Role of Chloride Channels in Regulating the Volume of Acinar Cells of the Rabbit Superior Lacrimal Gland

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PURPOSE. To characterize the outward chloride currents (Cl_{OR}) in single acinar cells isolated from the rabbit superior lacrimal gland (RSLG) to investigate the hypothesis that Cl_{OR} may have a role in regulating the volume of RSLG acini.

METHODS. Cl_{OR} was characterized by using patch-clamp electrophysiology. Confocal microscopy was used to measure intracellular calcium concentration ([Ca^{2+}]_i) and cell volume. Cell volume was altered by superfusing the cells with a hypotonic solution.

RESULTS. The Cl_{OR} current contributed 33% of total membrane conductance. With normal osmotic conditions, the Cl_{OR} current was activated by [Ca^{2+}]_i with an EC_{50} of 10^{-8} M. A decrease in intracellular pH from 7.4 to 6.8 totally inhibited Cl_{OR} current activity. Continuous superfusion of hypotonic solution caused (1) an increase in cell volume that peaked within 4 minutes and gradually returned to baseline levels after 12 minutes, (2) an increase in [Ca^{2+}]_i that peaked between 6 and 8 minutes and gradually returned to baseline levels after 15 minutes, and (3) an increase the Cl_{OR} current that peaked within 6 minutes after commencement of perfusion and quickly returned to baseline levels.

CONCLUSIONS. The Cl_{OR} current appears to be triggered by an increase in cell volume and then deactivates within the period of raised [Ca^{2+}]_i, during hypotonic stress, suggesting that Cl_{OR} may be an initiating event for volume homeostasis. This effect would be important during RSLG tear secretion, which usually involves cell volume changes and is accompanied by intracellular pH changes in the presence of the raised [Ca^{2+}]_i to support secretion. (Invest Ophthalmol Vis Sci. 2008;49: 5517–5525) DOI:10.1167/iovs.07-0435

Tears provide a protective and lubricating film that covers the anterior surface of the eye. The bulk of tears is a salty serous fluid containing a variety of proteins associated with bacterial defense, which in rabbits is produced by the superior and inferior lacrimal glands. Secretion of the aqueous component of tears is dependent on the distribution and nature of ion channels in lacrimal acinar cells. Studies of ion channel activity in acinar cells from the exorbital gland of the rat, mouse, and rabbit have identified K^+ and Cl^− channels that are both Ca^{2+}-dependent. However, histochemical studies have shown that the rat lacrimal gland is an acid mucin gland, similar to the rabbit. Because this histochemical difference may indicate a difference in the secretory mechanisms, and as the rabbit is a widely used model for studying the phenomenon of dry eye associated with the disease keratoconjunctivitis sicca, we investigated the nature of the ion channels in the superior lacrimal gland of the rabbit. In an earlier study of rabbit superior lacrimal gland (RSLG) cells, we showed that TEA-sensitive K^+ currents comprised a significant portion of the outward currents. In the present study, the other TEA-insensitive component of outward currents was carried by an outwardly rectifying chloride channel (Cl_{OR}). Our identification of Cl_{OR} was based on functional studies, since only a limited number of chloride channel genes have been identified. From the functional point of view, Cl^− channels have been classified according to their gating mechanisms, which include (1) transmembrane voltage (the CLC family), (2) protein kinase or nucleotide mediated mechanism (CFTR), (3) an increase in intracellular Ca^{2+}-(Ca^{2+}-activated Cl^− channels, CaCC), (4) cell swelling (volume-regulated anion channels, VRAC), or (5) binding of a ligand (GABA-activated channels). Transepithelial movement of chloride ions in many secretory epithelia may occur through any of four general classes of Cl^− channels based on their modes of activation, including intracellular cAMP, cell swelling (volume changes), hyperpolarization, and intracellular Ca^{2+} ([Ca^{2+}]_i) levels. There most likely is some overlap of function, since apical Cl^− channels that are involved in fluid and electrolyte secretion are primarily activated by cAMP in some epithelia and by Ca^{2+} in other epithelia. Also, volume sensitive Cl^− channels have been identified in rat parotid cells and in rat lacrimal gland acinar cells. The voltage-gated Cl^− channel known as CLC-2 is activated by hyperpolarization and is found in a variety of secretory epithelia including mouse mandibular cells and rat parotid acinar cells. The rat submandibular gland cells and the rat lacrimal gland cells were found to express CLC-3 protein and contain mRNA for CLC-3 which has a controversial role as a channel regulating Cl^− current. We investigated the hypothesis that the Cl_{OR} channels that we identified in the RSLG acini may have a role in regulating the volume of lacrimal acinar cells, since it has been shown that salivary acinar cells undergo changes in volume during secretion.

