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The Role of Calcium in the Volume Regulation of Rat Lacrimal Acinar Cells

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Abstract. Earlier studies have suggested a role for Ca^{2+} in regulatory volume decrease (RVD) in response to hypotonic stress through the activation of Ca^{2+}-dependent ion channels (Kotera & Brown, 1993; Park et al., 1994). The involvement of Ca^{2+} in regulating cell volume in rat lacrimal acinar cells was therefore examined using a video-imaging technique to measure cell volume. The trivalent cation Gd^{3+} inhibited RVD, suggesting that Ca^{2+} entry is important and may be via stretch-activated cation channels. However, Fura-2 loaded cells did not show an increase in $[Ca^{2+}]_i$ during exposure to hypotonic solutions. The absence of any changes in $[Ca^{2+}]_i$ resulted from the buffering of cytosolic Ca^{2+} by Fura-2 during hypotonic shock and therefore inhibition of RVD. The intracellular Ca^{2+} chelator, BAPTA, also inhibited the RVD response to hypotonic shock. An increase in $[Ca^{2+}]_i$ induced by either acetylcholine or ionomycin, was found to decrease cell volume under isotonic conditions in lacrimal acinar cells. Cell shrinkage was inhibited by tetraethylammonium ion, an inhibitor of Ca^{2+}-activated K^+ channels. On the basis of the presented data, we suggest an involvement of intracellular Ca^{2+} in controlling cell volume in lacrimal acinar cells.

Key words: Regulatory volume decrease — Ca^{2+} signaling — Acetylcholine — Secretion — Fura-2

Introduction

Calcium is the major intracellular signal controlling fluid and electrolyte secretion in exocrine acinar cells. An increase in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) activates ion channels in luminal and basolateral plasma membranes (Petersen, 1992). In acinar cells, the loss of K^+ and Cl^- following the activation of Ca^{2+}-dependent K^+ and Cl^- channels (Marty, 1987), generates an osmotic gradient leading to the loss of water and subsequent cell shrinkage as part of the secretory response (Foskett, 1990).

Recent studies in this laboratory have shown that the Ca^{2+}-dependent K^+ and Cl^- channels in lacrimal acinar cells are also activated during cell swelling (Kotera & Brown, 1993; Park et al., 1994). The regulatory volume decrease (RVD) observed in response to swelling in these cells was inhibited by the Ca^{2+}-dependent K^+ channel blocker, tetraethylammonium ion (TEA^+; Park et al., 1994); and accelerated by a reduction of extracellular Cl^- concentration (Kotera & Brown, 1993). Activation of both K^+ and Cl^- channels by cell swelling, and the RVD, were dependent on the presence of extracellular Ca^{2+}. These data led to the suggestion that Ca^{2+} influx may be necessary for channel activation during RVD. Additional experiments showed that Cl^- channel activation was blocked by Gd^{3+} (Kotera & Brown, 1993). It was concluded that Ca^{2+} entry via Gd^{3+}-sensitive, stretch-activated channels may therefore have an important role in controlling RVD.

Using video-imaging techniques to measure cell volume changes, a number of criteria have been assessed to determine the involvement of Ca^{2+} in RVD in this study. These criteria include the effect of intracellular Ca^{2+} buffering on RVD, and the use of Gd^{3+} to inhibit Ca^{2+} entry. The effects of hyposmotic shock on $[Ca^{2+}]_i$ were also examined using microspectrofluorimetry and Fura-2.

Materials and Methods

Cell Preparation

Single lacrimal gland acinar cells were isolated using the procedure described by Kotera and Brown (1993). Briefly, small pieces of rat

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lacrimal gland were incubated with trypsin (type XI; Sigma, Poole, UK) and collagenase (type II; Sigma). To dissociate the tissue into a suspension of single cells, the tissue was triturated repeatedly through a pipette tip and the cell suspension was then filtered through a nylon mesh. Following centrifugation, the acinar cells were resuspended in Medium 199 (Sigma) and plated out on 15-mm diameter round glass coverslips (washed in ethanol) placed on the bottom of 35-mm culture dishes for volume measurements. The dishes were stored in an incubator at 37°C and gassed with 5% CO_2/95% O_2 for a minimum of 1 hr to allow the cells to adhere to the coverslips.

Cell Volume Measurements

The perfusion chamber containing the glass coverslip was then transferred to the stage of an inverted microscope (World Precision Instruments) and superfused with isotonic medium at a flow rate of 3 ml/min. The isotonic medium contained (in mM): NaCl 140; KCl 5; $CaCl_2$ 1; $MgCl_2$ 1; HEPES 5; glucose 5 (osmolality = 276 ± 1.0 mOsm/kg H_2O; $n = 5$). The pH was adjusted to 7.4 with NaOH, and the solution gassed with 100% O_2 prior to use.

Cell volume was measured by video imaging as described by Park et al. (1994). Cells were observed through the ×25 objective lens of the microscope, which was fitted with an EDC-1000 camera (Electrim, Princeton, NJ). The images were saved on hard disk at 30-sec or 1-min intervals. The pixel resolution of each image was 192 × 165 which corresponds to an area of 77.5 × 77.5 μm. The area of the image was estimated using an AVS software package (Hewlett Packard), where the number of pixels bounded by the perimeter of each cell was measured.

