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A Bicarbonate- and Weak Acid-permeable Chloride Conductance Controlled by Cytosolic Ca^{2+} and ATP in Rat Submandibular Acinar Cells

T. Ishikawa*
Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444 Japan

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Abstract. A Ca^{2+}-activated Cl^- conductance in rat submandibular acinar cells was identified and characterized using whole-cell patch-clamp technique. When the cells were dialyzed with Cs-glutamate-rich pipette solutions containing 2 mM ATP and 1 μM free Ca^{2+} and bathed in *N*-methyl-D-glucamine chloride (NMDG-Cl) or Choline-Cl-rich solutions, they mainly exhibited slowly activating currents. Dialysis of the cells with pipette solutions containing 300 nM or less than 1 nM free Ca^{2+} strongly reduced the Cl^- currents, indicating the currents were Ca^{2+}-dependent. Relaxation analysis of the ''on'' currents of slowly activating currents suggested that the channels were voltage-dependent. The anion permeability sequence of the Cl^- channels was: NO_3^- (2.00) > I^- (1.85) ⩾ Br^- (1.69) > Cl^- (1.00) > bicarbonate (0.77) ⩾ acetate (0.70) > propionate (0.41) ⩾ glutamate (0.09). When the ATP concentration in the pipette solutions was increased from 0 to 10 mM, the Ca^{2+}-dependency of the Cl^- current amplitude shifted to lower free Ca^{2+} concentrations by about two orders of magnitude. Cells dialyzed with a pipette solution (pCa = 6) containing ATP-γS (2 mM) exhibited currents of similar magnitude to those observed with the solution containing ATP (2 mM). The addition of the calmodulin inhibitors trifluoperazine (100 μM) or calmidazolium (25 μM) to the bath solution and the inclusion of KN-62 (1 μM), a specific inhibitor of calmodulin kinase, or staurosporin (10 nM), an inhibitor of protein kinase C to the pipette solution had little, if any, effect on the Ca^{2+}-activated Cl^- currents. This suggests that Ca^{2+}/Calmodulin or calmodulin kinase II and protein kinase C are not involved in Ca^{2+}-activated Cl^- currents. The outward Cl^- currents at +69 mV were inhibited by NPPB (100 μM), IAA-94 (100 μM), DIDS (0.03–1 mM), 9-AC (300 μM and 1 mM) and DPC (1 mM), whereas the inward currents at −101 mV were not. These results demonstrate the presence of a bicarbonate- and weak acid-permeable Cl^- conductance controlled by cytosolic Ca^{2+} and ATP levels in rat submandibular acinar cells.

Key words: Salivary acinar cells — Ca^{2+}-activated Cl^- channels — Bicarbonate — Weak acids — ATP — Fluid secretion

Introduction

In the currently accepted model for fluid and electrolyte secretion in salivary acinar cells, a rate-limiting step in secretion is the Cl^- conductance in the apical membrane (Cook et al., 1994). Although no direct evidence for the membrane domain in which the Cl^- channels are localized has been provided, a Cl^- selective microelectrode study has suggested that the permeability increase during secretion is due to opening of chloride channels located principally in the apical membrane of the acinar cells (Lau & Case, 1988). Recent patch-clamp studies in various secretory epithelia have demonstrated the presence of several types of Cl^- channels including Ca^{2+}-activated Cl^- channels, cAMP-regulated Cl^- channels (CFTR) and volume-sensitive (swelling-induced) Cl^- channels (Frizzell & Morris, 1994). Among these channels, the Ca^{2+}-dependent Cl^- channels are probably the most important ones in the context of salivary acinar fluid and electrolyte secretion, since cytosolic Ca^{2+} concentration, but not cAMP, has been shown to be an essential regulator of secretion (*see* for review, Cook et al., 1994). Furthermore, whole-cell patch-clamp studies have shown that like other exocrine cells, salivary acinar cells exhibit a

* *Present address*: Division of Respiratory Research, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

Correspondence to: T. Ishikawa

Cl^- conductance that is activated by an increase in cytosolic Ca^{2+} concentration (Evans & Marty, 1986; Cook et al., 1988*a;* Cook et al., 1988*b;* Martin, 1993; Ishikawa & Cook, 1993).

With respect to the biophysical properties of the Ca^{2+}-activated Cl^- channels located in the apical membrane of submandibular acinar cells, it has been postulated that they may also be permeable to bicarbonate and other weak acids (Brown, Elliott & Lau, 1989). This hypothesis is based on the indirect evidence that the acetylcholine (ACh)-induced intracellular acidosis attributed to the efflux of HCO_3^- or weak acids such as acetate is inhibited by relatively high concentrations of Cl^- channel blockers such as diphenylamine-2-carboxylic acid (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (Lau, Elliott & Brown 1988; Brown et al., 1989). The presence of this kind of Cl^- channels could explain why either HCO_3^- or acetate is able to support fluid secretion in the intact perfused rat and rabbit mandibular glands when Cl^- in the perfusate is replaced with these anions (Case et al., 1984; Novak, Davé & Young, 1984; Novak & Young, 1989). There is, however, no direct electrophysiological evidence for HCO_3^- or weak acid permeability in salivary submandibular acinar cells.

Using the whole-cell patch-clamp technique, I report here that a Ca^{2+}-activated Cl^- conductance in rat submandibular acinar cells is permeable to bicarbonate and acetate and is controlled by cytosolic ATP levels. Furthermore, the present study also shows that, unlike other epithelial cells, Ca^{2+}/Calmodulin-dependent pathways are not involved in the activation of the channels by Ca^{2+}.

Methods and Materials

Submandibular Acinar Cells

Acinar cells were isolated from the submandibular glands of rats (Wistar-Hamamatsu, weighing 250–400 g) anaesthetized with pentobarbital sodium (50 mg/kg body weight *i.p.*). The submandibular glands were excised, placed in a physiological saline solution, diced, and incubated in a standard NaCl bathing solution containing collagenase (150 unit/ml, Yakult, Japan or 100 unit/ml, Nitta zeratin, Japan) for 20–30 min at 34°C in a shaking water bath. In some experiments, to facilitate the dissociation of single cells, the tissue was subsequently incubated for 5 min in a magnesium- and calcium-free bathing solution containing dispase (500–1000 unit/ml, Goudo, Tokyo). The tissue was dissociated by trituration, and then filtered through 125 μm Nylon mesh. The acinar cells were centrifuged and washed with the standard NaCl bath solution.

Patch-Clamp Methods

The cell preparations were transferred to a chamber mounted on a Nikon inverted microscope and viewed using Nomarski optics, after they had adhered to a glass coverslip coated with Cell-Tak (Collaborative Research, USA). Current recordings were made from acinar cells using the standard fast whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). The patch-clamp pipettes, which were pulled from glass capillaries (Narishige, Japan) using a horizontal puller (model P-87, Sutter Instrument, San Rafael, CA), had resistances of about 2–3 MΩ when filled with the standard Cs-glutamate-rich solution.

An Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) was used to measure whole-cell currents. The reference electrode was an Ag/AgCl electrode which was connected to the bath via an agar bridge (10 mg/ml) prepared with normal NaCl-rich bathing solution. The amplifier was driven by pClamp software to allow the delivery of voltage-step protocols with concomitant digitization of the whole-cell current. The whole-cell currents were filtered at 1 kHz and sampled at 2 kHz. The subsequent analysis of the current was done using programs supplied with Axograph.

The capacitance transient in most experiments was compensated by using the features of the Axopatch-200A amplifier. The cell capacitance was 30.8 ± 1.2 pF (n = 182). When constructing *I/V* plots, the steady-state whole-cell current was measured at 350 msec after onset of the voltage pulse. This procedure has the effect of underestimating the true steady-state whole-cell currents in situations when the whole-cell current had not been stabilized after 350 msec. Since this only occurred during the most extreme depolarizations, it has no effect on the estimates of reversal potentials but may produce underestimation of conductances at voltages more positive than +40 mV. The currents were not leak corrected. The series resistance (R_s) in these studies, 17.1 ± 0.5 MΩ (n = 182), was compensated as much as possible (40 to 70%) using the features of the amplifier in some experiments. However, the conductances of currents in the nanoampere range will be underestimated as a result of the voltage drop across the residual series resistance. The pipette potential was corrected for the liquid junction potentials between the pipette solution and the external solution, and between the external solution and the agar bridge as described by Barry and Lynch (1991).

