotonic as compared to isotonic solutions. The rate of loss for FAA or Glu between 5 and 20 min of incubation (Table 3; Figure 5) was similar under iso- or hypotonic conditions (i.e., FAA: 1.4 ± 0.4 vs. 1.1 ±

Table 2. Intracellular osmolyte contents

	time min.	**0K** µMol/g(dry wt)	**3K** µMol/g(dry wt)	**6K** µMol/g(dry wt)	**10K** µMol/g(dry wt)
Cl^-	5	343 ± 49	428 ± 99	332 ± 43	286 ± 58
	20	***194[1]^ ± 13↓***	235 ± 25	280 ± 23	227 ± 33
K^+	5	428 ± 46	443 ± 81	381 ± 55	406 ± 29
	20	509 ± 51	498 ± 37	380 ± 20	427 ± 23
Na^+	5	106 ± 11	115 ± 18	107 ± 12	105 ± 7
	20	*81^ ± 8*↓	92 ± 11	116 ± 7	113 ± 8
FAA (total)	5	75 ± 12	76 ± 12	86 ± 16	91 ± 13
	20	63 ± 7	**55 [1]± 7↓**	65 ± 9	*55^ ± 7*↓
Glu	5	29 ± 4	24 ± 6	27 ± 4	28 ± 6
	20	***19[1]^ ± 2↓***	**14[1] ± 12↓**	19 ± 3	***16[1]^ ± 2↓***
Urea	5	9 ± 1	8 ± 1	9 ± 1	10 ± 1
	20	9.5 ± 0.3	8 ± 1	**11 ± 1↑**	9 ± 1
NH_3^+	5	3.2 ± 0.8	2.5 ± 0.6	2.5 ± 0.6	1.9 ± 0.3
	20	3.5 ± 0.6	4.0 ± 0.5	***4.1[1]^ ± 0.6↑***	2.3 ± 0.4
Δ Volume[#]	5	**↑**	---	---	---
	20	**↑**	---	**↓**	**↓**

Intracellular osmolyte contents was measured under isotonic conditions measured in cells exposed to 0, 3, 6 or 10 mM K^+_o Ringer's for 5 or 20 min. Values in **bold** with ([1]) indicate significant change ($p \leq 0.05$, Student's t-test) in ion or amino acid content as compared to that in cells exposed to isotonic 6 mM K^+_o Ringer for 5 min. Values in *italics* with karot (^) indicate significant change ($p \leq 0.05$, Student's t-test) when comparing 5 min. to 20 min. times points for a given ion or amino acid content. Arrows indicate an increase or decrease in content. [#]Arrow indicates increase or decrease in volume as compared to initial volume at t = 0.

Table 3. Intracellular osmolyte contents

	time min.	**0K** µMol/g(dry wt)	**3K** µMol/g(dry wt)	**6K** µMol/g(dry wt)	**10K** µMol/g(dry wt)
Cl^-	5	**200[1] ± 14↓**	237 ± 27	**213[1] ± 13↓**	249 ± 20
	20	217 ± 31	**203[1] ± 16↓**	275 ± 65	213 ± 15
K^+	5	**564[1] ± 20↑**	**550[1] ± 14↑**	494 ± 72	**557[1] ± 8↑**
	20	488 ± 30	449 ± 56	366 ± 101	*398^ ± 67*↑
Na^+	5	***70[1] ± 8↓***	81 ± 26	79 ± 13	86 ± 11
	20	82 ± 4	79 ± 3	96 ± 9	94 ± 10
FAA (total)	5	75 ± 11	66 ± 20	73 ± 13	93 ± 11
	20	***25[1]^ ± 10↓***	**42[1] ± 5↓**	56 ± 12	***41[1]^ ± 9↓***
Glu	5	26 ± 6	25 ± 9	27 ± 6	38 ± 8
	20	***7[1]^ ± 3↓***	**11[1] ± 2↓**	24 ± 13	***8[1]^ ± 7↓***
Urea	5	9 ± 1	9 ± 2	9 ± 1	10 ± 1
	20	7 ± 1	9 ± 2	10 ± 2	11 ± 1
NH_3^+	5	3.5 ± 0.6	1.38 ± 0.03	1.7 ± 0.4	1.6 ± 0.4
	20	3.4 ± 0.9	***3.8[1]^ ± 0.4↑***	*3.8^ ± 0.7*	*4.2^ ± 0.9*
Δ Volume[#]	5	**↑**	**↑**	**↑**	---
	20	**↑**	**↑**	**↓**	**↓**