In all experiments cell volume was normalized to the volume measured during an initial 1- to 3-min superfusion with isotonic solution. Changes in cell volume in response to exposure to a hypotonic solution, or to stimulation with either acetylcholine (ACh) to ionomycin were then examined. The hypotonic solution was similar to the isotonic solution except that it contained only 90-mM NaCl (osmolality = 190.2 ± 1.1 mOsm/kg H_2O; $n = 5$). ACh (Sigma) and ionomycin (Sigma) were simply added to the isotonic solution.

The effect of various compounds on the observed changes in cell volume were examined, e.g., atropine (Sigma), TEA^+ (Sigma), $GdCl_3$ (Sigma), Fura-2/AM (acetoxymethyl ester; Molecular Probes, Leiden, Netherlands) and BAPTA/AM (Molecular Probes). In those experiments where the cells were loaded with BAPTA to buffer intracellular Ca^{2+}, 1-ml portions of the cell suspension following isolation were incubated with 20-μM BAPTA/AM for 90 min at room temperature. Loaded cells were washed twice and resuspended in isotonic medium. Cells were loaded with Fura-2/AM as described below. The osmolality of the solutions was measured by freezing depression point method using a Roebling micro osmometer (Camlab, Cambridge UK). Experiments were performed at room temperature (20–24°C).

Where appropriate, the data are expressed as the mean relative cell volume ± SE and n represents the number of cells tested. Unless otherwise stated, the significance of volume changes was assessed by comparing the mean maximum volume (non-normalized data) reached during an osmotic shock with the mean volume seen at the end of the osmotic shock using the Student's t-test for paired data.

Fluorescence Measurements of $[Ca^{2+}]_i$

Following isolation, 1-ml portions of the cell suspension were loaded with 1-μM Fura-2/AM for 30 min at room temperature. Loaded cells were then washed twice and resuspended in HEPES-buffered isotonic medium. Dye fluorescence was measured using a system based on a Nikon Diaphot inverted microscope equipped for epifluorescence with quartz optics. Acinar cells loaded with Fura-2 were allowed to adhere to a coverslip forming the base of a perfusion chamber, and were then observed with a ×40 oil immersion lens (N.A. 1.3). An adjustable diaphragm was used to isolate individual cells from the remaining cells in view. Cells were excited with light at wavelengths of 350 and 380 nm for Fura-2 using a filter wheel spinning at 40Hz (Cairn Research, Kent, UK). Emitted fluorescence (500 ± 20 nm) was detected by a photomultiplier tube (Thorn EMI Electron Tubes, Ruislip, UK), the signal from which was fed to the virtual ground current pre-amplifier of a spectrophotometer system (Cairn Research Ltd.). Demodulated fluorescence signals and ratio signals (calculated online) were displayed on a chart recorder (Gould Electronics, Ilford, UK) and recorded on VHS videotape using a VCR adaptor (PCM-8, Medical Systems, Greenvale, NY).

Changes in $[Ca^{2+}]_i$ in response to either stimulation with ACh or ionomycin, and during exposure to hypotonic solutions were examined. Acinar cells were perfused with isotonic control solution prior to stimulation with either ACh or ionomycin for a 5-min period, or hypotonic solutions for a period of 15 min.

In those figures illustrating changes in $[Ca^{2+}]_i$, the uncalibrated 350:380 nm ratio signal is shown as an index of $[Ca^{2+}]_i$. Absolute estimates of $[Ca^{2+}]_i$ were not routinely derived from the values of the 350:380 nm fluorescence ratio. However, in a previous study using the same fluorescence system and isolated lacrimal acinar cells (Yodozawa, Speake & Elliott, 1997), a two-point calibration of the Fura-2 signal was carried out (Grynkiewicz, Poenie & Tsien, 1985). The 350:380 nm ratio signal varied between 0.9 and 1.2 in resting cells, corresponding to an estimated $[Ca^{2+}]_i$ of between 20 and 75 nM. These values are comparable with earlier estimations for lacrimal acinar cells (Marty & Tan, 1989; Berrie & Elliott, 1994).

Results

The Role of Ca^{2+} in RVD

Figure 1*A* shows the effects of exposing lacrimal acinar cells to the hypotonic solution in control conditions, i.e., in the presence of 1-mM extracellular Ca^{2+}. On exposure to the hypotonic solution the cells rapidly swell to a maximum volume of 1.32 ± 0.01 ($n = 7$) within 1 min. A RVD is then observed over the next 19 min, so that the cell volume at the end of a 20 min exposure to the hypotonic solution is 1.18 ± 0.01, a significant decrease from the maximum volume ($P < 0.01$, by paired t-test). Similar results were obtained when an isotonic solution containing 100-mM mannitol and 90-mM NaCl was replaced by the hypotonic solution (Park et al., 1994), therefore eliminating any effect of altered driving forces on cell volume.

In our earlier study, a Ca^{2+}-free hypotonic solution significantly inhibited the RVD (*see* Fig. 2*B;* Park et al., 1994), suggesting the importance of extracellular Ca^{2+}. In Fig. 1*B*, 20 μM $GdCl_3$ was added to the hypotonic solution. In six experiments under these conditions, cells swelled to 1.31 ± 0.02 within 3 min. After 20-min exposure to the hypotonic solution, cell volume was 1.27 ± 0.02. Thus in the presence of Gd^{3+}, the cell volume at

the end of exposure to a hypotonic solution, was not significantly different from the peak volume observed ($P > 0.05$, by paired *t*-test).