Most relaxations were fitted with a single exponential using a least-squares method. To permit capacitive transients to die out, only data collected more than 10 msec after the potential change were used for fitting the exponentials. The amplitude of the relaxation was defined as the ratio of the steady-state current (estimated as the asymptote of the exponential) to the initial current immediately following the potential change (estimated by extrapolation of the exponential to the time of the potential change (*cf.* Evans & Marty, 1986).

Experiments were performed at room temperature (20–25°C). Bath solution changes were accomplished by gravity feed from reservoirs.

Solutions and Chemicals

The compositions of the standard pipette and bath solution were as follows. The pipette solution contained (in mmol/l): Cs-glutamate (100), $MgCl_2$ (1), HEPES (10), EGTA (10), Na_2-ATP (2) and D-glucose (5) (pH 7.4). The free Ca^{2+} concentration of the pipette solution was varied between 10^{-6} and less than 10^{-9} mol/l using 10 mmol/l bis(β-aminoethylether)-*N,N,N′N′*-tetraacetic acid (EGTA) as a Ca^{2+} buffer. The free concentration of Ca^{2+} was calculated from a formula which takes into account the concentrations of Mg^{2+}, Ca^{2+}, EGTA (96% purity), ATP and pH (Oiki & Okada, 1987) and an appropriate amount of $CaCl_2$ was added to the solution. When the free Ca^{2+} concentration of the pipette solution was fixed at less than 10^{-9} mol/l, no $CaCl_2$ but CsCl (15 mM) was added to the solution. The pH of the solution was adjusted with CsOH. The cells were initially immersed in

a bath solution containing (in mmol/l) NaCl (140), KCl (4.3), $CaCl_2$ (1), $MgCl_2$ (1), HEPES (10) and D-glucose (5) (pH 7.4). After the formation of the whole-cell configuration, the bath solution was changed to one containing (in mmol/l) NMDG-Cl (144.3), $CaCl_2$ (1), $MgCl_2$ (1), HEPES (10) and D-glucose (5) (pH 7.4). Relative permselectivities for most of the anions were determined using external solutions containing equimolar amounts of the $NMDG^+$ salts of the test anions. The pH of the bath solutions was adjusted with NMDG-OH. For determination of the relative permselectivities for I^- and HCO_3^-, after the formation of the standard whole-cell configuration, the bath solution was changed to one containing (in mM) choline-Cl (144.3), $CaCl_2$ (1 or 0), $MgCl_2$ (1), HEPES (10) and D-glucose (5) and then to external solutions containing equimolar amounts of the $choline^+$ salts of the test anions. In bicarbonate substitution experiments (144.3 mM HCO_3^-), the bath solutions were continuously gassed with 32% CO_2/68% O_2 and the pH of the solutions were also checked. The osmolarity of the pipette solution was set lower (by about 20 mosmol/kg H_2O) than that of the isotonic bathing solutions (about 290 mosmol/kg H_2O) to prevent spontaneous cell swelling due to poorly diffusible cytosolic constituents (Worrell et al., 1989).

The following drugs were used: DIDS (Dojindo, Kumamoto), 9-AC (Sigma, St. Louis), NPPB (RBI, MA), IAA-94 (RBI, MA), DPC (Fluka, Switzerland), glibenclamide (Sigma, St. Louis), KN-62 ({-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine}) (Seikagaku, Tokyo), trifluoperazine (Sigma, St. Louis) calmidazolium (Sigma, St. Louis), and staurosporin (Wako, Osaka). 9-AC, NPPB, DPC, KN-62, Calmidazolium, staurosporin and glibenclamide were prepared in dimethyl sulfoxide (DMSO) and IAA-94 in ethanol. The vehicle alone did not affect the Ca^{2+}-activated Cl^- currents.

Ther results are reported as means ±SEM of several experiments, and statistical significance was evaluated by using the two-tailed paired and unpaired Student's *t* test. A value of $P < 0.05$ was considered significant.

Results

Ca^{2+}-dependent Cl^- currents in Rat Submandibular Acinar Cells

Previous studies have shown that rat submandibular acinar cell membranes contain several types of K^+ channels and nonselective cation channels (Cook et al., 1988, Ishikawa, Murakami & Seo, 1994, Ishikawa & Murakami, 1995). Thus, to isolate the Cl^- currents, the K^+ currents were suppressed by employing Cs^+ as the major cation in the pipette solutions, and inward currents attributable to K^+ and nonselective-cation currents were minimized by using $NMDG^+$ as the major cation in bath solutions containing no K^+ ions. Cl^- concentrations in the standard bath and pipette solutions were 148.3 mM and 20 mM (Nernst potential for Cl^- in these studies was −51 mV) respectively, thereby allowing an increase in the leak conductance to be identified as a depolarizing shift in the zero current potential. Whole-cell currents were elicited by applying hyperpolarizing and depolarizing voltage pulses from a holding potential of −71 mV to potentials between −101 mV and +69 mV.

Figure 1*A* shows typical recordings of the Cl^- currents evoked by voltage jumps when the pipette solution contained three different free Ca^{2+} concentrations. The average current at −1 mV in cells dialyzed with pCa = 6 was 0.44 ± 0.06 nA ($n = 51$). Figure 1*A* also illustrates representative tracings of the whole-cell currents observed from cells dialyzed with the pipette solution containing less than 1 nM or 300 nM free Ca^{2+}. Under these conditions, outwardly rectifying current-voltage relations with a reversal potential close to −25 mV were observed. The average currents at −1 mV in cells dialyzed with pCa = 6.5 and pCa > 9 pipette solutions were 55.4 ± 39.4 pA ($n = 11$) and 28.1 ± 15.0 pA ($n = 11$) respectively.

In addition to the slowly activating Cl^- currents, rapidly activating currents were infrequently observed and shown to be mainly carried by Cl^-, since the glutamate substitution for Cl^- in the bath solution caused a marked reduction in the outward currents and a shift of the reversal potential towards a positive potential (*data not shown*).

Kinetic Properties of the Currents

Figure 2*A* shows a plot of the 'on' relaxation time constant as a function of membrane potential when the Ca^{2+} concentration in the pipette was 10^{-6} M. The time constant did not change at voltages between −101 mV and +69 mV: the values at −101 mV and +69 mV are 76.5 ± 9.5 msec ($n = 12$) and 74.1 ± 5.5 msec ($n = 13$), respectively. A plot of the open probability of the channels carrying the Cl^- current, estimated from the amplitude of the relaxation (*see* Materials and Methods and Evans & Marty, 1986), as a function of membrane potential is shown in Fig. 2*B*. The estimate of open probability was normalized to a value of 1 at +69 mV. The relative open probability at −81 mV was 0.56 ± 0.03 msec ($n = 13$) and at −1 mV 0.89 ± 0.03 msec ($n = 13$).