Most cells placed in an hypotonic environment swell and then undergo regulatory volume decrease (RVD), which is usually accomplished by the extrusion of K^+ and Cl^− ions via Ca^{2+}-activated K^+ and Cl^− channels. However, K^+/Cl^− cotransporters mediate the RVD regulation in fish, dog, and human red blood cells (RBCs). Cells exposed to a hypotonic environment will shrink and then undergo regulatory volume increase (RVI). Previously, ionic channels have not been implicated in RVI, which is thought to be mediated either.
by Na\(^+\)/Cl\(^-\) cotransporters.\(^{25}\) Na\(^+\)/H\(^+\) exchangers linked to Cl\(^-\)/HCO\(_3\)\(^-\) exchangers,\(^{26}\) or Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporters.\(^{27}\)

In the present study, we characterized the Cl\(_{OR}\) in the RSLG and showed that activation of this Cl\(_{OR}\) was triggered by an increase in cell volume. The Cl\(_{OR}\) may be involved in RVI as the initiating event for restoring volume homeostasis after hypotonic stress or secretion.

**METHODS**

**Preparation of Lacrimal Gland Cells**

New Zealand White rabbits of both sexes (1.5–2.5 kg) were killed by an overdose of pentobarbital (45 mg/kg) administered IV in accordance with Australian National Health and Medical Research Council guidelines for animal ethics and the tenets of the ARVO Statement of the Use of Animals in Ophthalmic Vision Research. The superior lacrimal glands were excised and placed in a Petri dish containing 5 mL of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, 10%; PA Biologicals Co Pty Ltd., Melbourne, Australia), using an excitation wavelength of 488 nm and detecting excitation at 500–530 nm. The footprint area of cells was measured from the optical section of the cell closest to its adhesion with the surface of the culture dish (Quantsight 570 Image Analyzer; Cambridge Instruments, North Ryde, NSW, Australia), using an excitation wavelength of 488 nm and detecting an emission wavelength of 525 to 530 nm.

Confocal Microscopy

Changes in [Ca\(^{2+}\)]\(_{i}\) concentration in single cells were measured by loading the cells with the acetoxy-methyl (AM) ester form of the Ca\(^{2+}\) indicator dye Fluo-3 (F-1242; Invitrogen-Molecular Probes, Eugene, OR). The cells were loaded with Fluo-3 AM (1.5 \(\mu\)M) by incubation in the dark for 25 minutes at room temperature. They were then washed with four changes of the extracellular bath solution to remove uncleaved Fluo-3 AM molecules. The cells and the Fluo-3-labeled [Ca\(^{2+}\)]\(_{i}\) were visualized with a 32× objective with a confocal laser scanning microscope (Model TCS-4D; Leica Microsystems Pty Ltd., North Ryde, NSW, Australia), using an excitation wavelength of 488 nm and detecting an emission wavelength of 525 to 530 nm.

**Solutions and Chemicals**

The standard pipette solution (pH 7.4) contained (in mM): KCl (140), MgCl\(_2\) (1.13), HEPES (10), and EGTA (5). The standard bath solution (pH 7.4) contained (in mM): NaCl (145), MgCl\(_2\) (1.13), KCl (5), CaCl\(_2\) (1), glucose (10), and HEPES (10). In the ion substitution experiments, the following pipette and bath solutions were used. Pipette solution (pH 7.4) contained (in mM): NaCl (140), MgCl\(_2\) (1.13), HEPES (10), glucose (10), CaCl\(_2\) (0.97), and EGTA (1.92). The bath solution (pH 7.4) contained (in mM): NaCl (145), KCl (5), CaCl\(_2\) (1.13), and glucose (10). To determine the ion selectivity of this channel for various anions (SCN\(^-\), I\(^-\), Br\(^-\), and NO\(_3\)\(^-\)) we