The Effects of Hypotonic Solutions on $[Ca^{2+}]_i$

If Ca^{2+} is important in RVD then one might expect to see an increase in $[Ca^{2+}]_i$ during exposure to hypotonic solutions. Lacrimal acinar cells were therefore loaded with Fura-2 to determine the effect of hypotonic shock on $[Ca^{2+}]_i$. Figure 2*A* shows that replacing the isotonic solution with the hypotonic solution did not change the 350:380-nm fluorescence ratio signal. Similar results were observed in ten experiments at room temperature. Hypotonic solutions were also found to have no effect on $[Ca^{2+}]_i$ in experiments performed at 37°C ($n = 10$; *data not shown*). The light emitted in response to excitation of 350 nm light is shown in Fig. 2*B*. There was a slight decrease in this signal in response to the hypotonic solution, indicating that the cell was swelling, i.e., there was dilution of the cytosolic dye ($n = 10$). On return to the isotonic solution, the 350-nm signal increased again as the cell returned to its normal volume. During the exposure to the hypotonic solution, however, the 350-nm signal did not change suggesting that the RVD may be inhibited by the presence of Fura-2.

Loading Lacrimal Cells with Fura-2 or BAPTA Inhibits RVD

To investigate the possible inhibition of RVD by Fura-2, the volume of cells loaded with Fura-2 was measured by the video-imaging method. Figure 3*A* shows the RVD in cells which were loaded with Fura-2 (▲; $n = 5$) and in control (unloaded) cells (■; $n = 8$) from the same preparations. Exposure of the Fura-2 loaded cells to the hypotonic solution caused an increase in volume to 1.22 ± 0.03, which is not significantly different to that observed in the control cells (1.25 ± 0.02; $P > 0.1$ by unpaired *t*-test). In control cells a clear RVD was observed (volume after 15 min, 1.09 ± 0.01). However, in the cells loaded with Fura-2, the RVD was inhibited, i.e., after a 15-min exposure to the hypotonic solution, the cell volume had decreased to 1.17 ± 0.02 (not significantly lower than the peak volume, $P > 0.1$ by paired *t*-test).

The most likely explanation for the inhibition of the RVD in Fig. 3*A* is that the Fura-2 is acting as a Ca^{2+} buffer. To test this hypothesis the RVD in cells loaded with another Ca^{2+} buffer, BAPTA, was examined. Figure 3*B* shows the effects of hypotonic solutions on control cells (■; $n = 8$) and cells loaded with BAPTA (▲; $n = 8$). Under control conditions an RVD was observed with a volume recovery from 1.25 ± 0.01 to 1.11 ± 0.02 during the 15-min exposure to the hypotonic solution. In the presence of BAPTA the RVD was completely

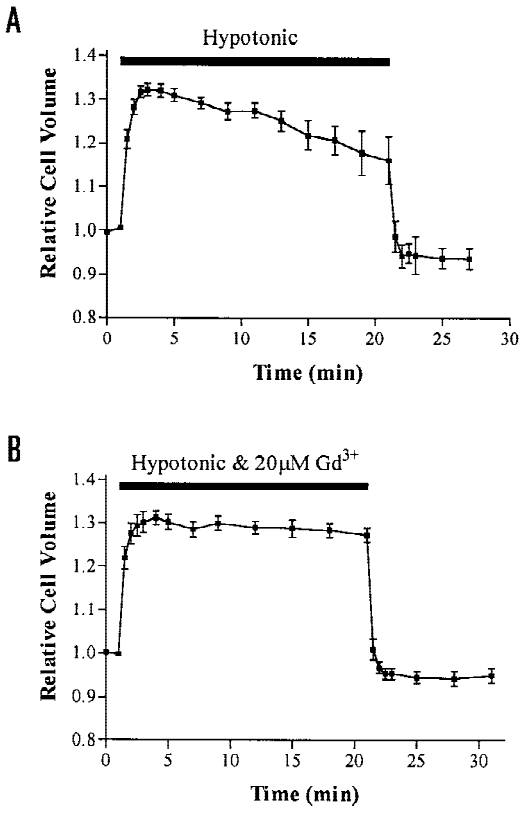

Fig. 1. The role of Ca^{2+} in RVD in lacrimal acinar cells. *(A)* The extracellular medium was made hypotonic by the removal of 50-mM NaCl (causing a reduction in osmolality from 276 to 190 mOsm/kg H_2O) for the period indicated by the bar. *(B)* The extracellular medium was made hypotonic in the presence of 20-μM Gd^{3+}. Cell volume was monitored by measuring the area of a video image of the cell. Volumes were then calculated assuming the cell to be spherical, and expressed as a fraction of the control volume measured over the first minute of the experiment. Data are mean ± SEM of *(A)* 7, and *(B)* 6 experiments respectively. The mean pixel value ± SEM for each group of cells at the beginning of the experiments in *(A)* and *(B)* were 4158 ± 150 and 3513 ± 38 respectively.

inhibited with the cell volume increasing to and remaining at 1.25 ± 0.02 throughout the exposure to the hypotonic solution (volume at end of osmotic shock not significantly different from peak volume, $P > 0.1$ by paired *t*-test).

