Gramicidin-perforated Patch Recording Revealed the Oscillatory Nature of Secretory Cl\textsuperscript{−} Movements in Salivary Acinar Cells

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ABSTRACT Elevation of cytoplasmic free calcium concentrations ([Ca\textsuperscript{2+}]) evoked by cholinergic agonists stimulate isotonic fluid secretion in salivary acinar cells. This process is driven by the apical exit of Cl\textsuperscript{−} through Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels, while Cl\textsuperscript{−} enters the cytoplasm against its electrochemical gradient via a loop diuretic-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter (NKCC) and/or parallel operations of Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} and Na\textsuperscript{+}-H\textsuperscript{+} exchangers, located in the basolateral membrane. To characterize the contributions of those activities to net Cl\textsuperscript{−} secretion, we analyzed carbachol (CCh)-activated Cl\textsuperscript{−} currents in submandibular acinar cells using the “gramicidin-perforated patch recording configuration.” Since the linear polypeptide antibiotic gramicidin creates monovalent cation-selective pores, CCh-activated Cl\textsuperscript{−} currents in the gramicidin-perforated patch recording were carried by Cl\textsuperscript{−}-efflux via Cl\textsuperscript{−} channels, dependent upon Cl\textsuperscript{−} entry through Cl\textsuperscript{−} transporters expressed in the acinar cells. CCh-evoked oscillatory Cl\textsuperscript{−} currents were associated with oscillations of membrane potential. Bumetanide, a loop diuretic, decreased the CCh-activated Cl\textsuperscript{−} currents and hyperpolarized the membrane potential. In contrast, neither methazolamide, a carbonic anhydrase inhibitor, nor elimination of external HCO\textsubscript{3}\textsuperscript{−} had significant effects, suggesting that the cotransporter rather than parallel operations of Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} and Na\textsuperscript{+}-H\textsuperscript{+} exchangers is the primary Cl\textsuperscript{−} uptake pathway. Pharmacological manipulation of the activities of the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel and the NKCC revealed that the NKCC plays a substantial role in determining the amplitude of oscillatory Cl\textsuperscript{−} currents, while adjusting to the rate imposed by the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel, in the gramicidin-perforated patch configuration. By concerting with and being controlled by the cation steps, the oscillatory form of secretory Cl\textsuperscript{−} movements may effectively provide a driving force for fluid secretion in intact acinar cells.

KEY WORDS: chloride secretion • exocrine glands • chloride channels • cation–chloride cotransporter • gramicidin

INTRODUCTION

Cholinergic agonists trigger several forms of [Ca\textsuperscript{2+}], signals in exocrine acinar cells, which include [Ca\textsuperscript{2+}], oscillations and Ca\textsuperscript{2+} waves, that are governed by the action of Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} pumps (Gray, 1988; Lee et al., 1997a,b). The elevation of [Ca\textsuperscript{2+}], stimulates fluid secretion by activating ion permeabilities (Foskett and Melvin, 1989; Petersen, 1992; Turner, 1993). Importantly, fluid secretion requires the polarized distribution of ion channels and transporters (He et al., 1997). Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels in the apical membrane as well as Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels in the basolateral membrane are believed to be essential for fluid secretion in the salivary glands (Petersen, 1992; Smith and Gallacher, 1992). In the currently accepted model, Cl\textsuperscript{−} is driven into the cytoplasm against its electrochemical gradient via Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter (NKCC) and/or via parallel operations of Na\textsuperscript{+}-H\textsuperscript{+} and Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} exchangers, using the energy in the Na\textsuperscript{+} gradient across the basolateral membrane that is maintained by the activity of Na\textsuperscript{+}-K\textsuperscript{+} ATPase (Pirani et al., 1987; Lau et al., 1990; Petersen, 1992; Turner, 1993). These transporters raise intracellular Cl\textsuperscript{−} concentration ([Cl\textsuperscript{−}],) to levels (~60 mM) that are significantly greater than the electrochemical equilibrium level (Foskett, 1990), providing a driving force for Cl\textsuperscript{−} exit to the cell across the apical membrane via a Cl\textsuperscript{−} channel. The secretory isoform of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter (NKCC1) was suggested to play a key role in the movement of electrolytes and water across secretory epithelia, and the control of [Cl\textsuperscript{−}], (Soltoff et al., 1989; Evans and Turner, 1997; Russell, 2000). Reduction of [Cl\textsuperscript{−}], resulting from secretory Cl\textsuperscript{−} loss appears to stimulate NKCC activity, presumably via the cotransporter phosphorylation, whereas elevation of [Cl\textsuperscript{−}], has been shown to inhibit NKCC activity in secretory epithelia (Foskett, 1990; Lytle and Forbush, 1992; Robertson and Foskett, 1994; Haas et al., 1995;