an upward deflection in all traces by adopting the convention of positive ions leaving the pipette. The membrane conductance (G\(_m\)) was calculated by dividing the current (I\(_m\)) by the electrical driving force (V\(_m\) – V\(_{rev}\)), with G\(_m\) = I\(_m\)/(V\(_m\) – V\(_{rev}\)). Relaxations in the current recordings were fitted with a single exponential using the least-squares fitting routine available in the pClamp software (Axon Instruments, Inc.). The fit was commenced approximately 20 ms after each voltage step to allow enough time for the slow capacitative currents to decay.\(^{3}\) Results are reported as the mean ± SEM, with \(n\) the number of observations, in parentheses. Statistical comparisons were performed using Student’s t-test, with the appropriate corrections (Bonferroni) when used for multiple comparisons.

**Membrane Capacitance Measurements**

Voltage clamp experiments provide for measurement of the transmembrane current (I) required to maintain an applied membrane voltage. A step to a new clamp voltage (V) results in a brief transient current spike, due to the membrane capacitance, that decays exponentially. The membrane capacitance (C) was calculated from the area-under-the-curve (AUC) of that transient capacitative current spike after a voltage step (ΔV) in the following way:

\[
I = C \frac{\Delta V}{\Delta t},
\]

with

\[
AUC = tA,
\]

to give

\[
C = \frac{AUC}{\Delta V}.
\]
replaced all but 16 mM of the Cl\textsuperscript- in the bathing solution with the monovalent anions.

The free Ca\textsuperscript{2+} in the pipette solutions was adjusted by using different amounts of EGTA\textsuperscript{3-} to achieve concentrations of 1.0 \times 10^{-6} M (1.54 mM Ca\textsuperscript{2+} and 1.73 mM EGTA), 1.5 \times 10^{-7} M (0.97 mM Ca\textsuperscript{2+} and 1.92 mM EGTA) or 1.0 \times 10^{-8} M (no added Ca\textsuperscript{2+}, 5 mM EGTA).

Hyposmotic bath solutions (217 mOsm) were prepared by adding deionized water to the standard bath solution (310 mOsm). Hyperosmotic bath solutions (398 mOsm) were prepared by adding 90 mM mannitol (Sigma-Aldrich) to the standard bath solution (310 mOsm).

Furosemide, DIDS, tetraethylammonium chloride (TEA) and Na-gluconate were obtained from Sigma-Aldrich and tetrathymelammonium chloride (TMA) and diphenylamine-2-carboxylic acid (DPC) were obtained from Fluka-Sigma-Aldrich (Buchs, Switzerland). All chemicals used in the experiments were of AR grade or higher.

RESULTS

Identification of the Outward Chloride Currents, Cl\textsubscript{OR}

The series resistance of 8.9 \pm 0.3 MΩ (n = 17) was not compensated for electronically during the experiments, and the average cell capacitance was 9.0 \pm 0.2 pF (n = 17). The resting cell membrane potential with the standard solutions in the pipette (KCl-rich) and in the bath (NaCl-rich) was \(-40 \pm 2\) mV (n = 17), similar to the resting membrane potential of \(-37 \pm 8\) mV (n = 50) obtained with intracellular electrodes.\textsuperscript{31,34}

The inset in Figure 1A shows a typical example of whole-cell currents evoked by voltage steps between \(-60\) mV and +80 mV. The steady state whole-cell current-voltage relation (I-V) from a sample of 12 cells showed a dominant outwardly rectifying current, which activated at potentials more positive than \(-40\) mV (Fig. 1A). The conductance of these currents was 24 \pm 1 nS (n = 12) in the range from \(+10\) to +80 mV. The addition of 10 mM TEA extracellularly to block K\textsuperscript+ channels shifted the I-V reversal potential from \(-39 \pm 2\) to \(-1 \pm 2\) mV (n = 8) (Fig. 1A) and reduced the chord conductances for inward currents from 3.9 \pm 0.4 to 1.8 \pm 0.2 nS by (54%, n = 8) and for outward currents from 24 \pm 1 to 16.2 \pm 1.5 nS by (33%, n = 8).