An increase in $[Ca^{2+}]_i$ Causes a Decrease in Cell Volume

The data in Figs. 1 and 3 suggest that Ca^{2+} may play a role in RVD in lacrimal gland acinar cells. However, the fact that Fura-2 inhibits RVD makes it difficult to confirm that an increase in $[Ca^{2+}]_i$ is necessary for RVD. To further examine the possible role of Ca^{2+} in controlling cell volume, experiments were performed to inves-

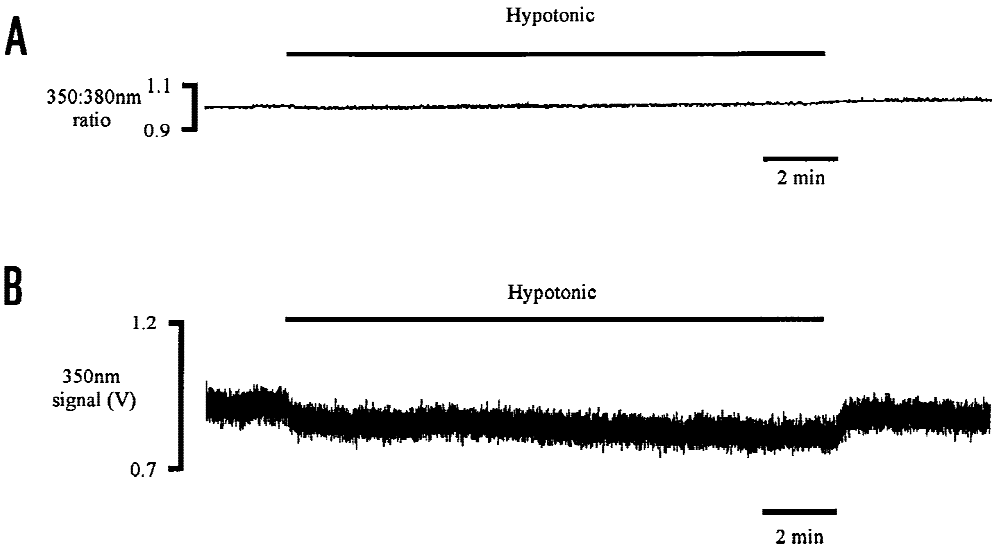

Fig. 2. *(A)* Cell swelling does not increase $[Ca^{2+}]_i$. Cells were loaded with the Ca^{2+}-sensitive dye, Fura-2 to measure $[Ca^{2+}]_i$. The extracellular medium was made hypotonic by the removal of 50-mM NaCl for the period indicated by the bar. The 350:380-nm ratio signal is shown as an index of $[Ca^{2+}]_i$. *(B)* The individual 350-nm signal did decrease during cell swelling. These data are representative of 9 other experiments.

tigate the effects of increasing $[Ca^{2+}]_i$ on cell volume in isotonic conditions.

Figure 4*A* shows the effect of 1-μM ACh on $[Ca^{2+}]_i$ in lacrimal gland acinar cells. ACh produced a biphasic increase in $[Ca^{2+}]_i$ to a maximum value followed by a decrease to a sustained 'plateau' level, as previously described by Kwan et al. (1990). The initial increase in the 350:380-nm ratio with ACh in Fig. 4*A* corresponds to an increase in $[Ca^{2+}]_i$ from approximately 95 nm (resting $[Ca^{2+}]_i$) to 300-nM Ca^{2+} [1]. Similar changes in $[Ca^{2+}]_i$ were observed in 5 cells in the presence of ACh. The increase in $[Ca^{2+}]_i$ was inhibited by 0.1 μM atropine indicating the involvement of muscarinic receptors (n = 6; *data not shown*).

Figure 4*B* illustrates the changes in $[Ca^{2+}]_i$ associated with the application of 0.3-μM ionomycin, a Ca^{2+} ionophore. There is a biphasic increase in $[Ca^{2+}]_i$ with an initial large increase followed by an elevated Ca^{2+} 'plateau' phase (similar results were obtained in 5 experiments). Thus, the increase in $[Ca^{2+}]_i$ stimulated by 0.3-μM ionomycin is similar to that observed with 1-μM ACh, both in terms of the size and time-course of the changes.

Figure 5 shows the effects of ACh and ionomycin on lacrimal gland acinar cell volume. During a 3-min exposure to 1-μM ACh at room temperature, the relative cell volume decreased to 0.92 ± 0.01 (n = 4; Fig. 5*A*). On removal of ACh, the cell volume did not recover and remained at this reduced level. In similar experiments performed at 37°C, ACh caused a similar decrease in cell volume (*data not shown*). In these experiments, however, cell volume recovered on the removal of ACh. This recovery was inhibited by bumetanide, an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter (*data not shown*)[2].

Figure 5*B* shows that in the presence of 0.1-μM atropine, 1-μM ACh failed to cause cell shrinkage (n = 4). This indicates that the ACh-induced cell shrinkage is caused as a result of ACh acting at muscarinic receptors in lacrimal acinar cells.

Figure 5*C* shows that 0.3-μM ionomycin causes a decrease in cell volume. During a 5-min exposure to ionomycin, the relative cell volume decreased to 0.90 ± 0.01 (n = 8).

The Role of K^+ Channels in Cell Shrinkage

The data in Fig. 5 show that an increase in $[Ca^{2+}]_i$ in isotonic conditions causes a decrease in cell volume. One possible mechanism is that the increase in $[Ca^{2+}]_i$ activates K^+ and Cl^- channels leading to a loss of K^+ and Cl^- from the cell. To assess the possible involvement

[1] The values quoted for $[Ca^{2+}]_i$ may be underestimated when considering that Fura-2 inhibits RVD through the buffering of intracellular Ca^{2+}.