Abbreviations used in this paper: BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CCh, carbachol; DPC, diphenylamine-2-carboxylate; KHR, Krebs-Henseleit Ringer; NHE, Na\textsuperscript{+}-H\textsuperscript{+} exchanger; NKCC, Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
Furthermore, the nature of transcellular Cl− measuring intracellular pH and the saliva flow rate. Reports, although the secretion rate was estimated by the net Cl− and [Cl−] i1989. Thus, it is likely that driving forces, transport mechanisms, governed by the concerted effort of NKCC and the deduced characteristics of secretory Cl− secretion, suggest that the steep control of their transport activities by [Ca2+] i and [Cl−] i may underlie the efficient driving of oscillatory Cl− secretion in intact submandibular acinar cells.

MATERIALS AND METHODS

Materials

CCh, gramicidin, bumetanide, methazolamide, genistein and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were purchased from Sigma-Aldrich. Diphenylamine-2-carboxylate (DPC) was purchased from Wako Pure Chemical. The media used were based on Krebs-Henseleit Ringer (KHR) solution containing 103 mM NaCl, 4.7 mM KCl, 2.56 mM CaCl2, 1.13 mM MgCl2, 25 mM NaHCO3, 1.15 mM NaH2PO4, 2.8 mM glucose, 4.9 mM sodium pyruvate, 2.7 mM sodium fumarate, 4.9 mM sodium glutamate, and 0.1% BSA, buffered with 12.5 mM HEPES at pH 7.4. The solution was thoroughly gassed with 95% O2 and 5% CO2 before each experiment.
Isolation of Acinar Cells

This study was approved by the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences), and the Guide for Animal Experimentation, Hiroshima University, and extra care was taken to avoid animal suffering. Male Wistar rats (~300 g) were anesthetized by the intraperitoneal injection of sodium pentobarbital (Nembutal, 70 mg/kg). Isolated rat submandibular acinar cells were prepared using collagenase (260 U/ml; type S-1, Nitta Gelatin) digestion as previously described (Sugita et al., 2000). The acinar cells were allowed to stick onto coverslips coated with poly-L-lysine (Sigma-Aldrich).

Gramicidin-perforated Patch Recording from Isolated Acinar Cells

The acinar cells on the cover glass were placed in a 0.2-ml chamber containing the KHR solution connected to a bath electrode by a 150 mM KClagar bridge. The chamber was constantly perfused at a rate of 1 ml/min with KHR solution without BSA at room temperature. Patch-clamp pipettes (2–4 MΩ) were pulled from borosilicate glass capillaries (Garner Glass Company). The gramicidin-perforated patch pipette solution contained 150 mM KCl and 10 mM HEPES (adjusted to pH 7.4 with KOH). Gramicidin was first dissolved in methanol to a concentration of 10 mg/ml and then diluted in the pipette solution to a final concentration of 100 µg/ml immediately before use. Before backfilling the pipette with the gramicidin-containing solution, the pipette tip was filled with gramicidin-free pipette solution by a brief immersion. After obtaining a gigaseal, the command potential was set at −40 mV, so that before membrane perforation, the transpatch potential was close to 0 mV, and after perforation, the cell was roughly at resting potential. Gramicidin-perforated patch recording was started after the stabilization of the capacitive currents (~15–25 min after cell attachment) (Ebihara et al., 1995; Sugita et al., 2000). The membrane potential of isolated acinar cells was measured under the current-clamp condition in the gramicidin-perforated patch configuration. Ionic currents were measured under voltage clamping at the holding potential of −80 mV, a value close to the potassium equilibrium potential. In experiments testing the effect of external anions, the perfused solution was modified by replacing 120 mM NaCl with equimolar quantities testing the effect of external anions, the perfused solution contained 140 mM KCl, 1 mM MgCl2, 10 mM HEPES, 0.5 mM EGTA, 10 mM glucose, and 1 mM ATP (adjusted to pH 7.4 with KOH). To establish whole-cell recording, additional suction was performed to rupture the patch membrane after the gigaseal formation. Chloride and potassium currents of the cells were isolated by changing the membrane potential from the holding potential (~80 mV, a value close to the potassium equilibrium potential) to 0 mV, a value close to the chloride equilibrium potential, briefly (300 ms) at 2-s intervals.