We studied the nature of the TEA-insensitive component of the current by substituting Na\textsuperscript+ for K\textsuperscript+ in the pipette solution and adding 5 mM CsCl to the bath solution. The Cl\textsuperscript- concentrations in the bath and pipette solutions were 159 and 142 mM, respectively. Under these conditions the whole-cell I-V had a reversal potential of \(-1.0 \pm 0.4\) mV (n = 25), suggesting that it was carried by either Na\textsuperscript+ or Cl\textsuperscript- ions. In five subsequent experiments, replacement of pipette Na\textsuperscript+ with the larger cation tetramethylammonium (TMA) had no effect on the reversal potential (Fig. 1B). We concluded that the current was carried by Cl\textsuperscript- ions, which we confirmed by substituting 125 mM of the extracellular NaCl with Na-gluconate, which shifted the reversal potential to \(-39 \pm 2\) mV (n = 5; Fig. 1B). The Nernst potential for Cl\textsuperscript- was 48 mV under these conditions, indicating that Cl\textsubscript{OR} channels carried the TEA-insensitive component of the macroscopic whole-cell current.

Pharmacologic confirmation that this TEA-insensitive current was carried by Cl\textsuperscript- ions was observed by the use of several known chloride channel blockers which markedly inhibited Cl\textsubscript{OR} current activity (Fig. 2). The blockers used were furosemide (1 mM) which is also known to inhibit Na\textsuperscript+-K\textsuperscript-Cl\textsuperscript- cotransporters, DPC (1 mM), and DIDS (2 mM).

Ca\textsuperscript{2+} and Voltage Activation of the Cl\textsubscript{OR} Currents

Figure 3A shows the whole-cell I-V evoked by voltage steps between \(-60\) and +80 mV when the free Ca\textsuperscript{2+} concentration in the pipette solution was either 1.0 \times 10^{-9} (n = 6), 1.5 \times 10^{-9} (n = 8), or 1.0 \times 10^{-7} M (n = 6). The pipette solution for these experiments contained 5 mM CsCl with KCl replaced by NaCl. Figure 3B shows that the total membrane conductance of the outward currents increased by 117\% when the [Ca\textsuperscript{2+}] was changed from 1.0 \times 10^{-9} to 1.5 \times 10^{-7} M, but only increased a further 16\% when the [Ca\textsuperscript{2+}] was changed from 1.5 \times 10^{-7} to 1.0 \times 10^{-6} M. Within that range of [Ca\textsuperscript{2+}], it appeared that a half-maximum increase in conductance is on the order of 10^{-6} M.

Figure 3B also demonstrates that the Cl\textsubscript{OR} conductance was voltage-activated. At the [Ca\textsuperscript{2+}] of 1.0 \times 10^{-7} M, the Cl\textsubscript{OR} conductance increased at membrane potentials more positive than +10 mV. The modulating effect of Ca\textsuperscript{2+} on this voltage-activation was to shift the potential at which the Cl\textsubscript{OR} conductance increased toward more negative potentials. For example, this potential was around \(-20\) mV at 1.5 \times 10^{-7} M [Ca\textsuperscript{2+}], and around \(-40\) mV at 1.0 \times 10^{-6} M [Ca\textsuperscript{2+}].

Analysis of Cl\textsubscript{OR} Current Relaxations

Figure 4 shows the relation between the “on” relaxation time constant (\(\tau_{ON}\)) and membrane potential with different levels of
The \( \tau_{\text{ON}} \) decreased from \( 61 \pm 9 \text{ ms} \) (\( n = 6 \)) to \( 23 \pm 3 \text{ ms} \) (\( n = 6 \)) over the same voltage range. However, there was no statistically significant difference between the effect of \( [\text{Ca}^{2+}]_i \) on \( \tau_{\text{ON}} \) within the voltage range \(-20\) to \(+50 \text{ mV} \). The activation kinetics were significantly slower (\( \tau_{\text{ON}} \) larger) at \( 1.0 \times 10^{-6} \text{ M} \) \( [\text{Ca}^{2+}]_i \), compared with \( 1.5 \times 10^{-7} \text{ M} \) \( [\text{Ca}^{2+}]_i \) when the cell potential was clamped at \( +60 \text{ mV} \) (\( P = 0.046 \)), \( +70 \text{ mV} \) (\( P = 0.04 \)), or \( +80 \text{ mV} \) (\( P = 0.001 \)). The relaxation of the current could not be reliably fitted with exponential curves when the \( \text{Ca}^{2+} \) concentration was \( 1.0 \times 10^{-9} \text{ M} \).

