Am J Physiol Cell Physiol 286: C1399–C1409, 2004.
First published January 21, 2004; 10.1152/ajpcell.00198.2003.

Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels

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Submitted 14 May 2003; accepted in final form 15 January 2004

Ordaz, B., L. Vaca, R. Franco, and H. Pasantes-Morales. Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels. *Am J Physiol Cell Physiol* 286: C1399–C1409, 2004. First published January 21, 2004; 10.1152/ajpcell.00198.2003.—Volume changes and whole cell ionic currents activated by gradual osmolarity reductions (GOR) of 1.8 mosM/min were characterized in C6 glioma cells. Cells swell less in GOR than after sudden osmolarity reductions (SOR), the extent of swelling being partly Ca^{2+} dependent. In nominally Ca^{2+}-free conditions, GOR activated predominantly whole cell outward currents. Cells depolarized from the initial −79 mV to a steady state of −54 mV reached at 18% osmolarity reduction [hyposmolarity of −18% (H-18%)]. Recordings of Cl^- and K^+ currents showed activation at H-3% of an outwardly rectifying Cl^- current, with conductance of 1.6 nS, sensitive to niflumic acid and 5-nitro-2-(3-phenylpropylamino)benzoic acid, followed at H-18% by an outwardly rectifying K^+ current with conductance of 4.1 nS, inhibited by clofilium but insensitive to the typical K^+ channel blockers. With 200 nM Ca^{2+} in the patch pipette, whole cell currents activated at H-3% and at H-13% cells depolarized from −77 to −63 mV. A K^+ current activated at H-1%, showing a rapid increase in conductance, suppressed by charybdotoxin and insensitive to clofilium. These results show the operation of two different K^+ channels in response to GOR in the same cell type, activated by Ca^{2+} and osmolarity and with different osmolarity activation thresholds. Taurine and glutamate efflux, monitored by labeled tracers, showed delayed osmolarity thresholds of H-39 and H-33%, respectively. This observation clearly separates the Cl^- and amino acid osmosensitive pathways. The delayed amino acid efflux may contribute to counteract swelling at more stringent osmolarity reductions.

volume regulation; taurine; hyposmolarity; isovolumetric regulation; regulatory volume decrease

MOST CELLS RESPOND TO DECREASES in external osmolarity by rapid cell swelling followed by an active process of volume regulation accomplished by extrusion of intracellular osmotically active solutes (5). This process, known as regulatory volume decrease (RVD), has been examined extensively in a variety of cells, including brain cells (14). In most studies, however, cells are exposed to abrupt and large osmolarity reductions, which seldom occur in vivo, even in conditions of acute hyponatremia. An approach closer to the physiological situation is that devised by Lohr and Grantham (7), in which renal cells are exposed to small and gradual changes in external osmolarity. Under these conditions, cells are able to maintain a constant volume over a wide range of tonicities if the rate of change in osmolarity is <2.2 mosM/min. This response was named "isovolumetric regulation" (IVR), a term reflecting the active nature of this process, because the unchanged volume seems not to be due to the absence of swelling but to a continuous volume adjustment accomplished by the efflux of intracellular osmolytes. This notion is based on the shrinkage observed in cells returned to an isosmotic medium, which, because of the decrease of intracellular osmolytes, is now hyperosmotic with respect to the intracellular medium (6, 7). The ubiquity of IVR as a mechanism of cell volume control has not been extensively explored. IVR has been described so far in two types of renal cells (6, 7, 22), in cerebellar granule neurons (21), and in a subset of hippocampal neurons (24). Other cell types such as C6 cells and cultured myocytes show IVR only over very short ranges of osmolarity and/or when the change in osmolarity is very small (8, 18). It cannot be ruled out, however, that gradual exposure to hyposmolarity results in better mechanisms of cell volume control, i.e., less swelling or more rapid volume recuperation. The contribution of the different osmolytes and the nature of the translocation pathways activated in volume regulation in the gradual osmolarity reduction (GOR) condition or during IVR are not well known. K^+ and taurine efflux occurs during GOR (18, 21, 22), whereas in trout erythrocytes, in which GOR leads to continuous swelling, there is no evidence of rapid taurine or K^+ loss (1). All studies so far of the K^+ and Cl^- efflux on GOR have been carried out by monitoring ionic fluxes and final changes in ion intracellular content, but no electrophysiological studies have been carried out. In the present work we examined the changes in cell volume during GOR in glioma C6 cells and characterized the ionic currents activated under these conditions. Efflux of taurine and glutamate, monitored by the release of radioactive tracers, was also examined.

MATERIALS AND METHODS

Cells. The glioma C6 cell line (American Type Culture Collection, Rockville, MD) was grown in Eagle's medium (GIBCO Life Technologies) supplemented with 15% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture dishes were kept at 37°C in a humidified atmosphere (5% CO_2-95% O_2).

Solutions and gradually diluted solutions. The isosmotic medium contained (in mM) 135 NaCl, 1.0 $CaCl_2$, 1.17 $MgSO_4$, 4.7 KCl, 5 dextrose, and 10 HEPES (300 mosM, pH 7.4). The gradually diluted solutions (−1.8 mosM/min) were obtained by the procedure described

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by Lohr and Grantham (7) and by Van Driessche et al. (22). Briefly, the system consisted of two identical glass cylinders interconnected at their bases by a tube with an interrupting valve. The first container was filled with isosmotic medium and the second with the same volume of hyposmotic medium (50%). Media were kept at 39°C by placing the cylinders on a temperature-controlled hot plate and stirring. Superfusion medium was pulled from the first container with a polystaltic pump, allowing the hyposmotic medium to begin to enter this cylinder, mixing gradually and continuously with the isosmotic medium. In this way, a solution with linear dilution of −1.8 mosM/min was produced, which at the end of the experiment (82 min later) reached 150 mosM (50% hyposmotic). The linearity of the diluted solutions and the osmolarity of all solutions were verified in a freezing-point osmometer (Osmete A, Precision Systems). Reductions in osmolarity are indicated throughout the manuscript as hyposmolarity of negative percent change in each case. For instance, a 3% reduction is expressed as H-3%.

Estimation of changes in cell volume. Cell volume measurements were performed by estimating the changes in relative cell volume with a large-angle light-scattering system (12, 16). C6 cells were cultured on rectangular cover glasses (10 × 50 mm) at 90% confluence at the time of experiments. Cover glasses were placed at a 50° angle relative to the excitation light in a cuvette filled with isosmotic medium (300 mosM) in an Aminco-Bowman Series 2 luminescence spectrometer. Cells were excited at 585 nm with an argon arc lamp, and emission was detected at the same wavelength. Data are expressed as the inverse of the emission signal, because light intensity inversely correlates with cell volume. Cell volume changes were calculated according to the equation l_o/l_t, where l_o is isosmotic emission signal average and l_t is emission signal at time *t*.