[2] It has previously been shown that the Na^+-K^+-$2Cl^-$ cotransporter contributes to regulatory volume increase (RVI) in lacrimal acinar cells at 37°C but not at room temperature (Douglas & Brown, 1996).

of Ca^{2+}-activated K^+ channels in cell shrinkage, experiments were performed in the presence of TEA^+. Calcium-activated K^+ channels in lacrimal acinar cells have previously been reported to be sensitive to TEA^+ (Trautmann & Marty, 1984; Lechleiter, Dartt & Brehm, 1988; Park et al., 1994). In Fig. 6*A*, lacrimal acinar cells were exposed to 2-mM TEA^+ for 5 min prior to the addition of 1-μM ACh to the superfusate. In these conditions, ACh failed to cause a significant decrease in cell volume ($n = 10$; $P > 0.1$), suggesting that activation of K^+ channels are involved in ACh-induced cell shrinkage. Since TEA^+ has been reported to partially block muscarinic-evoked secretion in the sheep parotid gland (Cook et al., 1992), we therefore also used ionomycin to increase $[Ca^{2+}]_i$ and to eliminate any possible antagonistic effects (Fig. 6*B*). TEA^+ (2 mM) also inhibited the effects of ionomycin on cell volume ($n = 5$).

Discussion

Evidence for a Role of Ca^{2+} in RVD

Calcium is thought to have a role in controlling RVD in many cells. There is, however, confusion in the literature as to its precise importance. This has probably arisen because of discrepancies between results produced by a variety of experimental methods (McCarty & O'Neil, 1992). Foskett (1994), therefore proposed that a number of criteria must be satisfied in order to establish the role of Ca^{2+} in RVD. In our studies of lacrimal acinar cells we have examined several criteria by different methods, and the significance of the results obtained are discussed below.

Inhibition of RVD By Removing Ca^{2+} From the Extracellular Medium

Our earlier study demonstrated that the RVD was inhibited in Ca^{2+}-free medium (Fig. 2*B;* Park et al., 1994). This maneuver has been shown to inhibit RVD in many other cells (McCarty & O'Neil, 1992; Foskett, 1994) and can be interpreted in two ways. The simplest explanation is that Ca^{2+} influx into the cell, leading to an increase in $[Ca^{2+}]_i$, is required for RVD. However, it may also be interpreted as showing that Ca^{2+} has a 'permissive role' in RVD, i.e., an increase in $[Ca^{2+}]_i$ is not a primary signal in controlling RVD, but that Ca^{2+}-dependent processes, which are inhibited by Ca^{2+} removal, are involved (*see* McCarty & O'Neil, 1992). In this second case, influx of Ca^{2+} and an increase in $[Ca^{2+}]_i$ are not necessary for RVD.

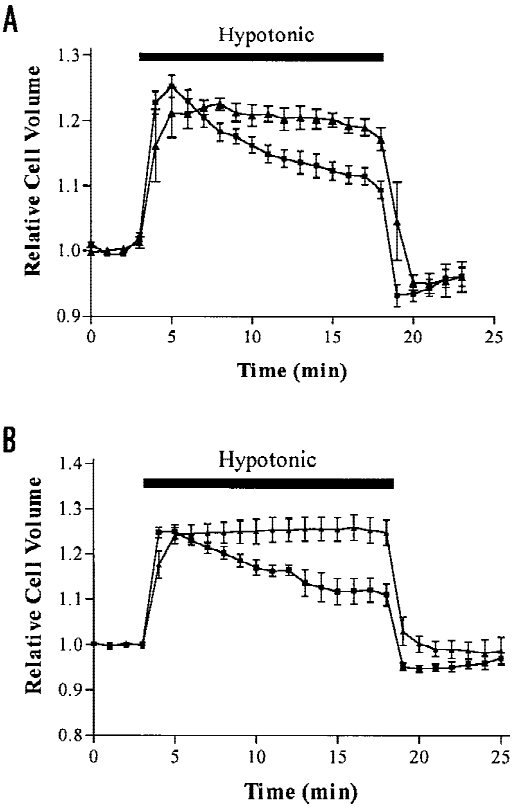

Fig. 3. Buffering $[Ca^{2+}]_i$ inhibits the RVD in hypotonic solutions. *(A)* RVD response to cell swelling is inhibited by loading the cells with Fura-2. (■) and (▲) symbols represent data from 8 control (pixel value, 3045 ± 353) and 5 Fura-2-loaded (pixel value, 2583 ± 205) cells respectively. *(B)* Intracellular Ca^{2+} chelation with BAPTA inhibits RVD in lacrimal acinar cells. The control cells (■) exhibited RVD, whereas RVD was inhibited by 20-μM BAPTA in loaded cells (▲). The extracellular medium was made hypotonic by the removal of 50-mM NaCl for the period indicated by the bar. Data are mean ± SEM of 8 experiments for both control (pixel value, 3517 ± 133) and BAPTA-loaded (pixel value, 3066 ± 250) cells.

RVD is Inhibited by Gd^{3+}

If Ca^{2+} influx from the extracellular solution is important in RVD, then there must be a pathway for Ca^{2+} entry during RVD. One such pathway could be via stretch-activated, nonselective cation channels which are activated by cell swelling (Christensen 1987; Kotera & Brown, 1993). These channels have been shown in patch-clamp experiments to be inhibited by the trivalent cation, Gd^{3+} (Yang & Sachs, 1989). In lacrimal cells, Gd^{3+} significantly reduced volume regulation during exposure to hypotonic solutions (Fig. 1*B*). This suggests that the route for Ca^{2+} entry during the RVD response may be via stretch-activated channels. Furthermore, the inhibition of RVD by Gd^{3+} indicates that Ca^{2+} influx is necessary for RVD to occur, i.e., the role of Ca^{2+} is not simply permissive.

