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Inhibition of Ca^{2+}-dependent Cl^- Channels from Secretory Epithelial Cells by Low Internal pH

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Abstract. The actions of intracellular pH (pH_i) on Ca^{2+}-dependent Cl^- channels were studied in secretory epithelial cells derived from human colon carcinoma (T84) and in isolated rat parotid acinar cells. Channel currents were measured with the whole cell voltage clamp technique with pipette solutions of different pH. Ca^{2+}-dependent Cl^- channels were activated by superfusing ionomycin to increase the intracellular calcium concentration ($[Ca^{2+}]_i$) or by using pipette solutions with buffered Ca^{2+} levels. Large currents were activated in T84 and parotid cells by both methods with pH_i levels of 7.3 or 8.3. Little or no Cl^- channel current was activated with pH_i at 6.4. We used on-cell patch clamp methods to investigate the actions of low pH_i on single Cl^- channel current amplitude in T84 cells. Lowering the pH_i had little or no effect on the current amplitude of a 8 pS Cl^- channel, but did reduce channel activity. These results suggest that cytosolic acidification may be able to modulate stimulus-secretion coupling in fluid-secreting epithelia by inhibiting the activation of Ca^{2+}-activated Cl^- channels.

Key words: Ca^{2+}-dependent Cl^- channels — pH_i — T84 cells — Parotid acinar cells — Cl^- secretion — Ionomycin

Introduction

Fluid and electrolyte secretion in many epithelia are linked to the movements of Cl^- ions. A major pathway for Cl^- flux in these epithelia is provided by Cl^- channel proteins located in the apical membrane. There are at least three general classes of Cl^- channels in secretory cells: channels activated by (i) intracellular Ca^{2+}, (ii) changes in cell volume, and (iii) cAMP (Findlay & Petersen, 1985; Evans & Marty, 1986; *see also* review by Petersen, 1992). All three types of channels have been identified in the T84 human colonic tumor cell line, whereas, only Ca^{2+}- and volume-sensitive channels appear to be expressed in rat parotid acinar cells (Cliff & Frizzell, 1990; Anderson & Welsh, 1991; Arreola, Melvin & Begenisich, 1995).

Control of fluid and electrolyte secretion by hormones and neurotransmitters is often mediated through activation of Ca^{2+}- and cAMP-dependent Cl^- channels (Frizzell & Halm, 1990). There are several observations that suggest that this secretory process may be inhibited by reductions in intracellular pH (pH_i) (Martinez & Cassity, 1985; Novak & Young, 1986; Pirani et al., 1987). The cAMP-activated Cl^- channels in T84 cells are not inhibited by low pH_i; indeed, low pH slightly increases the current through single channels of this class (Halm & Frizzell, 1992). Thus, the inhibition of secretion during cytosolic acidification may be due to alteration in the activity of Ca^{2+}-dependent channels.

To investigate this possibility, we measured the Ca^{2+}-dependent Cl^- current in T84 and rat parotid acinar cells. We found that the magnitude of this current was sensitive to pH_i. Low pH_i inhibited the activation of whole cell current without affecting the single channel current amplitude. Thus, Ca^{2+}-activated Cl^- channels have the necessary properties to account for the observed actions of cytosolic pH on secretion. Some of these results have been presented in abstract form (Arreola, Melvin, & Begenisich, 1993).

Materials and Methods

Cell Culture of Human Colon Carcinoma Cell Line

For many of the experiments in this study, we used cells derived from a human colon carcinoma cell line (T84). This cell line is capable of

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maintaining vectorial electrolyte transport (Dharmsathaphorn et al., 1984), and carbachol induces Cl^- secretion in a Ca^{2+}-dependent manner (Dharmsathaphorn & Pandol, 1986; Dharmsathaphom, Cohn, & Beuerlein, 1989). Moreover, Ca^{2+} and cAMP act synergistically on Cl^- secretion (Cartwright et al., 1985) suggesting the presence of different Cl^- efflux pathways, recently demonstrated using the patch clamp technique (Cliff & Frizzell, 1990).

T84 cells were obtained from the American Type Culture Collection (ATCC No. CCL 248, Rockville, MD) and grown at 37°C in a humidified chamber gassed with 95% air and 5% CO_2. Monolayers of aggregated cells were cultured in a solution composed of 50% Ham's F12 medium and 50% Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. For patch clamp experiments, monolayers of cells were dispersed by incubating the cells for 20 min with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline. Dispersed cells were washed with fresh media and plated on 10 mm diameter glass coverslips (Rochester Scientific, Rochester, NY). Cells were incubated for an additional 48 hr before use. The dispersion protocol and the age of the plated cells on coverslips were critical factors. Trypsin-dispersed cells that were incubated on coverslips for less than 24 hr did not show a significant increase in the Cl^- current upon stimulation with 1–10 μM ionomycin. However, cells that were dissociated with trypsin and plated on coverslips for >36 hr, or cells that were dissociated without trypsin and plated for less than 24 hr, did respond to 1 μM ionomycin (*unpublished results*). All T84 cells used in this study were dissociated without trypsin and grown as single cells for at least 48 hr.

Single Rat Parotid Cell Dissociation

Details of the technique used to dissociate single parotid acinar cells has been published elsewhere (Arreola et al., 1995). Male 150–250 g Wistar strain rats (Charles River, Kingston, NY) were used in these experiments. Dissected glands were minced in minimum essential medium (MEM)-Ca^{2+} free (Gibco BRL., Gaithersburg, MD) + 1% bovine serum albumin (BSA) (Fraction V, Sigma Chemical, St. Louis, MO). The minced tissue was treated for 20 min (37°C) with a 0.02% trypsin solution (MEM-Ca^{2+} free + 1 mM EDTA + 2 mM glutamine + 1% BSA). After stopping the reaction with 2 mg/ml of soybean trypsin inhibitor (Sigma), the tissue was further dispersed with collagenase (100 units/ml of type CLSPA, Worthington Biochemical, Freehold, NJ) in MEM-Ca^{2+} free + 2 mM glutamine + 1% BSA. After 60 min, the acinar suspension was centrifuged, the supernatant discarded and the pellet resuspended in fresh collagenase solution for an additional 60 min. This material was then centrifuged and washed with basal medium eagle (BME) (Gibco)/BSA-free. The "single cell" pellet was resuspended in 2–5 ml of BME/BSA-free + 2 mM glutamine and plated onto poly-l-lysine coated glass coverslips.