Electrophysiological recordings. Currents were monitored with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). All recordings were performed at 35°C with a diluted solution-generating system. Whole cell membrane currents were measured by using ruptured patches. The time course of whole cell currents was obtained by following voltage protocols of holding potential of −70 mV to potentials ranging from −120 to +100 mV in 20-mV increments for 350 ms. Electrophysiological recording was carried out on cells seeded on 35-mm petri dishes, as described above. Once the whole cell configuration was obtained, cells were perfused for 5 min with isosmotic solution before the gradual dilution in osmolarity was initiated. The voltage protocol was carried out every 5 min for the duration of the experiment while the dilution gradient continued. This manipulation did not interfere with the whole cell recordings, because the entire whole cell voltage protocol lasted only 12 s. The voltage protocol was carried out fast enough to be concluded while the osmolarity reduction was only 0.36 mosM (H-0.2%) during the 12-s period. This protocol was repeated every 5 min for the duration of the experiments, as indicated. Patch electrodes were prepared from 1.5-mm OD, 1.5-mm ID borosilicate glass (World Precision Instru-

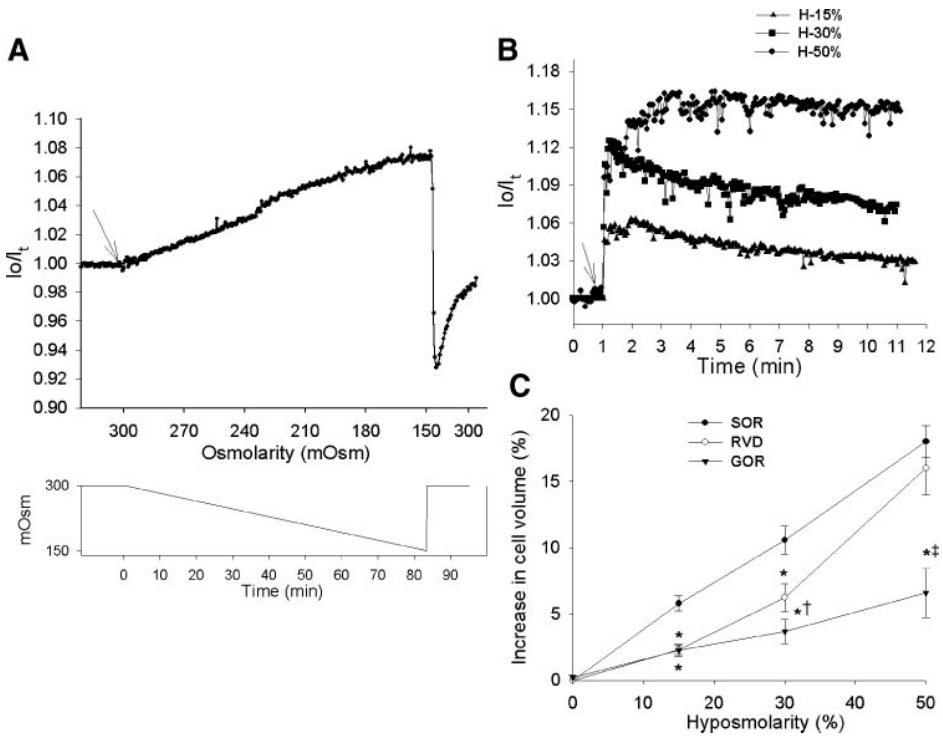

Fig. 1. Cell volume changes of C6 cells exposed to sudden (SOR) or gradual osmolarity reduction (GOR). Estimation of relative cell volume change (l_o/l_t, where l_o is isosmotic emission signal average and l_t is emission signal at time *t*) was performed with large-angle light scattering as described in materials and methods. Data are given as the inverse of the emission, because emitted light intensity inversely correlates with cell volume. *A*: *top*, a representative experiment of cell volume changes elicited by GOR. Cells were superfused (rate 2.0 ml/min at 37°C) for 10 min with isosmotic medium. At the indicated time (arrow), cells were exposed to the gradually diluted solution (−1.8 mosM/min) over 83 min (*bottom*), at which time the osmolarity had decreased 50% (150 mosM). At the end of the experiment, cells were returned to the isosmotic condition (300 mosM). *B*: representative traces of cell volume changes elicited by SOR. Cells were superfused with isosmotic medium, and then (at the arrow) sudden reduction in osmolarity was made by isosmotic medium dilution with distilled water until reaching −15% (H-15%), −30% (H-30%), and −50% (H-50%) hyposmotic medium. Cell volume changes were followed 10 min after hyposmotic stimulus. *C*: cell volume in C6 at H-15%, H-30%, and H-50% hyposmotic medium, reached in conditions of SOR (maximal swelling), after cell volume regulation in these conditions [regulatory volume decrease (RVD)], or when the same osmolarities were reached during exposure to GOR. Points are means ± SE; $n = 4$–10. *$P < 0.001$ with respect to SOR; †$P < 0.05$, ‡$P < 0.001$ with respect to RVD.

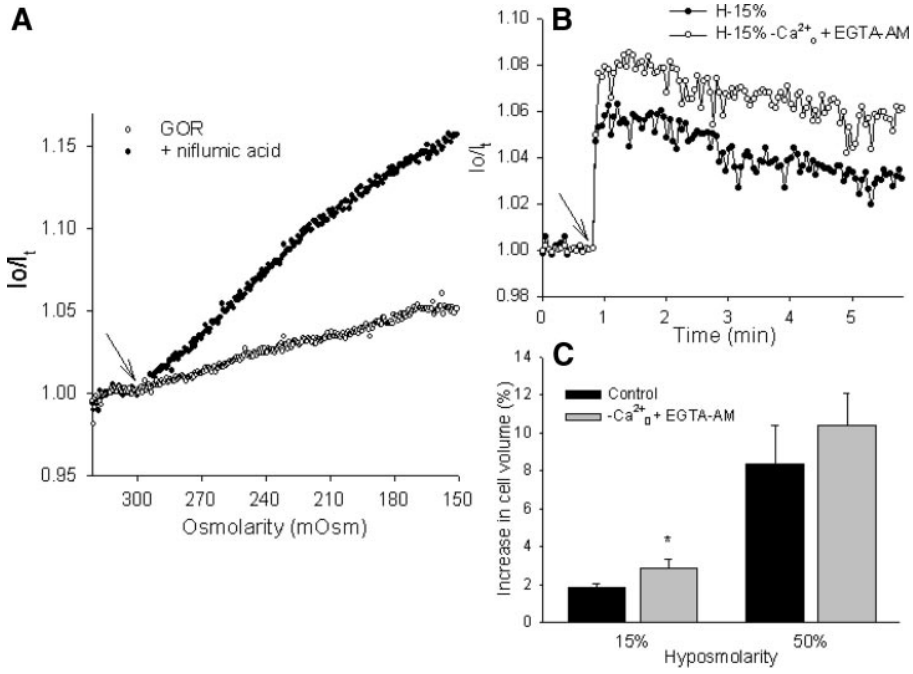

Fig. 2. Ca^{2+} dependence and effect of niflumic acid on cell volume changes of C6 cells exposed to SOR or GOR. Estimation and expression of relative cell volume change (l_o/l_t) as in Fig. 1. *A*: a representative experiment of cell volume changes elicited by GOR in the presence (●) or absence (○) of 600 μM niflumic acid. Niflumic acid was present throughout the experiment. *B*: representative traces of cell volume changes elicited by 15% SOR in the presence or absence of intracellular Ca^{2+}. Cells were superfused with isosmotic medium, and then (arrow) medium was made −15% hyposmotic (●) by addition of distilled water. Cell volume changes were followed 10 min after hyposmotic stimulus. ○, Cells were preloaded with 50 μM EGTA-AM 30 min before the experiment, and isosmotic medium was made nominally Ca^{2+} free by addition of 0.5 mM EGTA. *C*: effect of Ca^{2+}-free conditions (as described in *B*) on cell volume increase attained during GOR. Bars represent cell volume increase (% over isosmotic condition) reached at H-15% and H-50%. Solid bars, volume changes in the presence of Ca^{2+}; shaded bars, volume changes in cells in Ca^{2+}-free conditions as in *B*. Results are means ± SE; $n = 4$. *$P < 0.05$ vs. control.