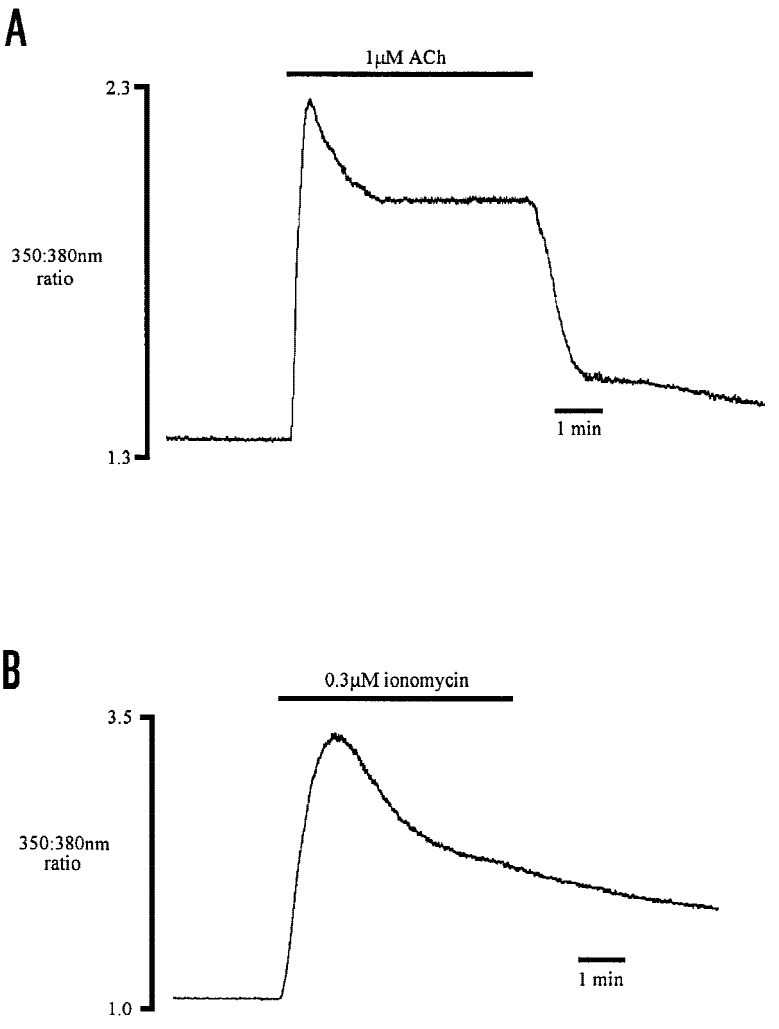

Fig. 4. *(A)* Effect of ACh (1 μM) on the fluorescence signal recorded from a single cell loaded with Fura-2. The ratio of the fluorescence emission obtained with dual excitation at 350 and 380 nm is shown as an index of $[Ca^{2+}]_i$. Four other experiments produced comparable results. *(B)* Ionomycin initially mobilizes Ca^{2+} from internal stores, followed by a sustained elevated plateau as Ca^{2+} entry is stimulated. This experiment is representative of 4 other experiments.

Ca^{2+}-Activated Channels are Involved in RVD

The activation of Ca^{2+}-activated Cl^- and Ca^{2+}-activated K^+ channels by cell swelling in lacrimal acinar cells is well documented (Kotera & Brown, 1993; Park et al., 1994), and it has previously been suggested that both channels are involved in RVD (Park et al., 1994). The present study provides additional evidence for the involvement of these channels in volume regulation, by showing that maneuvers which inhibit channel activation, also inhibit RVD, e.g., removal of extracellular Ca^{2+}, the addition of Gd^{3+} [3], and the buffering of Ca^{2+} with BAPTA. On the basis of these data, we suggest that Ca^{2+} entry via stretch-activated cation channels, leading to an increase in $[Ca^{2+}]_i$ is necessary for RVD in lacrimal gland acinar cells.

Cell Swelling Does Not Increase Fura-2 Fluorescence

Cell swelling caused by hypotonic solutions did not cause an increase in $[Ca^{2+}]_i$ in Fura-2 loaded cells (Fig. 2). These results are consistent with an earlier study on lacrimal acinar cells (Elliott, 1994). They are, however, surprising in view of the other evidence that Ca^{2+} is required for RVD. However, similar discrepancies between volume measurements and results obtained using fluorescent indicators exist elsewhere in the literature (for a review *see* McCarty & O'Neil, 1992).

There are several explanations as to why the Fura-2 fluorescence did not change in the present study. The first is that Ca^{2+} has only a permissive role in the control

[3] Gd^{3+} is not inhibiting RVD by directly blocking the K^+ and Cl^- channels, because it has been shown in patch clamp experiments that it neither blocks Ca^{2+}-activated Cl^- channels (Kotera & Brown, 1993), nor Ca^{2+}-activated K^+ channels (K.-P. Park and P.D. Brown, *unpublished observations*) in lacrimal acinar cells.