**Ion Selectivity of the \( \text{Cl}_{\text{OR}} \) Currents**

The selectivity of the \( \text{Cl}_{\text{OR}} \) channel to other anions was derived from reversal potential measurements when all but 16 mM of

\[ [\text{Ca}^{2+}]_i \] concentrations on whole-cell \( \text{Cl}^- \) currents. Steady state whole-cell I-V relations of freshly isolated single RSLG) acinar cells with free \( \text{Ca}^{2+} \) ion concentrations in the pipette solution of \( 1.0 \times 10^{-9} \text{ M} \) (\( \bullet \), \( n = 6 \)), \( 1.5 \times 10^{-7} \text{ M} \) (\( \circ \), \( n = 8 \)), and \( 1.0 \times 10^{-6} \text{ M} \) (\( \square \), \( n = 6 \)). The cells were held at \(-50 \text{ mV} \) between voltage steps of 10 mV increments over the range from \(-60\) to \(+80 \text{ mV} \). (B) Relation between the membrane potential and the total membrane conductance (\( \text{G}_{\text{m}} \)) for the \( \text{Cl}_{\text{OR}} \) channels, for free \( \text{Ca}^{2+} \) concentrations in the pipette solution of \( 1.0 \times 10^{-9} \text{ M} \) (\( \bullet \), \( n = 6 \)), \( 1.5 \times 10^{-7} \text{ M} \) (\( \circ \), \( n = 8 \)), and \( 1.0 \times 10^{-6} \text{ M} \) (\( \square \), \( n = 6 \)). The \( \text{Cl}_{\text{OR}} \) conductance increased as the membrane potential became more positive and as the \( \text{Ca}^{2+} \) concentration was increased. The potential at which the conductance started to increase shifted toward more negative values as the \( \text{Ca}^{2+} \) concentration increased, with \( +10 \text{ mV} \) at \( 1.0 \times 10^{-7} \text{ M} \), \( -20 \text{ mV} \) at \( 1.5 \times 10^{-7} \text{ M} \), and \( -40 \text{ mV} \) at \( 1.0 \times 10^{-6} \text{ M} \). Each experiment, the pipette solution contained \( \text{Na}^+ \) ions rather than \( \text{K}^+ \) ions and 5 mM \( \text{Cs}^+ \) was present in the bath solution. In all the experiments the bars represent the SEM when these were larger than the symbols.
Effect of pH on ClOR Current Activity

Figure 5A shows current profiles recorded from three cells by using pipette solutions of different pH and with the free [Ca\(^{2+}\)]\(_i\) set at 1.5 \times 10^{-7} \text{ M}. At pH 7.8, the time-dependent ClOR currents at depolarizing potentials were substantially larger than at pH 7.4, whereas at pH 6.8 the ClOR current was inhibited. Figure 5B is a plot of the current density as a function of membrane potential at various pH pipette solutions. The current density was determined by dividing the measured ClOR current by the capacitance of the membrane for each cell. It was important to use current density since that allowed us to separate the pH-induced changes in the magnitude of the ClOR current from those changes due simply to variations in cell size. The capacitance, and hence size, of the cells exposed to each pH was not significantly different (P < 0.05, n = 14). The mean (±SEM) cell capacitances were 13.1 ± 0.3 pF at pH 6.8, 13.0 ± 0.2 pF at pH 7.4, and 13.2 ± 0.2 pF at pH 7.8. At pH 7.4 and pH 7.8 the I–V relationship between -60 and +80 mV displayed outward rectification with increasing depolarizing potentials. Lowering intracellular pH (pH\(_i\)) from 7.4 to 6.8 markedly decreased outward current and the I–V relationship was almost linear. This inhibitory effect of lowering pH on ClOR current activity was independent of the Ca\(^{2+}\) concentration in the pipette solution. It was observed that the ClOR current was inhibited at pH 6.8 even at free [Ca\(^{2+}\)]\(_i\) concentrations of 1.0 \times 10^{-5}, 1.0 \times 10^{-4}, and 1.0 \times 10^{-3} \text{ M}. The results shown in Figure 5B indicate that changes in ClOR current activity are due to changes in RSLG and not due to cell size. This indicates that variation in current density between cells is not an artifact causing the changes in ClOR current activity, but in fact these variations in current activity are directly due to pH changes.