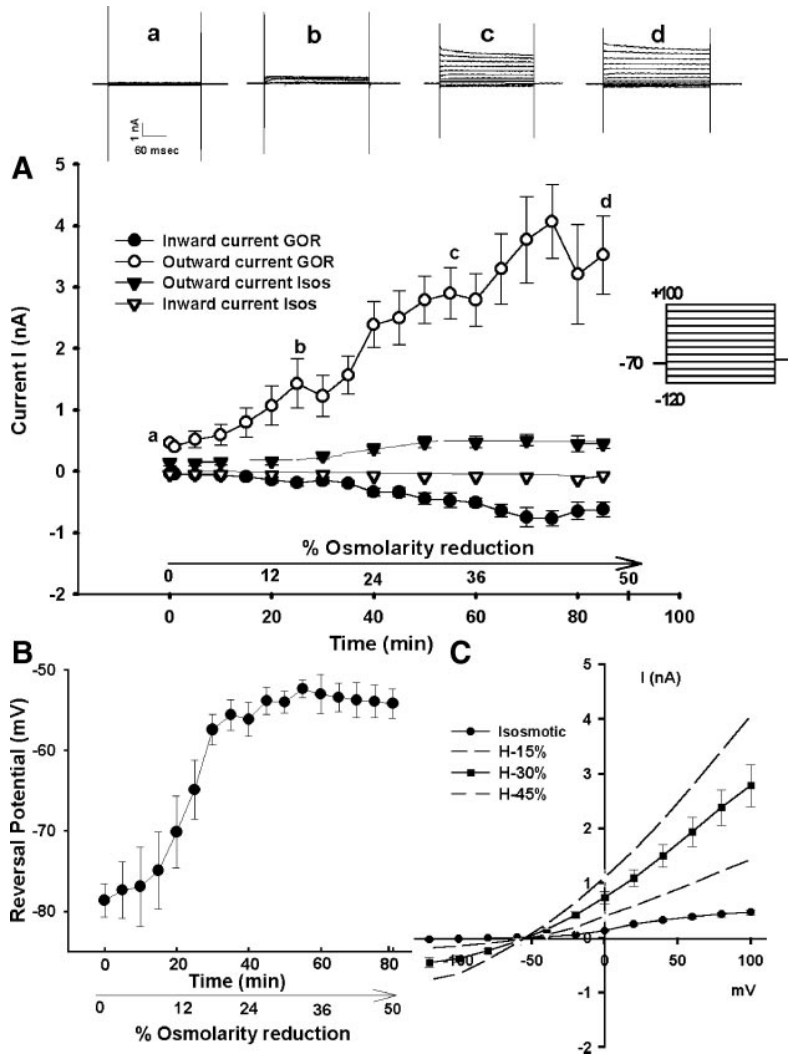

Fig. 3. Whole cell currents in C6 glioma cells induced by GOR in nominally intracellular Ca^{2+}-free solution. *A*: membrane currents (*I*) were measured in the whole cell recording mode of the patch-clamp technique with a holding potential of −70 mV. After GOR ($n = 17$): ●, inward currents; ○, outward currents. In isosmotic medium ($n = 4$): ▽, inward currents; ▼; outward currents. *Insets a–d* show the activation of currents at the time and osmolarity indicated by letters in the plot. *B*: change in the membrane potential (initial −79 ± 2 mV) through the effect of GOR. *C*: current-voltage (*I-V*) relationship in cells in isosmotic medium (●) and at the indicated osmolarity reductions.

ments), and the resistance was between 3 and 5 MΩ when filled with the pipette solution. The recorded signal was filtered at 10 kHz with a low-pass Bessel filter and transferred to a computer with the Digidata 1200 interface (Axon Instruments). Whole cell currents were analyzed with pCLAMP6 software (Axon Instruments).

The standard pipette solution contained (in mM) 110 K^+ aspartate, 30 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, and 5 Mg ATP, pH 7.4 adjusted with KOH (300 ± 3 mosM). In the anion-substitution experiments, K-aspartate and KCl were fully replaced by 140 mM CsCl. In experiments to isolate K^+ currents, aspartate was the major anion in the pipette and only 10 mM Cl^- was present in the intracellular solution.

Efflux of amino acids. Cells were incubated for 60 min in culture medium containing the labeled amino acids D-[^{3}H]aspartate (as a tracer for glutamate) and [^{3}H]taurine (0.5 μCi/ml). After the loading period, cultured dishes were superfused for 20 min with isosmotic medium, and the superfusion continued either with isosmotic medium or with the gradually diluted solutions (−1.8 mosM/min) during the time indicated. Samples were collected every minute, and radioactivity in samples and in cells after the experiment was quantified by scintillation. Labeled amino acid fluxes were calculated as efflux rate constants, i.e., the amount of radioactivity released in any given fraction divided by the total amount of label present in cells at that moment.

RESULTS

Cell volume changes after exposure to sudden decrease in osmolarity and GOR. C6 cells exposed to a sudden decrease in osmolarity (SOR) of H-15% and H-30% exhibited the typical RVD, as reported previously by Lohr and Yohe (8). As shown in Fig. 1*B*, the volume correction was more efficient at small osmolarity reductions. Cells exposed to GOR (−1.8 mosM/min) exhibited a progressive increase in cell volume, which continued over the time of the experiment, up to 83 min, when the osmolarity was decreased 50% (Fig. 1*A*). These results indicate the absence of IVR in the GOR model in C6 cells. However, cells swelled significantly less than those exposed to SOR at the same osmolarities (H-15%, H-30%, and H-50%), as shown in Fig. 1*C*. Moreover, at H-30%, cell volume in the GOR condition was still significantly lower than that in SOR after regulation, ~10 min later (Fig. 1*B*). At H-50%, no RVD was observed after SOR (Fig. 1*B*), but during GOR cells were notably less swollen (Fig. 1*C*). These results indicate a more rapid and efficient mechanism of volume regulation in GOR. Returning cells to isosmotic medium after GOR resulted in immediate decrease in cell volume to values lower than the

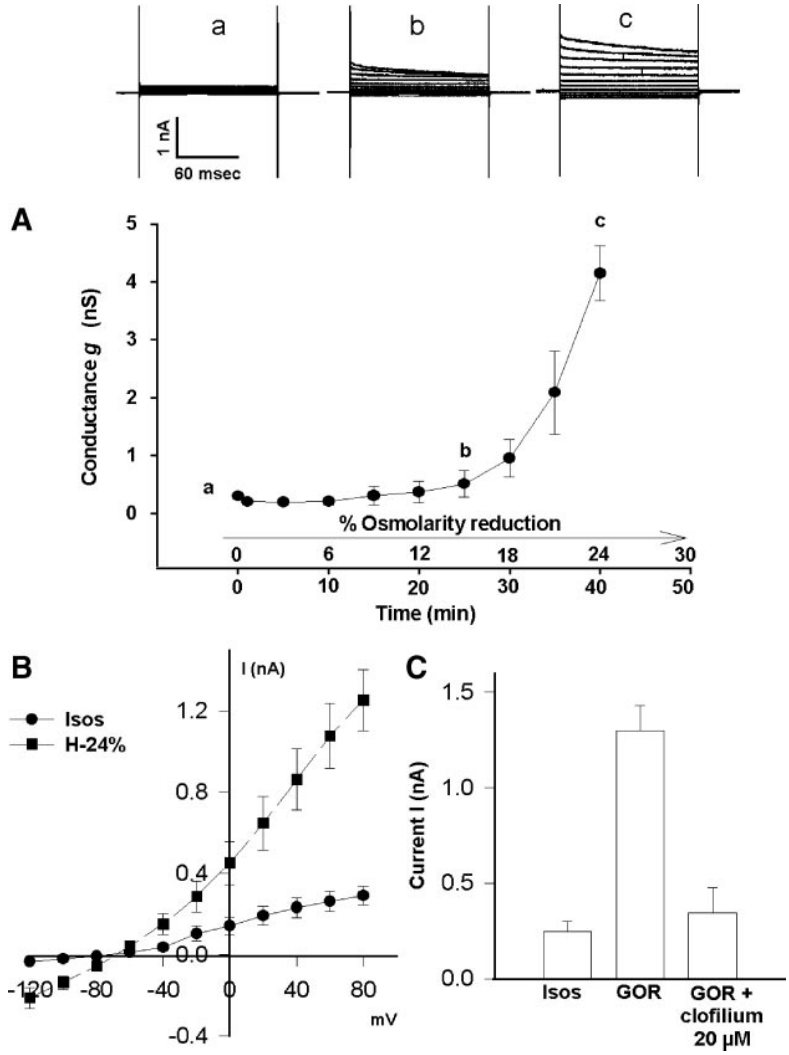

Fig. 4. K^+ conductance (*g*) elicited by GOR in intracellular Ca^{2+}-free conditions. The pipette was filled with 130 mM K-aspartate. The holding potential was −70 mV (n = 6). *A*: *insets a–c* show the currents obtained at the osmolarity indicated by letters in the plot. *B*: *I-V* relationships for K^+ currents (n = 6) under isosmotic conditions and at H-24%. *C*: effect of clofilium on the GOR-elicited K^+ current. Bars represent means ± SE (n = 5) of the current measured at +80 mV.