Electrophysiological Recordings

Whole Cell Recordings

The whole cell voltage clamp configuration of the patch clamp technique (Hamill et al., 1981) was used to record ionic currents with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Electrodes were fabricated with KG-12 glass (Garner Glass, Claremont, CA) and had resistances of 2–4 MΩ when filled with the internal solutions. The current was filtered at 5 kHz using an 8 db/decade low pass Bessel filter. Macroscopic currents were digitized at 0.5 kHz with a 12-bit analog-to-digital converter. All the experiments were done at room temperature (approximately 22–24°C). Mean ± SEM values are given or plotted.

Single Channel Recordings

Single channel currents from T84 cells were obtained with the on-cell configuration of the patch clamp technique. The cell resting potential was taken to be near zero mV in the high K^+ solution bathing the cells (*see below*). Currents were filtered at 200 Hz. Single channel current levels were determined from fits of a Gaussian function to amplitude histograms.

To study the effects of intracellular acidification on single Cl^- channel currents activated by Ca^{2+}, we set the pH_i to either 7.3 or 6.5 utilizing 5 μM nigericin for equilibrating $[H^+]_e = [H^+]_i$ (Thomas et al., 1979). Equilibration of pH_i with nigericin was complete within 5 min as verified with the pH-sensitive dye, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), as previously described (Melvin, Moran & Turner, 1988). Cells were bathed with the following external solution to abolish the resting potential (in mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, nigericin 0.005, pH of 7.3 or 6.5. The pipette solution composition was (in mM): CsCl 85, CsF 40, $CaCl_2$ 2, HEPES 10, pH = 7.3. The $[Ca^{2+}]_i$ was raised by perfusing the cell with the above external solution containing 1 or 0.5 μM ionomycin. A period of 8 min for intracellular pH equilibration was allowed between changes of bath solution pH. Single channel currents were recorded 20 sec following the equilibration period.

Whole Cell Recording Solutions

External Solutions

The regular external solution of pH 7.3 contained (in mM): NaCl 144, KCl 5, $CaCl_2$ 5 or 2, $MgCl_2$ 1, HEPES 10, and glucose 10. The Ca^{2+}-free external solution was the same but with 3 mM $MgCl_2$ and no $CaCl_2$. Ca^{2+}-dependent Cl^- channels were (usually) activated by 1 μM ionomycin (from a 5 or 10 mM stock in dimethyl sulphoxide, DMSO) to increase the $[Ca^{2+}]_i$. DMSO had no effect on the currents at the concentration used.

Internal Solutions, pH_i and $[Ca^{2+}]_i$, for T84 cells

The standard pipette solution (EGTA, pH 7.3) for recording ionomycin induced currents in T84 cells consisted of (in mM): CsCl 85, CsF 40, $CaCl_2$ 5, EGTA 5, HEPES 10, pH 7.3. The presence of Cs^+ in this solution served to minimize the contribution of K^+ currents (Yellen, 1984). The free Ca^{2+} concentration in this solution was determined to be 384 nM using fura-2 as described by Grynkiewicz, Poenie, & Tsien (1985). To ensure equilibration of the pipette solution in the cytosol, cells were allowed 8 min of stabilization after the patch membrane was ruptured.

To examine the effects of pH_i on the Ca^{2+}-activated Cl^- currents in T84 cells, we designed two additional solutions (after Tsien & Pozzan, 1989) with pH levels of 8.3 and 6.4. These solutions contained BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) as the Ca^{2+} buffer since the binding of Ca^{2+} to this buffer is much less pH sensitive than to EGTA (Harrison & Bers, 1987). One set of such solutions (Table) was designed for a low, nominal, free Ca^{2+} concentration of 100 nM and a free BAPTA concentration of 0.5 mM. The measured free Ca^{2+} concentrations were close to this nominal value (Table, last column).

As described above, we used the Ca^{2+} ionophore to increase intracellular Ca^{2+} levels and so activate the Ca^{2+}-dependent Cl^- channels. To eliminate the added complexity introduced by ionomycin and any possible pH sensitivity of this process (Fasolato & Pozzan, 1989), we also designed solutions of pH 8.3 (buffered with EPPS) and

Table 1. Pipette solutions for whole cell recording from T84 cells (mM)

Solution	pH	CsCl	CsF	$CaCl_2$	BAPTA	EPPS	MES	Ca^{2+} (nM)
Low Ca^{2+}, pH 8.3	8.3	85	20	0.2	0.7	30		103
Low Ca^{2+}, pH 6.4	6.4	85	20	0.13	0.63		30	83
High Ca^{2+}, pH 8.3	8.3	85	20	1.25	1.75	30		420
High Ca^{2+}, pH 6.4	6.4	85	20	0.625	1.125		30	300

6.4 (buffered with MES) with high (nominally 500 nM) free Ca^{2+} to directly activate the Cl^- channels. The free Ca^{2+} concentrations were determined using Ca^{2+}-fura-2 (Grynkiewicz et al., 1985) K_d values of 290 nM and 153 nM at pH 6.4 and 8.3, respectively (Lattanzio, 1990 and *personal communication* from Tom Gunther, Department of Biophysics, University of Rochester School of Medicine). While there is some experimental uncertainty associated with these K_d values and the technique in general, the levels of free Ca^{2+} determined with fura-2 (*see* Table) are not far from the nominal values. The somewhat lower levels in the high Ca^{2+} solutions are likely due to the formation of complexes with F^- ions.

Internal Solutions for Parotid Acinar Cells

We found that the Ca^{2+}-activated Cl^- channels in parotid acinar cells were more sensitive to intracellular Ca^{2+} than the channels in T84 cells. Consequently, intracellular solutions with very low Ca^{2+} levels were required for these cells. These solutions contained 0.5 mM BAPTA without added Ca^{2+} along with 135 mM TEA-Cl and 5 mM TEA-F. Two such solutions were used: one with pH 7.3 (buffered with 20 mM TES) and one at pH 6.4 (20 mM MES). The TEA^+ in these solutions, like Cs^+ in the solutions for T84 cells, do not pass through K channels and so enhance the recording of Cl^- channel currents.