Measurement of Intracellular pH (pHi) and Determination of NKCC Activity

The isolated acinar cells were loaded with the pH-sensitive indicator, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), by incubation in the presence of 2 µM BCECF-AM (Dojindo) for 15 min in the KHR solution containing 0.1% BSA. The cells were attached to the coverslips coated with poly-L-lysine, placed in a 0.5-ml chamber, and constantly perfused at a rate of 5 ml/min with KHR solution without BSA. Intracellular BCECF fluorescence was monitored using an inverted epifluorescence microscope equipped with an image analysis system, ARGUS-HiSCA (Hamamatsu Photonics). Cells were sequentially excited at 490 and 450 nm for 272 ms at 2-s intervals, and the emitted fluorescence images were stored in a computer. After each experiment, cells were perfused with the solution containing 140 mM KCl, 10 mM NaCl, 1 mM MgSO4, 10 mM HEPES, and 5 µM nigericin at various pH values (6.8 ~ 7.8) to monitor the fluorescence for pH calibration. Fluorescence ratios (F490/F450) were then calculated and converted to pH. The pH measurement was performed within 2 h after the cell isolation. During the period, the isolated cells seemed to be healthy, judged by the fact that the Ca2+ pumping activity, required for oscillatory Cl− movement, remained unchanged.

The NKCC activity of isolated acinar cells was measured by determining the initial rate of pH recovery from an NH4Cl-induced acute alkaline load, as described previously (Paulais and Turner, 1992; Evans and Turner, 1997). Following various experimental treatments, the acinar cells were subjected to a 30 mM NH4Cl challenge. The cells first rapidly alkalized and then recovered exponentially to a final pH. By using the program DeltaGraph PRO3 (DeltaPoint), the recovery traces were fitted to an exponential equation

\[ \text{pHi} = (\text{pH}_{\text{initial}} - \text{pH}_{\text{final}}) e^{-kt} + \text{pH}_{\text{initial}}. \]

where pHi is the value of pHi at time t, pH_{initial} is the initial alkaline pH induced by the addition of NH4Cl (at t = 0), pH_{final} is the final resting pH after NH4Cl addition, and k is the recovery rate constant. The recovery traces were fitted by using least-squares curve-fitting techniques and treating pH_{initial}, pH_{final}, and k as free parameters. The initial pHi recovery rate is given by k(\text{pH}_{\text{initial}} - \text{pH}_{\text{final}}).

Results are presented as means ± SEM of n observations. Unless noted otherwise, statistical significance was determined using unpaired t test. P values <0.05 were considered statistically significant.

RESULTS

CCh Induces Repetitive Spikes of Cl− Currents in the Gramicidin-perforated Patch Configuration