initial values, indicative of loss of intracellular osmolytes (Fig. 1*A*). Treatment with 600 μM niflumic acid increased cell volume to the same extent as in SOR at all osmolarities (Fig. 2*A*), further supporting the operation of an active mechanism of cell volume regulation. In cells incubated with EGTA-AM to reduce intracellular Ca^{2+}, a sudden osmolarity decrease of 15% resulted in higher cell swelling and less efficient RVD compared with cells not treated with EGTA-AM (Fig. 2*B*). In Ca^{2+}-free conditions, a significant increment in cell volume during GOR was observed at external osmolarity of H-15% but not at H-50% (Fig. 2*C*).

Studies in jejunal villus epithelial cells showed the operation of two mechanisms for cell volume regulation in response to small or large changes in cell volume, elicited either by nutrient uptake (9) or by hyposmolarity (10). The main differences were found in the volume regulatory response depending on the extent of cell swelling regarding Ca^{2+} dependence and sensitivity to charybdotoxin (10), thus suggesting the involvement of different K^+ channel types. To investigate a possible difference in whole cell currents depending on the extent of cell swelling and the influence of Ca^{2+}, experiments were carried out in either the presence or the absence of Ca^{2+} in the intracellular solution.

Whole cell currents activated during GOR in nominally Ca^{2+}-free intracellular solution. We first explored the currents activated by GOR in the patch-clamp whole cell configuration with 5 mM EGTA in the patch pipette. Figure 3*A* illustrates the activation and progressive increase with time of whole cell currents in this condition. As indicated in Fig. 3*A*, the outward current activated by changes in osmolarity was more prominent than the inward current, even though the time course was similar in both cases. No whole currents were activated when cells were maintained in isosmotic medium for the same time as those exposed to GOR (Fig. 3*A*). The membrane potential progressively depolarized within the first 30 min of the osmolarity decrease, then reached a steady-state level of about −54 mV at H-18% (Fig. 3*B*). Figure 3*C* illustrates the current-voltage (*I-V*) relationship for control (isosmotic) and 25, 50, and 75 min after GOR onset (H-15%, H-30%, and H-45%, respectively).

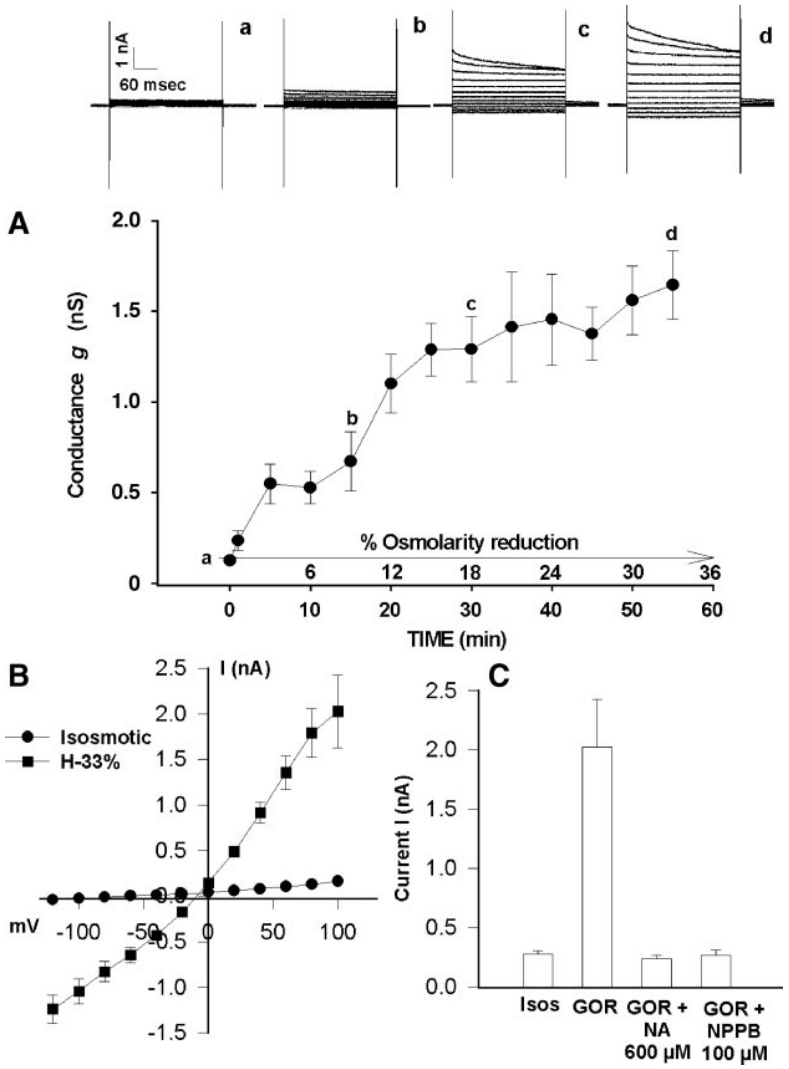

Fig. 5. *A*: Cl^- conductance elicited by GOR in intracellular Ca^{2+}-free conditions. The pipette was filled with 140 mM CsCl, the holding potential was −70 mV ($n = 6$). *Insets a–d* show the current activation at the osmolarity indicated by letters in the plot. *B*: *I-V* curves in cells in isosmotic medium and at H-33%. *C*: effect of the Cl^- channel blockers niflumic acid (NA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on the GOR-elicited Cl^- current. The blockers were present in the solutions used to generate the media with gradually decreased osmolarities. Bars represent means ± SE (n = 4) of the current measured at +100 mV.

Using ionic replacements, we separated Cl^- and K^+ currents to study the relative contribution of each to the whole cell currents activated by GOR. K-current was examined by using 130 mM K-aspartate in the patch pipette to decrease the contribution of Cl^-. Figure 4*A* illustrates the time course of K^+ current activation after GOR. The activation of the K^+ conductance was delayed compared with the activation of the whole cell currents observed in Fig. 3*A*, because a significant increment in K^+ conductance was observed only after 30 min of GOR onset (H-18%). After this delay period, K^+ conductance activated completely within the next 15 min, to reach a maximum in the following 10 min (H-24%) (Fig. 4*A*). Figure 4*B* compares the *I-V* relationships in control conditions (isosmotic) and at the maximal current observed after GOR onset (H-24%). The cell membrane potential was not depolarized under these experimental conditions. The reversal potential observed at H-24% was −70 mV, a value close to the expected reversal potential for a K^+ electrode calculated from the Nernst equation (−84 mV). The K^+ current was inhibited in the presence of extracellular 20 μM clofilium (Fig. 4*C*) but was insensitive to 5 mM TEA, 2 mM barium, 1 mM 4-aminopyridine (4-AP), and 600 μM quinidine (not shown).