### TABLE 1. Relative Anion Permeability and Conductivity Sequences for the ClOR Channel

<table>
<thead>
<tr>
<th>Anion</th>
<th>(\Delta E_{rev}) (mV)</th>
<th>Relative Permeability</th>
<th>Relative Conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO(_3^-)</td>
<td>-6.5 ± 1.7</td>
<td>1.32 ± 0.1</td>
<td>1.30 ± 0.1</td>
</tr>
<tr>
<td>I(^-)</td>
<td>-10.7 ± 2.4</td>
<td>1.58 ± 0.1</td>
<td>0.70 ± 0.1</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>-14.0 ± 1.1</td>
<td>1.80 ± 0.1</td>
<td>1.24 ± 0.1</td>
</tr>
<tr>
<td>SCN(^-)</td>
<td>-20.1 ± 2.2</td>
<td>2.32 ± 0.2</td>
<td>1.14 ± 0.1</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, we showed that the RSLG acinar cells contain a ClOR channel, which we found to be both Ca\(^{2+}\)- and voltage-activated and which responds to hyposmotic stress. Those characteristics are relevant to the cell homeostasis after the secretory functions of the RSLG. By analogy, there is a decrease in cell volume during secretion in other glands.\(^{20,32}\)

The Cl\(^-\) channel that we characterized carries approximately one-third of the macroscopic outward current in the rabbit lacrimal gland acinar cells, which we identified as the ClOR current. We identified the ClOR channels by ion substitution experiments, in which the I–V reversal potential shifted from -1 to +39 mV, and then with the use of the chloride channel blockers furosemide, DIDS and DPC. The ClOR channels were activated by cell depolarization and by [Ca\(^{2+}\)]\(_i\) in the range 1.0 \times 10^{-9} to 1.0 \times 10^{-4} \text{ M} (with an EC\(_{50}\) approximately 1.0 \times 10^{-8} \text{ M}). The conductance, permeability properties and activation kinetics of the ClOR Channel in the RSLG distinguished it from Cl\(^-\) channels in other epithelial cell types. Furthermore, the permeability sequence and sensitivity was almost linear. This inhibitory effect of lowering pH on ClOR current activity was independent of the Ca\(^{2+}\) concentration in the pipette solution. It was observed that the ClOR current was inhibited at pH 6.8 even at free [Ca\(^{2+}\)]\(_i\) concentrations of 1.0 \times 10^{-5}, 1.0 \times 10^{-4}, and 1.0 \times 10^{-3} \text{ M}. The results shown in Figure 5B indicate that changes in ClOR current activity are due to changes in RSLG and not due to cell size. This indicates that variation in current density between cells is not an artifact causing the changes in ClOR current activity, but in fact these variations in current activity are directly due to pH changes.

**Effect of Osmotic Stress on ClOR Current Activity, Cell Volume, and [Ca\(^{2+}\)]\(_i\)**

A sample of cells (n = 8) exposed to a hyposmotic solution (217 mOsM) showed a gradual increase in ClOR current, measured at a holding potential of +80 mV, which reached a peak at 6 minutes and then quickly reduced to the baseline level (Fig. 6A). Analysis of the activation kinetics of the maximal ClOR current at 6 minutes showed that the time constant had decreased to 15 ± 2 ms (n = 11) from the baseline value of 68 ± 6 ms (n = 11).

The volume, measured using confocal microscopy, increased when a sample of cells (n = 22) was exposed to the hyposmotic solution (Fig. 6B). The cell volume reached a peak at 4 minutes and then the volume oscillated to follow a gradual return to the baseline volume at around 18 minutes after exposure to the hyposmotic solution. [Ca\(^{2+}\)]\(_i\) in a sample of cells (n = 18) exposed to the hyposmotic solution increased to reach a peak at 6 minutes (Fig. 6C). The [Ca\(^{2+}\)]\(_i\) oscillated to follow a gradual return to the baseline level around 15 minutes after exposure to the hyposmotic solution.
permeability sequence of CFTR is different. Furthermore, the sequence (Table 2) is quite different from the ClOR channel found in the superior lacrimal gland of the rabbit. The Cl channel was inhibited by furosemide, but its anion selectivity sequence differed from the ClOR channel in the RSLG. Similarly, the Cl channel in the rat lacrimal gland35 is also activated by depolarization and \([Ca^{2+}]_{i}\) concentration was 0.5 \(\times 10^{-7}\) M free \(Ca^{2+}\) ions and 5 mM Ca was present in the bath solution.