First, submandibular acinar cells were voltage clamped in the conventional whole-cell patch configuration and stepped between −80 mV and 0 mV. CCh, a Ca2+-mobilizing agonist, induced repetitive spikes of inward cur-
rent and a sustained increase in outward current (Fig. 1 A). The inward and outward currents were expected to be Ca\textsuperscript{2+}-activated IC\textsubscript{L} and IK, respectively, as previously reported in rat salivary acinar cells (Hirono et al., 1998). DPC (500 \mu M), a nonspecific Cl\textsuperscript{−} channel inhibitor (Poronnik et al., 1992; Zeng et al., 1997), markedly suppressed the oscillatory Cl\textsuperscript{−} currents induced by CCh without affecting the sustained K\textsuperscript{+} currents (unpublished data). In the gramicidin-perforated patch configuration, the membrane potential of isolated acinar cells was measured under the current-clamp conditions, and ionic currents were measured at the holding potential of \(-80\) mV, a value close to the K\textsuperscript{+} equilibrium potential. Representative traces of the ionic current and membrane potential, recorded by repetitively switching between the voltage clamp mode and the current clamp mode, are shown in Fig. 1 B. The average current and membrane potential under resting conditions were 0.09 \pm 0.01 nA and \(-45.23 \pm 0.88\) mV (n = 19), respectively. CCh induced repetitive spikes of inward current that were associated with synchronous oscillations of membrane potential (Fig. 1 C). Although variability in the magnitude and kinetics of the inward current spikes was observed, CCh-triggered repetitive spikes of inward current and membrane potential oscillations had an average frequency of \(\sim 0.4\) Hz (Fig. 1 C). DPC (500 \mu M) inhibited the oscillatory inward current and eliminated the membrane potential oscillations, while causing the voltage to hyperpolarize in the direction of the K\textsuperscript{+} equilibrium potential (P < 0.05, n = 3, paired t test) (Fig. 1 D). Substitution of external Cl\textsuperscript{−} with gluconate suppressed the CCh-induced inward current spikes (Fig. 1 E), consistent with the inward current being carried by Cl\textsuperscript{−}, dependent upon the activity of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels and Cl\textsuperscript{−} influx into the cell. In intact acinar cells, the Na\textsuperscript{+}-K\textsuperscript{+} ATPase and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels are essential for salivary secretion, and their concerted actions, together with anion channels and transporters, play key roles in determining the rate of Cl\textsuperscript{−} secretion. However, because the gramicidin-perforated patch configuration allows permeation of monovalent cations, the requirement for K\textsuperscript{+} or Na\textsuperscript{+}-K\textsuperscript{+} ATPase activities is abrogated by voltage- and monovalent cation-clamping. In accord, neither the specific K\textsuperscript{+} channel blocker, charybotoxin, nor the Na\textsuperscript{+}-K\textsuperscript{+} ATPase inhibitor, ouabain, suppressed the CCh-induced inward currents (Fig. 1 F). Thus, the anion efflux rate, measured as the inward current, is determined solely by the activities of anion channels and transporters in the gramicidin-perforated patch configuration. Therefore, the gramicidin-perforated patch recording could enable dissection of the mechanisms specifically involved in mediating Cl\textsuperscript{−} transport across the apical and basolateral membranes. We have therefore characterized the sustained oscillatory anion currents under different conditions. The responses during the initial 2 min after the addition of CCh were excluded from the quantitative analysis below, since CCh may activate the anion channels possessing the distinct properties in the initial phase and the late phase of fluid secretion (Sugita et al. 2000), and CCh also induces nonoscillatory anion currents during the initial phase.

The Effect of Bumetanide and Methazolamide on the CCh-induced Cl\textsuperscript{−} Current in the Gramicidin-perforated Patch Configuration

Since gramicidin creates monovalent cation-selective pores, the CCh-activated Cl\textsuperscript{−} current in the gramicidin-perforated patch recording represents the Cl\textsuperscript{−} exit via Cl\textsuperscript{−} channels, dependent on Cl\textsuperscript{−} entry through membrane proteins expressed in the cells. Thus, to maintain the sustained Cl\textsuperscript{−} currents in the gramicidin-perforated patch configuration, [Cl\textsuperscript{−}], must be maintained in the cytoplasm above its electrochemical equilibrium value (Fig. 1 G). In the currently accepted model, Cl\textsuperscript{−} was suggested to enter the cytoplasm against its electrochemical gradient via a loop diuretic-sensitive NKCC and/or via parallel operations of Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} and Na\textsuperscript{+}-H\textsuperscript{+} exchangers (Pirani et al., 1987; Turner and George, 1988; Moore et al., 1995; He et al., 1997). In the gramicidin-perforated patch recording, the loop diuretic bumetanide (500 \mu M) reduced the CCh-activated Cl\textsuperscript{−} current by 87.8 \pm 10.1\% (P < 0.05, n = 4) (Fig. 2, A and B), and caused the membrane potential to hyperpolarize in the direction of the K\textsuperscript{+} equilibrium potential (P < 0.05) (Fig. 2, A and B). To inhibit Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} exchange activity, we employed methazolamide, a carbonic anhydrase inhibitor, to minimize the amount of HCO\textsubscript{3}\textsuperscript{−} available in the cytoplasm for transport. The previous study using parotid duct cells indicated that methazolamide suppressed the forskolin-activated anion current in the gramicidin-perforated patch configuration (Hirono et al., 2001). Although the addition of methazolamide (500 \mu M) after bumetanide application sometimes caused a slight inhibition of the CCh-activated Cl\textsuperscript{−} current (Fig. 2, A and B), there was no statistical difference between the currents in the experimental conditions of CCh + bumetanide, and CCh + bumetanide + methazolamide. Methazolamide alone had little effect on the CCh-activated Cl\textsuperscript{−} current or membrane potential oscillations (Fig. 2, C and D). In contrast, addition of bumetanide after methazolamide application markedly reduced the CCh-activated Cl\textsuperscript{−} current by 97.7 \pm 3.5\% (P < 0.05, n = 3) (Fig. 2, C and D), and caused the membrane potential to hyperpolarize in the direction of the K\textsuperscript{+} equilibrium potential (P < 0.05) (Fig. 2, C and D). Neither methazolamide nor bumetanide had significant effects on the frequencies of the current spikes (Fig. 2 G). Importantly, in the conventional
whole-cell patch configuration, neither bumetanide nor methazolamide affected the CCh-induced repetitive spikes of inward current or the sustained increase in outward current, expected to be Ca\(^{2+}\)-activated I\(_{\text{Cl}}\) and I\(_{\text{K}}\), respectively. In a different protocol, external HCO\(_3^\) (25 mM) was completely eliminated in the gramicidin-perforated patch configuration. The absence of HCO\(_3^\) was without effect on the CCh-induced inward current (Fig. 2 F). Thus, the present results suggest that Cl\(^{-}\)–HCO\(_3^\) exchange activity does not contribute significantly to the Cl\(^{-}\) uptake that maintains sustained Cl\(^{-}\) efflux currents. The use of
The Effects of the Modifier of NKCC Activity on the CCh-induced Cl\(^{-}\) Currents