Anion Selectivity

Figure 3*A* demonstrates that the currents evoked by hyperpolarizing and depolarizing potentials under these conditions are mainly Cl^- selective currents. In cells dialyzed with the standard pipette solutions (pCa = 6), a slow outward relaxation was observed in response to positive voltage jumps. The corresponding current-voltage curve shows that the currents were outwardly rectifying and that the reversal potential was very close to the Nernst potential for Cl^- (−51 mV) under those experimental conditions (Fig. 3*B*). Glutamate substitution for Cl^- in the bath solution dramatically reduced the outward currents and the outward relaxations almost disappeared. The corresponding current-voltage relationship (Fig. 3*B*) shows that the glutamate substitution shifted E_{rev} from −53 mV to +11.9 mV, indicating that

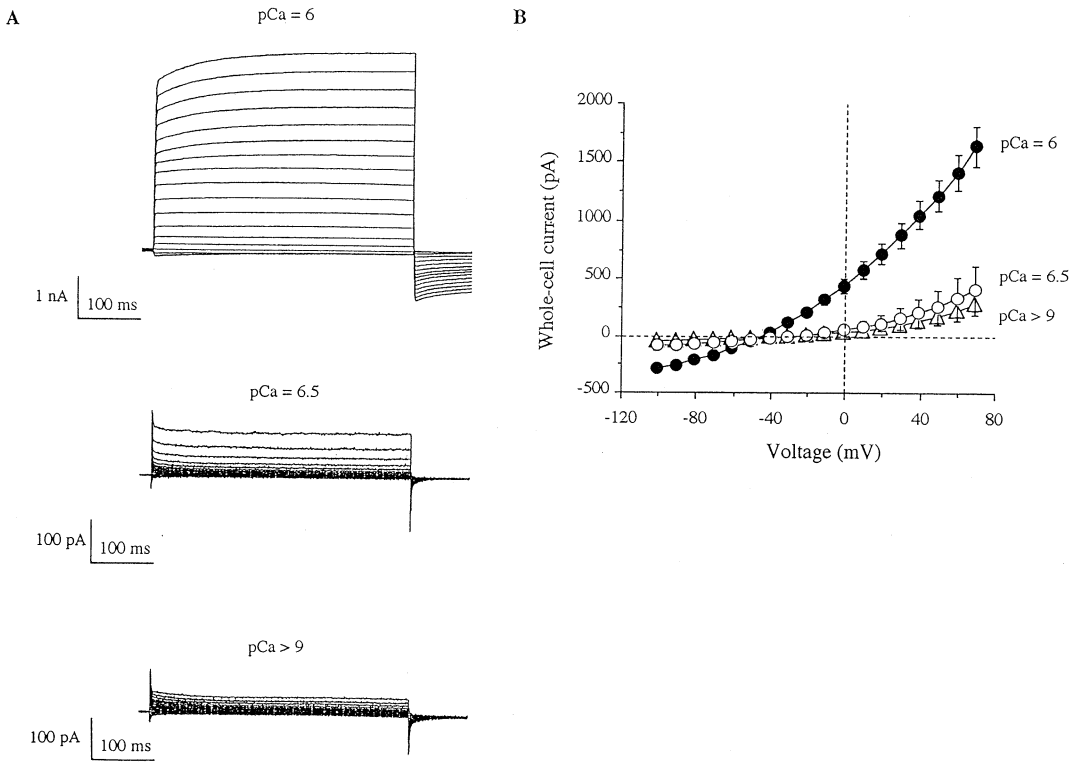

Fig. 1. Effect of free Ca^{2+} concentration in the pipette solution (ATP 2 mM) on the whole-cell currents. (*A*) Representative tracings of whole-cell current responses to voltage-steps from the cells dialyzed with pipette solutions having pCa = 6, pCa = 6.5 and pCa > 9, respectively. Hyperpolarizing and depolarizing pulses 400 msec in duration were applied from a holding potential of −71 mV to potentials between −101 and +69 mV in 10 mV intervals. (*B*) Steady-state current-voltage relations for the whole-cell currents from the cells dialyzed with pipette solutions having pCa = 6 (n = 51), pCa = 6.5 (n = 11) and pCa > 9 (n = 11), respectively.

the permeability for glutamate was much smaller than that for Cl^-. In seven experiments, the average reversal potential shift was 54.3 ± 3.7 mV (n = 7) (Table 1). The conclusion that the channels responsible for the currents are Cl^--selective was further supported by a tail current protocol experiment illustrated in Fig. 4*A*. Main voltage pulses given after a +69 mV or +76 mV prepulses produced inward relaxation or outward relaxations depending on the voltage of the main pulse. Figure 4*B* shows the zero-time extrapolated tail currents, which reversed at around −35.8 mV. Glutamate substitution for Cl^- in the bath solution shifted the reversal potential from −35.8 mV to +22.7 mV (Fig. 4*B*), indicating that the main charge carrier was Cl^-.

To determine the ion selectivity of the Ca^{2+}-activated Cl^- channels in rat submandibular acinar cells, experiments were carried out in which 144.3 mM of the external Cl^- was replaced with equimolar concentrations of various anions, leaving a total of 4 mM Cl^- in the bath. In some experiments, where the effects of I^- or bicarbonate substitution for Cl^- on the Ca^{2+}-activated Cl^- currents were examined, a choline-rich bath solution was used instead of a NMDG-rich bath solution. The Ca^{2+}-activated Cl^- conductance in the choline-Cl^--rich solution was kinetically very similar to that in the NMDG-Cl^--rich solution and its reversal potential was very close to the Nernst potential for Cl^- as found in the NMDG-Cl^--rich bath solutions. Although a previous study showed that high concentrations of choline evoked fluid secretion by the rat mandibular gland via atropine-sensitive muscarinic receptors (Murakami, Novak & Young, 1986), no inhibitory effects of atropine (5 μM on the currents were observed under the present experimental conditions (n = 4). The whole-cell currents at +69 mV in the absence and the presence of atropine in the bath were 2.79 ± 0.33 nA and 3.39 ± 0.82 nA (n = 4; P > 0.35).

Figures 5 and 6 illustrate examples of the tracings before and after the replacement of bath Cl^- with bicarbonate or acetate. As shown in these figures, the shift of the reversal potential was not clear although the amplitudes of the outward currents were reduced, indicating that the permeabilities of these anions were very similar to that of Cl^-. The results of the Cl^- replacement experiments using various anions are summarized in Table 1. The magnitude of the shift in the reversal potential recorded with each anion was used to calculate the permeability of the test anion (X^-) relative to that of Cl^- (P_x/

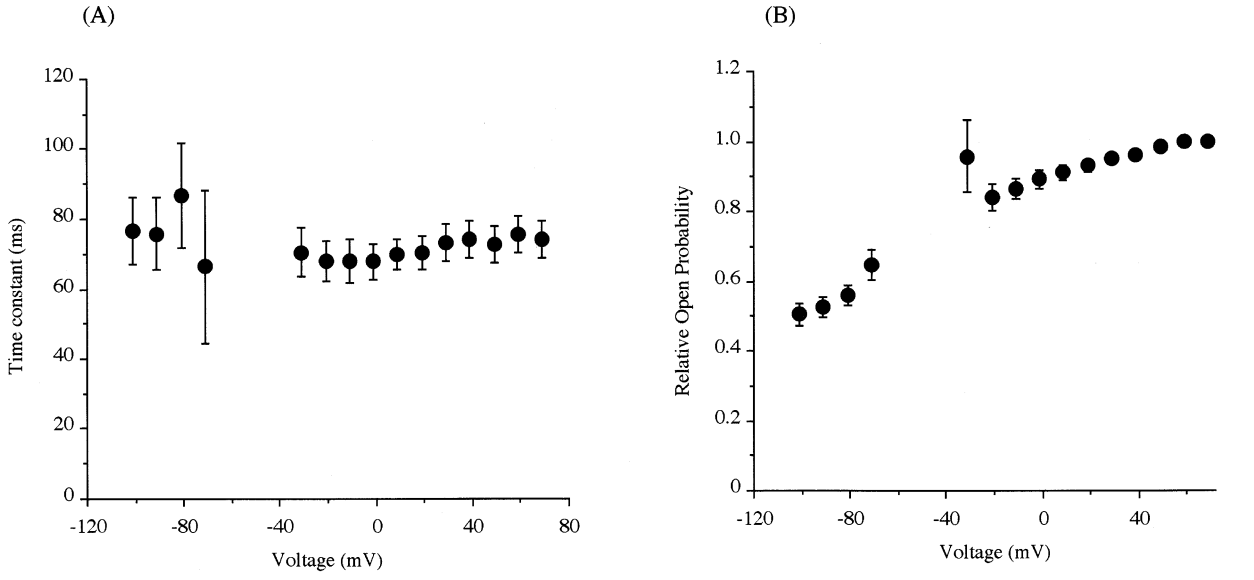

Fig. 2. Dependence of the relaxation time constant (*A*) and open probability relative to that at +68 mV (*B*) of the whole-cell Cl^- current measured by voltage pulses from a resting potential of −71 mV. The open probability (P_o) was assumed to be equal to the amplitude of the current relaxation and was normalized to a value of 1 at +69 mV. The Cs-glutamate pipette solution contained 1 μM free Ca^{2+}. Each point represents the mean of 13 experiments.