Intracellular osmolyte contents was measured under hypotonic conditions measured in cells exposed to 0, 3, 6 or 10 mM K^+_o Ringer's for 5 or 20 min. Values in **bold** with ([1]) indicate significant change ($p \leq 0.05$, Student's t-test) in ion or amino acid content as compared to that in cells exposed to isotonic 6 mM K^+_o Ringer for 5 min. Values in *italics* with karot (^) indicate significant change ($p \leq 0.05$, Student's t-test) when comparing 5 min. to 20 min. times points for a given ion or amino acid content. Arrows indicate an increase or decrease in content. [#]Arrow indicates increase or decrease in volume as compared to initial volume at t = 0.

0.4 µmol/g(dry wt)/min, respectively and Glu: 0.5 ± 0.2 vs. 0.2 ± 0.2 µmol/g(dry wt)/min).

4.5. Effect of Ouabain on cell volume and intracellular osmolyte content under iso- and hypotonic conditions

The effect of 30 µM ouabain on volume and osmolyte content was assessed under iso- and hypotonic condition in order to further assess the role of the Na^+/K^+ ATPase. Table 4 shows the effect of ouabain on the measured intracellular osmolyte content in isotonic or hypotonic 6 mM K^+ Ringer's. The intracellular content of Na^+ and K^+ increased in the presence of ouabain under both isotonic and hypotonic conditions. The intracellular content of Cl^-, FAA and Glu was also increased under both conditions with respect to their content in media while the Urea content increased in isotonic but decreased in hypotonic conditions. The increase in Na^+ and Cl^- can be explained by inhibition of the Na^+/K^+ ATPase. The increase in K^+ is unexpected and is difficult to explain (see Discussion). The increase in Glu and FAA was consistent with changes in intracellular content observed when cells were removed from culture media to isotonic Ringer's (Table 1).

Cells in isotonic 6 mM K^+ Ringer swelled in the presence of ouabain (Figure 6). Swelling was consistent with the observed increase in intracellular osmolyte content shown in Table 4. No additional swelling was observed in cells exposed to hypotonic 6K Ringer's (data not shown) which is consistent with the lack of further increase in intracellular osmolyte content,

Table 4. Intracellular osmolyte contents

	time min.	**ISO** µMol/g(dry wt)	**HYPO** µMol/g(dry wt)
Cl^-	5	219 ± 27	301 ± 55
	20	197 ± 33	286 ± 17
K^+	5	**605[1] ± 68↑**	406 ± 66
	20	***639[1]^ ± 46↑***	***669[1]^± 50↑***
Na^+	5	**334[1] ± 43↑**	**262[1]± 40↑**
	20	**374[1] ± 29↑**	***450[1]^ ± 44↑***
FAA (total)	5	114 ± 27	64 ± d5
	20	89 ± 5	51 ± 8
Glu	5	28 ± 5	22 ± 2
	20	40 ± 2	***17[1]^ ± 4↓***
Urea	5	**15[1] ± 3↑**	8.3 ± 0.8
	20	10.5 ± 0.5	**5[1] ± 1↓**
Δ Volume[#]	5	**↑**	**---**
	20	**↑**	**---**

Intracellular osmolyte contents was measured in the presence of 30 µM ouabain. measured in cells exposed to 6 mM K^+_o Ringer's for 5 or 20 min. Values in **bold** with ([1]) indicate significant change ($p \leq 0.05$, Student's t-test) in ion or amino acid content as compared to that in cells exposed to 6 mM K^+_o isotonic Ringer for 5 min. Values in *italics* with karot (^) indicate significant change ($p \leq 0.05$, Student's t-test) when comparing 5 min. to 20 min. times points for a given ion or amino acid content. Arrows indicate an increase or decrease in content. [#]Arrow indicates increase or decrease in volume as compared to initial volume at t = 0.