The gramicidin-perforated patch configuration allows us to evaluate the electroneutral transport of ions by NKCC, which was not detectable in the conventional electrophysiological measurements. The loop diuretic sensitivity of the CCh-activated Cl\(^{-}\) current and membrane potential oscillations suggests that, by comparison with the paired exchangers, NKCC is the dominant Cl\(^{-}\) influx pathway for supporting the oscillatory Cl\(^{-}\) efflux through the Cl\(^{-}\) channels in submandibular acinar cells. Next, we addressed the contributory role of the activity of the Ca\(^{2+}\)-activated Cl\(^{-}\) efflux channel and the Na\(^{+}\)-free solutions in the pipette in the perforated patch configuration increased the inward driving force for Cl\(^{-}\) uptake on both the cotransporter and the paired exchangers, compared with the normal in vivo conditions. However, addition of 5 mM Na\(^{+}\) to the pipette had little effect on the amplitude of the CCh-induced anion current or its sensitivities to methazolamide and bumetanide (Fig. 2 E). Taken together, these results indicate that NKCC activity is the predominant mediator of Cl\(^{-}\) influx across the basolateral membrane of submandibular acinar cells under the experimental conditions used, as well as when the physiological intracellular and extracellular concentrations of Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) are maintained.

To evaluate if NKCC activity limits the magnitude of the CCh-activated Cl\(^{-}\) current, we compared the duration elements of the oscillatory spikes of CCh-induced inward currents (Fig. 3, A and B). Bumetanide, but not methazolamide, significantly decreased T\(_{1/2}\) and T\(_{d}\) (P < 0.05) (Fig. 3 B), without effect on T\(_{1/2}\)/T\(_{d}\). Neither Ti\(_{1/2}\) nor Ti was affected by the application of either drug. These results indicate that the duration of a decreasing phase of the current spikes may be prolonged by the NKCC activity, possibly by supplying sufficient Cl\(^{-}\) to the channel efflux pathway.

The Effects of the Modifier of NKCC Activity on the CCh-induced Cl\(^{-}\) Currents