Chemicals

All the salts, as well as the pH buffers HEPES, EPPS, TES, and MES were purchased from Sigma (St. Louis, MO). EGTA and BAPTA were from Sigma and Fluka (Buchs, Switzerland), respectively. Glibenclamide was obtained from Research Biochemical Incorporated (Natick, MA). 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and ionomycin were obtained from Calbiochem (San Diego, CA). Nigericin and BCECF were from Molecular Probes (Eugene, OR).

Results

Ca^{2+}-Dependent Cl^- -Current

As described in Materials and Methods, the solutions used in this study were designed to optimize the recording of Cl^- channel currents. An example of the currents observed in T84 cells recorded with the standard pipette solution with EGTA at pH 7.3 is illustrated in Fig. 1. Control currents, recorded in the absence of ionomycin, are shown in the left panel of Fig. 1*A*. Superimposed are currents in response to voltages from −80 to +80 mV in 40 mV increments. The right panel contains currents recorded under identical conditions but in the presence of 1 μM ionomycin. The currents at all voltages were substantially enhanced by ionomycin and a characteristic time dependence became apparent. In six such experiments the relative increase in current (at +60 mV) induced by ionomycin ranged from a factor of 1.5 to 13.3.

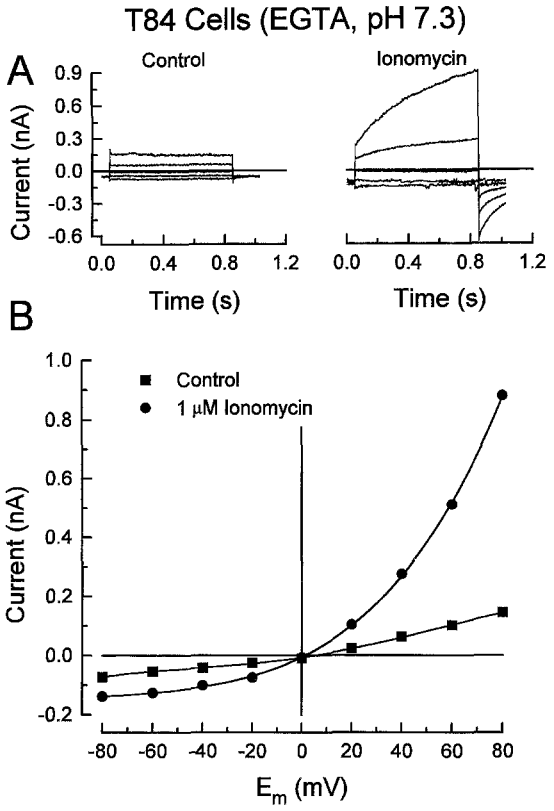

Fig. 1. Ionomycin-activated current in T84 cells. (*A*) Macroscopic currents recorded in control conditions, and in the presence of 1 μM ionomycin. Ionic currents were recorded at voltages of −80 to +80 mV in 40 mV steps from a holding potential of −50 mV. (*B*) Current-voltage relations in the control condition (■), and in the presence of 1 μM ionomycin (●). The standard EGTA, pH 7.3 pipette solution was used.

The current-voltage relations of the data in Fig. 1*A* are illustrated in Fig. 1*B*. Plotted are the current magnitudes at the end of the voltage clamp pulses. The current-voltage relation in the presence of ionomycin displayed the outward rectification previously observed under similar conditions (Cliff & Frizzell, 1990; Anderson & Welsh, 1991). The current reversal potential in the absence and presence of ionomycin was about −6 mV. The small difference between this value and the Cl^- equilibrium potential of −11 mV may be due to a small,

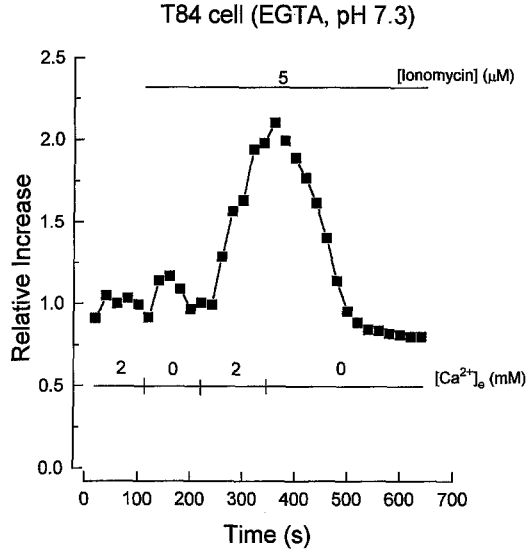

Fig. 2. Ca^{2+} dependency of the ionomycin-activated current from T84 cells. Time course of the relative increase of the ionic current amplitude induced by 5 μM ionomycin measured at +60 mV. Ionomycin was perfused during the time indicated by the solid line in the presence or absence of extracellular Ca^{2+}. Ionic currents were recorded every 20 sec. Current amplitudes were normalized to the average of the values obtained with 2 mM external Ca^{2+} in the absence of ionomycin (first 100 sec). The time course of the relative current increase is similar to the solution exchange time of the experimental chamber. The holding potential was −100 mV. The standard, EGTA, pH 7.3 pipette solution was used.