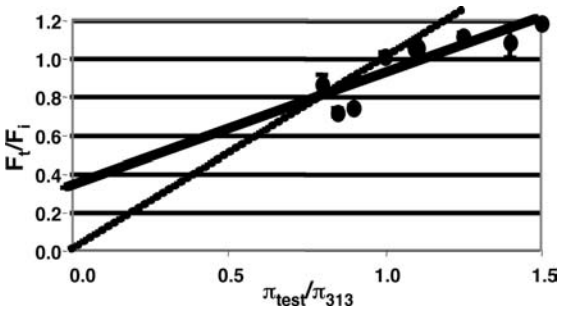

Figure 1. Peak change in relative fluorescence (F_t/F_i) versus relative osmotic pressure (p_t/p_{313}) in wild-type tsA201a cells. Cells were loaded with 2.5 mM calcein-AM and placed in isotonic 100 mM NaCl Ringer's bath solution prior to superfusion with NaCl Ringer's solution of the indicated osmolality. A linear regression of the data points is shown as a solid line ($y= 0.59x + 0.33$, $R^2 = 0.91$). Ideal osmometer behavior is shown as a dashed line ($y = x$). Each point represents the average peak change (F_t/F_i ± SEM) in fluorescence. n = 3 - 5 cells per experimental condition.

5. THE EFFECT OF GLUCOSE AND K_O ON CELL VOLUME

5.1. Isosmotic replacement of culture media by Ringer's solution

Culture cells are widely used in biological research for numerous purposes. The results here presented show that isotonic replacement of culture media by Ringer's solution produces significant, though transient increases in the osmolyte content (Table 1) and cell volume (Figure 2) in tsA201a cells. The increase in osmolytes was due to FAA, Na^+ and Cl^- and was accompanied by a decrease in K^+. A straightforward explanation for these results is that absence of glucose in the Ringer media may lead to a partial and transient decrease in the intracellular ATP levels. A decrease in intracellular ATP content would in turn produce partial inhibition of the Na^+/K^+ ATPase resulting in an increase in Na^+, followed by Cl^- to maintain electroneutrality and a decrease in K^+. The increase in FAA content may be explained by partial degradation of intracellular proteins in the absence of glucose. This hypothesis is supported by observations in nerve cells in which exposure to glucose-free conditions or inhibition of glucose utilization by exposure to high concentrations of CO_2 lead to proteolysis and NH_4^+ production (20, 21).

The reversibility (from 20 to 60 min) of the effects on intracellular osmolyte content and cell volume (Table 1 and Figure 3) upon continuous exposure to Ringer solution suggests a time-dependent restoration of the intracellular ATP levels. It is interesting that restitution of the Na^+, K^+ and Cl^- levels was more pronounced in cells exposed to low K_o (0 and 3 mM; Table 1) as compared to cells exposed to 6 or 10 mM K^+. This result may be explained by activation of the Na^+/K^+ ATPase resulting form cell swelling at 0 mM K^+ (see Table 1). Swelling-induced stimulation of the Na^+/K^+ ATPase results from activation of a messenger cascade leading to dephosphorylation of pump units (19). This hypothesis, however, requires justification of how a nominal 0 mM K^+ media can support activation of the Na^+/K^+ ATPase since activation of the ATPase necessitates presence of K^+ at the extracellular cell surface. The sources for K^+ could be trace contamination of K^+ from the Ringer solution, and leak of K^+ from the cell's cytosol under nominal 0 mM K^+ conditions. This has been reported in other cell types (22). The hypothesis linking glucose, ATP, FAA, Na^+, K^+ and Cl^- will be tested in future experiments.