Figure 2. The effects of bumetanide and methazolamide on the CCh-induced anion current and oscillations of membrane potential in the gramicidin-perforated patch configuration. (A and C) Representative traces of the anion current and membrane potential of isolated acinar cells during the application of CCh (500 nM), bumetanide (Bum, 500 μM), and methazolamide (Met, 500 μM) added to the perfusate during the period indicated by the horizontal bars. (B and D) Comparison of the CCh-induced anion current and the average membrane potential in the presence of bumetanide and/or methazolamide. The average current and membrane potential in B and D were determined in the final 20 s before switching between the voltage and current clamp modes in the absence (control) or presence of CCh, followed by the sequential addition of bumetanide and/or methazolamide, as indicated in A and C, respectively (n = 3–5). (E) The effects of the addition of Na\(^{+}\) to the pipette on the CCh-induced anion current. The CCh-induced anion current was recorded using the pipette solution containing 145 mM KCl, 5 mM NaCl, and 10 mM HEPES (pH 7.4), and the effects of bumetanide (500 μM) and methazolamide (500 μM) on the current were determined. (F) The effect of the external HCO\(_{3}\)\(^{-}\) depletion on the CCh-induced anion current. CCh (500 nM) and the HCO\(_{3}\)\(^{-}\)-free external solution, in which 25 mM HCO\(_{3}\)\(^{-}\) was replaced by Cl\(^{-}\) and CO\(_{2}\) was removed, were applied during the period indicated by the horizontal bars. (G) The representative time-resolved traces of the CCh-induced repetitive spikes of inward current in the presence of methazolamide and bumetanide. The traces of the CCh-induced inward currents before (a, closed circles) and after (b, closed triangles) the application of methazolamide, followed by the simultaneous addition of methazolamide and bumetanide (c, open diamonds), were deduced from a single gramicidin-perforated patch recording.
Cl⁻ influx activity via the cotransporter in the transcellular Cl⁻ movement. To characterize the involvement of the NKCC activity in determining the amplitude of oscillatory Cl⁻ currents, we investigated the effects of reagents that modify cotransporter activity. Genistein, a flavonoid contained in soybean and other various foods, was reported to stimulate Cl⁻ transport in epithelial tissues through the activation of cystic fibrosis transmembrane conductance regulator, an epithelial Cl⁻ channel, and NKCC (Wang et al., 1998; Nisato et al., 1999). To determine if genistein stimulates Na⁺-K⁺-2Cl⁻ transport in submandibular acinar cells, we analyzed NKCC activity by determining the rate of pHᵢ recovery of cells from an NH₄Cl-induced alkaline load, as previously described (Paulais and Turner, 1992; Evans and Turner, 1997). Application of CCh, before an NH₄Cl-induced alkaline load, induced the cytosolic acidification created by the initial HCO₃⁻ efflux. The recovery from the acid load, previously reported in salivary acinar cells, was not clearly observed in our experiments. The different response may be derived from the difference in the CCh dose used in the experiments, and the resultant differences in elevations of [Ca²⁺]; and the activities of NHEs and the other ion transporters. Of note, the dose-dependent changes in the pHᵢ of submandibular glands in the HCO₃⁻-containing medium was previously reported as stimulation with a submaximal concentration of acetylcholine caused a fall in pHᵢ in sharp contrast to the effect of a maximal concentration (Pirani et al., 1987). The initial rate of pHᵢ recovery from an NH₄Cl-induced alkaline load, calculated from the pHᵢ traces of the CCh-preincubated acinar cells in the presence of external HCO₃⁻, was 0.43 ± 0.03 pH U/min (n = 27) (Fig. 4, A and C). The initial pHᵢ recovery from the alkaline load was markedly slowed by bumetanide (0.12 ± 0.01 pH U/min, P < 0.05; n = 11) (Fig. 4, A and C). Genistein (100 μM) accelerated the initial rate of pHᵢ recovery of the CCh-preincubated acinar cells (0.59 ± 0.04 pH U/min, P <0.05; n = 14) (Fig. 4, B and C). The genistein-stimulated recovery was inhibited by bumetanide to a rate of 0.10 ± 0.01 pH U/min (Fig. 4, B and C), suggesting that it stimulated NKCC activity. In the conventional whole-cell patch configuration, the addition of genistein had little effect on the CCh-induced repetitive spikes of inward current and sustained increase in outward current, expected to be Ca²⁺-activated K⁺ and BK, respectively (Fig. 4, D and F). In contrast, genistein significantly increased the CCh-induced Cl⁻ current in the gramicidin-perforated patch configuration (P < 0.05; n = 3, paired t test) (Fig. 4, E and F). The genistein-induced enhancement was sensitive to bumetanide (Fig. 4 E). Thus, genistein enhanced CCh-induced Cl⁻ currents indirectly, by enhancing NKCC activity without affecting the activity of Ca²⁺-activated Cl⁻ channels directly.