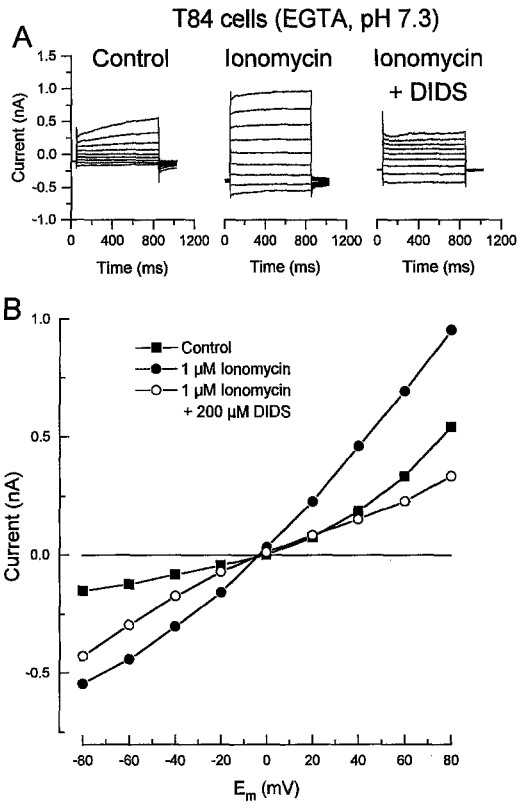

Fig. 3. Ca^{2+}-dependent currents were DIDS sensitive. (*A*) Macroscopic currents recorded in control conditions, in the presence of 1 μM ionomycin, and in the presence of 1 μM ionomycin + 200 μM DIDS. Ionic currents were recorded at voltages of −80 to +80 mV in 20 mV steps from a holding potential of −50 mV. (*B*) Current-voltage relations in the control condition (■), in the presence of 1 μM ionomycin (●), and in the presence of 1 μM ionomycin + 200 μM DIDS (○). The standard EGTA, pH 7.3 pipette solution was used.

uncompensated junction potential or to a small channel permeability to F^-.

The data of Fig. 2 demonstrate that the enhancement of current by ionomycin required the presence of extracellular Ca^{2+}. This figure shows the relative membrane current increase (recorded at +60 mV every 20 sec) during superfusion with Ca^{2+} free or Ca^{2+} containing (2 mM) solutions with or without 5 μM ionomycin, as indicated. In the absence of external Ca^{2+}, ionomycin did not increase the current. However, with 2 mM extracellular Ca^{2+}, ionomycin produced an enhancement of about 2.2 over the basal current. Subsequent removal of external Ca^{2+} in the continued presence of ionomycin caused the current to decline back to control levels. The decline of the current amplitude after external Ca^{2+} removal is likely due to a reduction of $[Ca^{2+}]_i$ in the continuous presence of ionomycin. The current activated by ionomycin was also sensitive to the amount of EGTA/Ca^{2+} in the pipette solution. In the presence of external Ca^{2+}, ionomycin did not increase the current when the pipette solution contained 5 mM EGTA and no added Ca^{2+} (*data not shown*).

The increase in current induced by ionomycin treatment was inhibited by the Cl^- channel blocking agent, DIDS (Fig. 3). The first two panels of Fig. 3*A* illustrates, as in Fig. 1, the enhancement of current by 1 μM ionomycin. The addition of 200 μM DIDS in the continuous presence of ionomycin reduced the amplitude of the ionic currents (right panel of Fig. 3*A*). The corresponding current-voltage relations of the data in Fig. 3*A* are shown in Fig. 3*B*. It is apparent from these current-voltage relations that DIDS inhibited the currents in the presence of ionomycin at positive more than at negative potentials. This apparent voltage sensitivity of DIDS inhibition was a consistent finding. For example, in three T84 cells treated with 200 μM DIDS, the currents at +50 mV were inhibited by 52 ± 12% but only by 20 ± 14% at −50 mV.

We investigated the anion selectivity of the current induced by ionomycin by substituting I^-, NO_3^-, or aspartate for 144 mM of the Cl^- in the bath solution. The permeability ratios for I^- and NO_3^- were calculated from the shift of the current reversal potentials using the Goldman, Hodgkin and Katz equation (Hille, 1971). The shifts for I^- and NO_3^- were −19 ± 4 mV (n = 6) and −17 ± 4 mV (n = 5) and the computed P_I/P_{Cl} and P_{NO3}/P_{Cl} values were 2.2 and 2.1, respectively. Outward currents in the presence of external aspartate were very small and the current reversal potential was approximately 20 mV

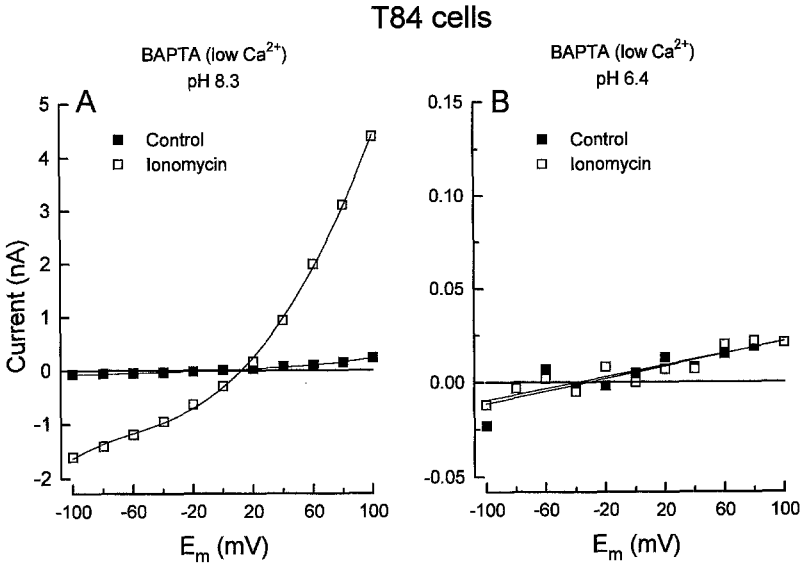

Fig. 4. Effect of high and low pH_i on the ionomycin-activated Cl^- current from T84 cells recorded with BAPTA-buffered internal solutions. Current-voltage relationships of the Ca^{2+}-dependent Cl^- current before (■) and after addition of 1 μM ionomycin (□) at pH_i of 8.3 (*A*) and 6.4 (*B*). Currents were recorded from −100 to +100 mV in 20 mV steps from a holding potential of −50 mV.

(n = 2) more positive than currents in the presence of Cl^-. The observed higher permeability to I^- and NO_3^- than to Cl^- is a property of Ca^{2+}-activated but not voltage- or cAMP-gated Cl^- channels (Evans & Marty, 1986; Cliff & Frizzell, 1990; Anderson & Welsh, 1991).