We next performed similar experiments but with solutions that minimize the contribution of K^+ channels to whole cell currents (140 mM CsCl in the patch pipette). Figure 5*A* illustrates the Cl^- conductance elicited by GOR. The Cl^- conductance activated early during the gradual hyposmotic reduction. Significant increments in conductance were detected as early as 2 min after GOR initiation (H-3%). The Cl^- conductance increased continuously with progressive osmolarity reduction.

Figure 5*B* illustrates the *I-V* relationship for the Cl^- current under isosmotic conditions (control) and at the maximal current observed after GOR onset (H-33%). In this case, the Cl^- current showed a reversal potential near 0 mV, a value expected when using symmetrical Cl^- concentrations. The Cl^- current was completely inhibited in the presence of extracellular 600 μM niflumic acid and 100 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Fig. 5*C*).

All of these results show that the initial increments of whole cell currents induced by GOR in the intracellular Ca^{2+}-free condition result from the activation of a Cl^- conductance, followed minutes later by the activation of a K^+-selective conductance. The maximal conductance values obtained were 1.6 ± 0.18 nS (n = 7) for Cl^- and 4.1 ± 0.47 nS (n = 6) for K^+.

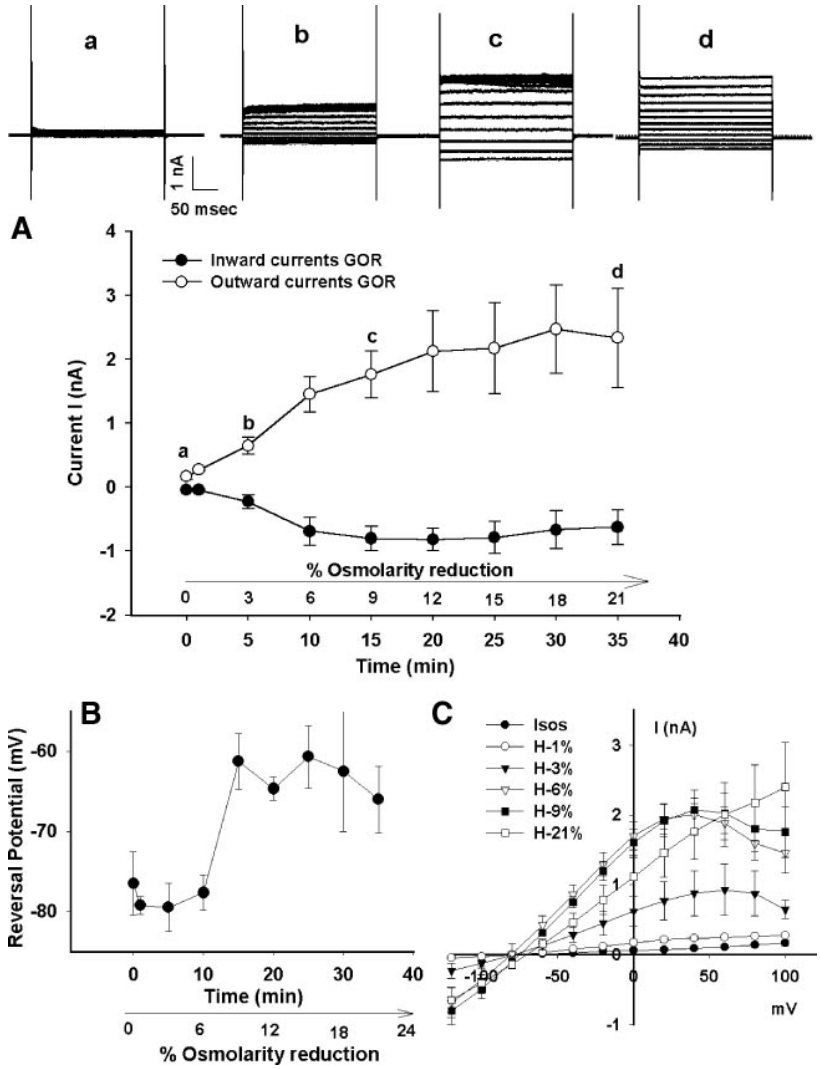

Fig. 6. Whole cell currents activated by GOR in the presence of intracellular Ca^{2+}. *A*: membrane currents measured as in Fig. 3 but with 200 nM Ca^{2+} in the patch pipette (n = 6). *Insets a–d* show the activation of currents at the time and osmolarity marked by letters in the plot. *B*: change in the membrane potential (initial −78 ± 3 mV) through the effect of GOR. *C*: *I-V* relationship in cells in isosmotic medium and at osmolarity reductions as indicated.

Whole cell currents activated by GOR in presence of intracellular Ca^{2+}. All of the above described recordings were performed in nominally intracellular Ca^{2+}-free conditions, and therefore all observed currents occurred through Ca^{2+}-independent channels. To investigate whether Ca^{2+}-dependent currents are elicited during the first minutes of osmolarity reduction and can contribute to cell volume control in cells showing small volume increase, whole cell currents were recorded in the presence of 200 nM Ca^{2+} in the patch pipette. As indicated in Fig. 6*A*, the change in osmolarity evoked inward and outward currents with similar time courses, the outward current being more prominent than the inward current at all osmolarities. Activation of these currents occurs at changes in osmolarity as low as H-3%. Figure 6*B* illustrates the change in membrane potential during osmolarity reductions. The potential remained hyperpolarized during the first 10 min (H-6%). After H-6%, the membrane potential depolarized to reach approximately −63 mV at H-9% and remained constant for the duration of the experiment. Figure 6*C* shows the *I-V* relationship for control (isosmotic) and at H-1%, H-3%, H-6%, H-9%, and H-21%. From H-6%, the maximal amount of current was reached. It is noteworthy that the total current at all points explored was lower in the Ca^{2+}-free condition than in the presence of Ca^{2+} (Figs. 3 and 6), indicating the superposition of Ca^{2+}-activated conductances.

We next examined the effect of GOR on K^+ currents in the presence of 200 nM Ca^{2+} in the patch pipette. K^+ conductance activated as early as H-1% and increased rapidly to reach a maximal value at H-3%, remaining at this value for the duration of the experiment (Fig. 7*A*). The *I-V* curves in Fig. 7*B* show a reversal potential of −76 mV, near the expected reversal potential for a K^+ electrode. From H-3% to H-12%, the K^+ current was reduced at potentials more positive than +60 mV. At larger osmolarity reductions (above H-15%) the *I-V* relationship was linear throughout the voltages explored. We did not explore further the reason for the current inactivation observed at very positive potentials.

These experiments showed in Ca^{2+}-free conditions an early activation of Cl^-, but not of K^+ currents, until osmolarity was reduced to H-18%, whereas in the presence of Ca^{2+} both Cl^- and K^+ currents activated as early as H-3%, thus suggesting a different osmolarity threshold for the K^+ current in the absence of Ca^{2+}. To confirm these differences, we examined K^+ and Cl^- currents in cells after sudden exposure to H-15%. In Ca^{2+}-free medium the osmolarity change activated only a Cl^- current, abolished by NPPB (Fig. 8*A*), whereas no K^+ current could be detected (Fig. 8*B*). In contrast, in the presence of 200 nM intracellular Ca^{2+}, H-15% evoked a K^+ current with an *I-V* curve similar to that observed at the same osmolarity reached by GOR, i.e., about the same maximal value and the same inactivation at positive voltages (Fig. 9*A*). This current was insensitive to clofilium (up to 100 μM) but was abolished by 100 nM charybdotoxin (Fig. 9*B*). At H-30% in Ca^{2+}-free conditions, a K^+ current activates that is abolished by clofilium and insensitive to charybdotoxin (not shown). These results clearly demonstrate the presence of two K^+ conductances with different pharmacological properties, showing distinguishable osmolarity thresholds for activation. The Ca^{2+}-activated K^+ channel contributes to cell volume regulation during small osmolarity reductions, whereas the clofilium-sensitive K^+ channel activates after more severe reductions in osmolarity. These two K^+ currents are accompanied by an early activated Cl^- channel.