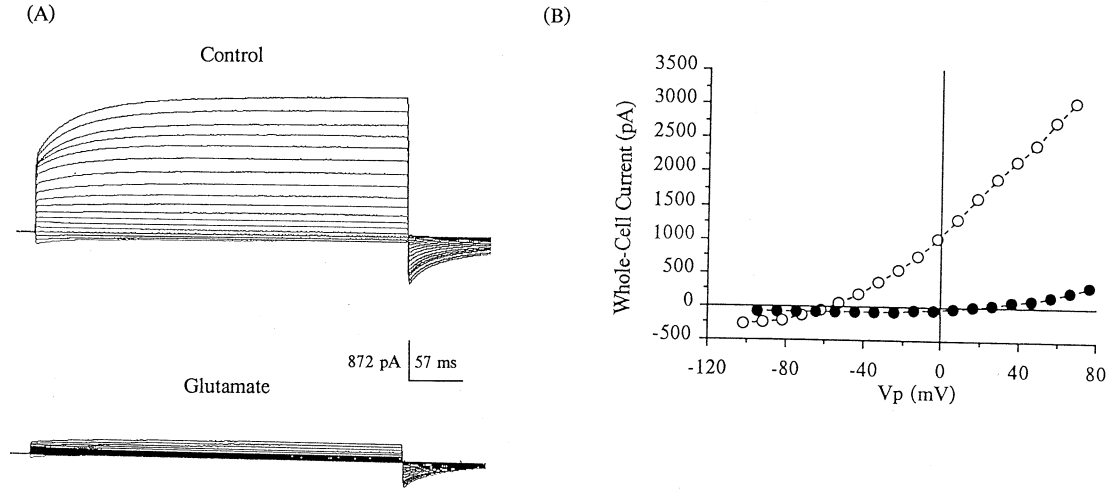

Fig. 3. Effect of glutamate substitution for Cl^- in the bath solution on the whole-cell currents. (*A*) Representative tracings of the whole-cell currents in response to the voltage-pulses before and after replacement of bath Cl with glutamate (but 4 mM Cl^-). The pipette solution contained the standard Cs-glutamate-rich solution with pCa = 6. (*B*) Corresponding current-voltage relations: open circles (Cl^- = 148.3 mM) and filled circles (Cl^- = 4 mM), respectively.

P_{Cl}). In this calculation, it was assumed that the currents measured under the conditions of the experiment were carried solely through Cl^- channels. The sequence of the relative permeabilities was: NO_3^- (2.00) ≥ I^- (1.85) ≥ Br^- (1.69) > Cl^- (1) > bicarbonate (0.77) ≥ acetate (0.70) > propionate (0.41) ≫ glutamate (0.09). When the chord conductances between the reversal potential and +69 mV were calculated, the sequence of the relative conductances was: NO_3^- (1.77) ≥ Br^- (1.46) ≥ I^- (1.31) ≥ Cl^- (1) > bicarbonate (0.67) ≥ Acetate (0.51) = Propionate (0.50).

Effect of Intracellular ATP

It was found that the addition of ATP to the pipette solution was required for the continuous activation of the Cl^- currents under the present experimental conditions

Table 1. Effect of Cl^- replacement by different anions on reversal potential

Anion	ΔE_{rev} (mV)	P_X/P_{Cl}	n
NO_3^-	−17.2 ± 3.1	2.00	5
I^-	−15.2 ± 6.0	1.85	4
Br^-	−13.0 ± 1.6	1.69	3
Bicarbonate	6.2 ± 3.6	0.77	5
Acetate	8.7 ± 1.4	0.70	4
Propionate	21.1 ± 2.0	0.41	4
Glutamate	54.3 ± 3.7	0.09	7

Results are means ± SEM of the number of experiments shown. P_X/P_{Cl} values were calculated from the Goldman-Hodgkin-Katz equation assuming the anions to be the only permanent ions. ΔE_{rev} is defined as the reversal potential in Cl^- minus the reversal potential in other anions.

(pCa = 6 in the pipette solution). Figure 7 shows the effect of three different concentrations of ATP in the pipette solution on the Ca^{2+}-activated Cl^- currents. As shown in the figure, when no ATP was included in the pipette solution, 1 μM free Ca^{2+} concentration could not activate the Cl^- currents. The average current at +69 mV was 0.45 ± 0.16 nA (n = 18). In one experiment, the currents were transiently activated and then rapidly decreased (*data not shown*). To examine further whether the ATP requirement in the pipette solution is absolute, the free Ca^{2+} concentration in the pipette solution was increased to pCa = 5. As shown in Fig. 7, even in the absence of ATP, cells dialyzed with a pipette solution of pCa = 5 exhibited Cl^- currents of similar magnitude to those observed in cells dialyzed with pCa = 6 under control conditions. The average current at +69 mV was 1.89 ± 0.51 nA (n = 7). In four experiments, where the cells in the NaCl bath solution were dialyzed with the ATP-free pipette solution of pCa = 5.5, the current amplitudes were not different from those observed in cells dialyzed with the ATP-free pipette solution of pCa = 6 (Fig. 7). When cells were dialyzed with pipette solutions containing a higher concentration of ATP (10 mM), the activation of the Cl^- currents became much more sensitive to cytosolic free Ca^{2+}-concentrations. Figure 8 summarizes the effects of ATP concentration in the pipette solution on the Ca^{2+}-activated Cl^- currents. These plots of Cl^--current amplitude at −1 mV against free Ca^{2+} concentration at different ATP concentrations in the pipette solution show that the response to Ca^{2+} is markedly dependent on ATP concentration.

The ATP dependency of the Ca^{2+}-dependent Cl^- current activation may be due to the ability of ATP to allosterically regulate Cl^- channels in rat submandibular acinar cells. It was therefore decided to investigate whether nonhydrolytic binding of ATP was a conditional requirement for Ca^{2+}-dependent channel activation by testing the ability of the poorly hydrolyzable ATP analogue, ATP-γS to activate Cl^- conductance. When the cells were dialyzed with a pipette solution containing ATP-γS (2 mM), it was found that the whole-cell current amplitude and kinetics were similar to those in the cells dialyzed with the ATP-containing a pipette solutions. In six experiments, the current amplitude at −1 mV with the ATP-γS-containing pipette solutions was 0.54 ± 0.15 nA (n = 6), which was not reduced compared with that obtained in control cells (0.44 ± 0.06 nA, (n = 51)).

Effects of Inhibitors of Ca^{2+}/Calmodulin, Calmodulin Kinase and Protein Kinase C

To assess the involvement of calmodulin in the Ca^{2+}-dependent stimulation of the Cl^- conductance, cells were exposed to the membrane-permeable calmodulin antagonists, trifluoperazine (TFP) and calmidazolium. Figure 9*A-C* show the effect of TFP (100 μM) on the Ca^{2+}-activated Cl^- conductance. The addition of TFP to the bath solution did not reduce the current amplitude. In four experiments, mean values of the current amplitudes after TFP addition were 124.7 ± 25.3% and 114.0 ± 26.6% (n = 4) of the control values at −101 mV and +69 mV, respectively. In two experiments, calmidazolium (25 μM) did not reduce the currents (*data not shown*). I also examined the effect of KN-62, a specific inhibitor of calmodulin kinase II (Okazaki et al., 1994) in the pipette solution on the Cl^- currents. Under these conditions, however, the normal current activation was observed. The current amplitude at +69 mV was 2.20 ± 0.95 nA (n = 6), which was not reduced compared with the corresponding control value of 1.64 ± 0.17 nA (n = 49). These results together suggest that Ca^{2+}/Calmodulin is probably not involved in the Ca^{2+}-induced Cl^- current activation in rat submandibular acinar cells.

I also evaluated the possible involvement of protein kinase C in mediating the Ca^{2+} (1 μM)-induced Cl^- channel by introducing staurosporin (10 nM), a potent protein kinase C inhibitor, into the cell via whole-cell dialysis. However, in four experiments, the Ca^{2+}-induced Cl^- currents in the presence of staurosporin were similar to those observed in the cells without the inhibitor, thus excluding the involvement of protein kinase C. The current amplitude at −1 mV in staurosporin-treated cells was 0.65 ± 0.17 nA (n = 4), which was not reduced compared with that obtained in control cells (0.44 ± 0.06 nA, n = 51).