The current induced by ionomycin in these experiments had many of the properties previously described in T84 cells and identified with Ca^{2+}-activated Cl^- channels (Cliff & Frizzell, 1990). These properties include: (i) a reversal potential near the Cl^- equilibrium potential; (ii) high selectivity for I^- and NO_3^- and a low permeability of aspartate; (iii) ionomycin and external Ca^{2+} sensitivity; and (iv) inhibition by DIDS. Taken together, these results support the view that under the experimental conditions used here, the current activated by ionomycin was due to the opening of Ca^{2+}-dependent Cl^- channels. Hereafter, the current activated by ionomycin will be referred to as the Ca^{2+}-activated Cl^- current.

Effects of pH_i on Macroscopic Chloride Currents

The increase in current induced by ionomycin seen in Figs. 1–3 was dependent upon the pH_i. As described in Materials and Methods, the effects of pH_i were evaluated in T84 cells by setting the pipette solutions to a pH of 6.4 or 8.3 with BAPTA (nominally 100 nM free Ca^{2+}). Figure 4 contains examples of Cl^- channel current-voltage relations from two different T84 cells at pH_i levels of 8.3 (Fig. 4*A*) and 6.4 (Fig. 4*B*). The filled symbols in this figure represent the current amplitude obtained in the absence and the open symbols the current amplitude obtained in the presence of 1 μM ionomycin. As with pH_i of 7.3 (Figs. 1 and 2), ionomycin substantially increased the Cl^- channel current with the pH_i set to 8.3 (Fig. 4*A*). In contrast, when pH_i was 6.4 (Fig. 4*B*) ionomycin did not increase the Cl^- channel current level. Ionomycin increased the current at +50 mV by an average factor of 5.3 ± 2.1 (n = 5) at pH 8.3. At pH_i of 6.4 ionomycin did not produce a statistically significant (at the 0.05 level) increase in current (n = 3). As in the example of Fig. 4*B*, current levels at pH 6.4 with or without ionomycin were less than 0.1 nA (at +50 mV).

It has been shown that ionomycin-mediated Ca^{2+} transport is decreased at low external pH (Fasolato & Pozzan, 1989). Our experiments like those illustrated in Fig. 4 involved changes in pH_i and so should not have compromised the ability of externally applied ionomycin to increase internal Ca^{2+}. Nevertheless, in order to address this possibility, we activated the Cl^- channels directly with buffered levels of intracellular Ca^{2+} (*see* Materials and Methods). Figure 5*A* shows two sets of Ca^{2+}-dependent Cl^- currents recorded from different T84 cells at pH_i of 8.3 (left) and 6.4 (right). Typical time dependent, outwardly rectifying currents were recorded at pH 8.3. However, nearly the same $[Ca^{2+}]_i$ at pH 6.4 produced little or no current activation. Figure 5*B* shows the average current-voltage relationships from several cells with high (nominally 500 nM) Ca^{2+} at pH_i levels of 6.4 (●) and 8.3 (■). Cl^- currents at high pH_i were more than 5 times larger than the currents recorded at low pH_i.

As described in Materials and Methods, we found that the free Ca^{2+} concentrations in the pH 8.3 and 6.4 solutions were somewhat less than the nominal 500 nM value and that the level of Ca^{2+} in the pH 6.4 solution was somewhat lower than that at pH 8.3 (300 nM compared to 420 nM). However, this difference in Ca^{2+} concentration cannot explain the reduced currents at pH 6.4. The data in Fig. 5*B* (□) shows that the current activated by 103 nM Ca^{2+} at pH 8.3 was larger than that activated by 300 nM Ca^{2+} at pH 6.4.

We also examined the influence of pH_i on Ca^{2+}-activated Cl^- currents in freshly dissociated rat parotid

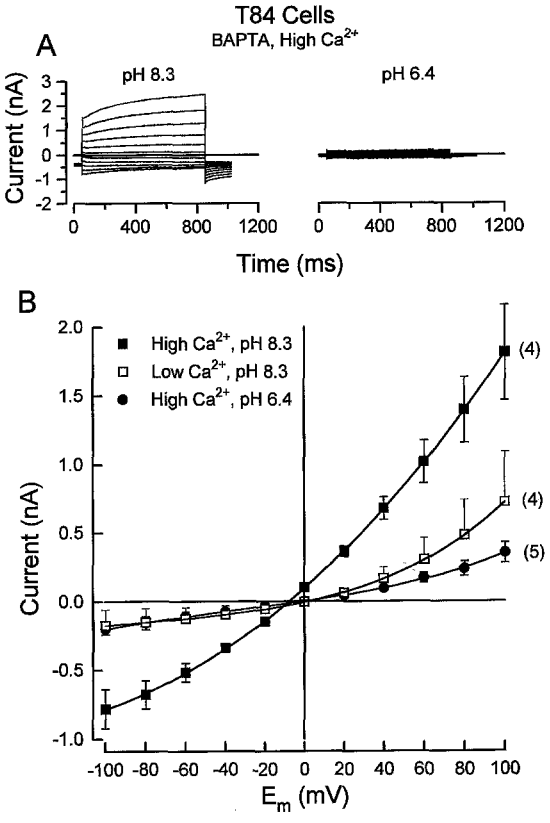

Fig. 5. Effect of high and low pH_i on the Ca^{2+}-dependent Cl^- currents from T84 cells activated by high internal Ca^{2+}. (*A*) Ionic currents recorded from −100 to +100 mV in 20 mV steps from a holding potential of −50 mV. BAPTA buffered high Ca^{2+} solutions at pH 8.3 (left) and 6.4 (right), respectively (*see* Table). (*B*) Current-voltage relations obtained with high Ca^{2+} at pH_i 8.3 (■, n = 4) and 6.4 (●, n = 5). Also shown are currents activated by low Ca^{2+} at pH 8.3 (□, n = 4). *See* Table for solution details.

acinar cells. Figure 6 shows that low pH_i inhibited the Ca^{2+}-dependent Cl^- currents from these cells just as in T84 cells. Figure 6 includes examples of the current enhancement induced by ionomycin (open symbols) from two different cells with internal solutions of pH of 7.3 (*A*) and 6.4 (*B*). With the intracellular pH maintained at 7.3 ionomycin produced a substantial increase in current over the entire voltage range. This large enhancement of current was substantially reduced with an internal pH of 6.4. The average current at an internal pH of 7.3 in three similar experiments was increased by a factor of 20 but by only 2.4-fold with a pH_i of 6.4. Thus, low pH_i inhibited the ability of ionomycin to activate the Ca^{2+}-dependent Cl^- channels in both T84 and rat parotid acinar cells.