An additional important question to be considered is whether the results here presented are particular to tsA201a

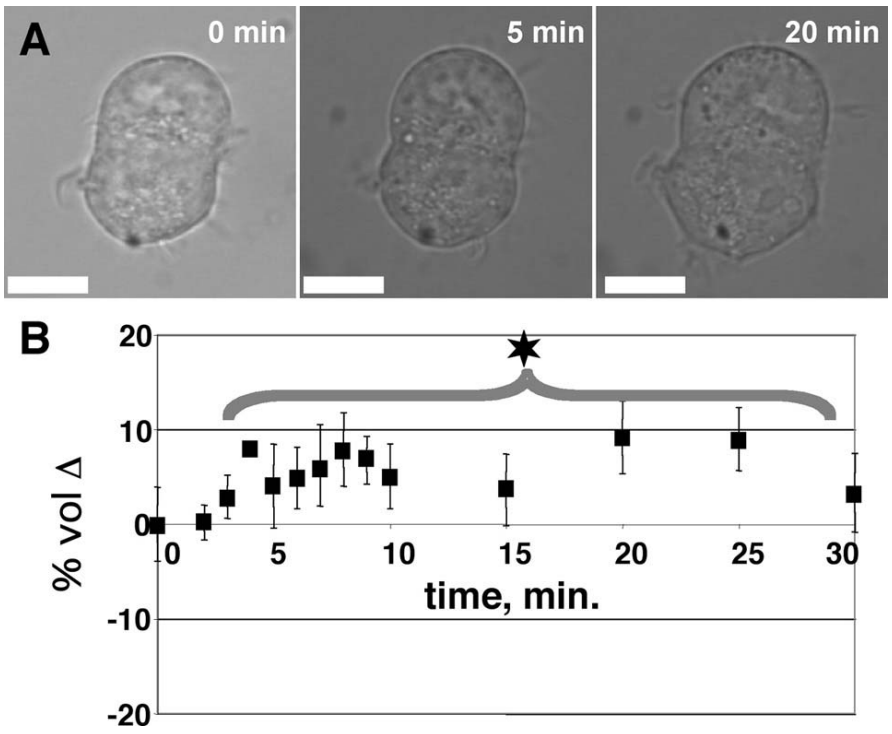

Figure 2. tsA201a cells grown in DMEM swell with exposure to isotonic 6 mM K^+_o Ringer. A. Panel shows a pair of tsA201a cells exposed to growth media (DMEM), 0 min., and then after 5 or 20 min. exposure to isotonic 6 mM K^+_o Ringer. (0 min image was taken with a 0.3 sec exposure, ND filter = 2: 5, 20 min images was taken with a 0.125 sec exposure, ND filter = 16. Difference in exposure times was due to coloration of media.) Volume was calculated by measuring diameter of cell at three difference points and calculating volume as $V = 4/3\pi r^3$. %Change in volume = $(V_t/V_{initial})(100)$. Average V ± SEM is shown, n ≥ 6. ✶P<0.05 vs. initial cell volume at t = 0 min**.**