Effects of DIDS, 9-AC, IAA-94, DPC, NPPB and Glibenclamide

To characterize the currents pharmacologically, several drugs that have been shown to block various Cl^- channels were tested on the Cl^- currents in rat submandibular acinar cells. Figure 10*A* and *B* shows representative,

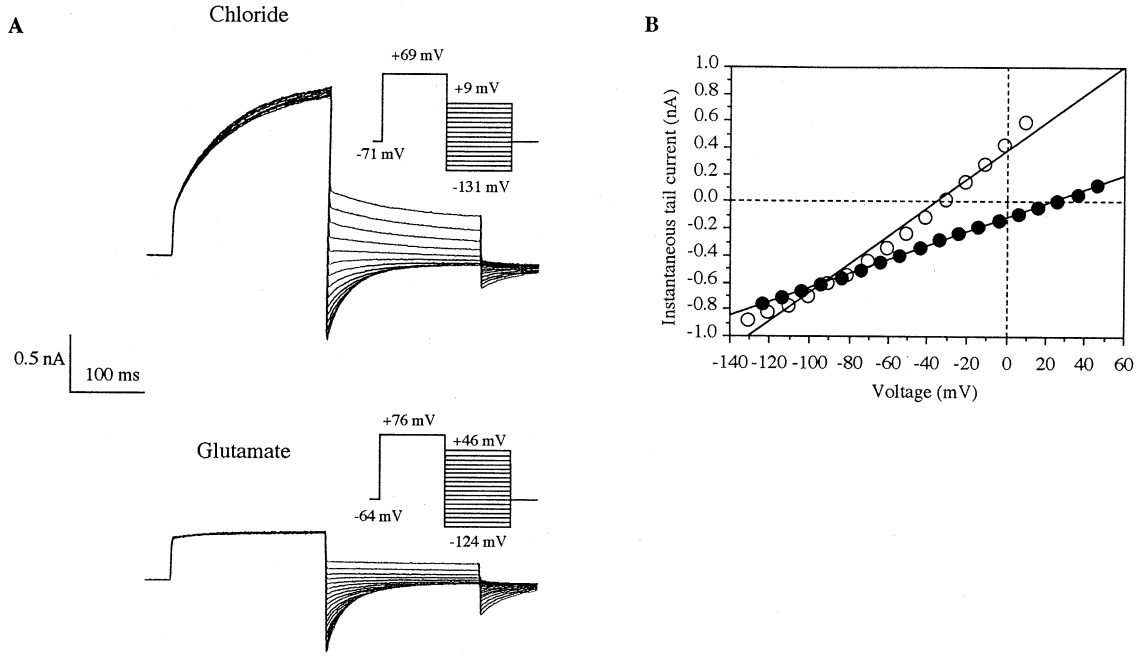

Fig. 4. Effect of glutamate substitution for Cl^- in the bath solution on the tail currents of the slowly activating currents. (*A*) Representative tracings before (open circles in *B*) and after replacement of the bath Cl^- with glutamate (but 4 mM Cl^-) (filled circles in *B*). Tail currents were obtained by the voltage-pulse protocols shown. The corresponding zero time amplitudes of the tail currents are plotted as a function of a membrane potential in *B*.

tracings before, during and after addition of DIDS (0.1 and 1 mM). The addition of DIDS to the bathing solution caused a reduction in the whole-cell Cl^- currents. At 0.1 mM, DIDS reduced the current amplitude at +69 mV to 59.5 ± 7.5% ($n = 6$) of the control level (Fig. 10*C*). The inhibitory effect was more pronounced at a higher concentration of DIDS (1 mM) which reduced the current at +69 mV to 27.9 ± 4.8% ($n = 6$) of the control value (Fig. 10*C*). The inhibitory effect of DIDS on the Cl^- current was largely reversible. However, it was noticed that the inhibition by DIDS was voltage-dependent, since it did not have a significant inhibitory effect at −101 mV over the concentration range tested (0.01–1 mM).

9-AC (0.3 and 1 mM) reduced the Cl^- current amplitude evoked by membrane depolarization (to +69 mV) to 74.8 ± 9.7% and 39.1 ± 13.0% ($n = 4$) of the control values, respectively. It was also found that the inhibitory effect of 9-AC was clearly voltage-dependent, since 9-AC (0.3 and 1 mM) at −101 mV only reduced the Cl^- current amplitude to 91.6 ± 4.4% ($n = 4$) and 83.2 ± 9.4% ($n = 4$), respectively.

Effects of glibenclamide, IAA-94, DPC, NPPB were also examined on the Cl^- currents (Table 2). NPPB (100 μM) and DPC (1 mM) significantly inhibited the outward Cl^- currents at +69 mV. IAA-94 (100 μM) caused a small but a significant reduction in the current, although the inhibitory effect at +69 mV was not more pronounced at a higher concentration of IAA-94 (300 μM) (70.7 ± 8.8% ($n = 4$) of the control value). As shown in Table 2, it should be noted that the inhibitory effect of these inhibitors is voltage-dependent, since none of these drugs blocked the inward current at −101 mV. Glibenclamide (50 μM), an inhibitor of CFTR (Sheppard & Welsh, 1992), had no effect on the Cl^- currents.

Discussion

The present study demonstrates the presence of a bicarbonate- and weak acid-permeable Cl^- conductance controlled by cytosolic Ca^{2+} and ATP levels in rat submandibular acinar cells. The view that the whole-cell currents under the present experimental conditions can be attributable to Ca^{2+}-activated Cl^- channels is strongly supported by the following observations: (i) glutamate substitution for Cl^- in the bath solution shifted the reversal potential of the steady-state whole-cell currents and that of the instantaneous tail currents of the depolarization-activated currents towards positive potentials and (ii) the current was activated by cytosolic Ca^{2+}. Furthermore, the Cl^- channel open probability also appeared to be dependent upon membrane potential as found in rat lacrimal and sheep parotid secretory cells (Evans & Marty, 1986; Ishikawa & Cook, 1993). This paper is, to my knowledge, the first report of the regulation of the Cl^- conductance in salivary acinar cells by cytosolic ATP concentration and of its permeability for bicarbonate and weak acids.

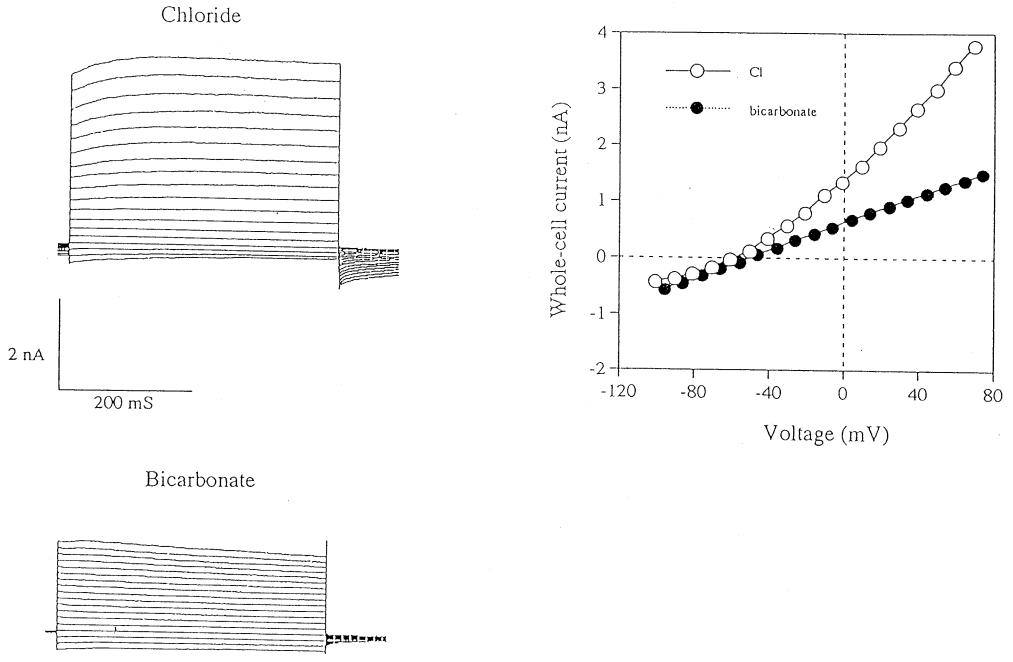

Fig. 5. Effect of bicarbonate substitution for Cl^- in the bath solution. Representative tracings of the whole-cell currents from a cell bathed in choline-Cl and choline-bicarbonate solutions (left panel). Right panel represents corresponding current-voltage relations derived from the experiments shown in left panel. Pipette solution was the ATP-free Cs-glutamate-rich solution with pCa = 5 and the bath contained choline-Cl-rich and choline-bicarbonate-rich bath solutions. In this series of experiments, the bath solutions contained no $CaCl_2$.