Low pH_i and Single Chloride Channels

To determine if the action of low pH_i solutions was due to a reduction in the amplitude of single channel current, we studied the effect of low pH_i on single chloride channels in T84 cells. We used cell-attached recording methods since channel activity was rapidly lost when patches were excised. The pH_i was set to either 7.3 or 6.5 with 5 μM nigericin as described in Materials and Methods. Recordings began 8 min after solution changes in order to allow internal pH to equilibrate (*see* Materials and Methods).

Without treatment with ionomycin, we rarely observed any channel activity, presumably because the resting intracellular Ca^{2+} level was too low to activate Ca^{2+}-dependent channels. This is illustrated in panel 1 of Fig. 7. Panels 2 and 4 show single channel openings activated by ionomycin recorded at +20 mV with the pH_i set to 7.3. Very little single channel activity was ob-

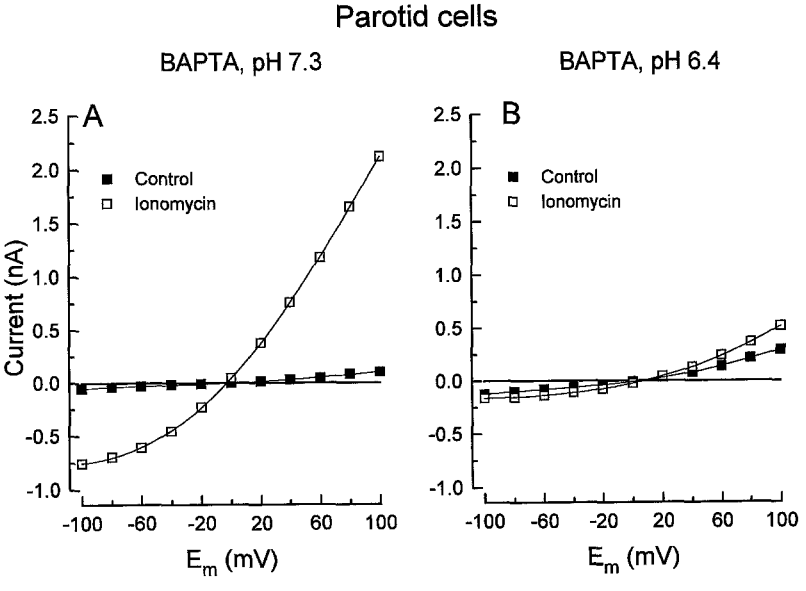

Fig. 6. Effect of high and low pH_i on the ionomycin-activated Cl^- current from rat parotid acinar cells. Representative current-voltage relations obtained from two different cells dialyzed with internal solutions of pH 7.3 (*A*) and 6.4 (*B*) containing BAPTA before (■) and during application of 1 μM ionomycin (□). Test pulses of 100-msec duration were used in these experiments.