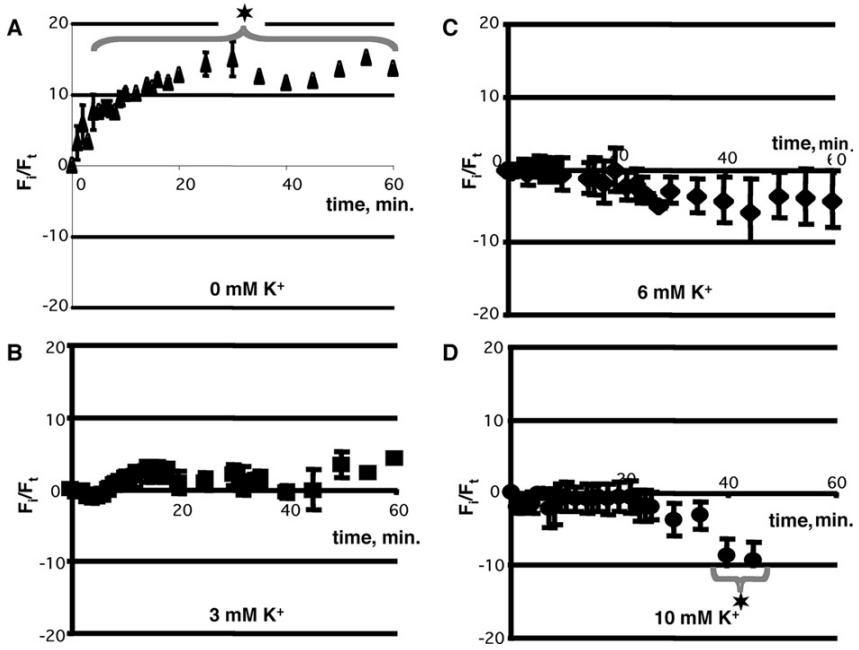

Figure 3. Relative cell volume change during superfusion with isotonic (A) 0, (B) 3, (C) 6 or (D) 10 mM K^+_o Ringer's solution. Each point represents the average (± SEM) percent change in F_i/F_t, n = 4. ✶P<0.05 vs. initial cell volume at t = 0 min**.**

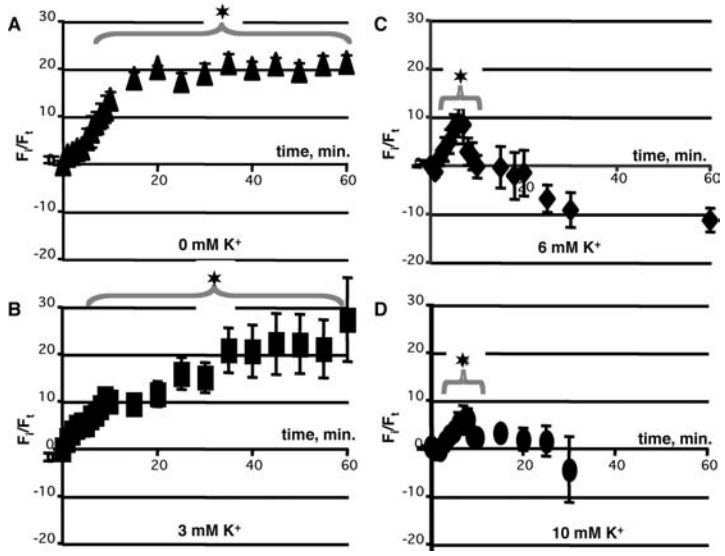

Figure 4. Relative cell volume change during superfusion with hypotonic (A) 0, (B) 3, (C) 6 or (D) 10 mM K^+_o Ringer's solution. Each point represents the average (± SEM) percent change in F_i/F_t, n = 4. ✶P<0.05 vs. initial cell volume at t = 0 min**.**