Amino acid efflux during GOR. Figure 10 shows the efflux from C6 cells of [^{3}H]taurine and D-[^{3}H]aspartate (as tracer or glutamate) in response to GOR. The amino acid efflux in this condition was compared at each point with the release from cells bathed with isosmotic medium. The efflux of taurine and D-aspartate showed a delayed activation threshold at external osmolarity reductions of H-39% and H-33%, respectively. The

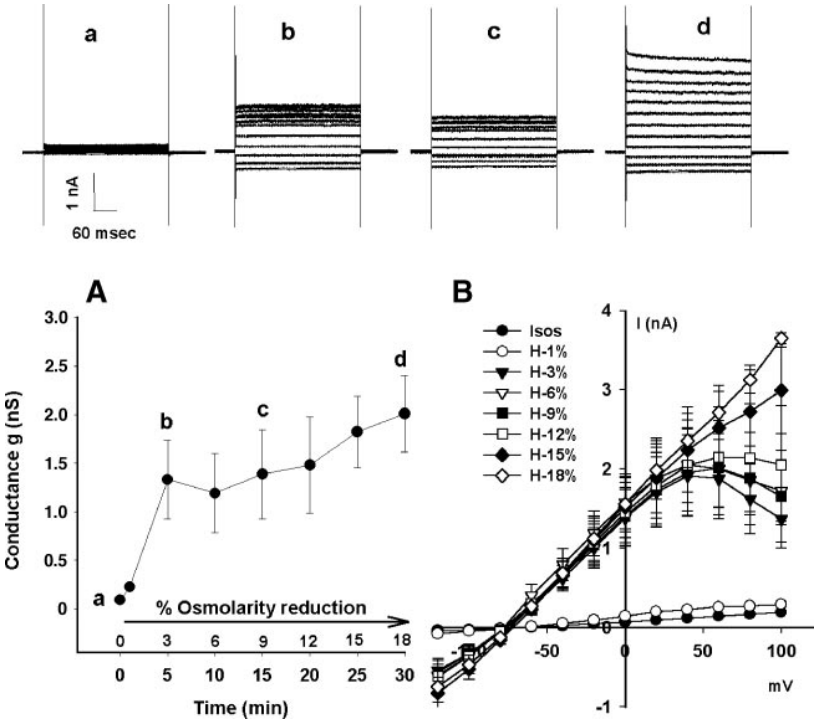

Fig. 7. *A*: K^+ conductance elicited by GOR in the presence of intracellular Ca^{2+}. The pipette was filled with 130 mM K-aspartate and contained 200 nM Ca^{2+}. The holding potential was −70 mV (n = 5). *Insets a–d* show the currents obtained at the osmolarity indicated by letters in the plot. *B*: *I-V* relationships for K^+ currents in cells in isosmotic medium and at osmolarity reductions as indicated (n = 5).

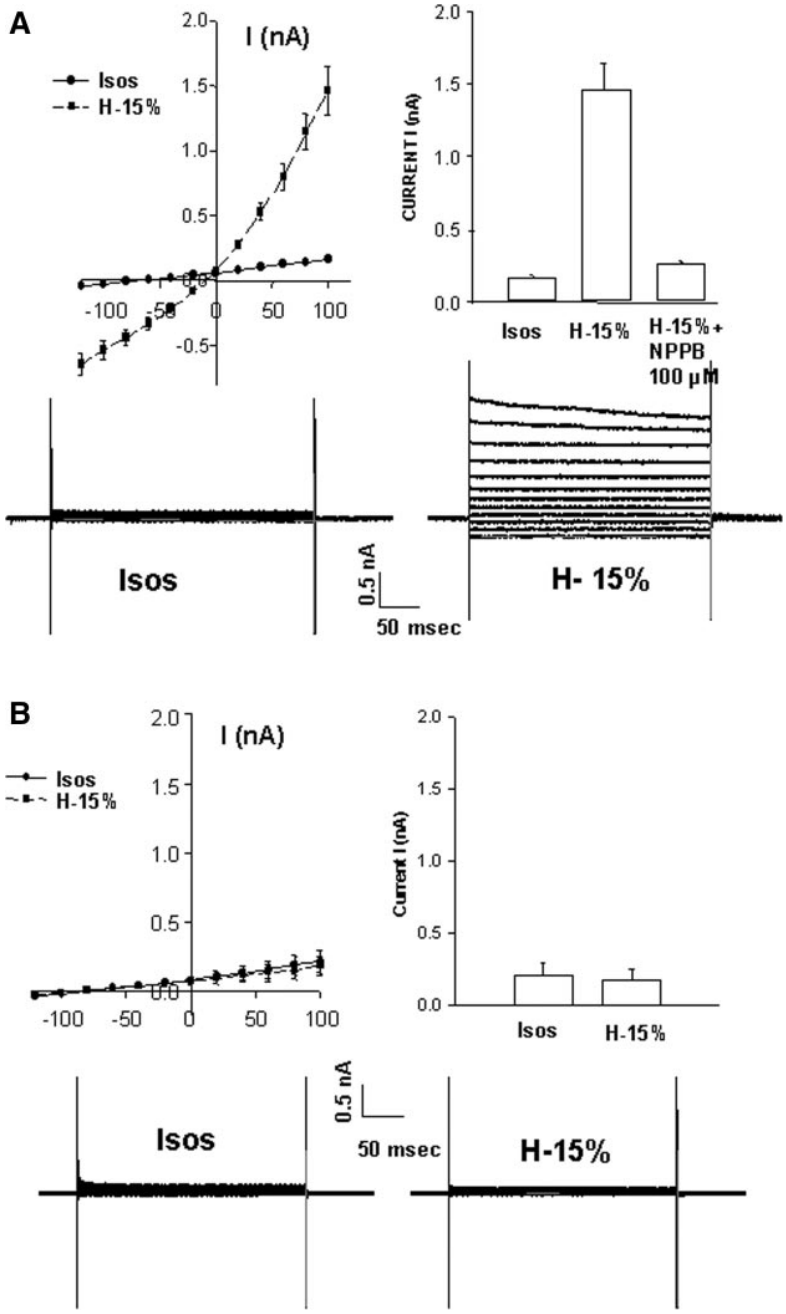

Fig. 8. K^+ and Cl^- currents activated by a sudden decrease in osmolarity of 15% in Ca^{2+}-free conditions. Cl^- (*A*) and K^+ (*B*) currents were measured as in Fig. 3, with ionic replacements and effect of Cl channel blockers as in Figs. 4 and 5; $n = 6$.

amino acid efflux in isosmotic and GOR conditions was followed from *minutes 1* to *82*, but because no difference was observed during the first 50 min, only the time course of efflux from *minute 40* to the end is shown (Fig. 10).

DISCUSSION

In the present study we compared the changes in cell volume evoked by sudden or gradual reductions in osmolarity in C6 cells. We found that cell swelling after GOR was consistently lower than that attained after SOR at the same osmolarities. Moreover, at H-15%, cell volume after GOR was still significantly lower than after 10 min of RVD subsequent to a sudden hyposmotic stimulus. Furthermore, at H-50%, when cells do not exhibit RVD after SOR, cell volume is notably lower. Thus the mechanisms activated by GOR in our conditions, although not sufficient to fully prevent swelling, can substantially reduce it. According to the study of Lohr and Yohe (8) in C6 cells, swelling is prevented only when the osmolarity decrease is of 0.3–0.4 mosM/min and the osmolarity reductions do not exceed 20%. Therefore, C6 cells possess mechanisms to counteract hyposmotic swelling, which, however, appear less efficient than those present in renal cells, A6 cells, and some neurons, which are able to maintain constant volume in the face of osmolarity reductions similar to those used in the present study (7, 21, 24). This may be due to the contribution of amino acids and possibly other organic osmolytes, as discussed below.