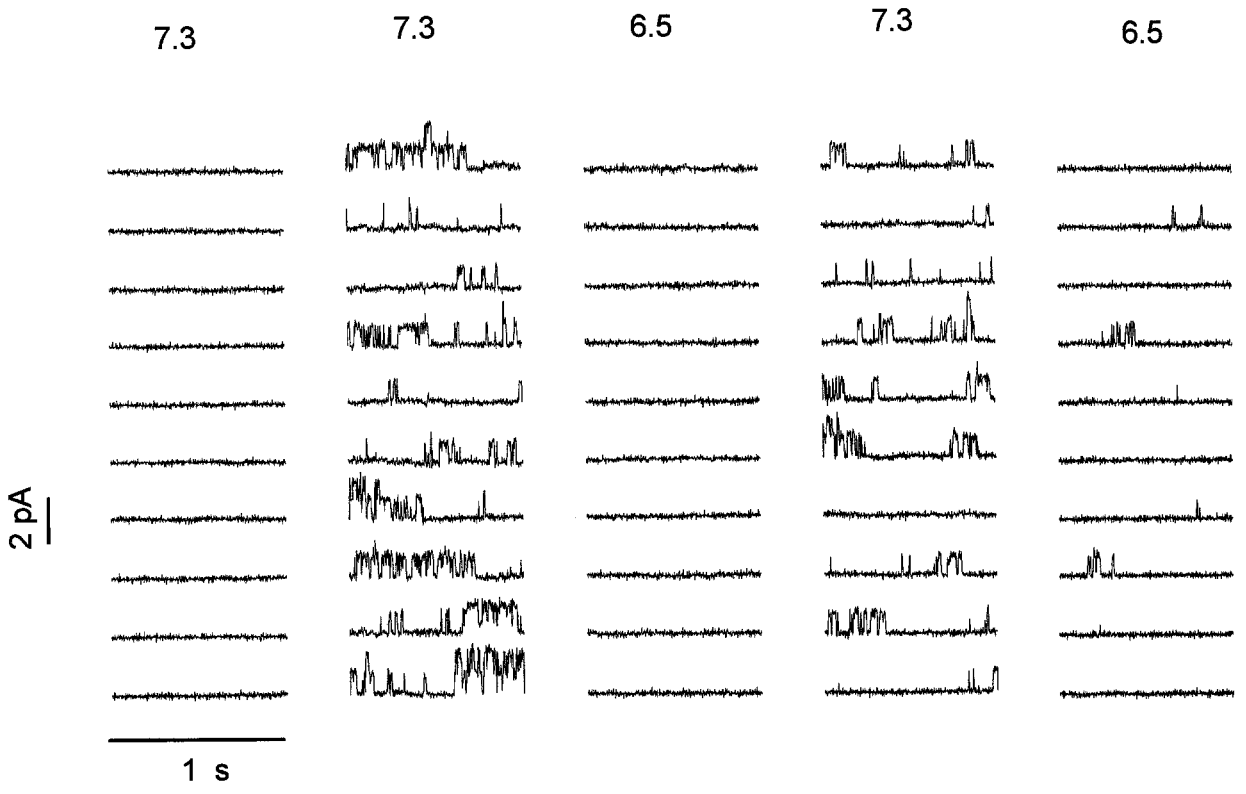

Fig. 7. Cell-attached single Cl^- channels recorded from a T84 cell. The cell was in the high K^+ solution + 5 μM nigericin for more than 10 min before ionomycin exposure. In the absence of ionomycin, no channel activity was recorded (panel 1). Single-channel openings can be seen after addition of 0.5 μM ionomycin (panel 2). This activity was eliminated by setting pH_i to 6.5, even in the continued presence of ionomycin. A return to pH 7.3 (panel 4) was accompanied by a return of channel activity. Some activity could occasionally be recorded at pH 6.5 in the presence of ionomycin (panel 5). The sequence of pH_i changes in the same cell was as indicated by the pH values at the top of each panel with a minimum of 8 min between each pH change (*see* Materials and Methods). The holding potential was +20 mV. Current records were filtered at 200 Hz. Single channel amplitudes were 0.93 and 0.87 pA at pH_i values of 7.3 and 6.5, respectively. Amplitude and time scales are the same for all panels.

served at a pH_i value of 6.5 (Fig. 7, panels 3 and 5). The single channel current amplitude for those openings observed at $pH_i = 6.5$ (panel 5) was not much different from that seen at pH 7.3.

A quantitative analysis of the single channel current amplitude recorded at internal pH levels of 7.3 and 6.5 is presented in Fig. 8. Examples of amplitude histograms at +20 mV obtained from the same cell at pH_i levels of 7.3 and 6.5 are shown in the top of the figure. The single channel current-voltage relation obtained from many patches is shown in the bottom of Fig. 8. No significant difference was seen in the current amplitude at these two pH values. Even though the whole-cell current-voltage relation exhibited outward rectification over the entire voltage range studied (e.g., Figs. 1 and 3), the single channel current data in Fig. 8 could be approximated by a constant 7.9 pS slope conductance.

It has been shown that T84 cells also express cAMP-dependent Cl^- channels with a conductance close to that described above (Tabcharani et al., 1990). Wagner et al. (1992) have shown that this conductance is due to expression of CFTR (cystic fibrosis transmembrane conductance regulator), the cAMP-activated Cl^- channel associated with cystic fibrosis disease. The single channel currents recorded here were activated by ionomycin and so are unlikely to arise from CFTR channels. Nevertheless, we repeated the experiment of Fig. 7 in the presence of an inhibitor of CFTR channels (glibenclamide, K_d = 21.8 μM; Sheppard & Welsh, 1992). As illustrated in Fig. 9, 0.5 μM ionomycin induced the activation of channels with an amplitude of 0.8 pA at +20 mV (right panel) even in the continued presence of 100 μM glibenclamide. In six cells patched with 100 μM glibenclamide the average single channel current was 0.7 ± 0.02 pA, essentially the same as observed in the absence of this CFTR channel inhibitor. The lack of effect of glibenclamide supports the conclusion that the Cl^- currents activated by ionomycin in these experiments are due to Ca^{2+}-dependent not cAMP-dependent Cl^- channels.

Discussion

Ca^{2+}-activated Cl^- channels are present in T84 (Cliff & Frizzell, 1990) and parotid acinar cells (Iwatsuki et al.,

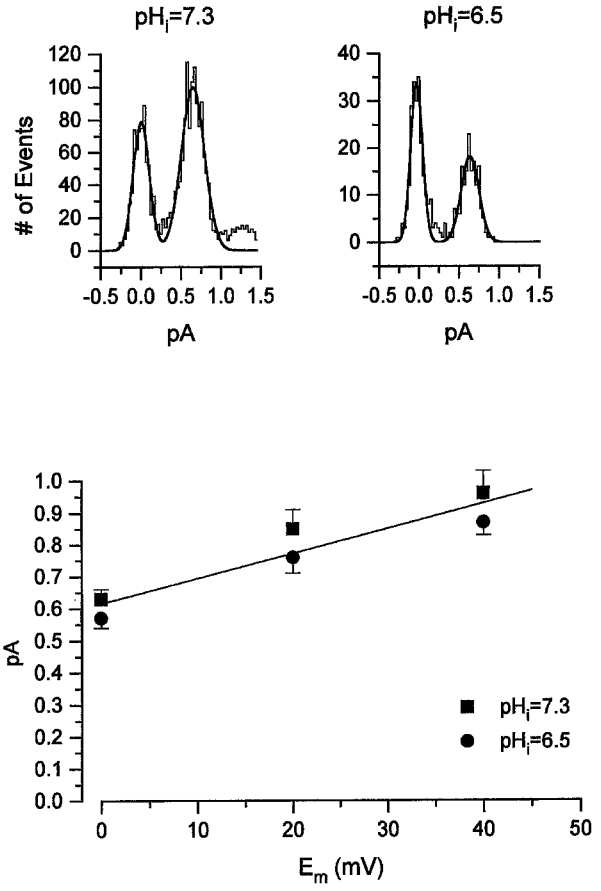

Fig. 8. Single Cl^- channel amplitude at different membrane potentials at pH_i 7.3 and 6.5. Top: Amplitude histograms of the single channel currents from the same cell at +20 mV are shown at pH_i 7.3 (left) and 6.5 (right). The ordinate is the number of observations of current in each current bin (0.01 pA wide). Bottom: single channel current vs. membrane potential relation at pH_i levels of 7.3 (■) and 6.5 (●). The solid line is a linear regression fit which yields a conductance of 7.9 pS. Mean ± SEM (n = 6 to 12) values are plotted.

1985). Channels with similar properties have been observed in other cell types (Evans & Marty 1986; Alton et al., 1991; Hume & Thomas, 1989; Anderson & Welsh, 1991; *see also* review by Anderson et al., 1992). These channels share the properties of (i) a reversal potential near the Cl^- equilibrium potential; (ii) high selectivity for I^- and NO_3^- and with little permeability to aspartate; (iii) ionomycin sensitivity; and (iv) inhibition by DIDS. In addition, we found that the activity of these channels in both T84 and parotid acinar cells is inhibited by internal acidic pH.