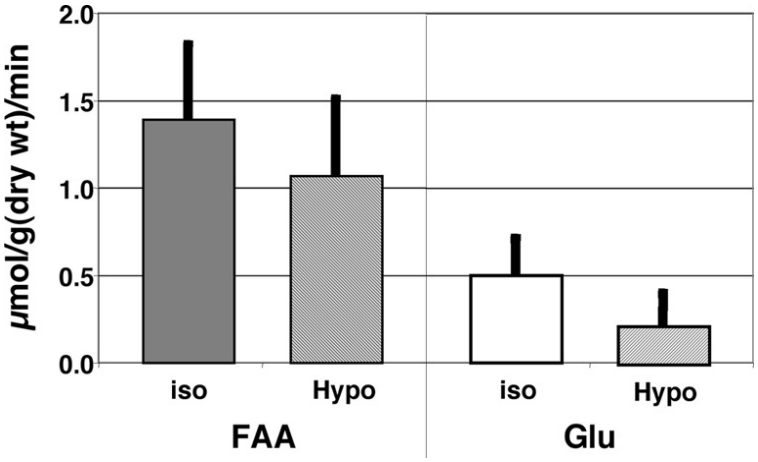

Figure 5. Rate of loss of FAA and Glu is similar under isotonic and hypotonic 6 mM K^+_o Ringer's. Each bar represents the average rate of change (µMol/g(dry wt)/min.) between 5 min and 20 min. time points (± SEM).

cells or may also occur in other culture cells. Although the answer to this question would require proper investigation, it is reasonable to expect that they should be mirrored in any culture cells utilizing glucose as their primary energy source (e.g., cells grown in media not containing fatty acids). In any event, these results should be of interest to researchers using cultured cells because alterations in intracellular osmolyte content and cell volume could have a significant impact on the interpretation of their results.

5.2. Effect of extracellular K^+ on cell volume regulation and intracellular osmolyte content

Tables 1 and 2 and Figures 3 and 5 show that K_o plays a key role in the maintenance and regulation of cell volume in tsA201a cells. Specifically: i) Under isosmotic conditions, low K_o prevented the volume recovery otherwise observed in 6 and 10 mM K^+ following the swelling upon replacement of culture by Ringer media; and ii) Low K_o (i.e., 0 and 3 mM) prevented RVD observed at 6 and 10 mM K^+ in response to a hypotonic challenge. Both results can be explained by a low K_o-promoted net gain of an as yet unidentified intracellular osmolyte. This osmolyte is not one of the inorganic ions or FAA measured, but its intracellular content must increase under low K_o conditions. Synthesis or degradation of intracellular proteins appears unlikely because there were no significant changes in protein concentration under the experimental conditions tested (data not shown). Intracellular content of free Ca^{++} and Mg^{++} also did not change (data not shown).

5.3. Nature of the Intracellular osmolyte(s) responsible for cell swelling

In addition to inorganic ions and FAA, polyhydric alcohols (e.g., sorbitol, inositol), methylamines (e.g., glycerophosphorylcholine, betaine) and urea are osmoregulators in kidney cells (reviewed by ref 15).

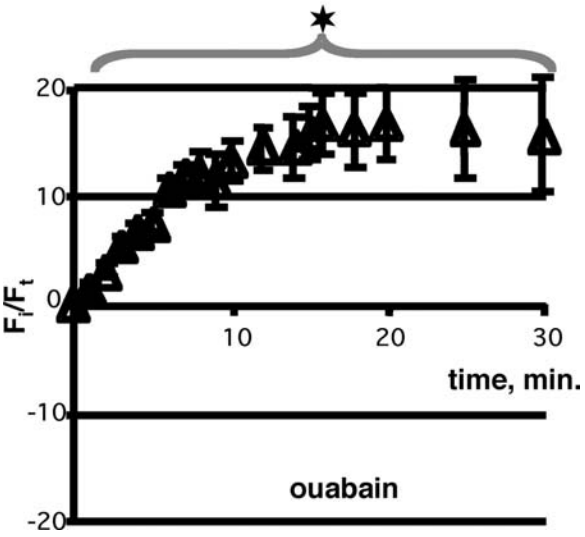

Figure 6. Relative cell volume change during superfusion with isotonic 6 mM K^+_o Ringer's solution + 30 µM ouabain. Each point represents the average (± SEM) percent change in F_i/F_t, n = 4. ✶P<0.05 vs. initial cell volume at t = 0 min**.**