Anion Selectivity and Blocker Sensitivity of the Ca^{2+}-activated Cl^- Currents

This study has demonstrated directly that the Ca^{2+}-activated Cl^- conductance in rat submandibular acinar cells is permeable to bicarbonate and other weak acids such as acetate and propionate. The sequence of the relative permeabilities for various anions obtained from the present studies was: NO_3^- (2.00) $\geq$ I^- (1.85) $\geq$ Br^- (1.69) > Cl^- (1) > bicarbonate (0.77) $\geqslant$ acetate (0.70) > propionate (0.41) $\geqslant$ glutamate (0.09). With respect to halide permeability, this series corresponds with Eisenman's series I, which is expected for an anionic channel containing weak binding sites. Similar sequences of halide permeabilities have been reported in previous whole-cell and single-channel recordings of Ca^{2+}-activated Cl^- channels in rat submandibular acinar cells (Cook et al., 1988; Martin, 1993). The sequence of halide and nitrate permeabilities is slightly different from that of the Ca^{2+}-activated Cl^- currents reported in other cell types: in rat lacrimal acinar cells: I^- (2.7) > NO_3^- (2.4) > Br^- (1.6) > Cl^- (1) (Evans & Marty, 1986); in sheep parotid endpiece cells (Ishikawa & Cook, 1993): I^- (1.07) > Cl^- (1) > NO_3^- (0.92) > Br^- (0.75); in rat epididymal cells (Huang et al., 1993): I^- (2.93) > Br^- (1.39) = NO_3^- (1.33) > Cl^- (1); in T84 cells (Cliff & Frizzell, 1990): I^- (2.2–2.4) > Br^- (1.3–1.6) > Cl^- (1).

It has been proposed that the anion channels on the apical membrane of submandibular acinar cells are also permeable both to HCO_3^- and to short-chain fatty acids (Lau & Case, 1988; Brown et al., 1989). The present experiments indicate that in rat salivary acinar cells the permeability ratios of bicarbonate, acetate and propionate for the Ca^{2+}-activated Cl^- conductance are 0.77, 0.70 and 0.46, respectively. These values seem to be relatively large compared with those in other types of Cl^- channels. It has been shown that P_{HCO3}/P_{Cl} ratios for outwardly rectifying Cl^- channels are 0.42 for HT29 (Kunzelmann et al., 1991), 0.5 and 0.44 for T84 (Kunzelmann et al., 1991; Halm & Frizzell, 1992), for 0.56 for respiratory epithelial cells (Kunzelmann et al., 1991), 0.11–0.25 and 0.25 for cAMP-activated Cl^- channels (CFTR) in rat pancreatic duct (Gray et al., 1990) and in wild-type CFTR-transfected NIH 3T3 cells (Poulsen et al., 1994) and 0.48 for volume-sensitive Cl^- currents in human airway epithelial cell lines 9HTEo- and CFNPE9o-(Rasola et al., 1992). For acetate permeability, it has been reported that $P_{acetate}/P_{Cl}$ ratios for outwardly rectifying Cl^- channels are 0.49 and 0.35 for T84 and a human submandibular duct cell line (HSG cells) (Halm & Frizzell, 1992; Ishikawa & Cook, 1994).

In the present study, it was found that the kinetic appearance of the currents in the presence of bicarbonate was different from that in the presence of chloride (Fig. 5) and that the current-voltage curve became strictly linear (Fig. 5). The phenomenon could be attributable to

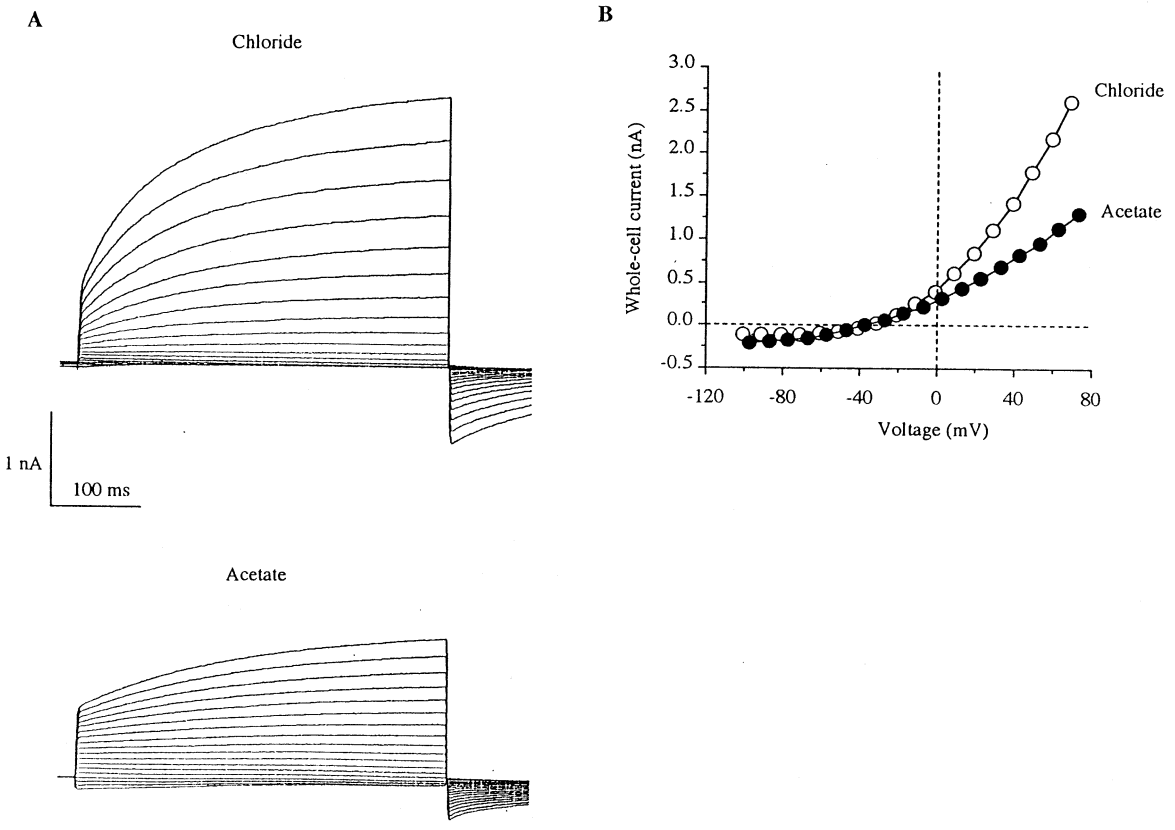

Fig. 6. Effect of acetate substitution for Cl^- in the bath solution. (*A*) Representative tracings of whole-cell current from a cell bathed in the standard NMDG-Cl and NMDG-acetate solution. (*B*) Corresponding current-voltage relations derived from the experiments shown in *A*.