The activation kinetics of the ClOR channels are faster than the Cl channels in either the sheep parotid or rat lacrimal gland acini. In sheep parotid and rat lacrimal glands, the time constant of the single exponential fitted to the “on” current relaxation \(\tau_{on}\) was generally greater at more positive potentials than for the ClOR channels in the RSLG. However, in rat lacrimal gland cells with a large \([Ca^{2+}]_{i}\) concentration (> 500 mM) the trend was for \(\tau_{on}\) to decrease slightly for more positive potentials, and under these conditions \(\tau_{on}\) was similar to the ClOR channels in the rabbit. We did not find significant differences between \(\tau_{on}\) when the \([Ca^{2+}]_{i}\) concentration was changed from 1.5 \(\times 10^{-7}\) to 1.0 \(\times 10^{-6}\) M. The interspecies variations in activation kinetics and the modulation of gating by \(Ca^{2+}\) suggest that there are differences in the structure of \(Ca^{2+}\)- and voltage-activated Cl channels present in different exocrine glands. These differences more importantly highlight the interspecies variation between rabbit and rat lacrimal glands.

Similar to the results presented here for the ClOR channel, the inhibition of \(Ca^{2+}\)-activated Cl channels by a decrease in pH, has been reported in rat lacrimal glands52 and rat parotid acinar cells.16 However, studies on T84 cells45 on a mutant form of the cystic fibrosis transmembrane conductance channel showed that a decrease in pH actually causes an increase in Cl channel activity. Other studies3 suggest that this may be due to a decrease in the interactions between charged sites within the channel pore and the permeating ions leading to an increase in channel conductance. Conversely, the decrease in ClOR current activity in rabbit superior lacrimal acinar cells may be due to an increase in the interaction between charged sites within the pore and the permeating H ions, resulting in conformational changes within the protein pore, affecting its structure and inhibiting the permeation of the Cl ions through the channel. There is limited previous evidence to suggest that pH plays a role in regulating channel activity in

Figure 5. Effect of pH on whole-cell currents in RSLG acinar cells. (A) Typical whole-cell currents recorded from three different acinar cells using pipette solutions containing 1.5 \(\times 10^{-7}\) M \([Ca^{2+}]_{i}\), with pH buffered at 6.8, 7.4, and 7.8. Voltage steps were applied over the range of –60 to 80 mV at 10-mV increments from a holding potential of –50 mV. In each experiment, the pipette solution contained Na ions rather than K ions, and 1.5 \(\times 10^{-7}\) free \(Ca^{2+}\) ions and 5 mM Ca were present in the bath solution. (B) Whole-cell steady state I–V relation between current density and membrane potential for the ClOR channel in freshly isolated RSLG acinar cells activated by an increase in pH. Currents were recorded between –60 and +80 mV in 10-mV voltage step increments at pH 6.8 (○, n = 14), 7.4 (●, n = 14), and 7.8 (□, n = 14) with each solution containing 1.5 \(\times 10^{-7}\) M free \(Ca^{2+}\) ions, and 5 mM Ca was present in the bath solution.
8.2. However, small fluctuations in pH have been observed in both lacrimal46 and salivary47–49 cells after stimulation with ACh, which causes a small transient intracellular acidosis followed by a sustained alkalinization. It has been suggested49 in rabbit salivary glands that the initial ACh induced transient acidosis may possibly be due to HCO$_3^-$ efflux through Cl$^-$ channels.