Sorbitol is synthesized from glucose and glycerophosphorylcholine from choline. Choline, along with inositol and betaine, are taken up from the extracellular media. These osmolytes, therefore, are not likely candidates for the unidentified osmolyte in tsA201a cells. Urea can be synthesized from oxidative deamination of Glu and ATP hydrolysis (see, ref. 23). Tables I and II show significant decreases in Glu content under iso and hypotonic conditions in the presence of low K_o and not under 6 mM K^+. Table 1 also shows an increase in Urea content when cells are exposed to isotonic Ringer for 5 min. This increase is maintained, or increased, under all subsequent conditions. The increase in Urea content when replacing media with Ringer solution is, in part, responsible for the observed cell swelling. The maintained increase in Urea is somewhat surprising. A high intracellular Urea content, however, did not interfere with RVD in cells exposed to 6 or 10 mM K^+ hypotonic Ringer.

5.4. Possible cellular effects of K_o

One of the most intriguing results here presented is that under iso or hypotonic conditions, reduction or removal of K_o produces the likely net gain of an unidentified osmolyte in tsA201a cells. Three possible cellular effects linking low K_o to osmolyte synthesis deserve consideration: i) Membrane potential (V_M); ii) Plasmalemmal transporters; and iii) Cytoplasmic pH. Reduction or removal of K_o increases the driving force of this ion and thus produces membrane hyperpolarization that would then affect voltage dependent ion channels and transporters. Changes in V_M are associated with cell swelling and RVD (24-26). Urea is an uncharged molecule at physiological pH and it is unlikely that changes in V_M could affect its synthesis and/or intracellular retention. It is well established that activity of the Na^+/K^+ ATPase, the plasmalemmal Ca^{++} ATPase, Na^+/Ca^{++} exchanger, KCl co-transporter and $Na^+/K^+/Cl^-$ co-transporter are either activated by or require intra- or extracellular K^+ (for reviews see refs. 27-29). Thus, reduction in K_o is likely to affect cell volume maintenance under isotonic conditions and maintenance under anisotonic conditions. At present there is no information available that could link these effects of K_o on membrane ionic transport and the synthesis of urea or other organic osmolyte. Finally, removal of K_o can produce cellular alkalinization in kidney cells (30). Enzymatic activity is susceptible to changes in pH. Thus, it is possible that changes in intracellular pH could affect the synthesis of urea or other organic osmolytes.

5.5. Effect of Ouabain on cell volume and intracellular osmolyte content

The results presented here clearly show involvement of the Na^+/K^+ ATPase in regulating isotonic and anisotonic volume in tsA201a cells. This result is consistent with numerous observations in many cell types including mammalian renal epithelial cells (31, 32). Table 3 shows an unexpected increase in the intracellular content of K^+ in response to addition of ouabain under isotonic conditions. One explanation for this result is the presence of a ouabain-insensitive Na^+/K^+ exchange in tsA201a cells. This possibility remains to be tested.

6. CONCLUDING REMARKS

The results here presented show that exposure of human embryonic kidney tsA201a cells grown in culture media to glucose-free Ringer solution significantly affects the intracellular osmolyte composition and cell volume. The lack of glucose in the Ringer media likely produced a partial and transient inhibition of the Na^+/K^+ ATPase leading to net gain of K^+, Cl^- and cell volume. Recovery of ATP levels would result in the recovery of Na^+/K^+ ATPase activity and intracellular ionic content. Interestingly, low K_o conditions resulted in a net gain of an unidentified intracellular osmolyte under iso and hypotonic conditions, and led to cell swelling. The synthesis of urea, at the expense of Glu, contributes to this swelling. The underlying mechanisms linking low K_o and urea synthesis require elucidation. These results should be taken in consideration for interpreting results of investigations in which culture cells are exposed to glucose free saline solutions.

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Key Words: cell volume regulation, regulatory volume decrease, intracellular osmolyte content, isotonic, hypotonic, Na^+/K^+ ATPase, cultured cells

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