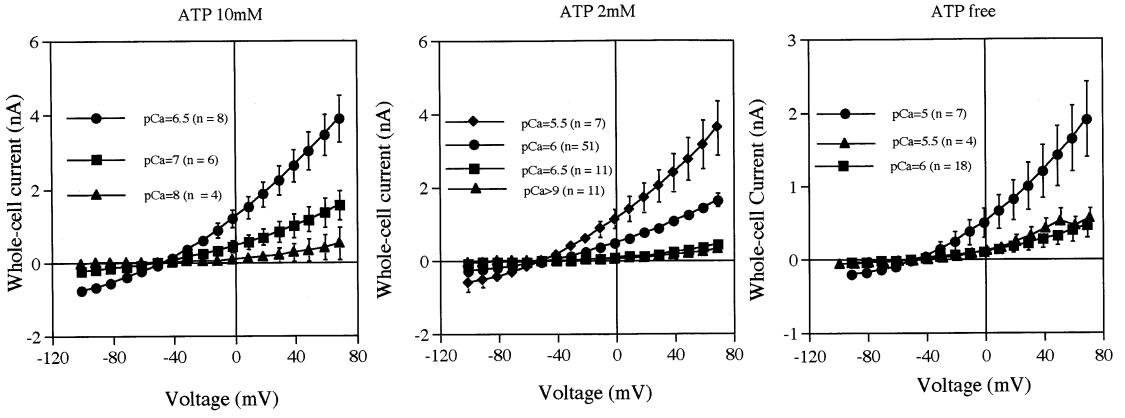

Fig. 7. Effect of inclusion of different concentrations of ATP in the pipette solution on the Ca^{2+}-activated Cl^- currents. Current-voltage relations from the cells dialyzed with various concentrations of Ca^{2+} and with three concentrations of ATP in the pipette solution. The voltage pulse protocol was the same as that in Fig. 1.

HCO_3^- from the extracellular solution entering the cell in response to depolarization and then interacting with the channel so as to alter its biophysical characteristic such as kinetics and conductance. This phenomenon could also be due to acidification of the cytosol by diffusion of CO_2 across the cell membrane, modifying the channel characteristics, since cytosolic pH would not be kept constant after replacement of extracellular Cl^- with HCO_3^- under the present experimental conditions. In fact, modulation of Ca^{2+}-dependent Cl^- channels by low internal pH has been demonstrated in secretory epithelial cells (Park & Brown, 1995; Arreola, Melvin &

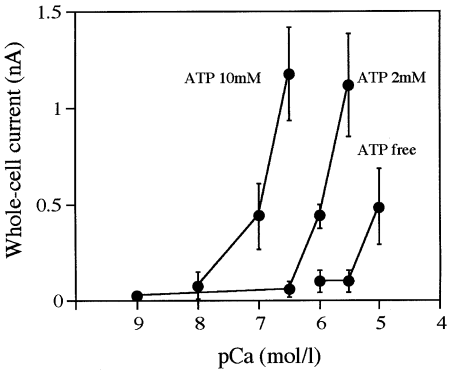

Fig. 8. Comparison of the dependency of current activation on free Ca^{2+}-concentrations in the pipette solution. The Cl^- current amplitudes at −1 mV are plotted against free Ca^{2+}-concentration at different ATP concentrations in the pipette solutions. Data were derived from those shown in Fig. 7.

Begenisich, 1995). Similar acidification of the cytosol via nonionic diffusion of CH_3COOH may be also applicable to the acetate permeability experiments (Fig. 6). Further studies will be required to test these possibilities.

Dependency of the Ca^{2+}-activated Cl^- Conductance on Cytosolic ATP Concentration

The present study has also shown that the Ca^{2+} sensitivity of the activation of the Cl^- current may be altered by cytosolic ATP levels. Even assuming the Cl^- conductance at 300 nM free Ca^{2+} concentration to be maximal at the highest ATP concentration (10 mM) tested in the present study, the EC_{50} is clearly in the mM range. This seems to be much higher than the K_m for ATP of protein kinases such as Ca^{2+}/calmodulin kinase II (9 to 22 μM) (Schulman, 1988), suggesting that phosphorylation is not the exclusive mechanism for regulation of Ca^{2+}-activated Cl^- currents by ATP in this tissues. However, it does not rule out a secondary role for protein kinases in modulating the currents once these currents are activated. Furthermore, the present study also showed that ATP can be replaced with a poorly hydrolyzable analogue, ATP-γS, suggesting that a nonhydrolytic mechanism may be involved in the phenomenon observed in the present study. Similar regulation of a Cl^- conductance by mmolar ATP concentrations has been demonstrated for CFTR in sweat duct cells (Quinton & Reddy, 1992). Further studies using other ATP analogues such as AMP-PNP will be necessary to conclude that the effect of ATP is truly via a nonhydrolytic mechanism.

The physiological significance of this phenomenon is unclear at this stage. However, it is possible that cytosolic ATP levels modulate not only active transport but also the Ca^{2+}-activated Cl^- conductance when the ATP levels change during secretion, thereby controlling the fluid secretory rate. A similar mechanism has been postulated for the control of the CFTR Cl^- conductance by ATP levels in sweat duct cells (Quinton & Reddy, 1992). In view of this, it is interesting to note that tachyphylaxis, the time-dependent decrease in the secretory response to continuous stimulation with 1 μM or higher concentration of ACh, is accompanied by a decrease in cytosolic ATP levels in perfused rat submandibular glands (Murakami, Seo & Watari, 1989). Further studies on the selectivity to other nucleotides and on the molecular structure of this type of Cl^- channel are required to answer the question of whether Ca^{2+}-activated Cl^- channel protein itself and/or its regulatory protein has the nucleotide-binding domains.

Minor Role of Ca^{2+}/Calmodulin-dependent Protein Kinase

Although Cl^- channel activation by Ca^{2+} in other epithelial cells has been shown to be mediated indirectly by a multifunctional Ca^{2+}/Calmodulin-dependent protein kinase (Wagner et al., 1991; Worrel & Frizzell, 1991), whether such mechanism is involved in salivary cells has not been well documented. Furthermore, the involvement of other cytosolic factors, in addition to cytosolic Ca^{2+} level, in the activation of the Cl^- conductance has not been well described in salivary acinar cells, although recent studies have shown that intracellular pH modulates the activity of Ca^{2+}-activated Cl^- channels in lacrimal and parotid acinar cells (Park & Brown, 1995; Arreola et al., 1995).

The present study has shown that the Ca^{2+}-activated Cl^- conductance in rat submandibular acinar cells is probably not mediated by either Ca^{2+}/Calmodulin or Ca^{2+}/Calmodulin kinase II, since the Cl^- currents were not affected by the addition of trifluoperazine or calmidazolium to the bath, or by addition KN-62, a specific inhibitor of calmodulin kinase II, to the pipette solution. This is contrary to the findings that Ca^{2+}/Calmodulin, acting via Ca^{2+}/Calmodulin kinase II, mediates the stimulation of a chloride conductance by calcium in T84 cells (Worrel & Frizzell, 1991), Jurkat T lymphocytes (Nishimoto et al., 1991), human neutrophils (Schumann, Gardner & Raffin, 1993), and HT-29 cells (Morris & Frizzell, 1993). However, the present data are in good agreement with the previous reports in rat submandibular glands that trifluoperazine does not affect the K^+ efflux induced by a calcium ionophore, A23187 (Kurtzer & Roberts, 1982) and that calmodulin inhibitors such as trifluoperazine, chlorpromazine and W-7 have no inhibitory effect on the increased oxygen consumption seen following stimulation by ACh or substance P (Komabayashi et al., 1984).