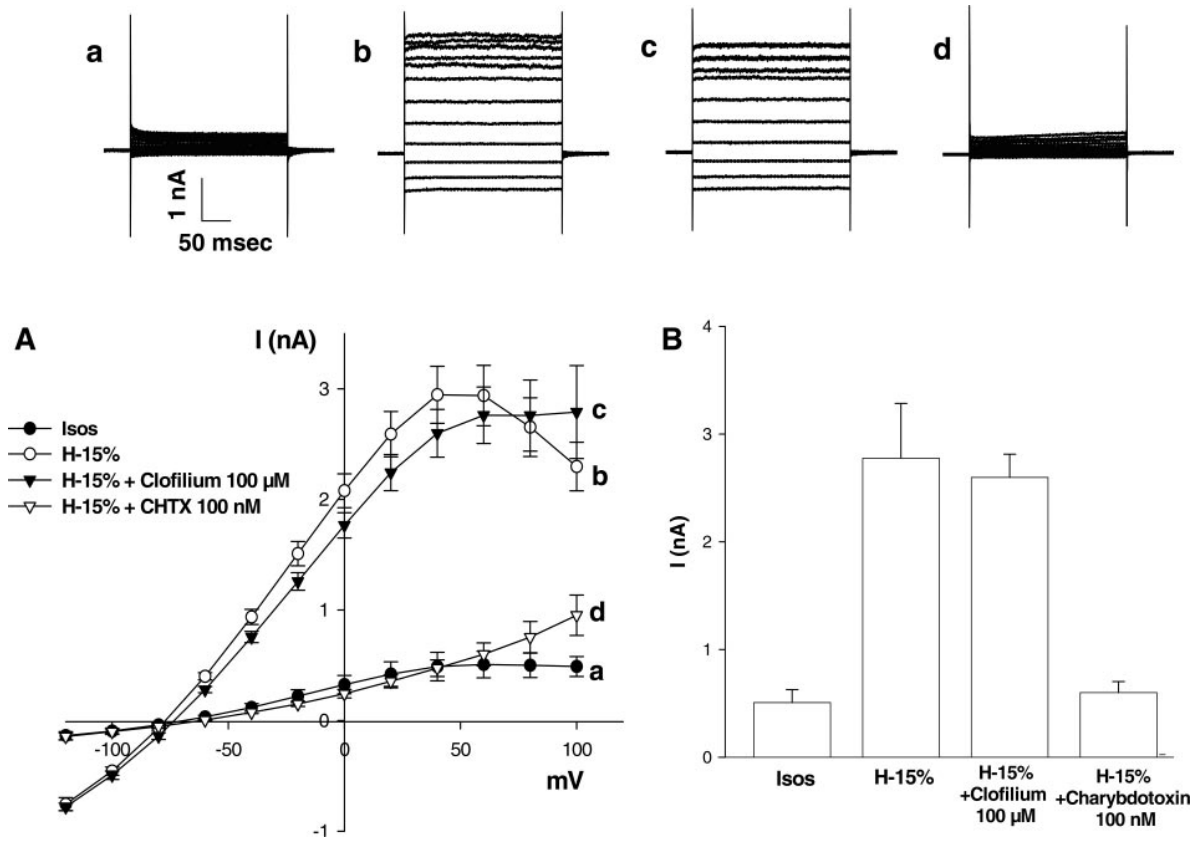

Fig. 9. K^+ current activated by SOR of H-15% in the presence of intracellular Ca^{2+}. The pipette was filled with 130 mM K-aspartate and contained 200 nM Ca^{2+}. The holding potential was −70 mV. *A*: *I-V* relationships for K^+ currents in cells in isosmotic medium and at H-15%, +100 μM clofilium, and 100 nM charybdotoxin (CHTX). *Insets a–d* show the currents obtained at isosmolarity and osmolarity of H-15% indicated by letters in the plot. *B*: effect of clofilium and charybdotoxin on the SOR-elicited K^+ current. Bars represent means ± SE (n = 5) of the current measured at +60 mV.

In both the sudden and the gradual model, at small reductions in osmolarity and less swelling cell volume control was more efficient in the presence of Ca^{2+}, suggesting a Ca^{2+}-dependent element in the volume-regulatory mechanism in this condition. The swelling-activated Cl^- channels, as well as the osmosensitive amino acid fluxes, are largely Ca^{2+} independent (15), thus making the K^+ efflux pathway the likely candidate to be influenced by Ca^{2+}. Volume-activated K^+ channels show marked differences with respect to Ca^{2+} dependence, depending on the cell type (reviewed in Ref. 15). In epithelial cells hyposmolarity activates Ca^{2+}-dependent K^+ channels, whereas in most nonepithelial cells the volume-sensitive K^+ channels are Ca^{2+} independent (15). Interestingly, in the present work we found the operation of both Ca^{2+}-dependent and Ca^{2+}-independent K^+ channels in the same cell type, which activate at different times and by different signals. For the Ca^{2+}-dependent channel, the activation signal may not primarily be the change in osmolarity but the increase in intracellular Ca^{2+} concurrent with swelling, known to occur in most cells (11), whereas the Ca^{2+}-independent channel may respond to the osmolarity reduction or the magnitude of the volume change. The two types of K^+ channels also differ in their pharmacological profile. The Ca^{2+}-dependent channel is sensitive to charybdotoxin and insensitive to clofilium, whereas the opposite is found for the Ca^{2+}-independent channel. This coincidence of two different types of K^+ channels activated by swelling in the same cell has not been previously reported, to our knowledge, but was first suggested by an interesting study in jejunal villus epithelial cells showing that volume regulation at small volume increases is Ca^{2+}- and charybdotoxin sensitive, although it turns to be Ca^{2+} independent and charybdotoxin insensitive at larger cell volume increases (10).

The relative contribution of the Ca^{2+}-dependent and the Ca^{2+}-independent K^+ channel to the total currents evoked by GOR can be estimated by the current magnitude as well as by the changes in membrane potential observed in the presence or absence of intracellular Ca^{2+}. In the Ca^{2+}-free condition, the first event elicited by GOR is an early activation (H-5%) of a Cl^- current, which increases as the external osmolarity drops. In the absence of a significant accompanying K^+ current, cells markedly depolarize from the resting potential of −79 ± 2 mV to −54 mV. Activation of a K^+ current at H-19% prevents further depolarization and stabilizes the membrane potential. A different response is observed in the presence of Ca^{2+}. During the first 10 min (up to H-6%), no significant change in membrane potential is observed, suggesting that Cl^- and K^+ currents already activated at that time stabilize the membrane potential. After this time, cells slightly depolarize, reaching −63 mV at H-9%, and this value remains unchanged for the duration of the experiment, suggestive of a predominant Cl^-