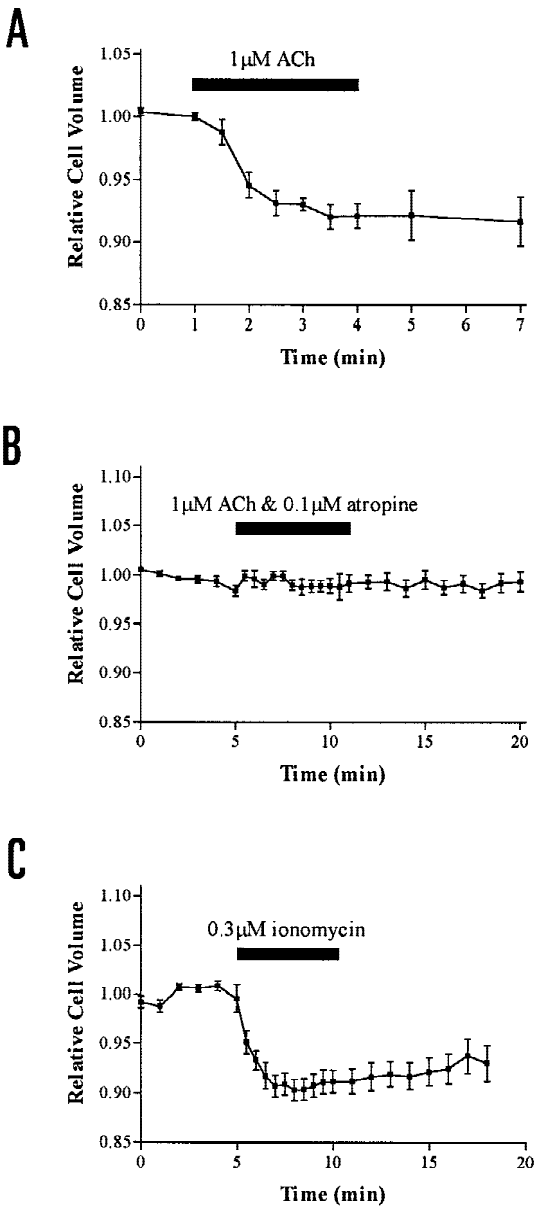

Fig. 5. Volume changes in lacrimal acinar cells exposed to *(A)* acetylcholine (ACh; 1 μM); *(B)* ACh and atropine (0.1 μM); and *(C)* ionomycin (0.3 μM). Cells were exposed to solutions containing ACh/ionomycin for the period indicated by the bar. Data are mean ± SEM of *(A)* 4 (pixel value, 2093 ± 187), *(B)* 4 (pixel value, 2254 ± 250), and *(C)* 8 (pixel value, 2162 ± 215) experiments.

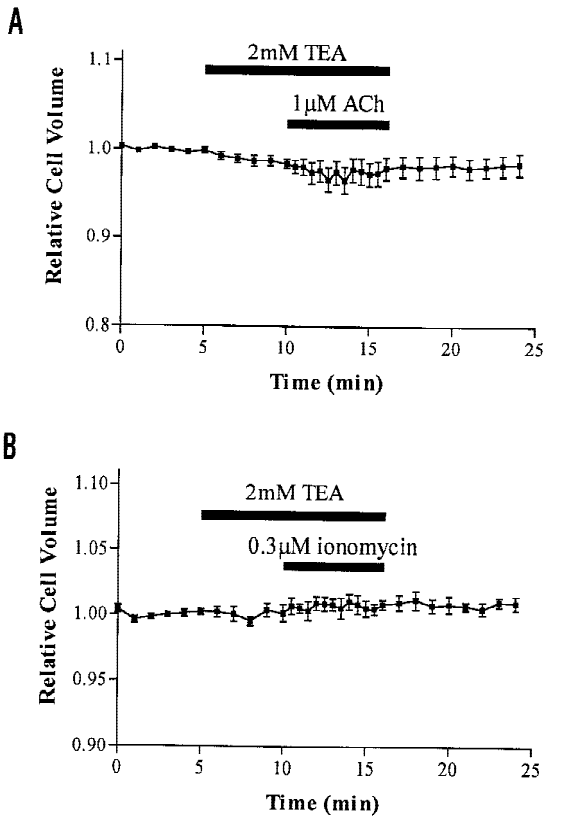

Fig. 6. Cell shrinkage is inhibited by the Ca^{2+}-activated K^+ channel blocker, tetraethylammonium (TEA^+; 2 mM). ACh *(A)* and ionomycin *(B)* were used to induce cell shrinkage in lacrimal acinar cells. Data are mean ± SEM of 10 experiments in *(A)* and 5 experiments in *(B)*. The mean pixel value was *(A)* 2442 ± 156; and *(B)* 2415 ± 241.

of RVD, and that other intracellular messengers may be involved in the actual inhibition of RVD, e.g., leukotrienes, protein kinases and phosphatases (Hoffmann & Dunham, 1995). This, however, seems unlikely for a number of reasons: (i) both the K^+ and Cl^- channels involved in RVD are known to be activated by an increase in $[Ca^{2+}]_i$, and they have not been reported to be activated by other intracellular messengers[4]; (ii) $[Ca^{2+}]_i$ does have a role in determining cell volume, e.g., increasing $[Ca^{2+}]_i$ with ACh or ionomycin causes cell shrinkage (Fig. 5); (iii) RVD is inhibited by Gd^{3+} suggesting that Ca^{2+} influx is necessary for volume regulation.