We investigated a role for the Cl$_{\text{OR}}$ channels in volume regulation because it has been shown in rat salivary glands49,52 that the acinar cells undergo volume changes during secretion. The response of this Cl$_{\text{OR}}$ current to osmotic stress is interesting, in that intuitively one might expect a decrease in Cl$_{\text{OR}}$ current under hyposmotic conditions. However, when cells are exposed to a hyposmotic solution they initially swell and subsequently undergo RVD.21,50–52 In many cells, this is observed by an increase in K$^+$ and Cl$^-$ channel activity leading to an extrusion of these ions with the passive movement of water afterward. After RVD, the decrease in cell volume then triggers an increase in the inward movement of Cl$^-$ which corresponds to the large transient increase in Cl$_{\text{OR}}$ current activity which causes an increase in cell volume. The cell volume returned to basal levels in an oscillatory manner after exposure to hyposmotic stress. This Cl$_{\text{OR}}$ channel in the RSLG which has some properties similar to those of the rat lacrimal1,3,53 and sheep parotid41 glands may be involved in the compensatory response to RVD initially induced by hyposmotic stress resulting in the influx of Cl$^-$ ions followed by the passive influx of water due to osmotic pressure. This type of RVI cellular response after RVD has been termed post-RVD RVI.5–7 Our experimental results support this post-RVD RVI phenomenon induced by hyposmotic stress, whereby we show that the increase in Cl$_{\text{OR}}$ channel activity is closely related to cell volume changes.

Confocal microscopy demonstrated that hyposmotic stress evoked an increase in [Ca$^{2+}$], concentrations which were maintained for up to 10 minutes. This increase in [Ca$^{2+}$], concentration may be the result of both release of Ca$^{2+}$ from intracellular stores and an influx of Ca$^{2+}$ from extracellular sources via stretch-activated cation channels located on the cell membrane. This response is not surprising since [Ca$^{2+}$], is involved in cell volume regulation54 and both Ca$^{2+}$-activated Cl$^-$ and K$^+$ channels are responsible for the oscillatory volume changes observed in returning cell volume back to basal conditions after hyposmotic shock. These changes in cell volume may be accompanied by fluctuations in pH, resulting in activation and deactivation of the Cl$_{\text{OR}}$ channel. Studies by Saito et al.55 showed that after stimulation of mouse lacrimal acinar cells by ACh, transient acidosis occurs followed by sustained alkalinization. Of interest, our experiments showed that Ca$^{2+}$-activated Cl$_{\text{OR}}$ channels are inhibited by a decrease in pH, and activated by an increase in pH. This continual competition between H$^+$ and Ca$^{2+}$ ions may lead to fluctuations in Cl$_{\text{OR}}$ current activity concomitant with changes seen in cell volume.

The Cl$_{\text{OR}}$ currents in the RSLG have two distinctive characteristics that are quite different from other volume sensitive Cl$^-$ currents in other epithelial cells. First, Cl$_{\text{OR}}$ currents were Ca$^{2+}$-dependent as shown by our patch-clamp studies, and calcium imaging using confocal microscopy showed that osmotic stress evoked an increase in [Ca$^{2+}$], concentrations. In contrast, patch-clamp studies in other epithelia including, human intestinal cells,55,56 human sweat glands,57 Madin Darby Canine Kidney cells,58 and T84 cells59 have identified Cl$^-$ channels that are activated by cell swelling due to hyposmotic solutions but are independent of [Ca$^{2+}$], concentrations. However, in some epithelia, cell swelling was shown to cause an increase in [Ca$^{2+}$], levels which activated K$^+$ channels, causing RVD.59,60,61

Second, the Cl$_{\text{OR}}$ current activity in the rabbit lacrimal gland is voltage dependent, with Cl$_{\text{OR}}$ currents being activated by
depolarization. A similar osmotically activated Cl_{OR} current in the rat lacrimal gland has also been found to be activated by depolarization. However, the investigators in those studies make no reference to its possible role in cell volume regulation. In contrast, volume sensitive Cl \textsuperscript{−} currents in other epithelia are inactivated at depolarizing potentials.\textsuperscript{57,59–61} Our functional characterization of the Cl_{OR} current in the RSLG shows that it shares the characteristics of both the swelling-activated (VRAC) and Ca\textsuperscript{2+}-activated (CaCC) Cl \textsuperscript{−} channel families. The recent reports that overexpression of CLC-3 produced a novel pH-sensitive current in HEK293T cells\textsuperscript{45} and that CLC-3 is expressed in rat lacrimal gland acini\textsuperscript{19} raise the intriguing possibility that Cl_{OR} may also share characteristics of CLC-3. Notwithstanding, the osmotically active Cl_{OR} current that we describe in RSLG acinar cells is rather unique and different from volume-sensitive Cl \textsuperscript{−} currents in other epithelial cells. Further studies are needed to accurately define the membrane domain in which the Cl_{OR} channels are localized.

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