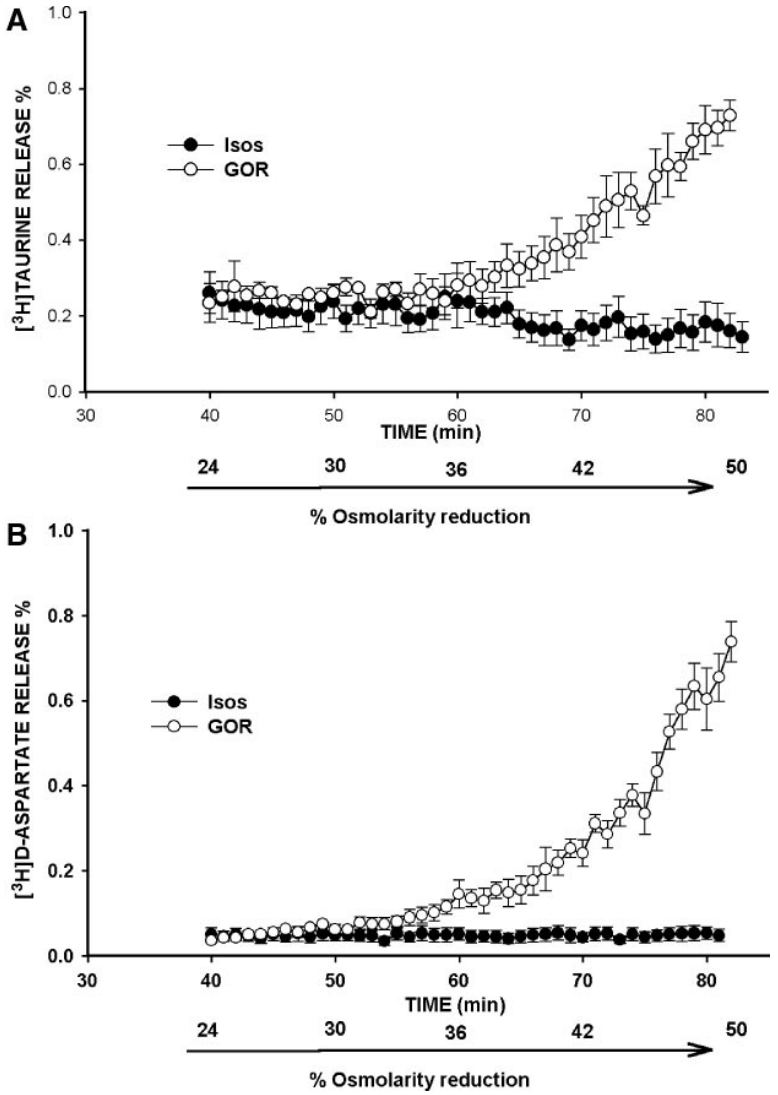

Fig. 10. Amino acid release from C6 glioma cells elicited by GOR. Cells were preloaded with [^{3}H]taurine (*A*) or D-[^{3}H]aspartate (*B*) as described in MATERIALS AND METHODS. After a basal collection period of 6 min with isosmotic medium, the external osmolarity was decreased at a rate of 1.8 mosM/min and superfusion continued until −50% reduction in osmolarity was achieved (final osmolarity 150 mosM). Controls were continuously superfused with isosmotic medium. Fractions were collected every minute from *minutes 1* to *82*, but because no difference was observed during the first 50 min, the points show only the time course of efflux from *minute 40* to the end. Data are expressed as efflux rate constants (min^{-1}) and are means ± SE of 6 experiments.

current. The maximal value of total currents is consistently higher in the presence of Ca^{2+} than in the Ca^{2+}-free condition at the same osmolarities, most likely reflecting the contribution of the Ca^{2+}-activated K^+ current. However, larger differences are observed at small osmolarity reductions, suggesting a more important contribution of the Ca^{2+}-dependent channel in this condition, in line with the observation in jejunal villus cells on Ca^{2+}-dependent RVD at small cell volume changes (10). The influence of the two types of K^+ channels with respect to Ca^{2+} dependence is also reflected in the cell volume regulation. At H-15%, volume regulation after SOR is less efficient and swelling is higher after GOR in the Ca^{2+}-free condition, whereas Ca^{2+} has no significant influence at H-30%. These results, similar to those reported in jejunal epithelium cells (10), stress the fact that volume regulatory mechanisms in conditions of physiological cell volume changes, such as those elicited by nutrient uptake or ionic gradients, differ from those observed at large cell volume changes, which may not occur even in pathological conditions. The type of K^+ channel activated appears to be determinant in the mechanisms operating in the two different situations. The fact that the same differences in the type of K^+ channel involved in volume regulation at small or large cell volume changes were observed in conditions of hyposmolarity (Ref. 10 and present results) or isosmolarity-evoked swelling (9) supports the notion that it is the change in cell volume rather than in osmolarity that determines the type of K^+ channel activation and the consequent mechanism of cell volume adjustment.

The Cl^- and K^+ efflux pathways during GOR may or may not be identical to those operating for the RVD after SOR. In A6 cells, the anion selectivity for the Cl^- efflux pathway in GOR and SOR is different, suggesting different mechanisms for Cl^- efflux (22). In C6 cells, the electrophysiological and kinetic properties of the Cl^- current activated by GOR, including an outwardly rectifying *I-V* relationship and current inactivation at hyperpolarizing voltages, as well as its pharmacological sensitivity, are similar to those of the Cl^- conductance activated in SOR described by Jackson and Strange (3). As to the K^+ channels, the sensitivity to charybdotoxin of RVD in jejunal epithelium cells (10) and of the K^+ current in C6 cells

(present results) suggests that the Ca^{2+}-dependent, swelling-activated K^+ channel is a maxi-K^+ channel, which is distinctively sensitive to this blocker. This type of channel appears similar to that involved in RVD in most epithelial cells (15). The Ca^{2+}-independent channel, which is apparently also voltage independent, activates only by osmolarity and shows a pharmacological profile characterized by insensitivity to typical K^+ channel blockers such as TEA, quinidine, 4-AP, and Ba^{2+} but sensitivity to clofilium. This feature relates this channel to the volume-activated K^+ channel recently described in Ehrlich ascites cells (2, 13). Putative candidates for this channel include members of the 4M2P family of channels (13).

The gradual decrease in osmolarity elicited the release of taurine and glutamate in C6 cells. However, the amino acid efflux was a delayed cell response, the fluxes being activated at very low osmolarities. This is in marked contrast with the early activation of a Cl^- current. This result strongly supports the notion of different permeability pathways for the two osmolytes (4, 20) and provides further evidence against a common pathway for amino acids and Cl^- efflux, which was based mainly on similarities in their pharmacological profile (17, 19). The sensitivity to Cl^- channel blockers, however, is suggestive of some interdependence of the two osmolyte pathways.

The contribution of amino acids, and possibly other organic osmolytes permeating through the same pathway, may be crucial for the efficacy of cell volume regulation elicited by small cell volume changes. According to their behavior in the face of GOR, three types of cell responses have been observed so far. In cells responding by IVR such as tubule renal cells, A6 cells, a subset of hippocampal neurons, and cerebellar granule neurons (7, 21, 22, 24), amino acid efflux activates early after the osmotic stimulus, as documented in brain cells (21). Other cells such as cultured myocytes (18) and C6 cells in the present study in conditions of GOR do not exhibit IVR but show a higher efficiency for volume regulation (evidenced as less swelling) in conditions of large cell volume increase, concomitant with a delayed activation of amino acid fluxes. Finally, in trout erythrocytes (1), cell swelling is the same in both SOR and GOR, with no efflux of taurine observed until very late. The importance of taurine, amino acids, and other organic osmolytes in preventing cell swelling in brain cells may operate also in vivo. The long-term swelling prevention in brain during chronic hyponatremia relies not on electrolytes but on the sustained decrease in the brain content of organic osmolytes, which may be as large as 90% in the case of taurine (23). The superior ability of neurons compared with C6 cells to resist to changes in external osmolarity, which seems to be based primarily on the contribution of organic osmolytes, may represent a protective mechanism to spare neurons from the deleterious consequences of swelling.

In summary, a main conclusion of the present study is that the mechanisms operating in response to small or large changes in cell volume differ essentially in the type of K^+ channel involved as well as in the sensitivity of amino acid efflux pathways.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Claudia Peña-Segura and Alicia Sampieri.

GRANTS

This work was supported by Grants IN204900 from DGAPA, UNAM and 3586-N and 34886-M from CONACYT, Mexico.

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