A second explanation is that any swelling-induced increases in $[Ca^{2+}]_i$ may be localized close to the cell membrane, so that they cannot be detected by Fura-2 which reports the $[Ca^{2+}]$from the entire cytoplasm. Evidence for this explanation comes from studies of signal-secretion coupling in pancreatic acinar cells (Osipchuk et al., 1990). In these experiments, oscillations in the activity of Ca^{2+}-activated Cl^- current could be recorded electrophysiologically, without any observable changes

[4] The only other factor known to activate the K^+ and Cl^- channels in lacrimal acinar cells is an increase in intracellular pH (Park & Brown, 1994, 1995). However, preliminary experiments show that pH remains constant during cell swelling (T. Speake & P.D. Brown, *unpublished observations*).

in the Fura-2 fluorescence (Osipchuk et al., 1990). Several recent studies have used a derivative of Fura-2, FFP-18, to measure changes in $[Ca^{2+}]_i$ localized at the plasma membrane (Etter, Kuhn & Fay, 1994; Vorndran, Minta & Poenie, 1995; Davies & Hallett, 1996). However, we were unable to test this hypothesis in this way, because the lacrimal gland acinar cells would not load with the acetoxymethyl ester form of FFP-18 in preliminary experiments (T. Speake and P.D. Brown, *unpublished observations*).

The final explanation for the absence of any swelling-induced changes in Fura-2 fluorescence, could be because the increase in $[Ca^{2+}]_i$ necessary for RVD is very small[5]; and as such may be buffered by the Fura-2. This possibility was assessed by monitoring the volume of cells loaded with Fura-2. The RVD in Fura-2 loaded cells was completely inhibited compared with control cells (Fig. 3). Fura-2 has also been reported to inhibit volume regulation in cells of the kidney proximal tubules (McCarty & O'Neil, 1990). The effects of buffering changes in $[Ca^{2+}]_i$ on RVD were further examined by loading the cells with the Ca^{2+} chelator, BAPTA. RVD in the lacrimal cells was completely abolished by BAPTA (Fig. 3*B*), and this is consistent with data from other epithelial cell types loaded with the chelator (Adorante & Cala, 1995; Fu, Kuwahara, & Marumo, 1995).

In contrast to the absence of any recognizable change in $[Ca^{2+}]_i$ during hypotonic exposure, a large change in $[Ca^{2+}]_i$ was observed with ACh and ionomycin (Fig. 4). These contrasting effects on $[Ca^{2+}]_i$ can similarly be related to the difference in magnitude of the cell shrinkage observed under both conditions — with ACh/ionomycin the decrease in cell volume was significantly greater than the RVD observed during cell swelling (*compare* Figs. 1 and 4).

To summarize, the data presented here support the hypothesis that Ca^{2+} has an important role in controlling the RVD in lacrimal acinar cells. However, it is not possible to measure an increase in $[Ca^{2+}]_i$ during cell swelling, because of the technical limitations of the present fluorescent indicators in measuring small changes in $[Ca^{2+}]_i$ which may also be localized to the plasma membrane.

Secretagogue-induced Volume Changes — A Physiological Role for Cell Shrinkage

Stimulation of lacrimal acinar cells with ACh resulted in cell shrinkage (Fig. 5*A*). The ACh-induced cell shrinkage was inhibited by atropine (a muscarinic antagonist; Fig. 5*B*), and could be mimicked by ionomycin (a Ca^{2+} ionophore; Fig. 5*C*). This suggests that the decrease in cell volume may occur as the result of a receptor-mediated increase in $[Ca^{2+}]_i$.

Similar effects of secretagogues on cell volume have been observed in other secretory epithelia, e.g., salivary gland acinar cells (Foskett & Melvin, 1989; Steward & Larcombe-McDouall, 1989: Nakahari et al., 1990), sweat gland secretory coil cells (Takemura et al., 1991), and the intestinal crypt cells (Walters et al., 1992). Simultaneous measurements of $[Cl^-]_i$ and cell volume in salivary acinar cells demonstrated that the initial cell shrinkage was due to Cl^- efflux (Foskett et al., 1994), which presumably occurs via Ca^{2+}-activated Cl^- channels which are activated by the secretagogue. Whereas, Walters et al. (1992) showed that the effects of vasoactive intestinal peptide on cell volume could be inhibited by the Cl^- channel blocker NPPB or by increasing the extracellular K^+ activity, suggesting that both secretagogue-activated Cl^- and K^+ channels contribute to the loss of cell volume. These data are therefore consistent with our own on events in the lacrimal gland.

It is not clear whether cell shrinkage has a specific role in the process of secretion, or whether it is simply a consequence of channel activation. However, a previous study of volume regulation in lacrimal acinar cells has shown that cell shrinkage, induced by hypertonic solutions, results in the activation of the Na^+-K^+-$2Cl^-$ co-transporter and Na^+-H^+ and Cl^--HCO_3^- exchangers (Douglas & Brown, 1996). These transporters are all thought to contribute to secretion in the lacrimal gland, i.e., they mediate Cl^- uptake across the basolateral membrane of the acinar cell. Secretagogue-induced cell shrinkage may therefore activate these pathways, and may be necessary in order to maintain secretion in the lacrimal gland.

In conclusion, the RVD in response to cell swelling was inhibited by maneuvers that either inhibit Ca^{2+} entry or buffer intracellular Ca^{2+}. Hypotonic shock did not increase $[Ca^{2+}]_i$ in Fura-2 loaded lacrimal cells as a result of intracellular Ca^{2+} buffering by Fura-2. In contrast, ACh stimulation or ionomycin produced large changes in $[Ca^{2+}]_i$ and an associated cell shrinkage. Cell shrinkage was inhibited by TEA^+, an inhibitor of Ca^{2+}-dependent K^+ channels. On the basis of these data, we suggest that intracellular Ca^{2+} is involved in controlling cell volume in lacrimal acinar cells.

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[5] An indication that the increase in $[Ca^{2+}]_i$ may be small, is given by the magnitude of the initial rate of the RVD (0.008/min), which is an order of magnitude slower than that induced by ACh or ionomycin (0.09/min).

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