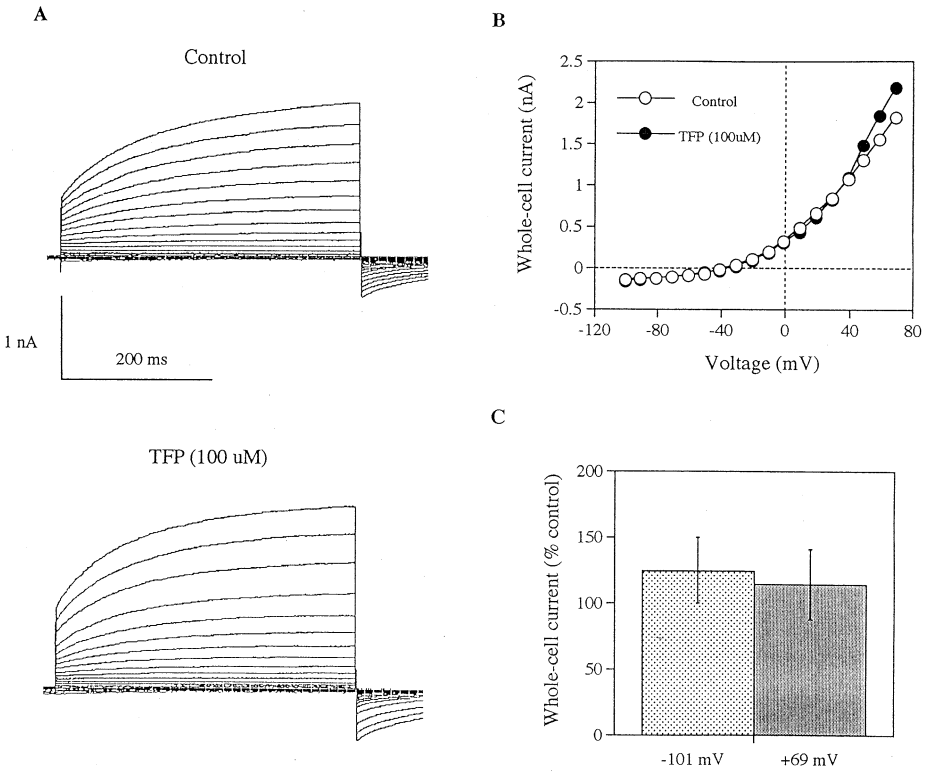

Fig. 9. Effects of a Ca^{2+}/Calmodulin inhibitor on the Ca^{2+}-activated Cl^- currents. (*A*) Whole-cell current responses of single rat submandibular acinar cells prior to and following trifluoperazine (100 μM) given extracellularly. (*B*) Corresponding steady-state whole-cell current-voltage relations derived from the experiments shown in *A*. (*C*) Summary of the effect of trifluoperazine (100 μM) on the Ca^{2+}-activated Cl^- currents. Data were derived from four experiments.

Pharmacological Characteristics of the Conductance

Finally, the present study has also provided pharmacological information about the Ca^{2+}-activated Cl^- channels in rat submandibular acinar cells. The outward currents during membrane depolarization were inhibited by 9-AC (0.3 and 1 mM), DIDS (0.3 − 1 mM), NPPB (0.1 mM) and IAA-94 (0.1 mM) and DPC (1 mM). These are in good agreement with previous reports in sheep parotid cells that DIDS and NPPB inhibited the outward Cl^- currents (Marty et al., 1988, Ishikawa & Cook, 1993). Under the present experimental conditions, however, none of the inhibitors tested significantly reduced the inward current at −101 mV. Such voltage-dependent blockage of current is not without precedent. A Ca^{2+}-activated Cl^- conductance in human macrophages is a good example of such a voltage-dependent blocking action for DIDS (Holevinsky, Jow & Nelson, 1994). In contrast, an opposite voltage-dependent blocking action for 9-AC and DIDS on Ca^{2+}-activated Cl^- currents has been found in guinea-pig hepatocytes (Koumi, Sato & Aramaki, 1994). Nevertheless, the present data suggest that the inhibitory effects of these agents may be largely dependent upon the membrane potential and do not necessarily allow them to block the currents significantly at physiological membrane potentials. In fact, it has been reported that DIDS did not block the acetylcholine-induced acidosis attributed to bicarbonate efflux through the channels on the apical membrane (Lau et al., 1989). It is also interesting to note that quite high concentrations of DPC (1 mM) and NPPB (0.5 mM) were required to block anion efflux pathways in salivary acinar cells (Melvin et al., 1987; Brown et al., 1989). Such concen-

Table 2. Effects of Cl^- channel blockers on the Cl^- currents

Blocker	% control (+69 mV)	% control (−101 mV)
DIDS (1 mM)	27.9 ± 4.8 (n = 6)[c]	99.0 ± 20.5 (n = 6)
9-AC (1 mM)	39.1 ± 13.0 (n = 4)[a]	83.2 ± 9.4 (n = 4)
NPPB (100 μM)	55.1 ± 24.7 (n = 5)[a]	155.5 ± 56.3 (n = 5)
DPC (1 mM)	66.0 ± 0.9 (n = 4)[c]	100.3 ± 17.1 (n = 4)
IAA-94 (100 μM)	78.2 ± 2.8 (n = 4)[b]	93.6 ± 3.9 (n = 4)
Glibenclamide (50 μM)	95.8 ± 2.5 (n = 3)	96.9 ± 4.4 (n = 3)

Results are means ± SEM of the number of experiments shown. The results are shown as the percent of control that remains. Tests for differences between group means were made by paired-t tests. [a] = P < 0.05, [b] = P < 0.01, [c] = P < 0.001.

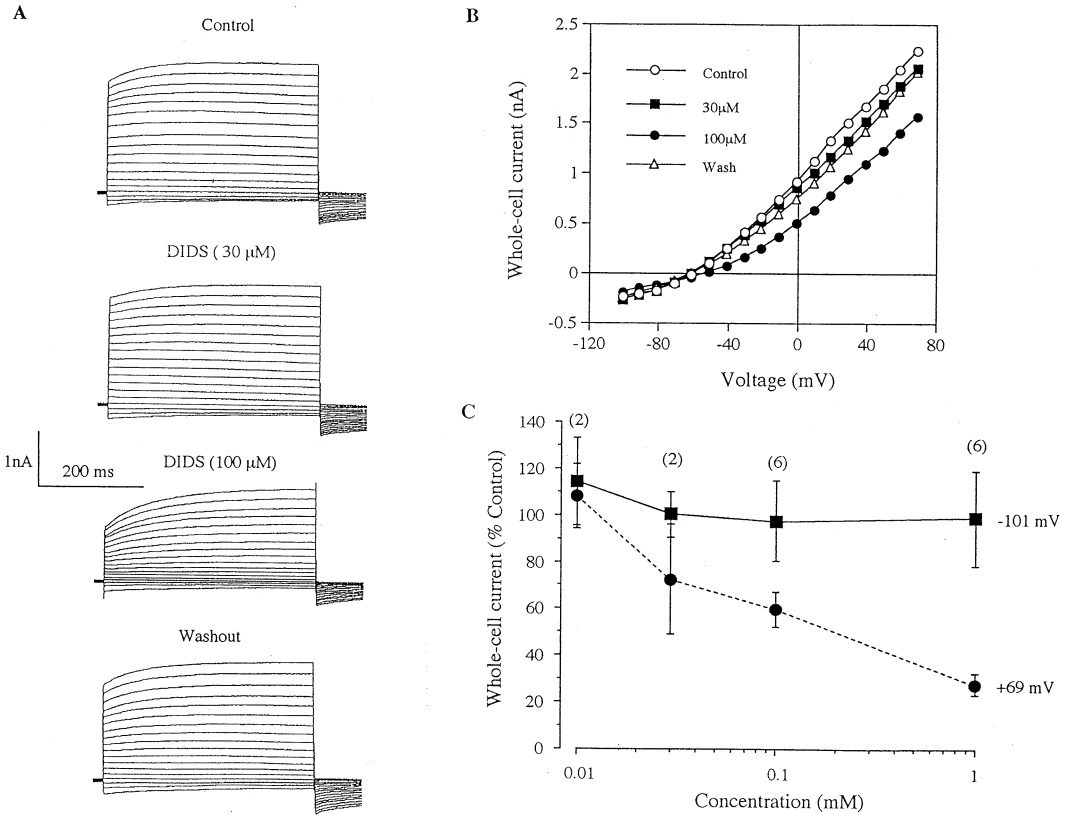

Fig. 10. Effect of DIDS on the Ca^{2+}-activated Cl^- currents. (*A*) Whole-cell current responses of single rat submandibular acinar cells prior to and following two doses of DIDS given extracellularly. (*B*) Corresponding steady-state whole-cell current-voltage relations derived from the experiments shown in A. (*C*) Dose-inhibition relation for the reduction of outward current at +69 mV and −101 mV produced by DIDS. The Cs-glutamate-rich pipette solution contained 1 μM free Ca^{2+} and the bath NMDG-Cl rich bath solution. Each point represents the mean of 2–6 experiments.

trations may be sufficiently high to raise the question of whether the drugs were having nonspecific effects. Therefore, blockers tested in the present study should be used with caution to elucidate the role of the Cl^- channels in the Ca^{2+}-activated anion efflux pathway in intact salivary cells. Further pharmacological characterization will be required in order to identify suitable voltage-independent blockers of the Ca^{2+}-activated Cl^- channels in salivary acinar cells.

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