Two classes of single Cl^- channels have been reported in T84 cells: one with a slope conductance near 9 pS at 0 mV and 37°C that is activated by cAMP (Tabcharani, et al., 1990), and a larger conductance channel with a chord conductance near 40 pS at 0 mV (Halm & Frizzell, 1992). The slope conductance of the single Cl^- channels we have observed was about 8 pS—quite close to that of the cAMP-activated channels. However, the single channel activity we observed (near 22°C) required ionomycin and the current amplitude was insensitive to glibenclamide, an inhibitor of cAMP-activated Cl^- channels (Sheppard & Welsh, 1992). Thus, the single channel currents recorded here are likely those underlying the macroscopic Ca^{2+}-activated current in T84 cells. These channels may be similar to the 15 pS Ca^{2+}-dependent Cl^- channel observed in the colonic cell line HT-29 (Morris & Frizzell, 1993).

The Ca^{2+}-dependent currents from T84 cells were sensitive to DIDS. Current inhibition was voltage dependent: the currents were preferentially inhibited at positive potentials. A similar voltage-dependent action of DIDS has been previously described on volume-sensitive Cl^- channels (Lewis, Ross, & Cahalan, 1993). In contrast, the cAMP-dependent Cl^- channel is not inhibited by this compound (100 μM; Tabcharani, et al., 1990).

We found that the activity of Ca^{2+}-dependent Cl^- currents in both T84 and parotid acinar cells was inhibited by low pH_i, similar to what has been reported in lachrimal gland acinar cells (Park & Brown, 1995). This action could have been due to a reduction of single channel current amplitude but the data and analyses associated with Figs. 7 and 8 showed that the single channel current amplitude was insensitive to pH_i. Hence, H^+ ions may modulate Ca^{2+}-dependent Cl^- channel activity in these cells by regulating the single channel open probability by competing with Ca^{2+} for binding sites on the channel protein. Alternatively, H^+ ions could act on a channel regulatory element like Ca^{2+}-calmodulin kinase type II which appears to be involved in the activation of Ca^{2+}-dependent Cl^- channels of T84 cells (Wagner et al., 1991).

The high sensitivity of the activation of Ca^{2+}-dependent Cl channels to pH changes near 7.3 may have important physiological significance. Ca^{2+}-dependent Cl^- channels in secretory epithelial cells control Cl^- efflux, and, thus, underlie fluid and electrolyte secretion. Therefore, changes in the activity of these channels will have direct consequences for the secretory process. For example, in salivary acinar cells, cytosolic acidification of 0.3–0.4 pH units induced by block of the Na^+/H^+ exchanger (Melvin, et al., 1988; Elliot, Lau, & Brown, 1991; Zhang, Cragoe, & Melvin, 1992) inhibits fluid secretion (Martinez & Cassity, 1985; Novak & Young, 1986; Pirani et al., 1987).

Many anion channels are permeable to both Cl^- and HCO_3^- (Bormann, Hamill, & Sakmann, 1987; Reinhardt, et al., 1987), including Ca^{2+}-activated anion channels (Light et al., 1990) and perhaps channels in salivary cells as well (Melvin, et al., 1988; Zhang, et al., 1992; Turner, 1993). This suggests that the acidification induced by Ca^{2+}-activated HCO_3^- efflux during secretion would act as a feedback mechanism to prevent an excessive drop in

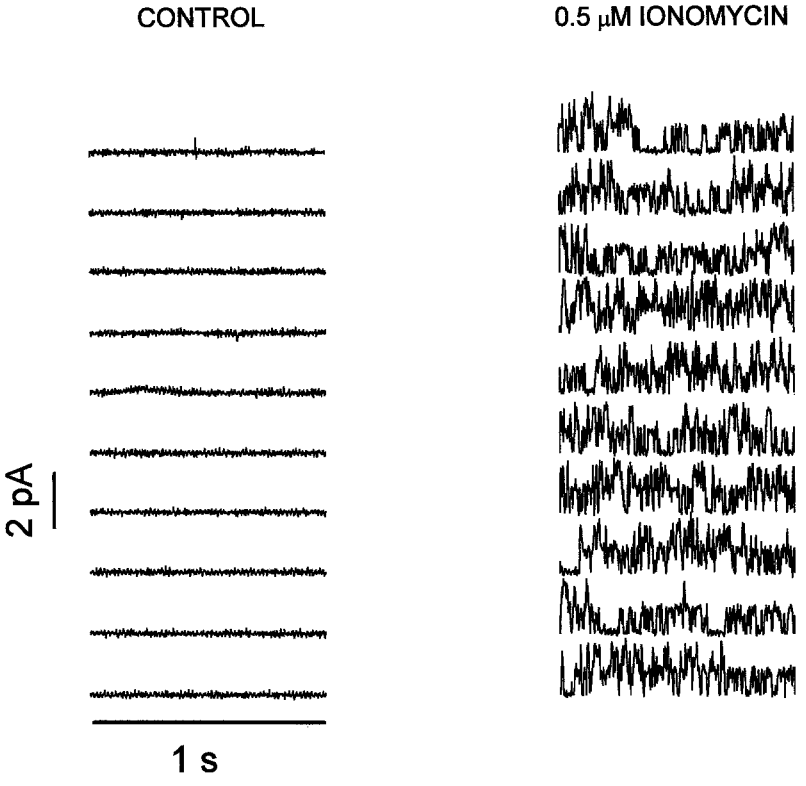

Fig. 9. Single channel currents activated by ionomycin in T84 cells were insensitive to glibenclamide. Left panel: Control records from a cell exposed to 0 ionomycin. Right panel: Single channels from the same cell superfused with 0.5 μM ionomycin. Channel activity was recorded at +20 mV from a cell attached patch with a pipette containing 100 μM glibenclamide. The single channel current amplitude was 0.8 pA. Experimental conditions were the same as in Fig. 7.

the pH_i by closing Ca^{2+}-dependent Cl^- channels and so preventing further HCO_3^- flux. Conversely, when the internal pH rises above the resting level during sustained stimulation (Melvin et al., 1988; Pirani et al., 1987), the Ca^{2+}-sensitivity of the Cl^- channel would increase, resulting in continued fluid production even as the cytosolic $[Ca^{2+}]$ decreases.

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