**The Effect of NPPB on the CCh-induced Cl⁻ Currents**

To further characterize the relative contributions of the activities of NKCC and the Ca²⁺-activated Cl⁻ channel to transcellular Cl⁻ movement, we took the converse approach of partially inhibiting the channel activity. We...
compared the sensitivities of the CCh-induced Cl− currents to the various concentrations of NPPB, a Cl− channel blocker, under either the conventional whole-cell or gramicidin-perforated patch configurations. Here, if the NKCC activity predominantly restricts the amplitude of oscillatory Cl− currents measured in the gramicidin-perforated patch configuration, we expect Cl− currents in this configuration to be less sensitive to Cl− channel inhibition than those observed in the whole-cell mode. In agreement with this prediction, 4 μM NPPB had little effect on the Cl− current measured in the gramicidin-perforated patch configuration (Fig. 5, B and C), whereas it inhibited the conventional whole-cell current by 43.6 ± 4.7% (P < 0.05; n = 3) (Fig. 5, A and C). These data suggest that partial block of the Cl− channel did not significantly alter the CCh-activated Cl− current under the gramicidin-perforated patch configuration. Together, these results suggest that NKCC activity may prevailingly determine the amplitude of the oscillatory Cl− currents, while adjusting to the rate imposed by the Cl− channel, in the gramicidin-perforated patch configuration.

**DISCUSSION**

The present results suggest that gramicidin-perforated patch recording enables direct evaluation of the contributions of both channels and transporters to the Ca2+-activated anion current in single exocrine acinar cells. Under the gramicidin-perforated patch configuration, the CCh-evoked repetitive spikes of inward current were associated with synchronous oscillations in membrane potential. The sensitivity of the current to DPC and gluconate substitution suggests that the CCh-induced spikes of the inward current were mainly carried by Cl−. Submandibular acinar cells express at least five distinct Cl− channels; Glc7-like, volume sensitive, inward rectifying, Ca2+-activated, and CFTR-like Cl− channels (Zeng et al., 1997). The oscillatory nature of the CCh-induced current suggests that the Ca2+-activated Cl− channel is the dominant Cl− efflux pathway, tightly regulated by the [Ca2+]i oscillations, presumably with only minor contributions of other channels to the CCh-induced inward current. In the currently accepted model of the roles of key membrane transport proteins in rat salivary acinar cells, activities of both NKCC and parallel opera-

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**Figure 4.** Effect of genistein on NKCC activity and the CCh-induced Cl− currents. (A) Representative pHi traces from isolated acinar cells challenged with NH4Cl. CCh (500 nM) and NH4Cl (30 mM) were added to the perfusate during the period indicated by the horizontal bars. The traces were from the acinar cells with (the thin line) or without (closed circles) bumetanide (Bum, 500 μM) added 20 s before the application of NH4Cl. (B) Representative pHi traces from isolated acinar cells preincubated with genistein and challenged with 30 mM NH4Cl. CCh (500 nM), genistein (Gen, 100 μM), and NH4Cl (30 mM) were added to the perfusate during the period indicated by the horizontal bars. The traces were from the acinar cells with (the thin line) or without (closed circles) bumetanide (500 μM) added 20 s before the application of NH4Cl. (C) Comparison of the initial rates of pHi recovery from an NH4Cl-evoked alkaline load, determined in CCh-pretreated acinar cells in the presence or absence of genistein. The initial recovery rates were calculated from pHi traces indicated as A and B, as described in MATERIALS AND METHODS. Results are presented as means ± SEM of the initial recovery rates from CCh-pretreated cells (n = 27), CCh- and Bum-pretreated cells (n = 8), CCh- and Gen-pretreated cells (n = 14), and CCh-, Gen-, and Bum-pretreated cells (n = 9). (D) The effect of genistein on the CCh-induced Cl− and K+ currents in the conventional whole-cell patch configuration. (E) The effect of genistein on the CCh-induced anion current recorded under the gramicidin-perforated patch configuration. CCh (500 nM), genistein (Gen, 100 μM), and bumetanide (Bum, 500 μM) were added to the perfusate during the period indicated by the horizontal bars. (F) Comparison of the effect of genistein on the CCh-induced anion currents under either the conventional whole-cell patch or the gramicidin-perforated patch configurations. The relative currents before (black columns) and after (white columns) the addition of genistein were determined in the final 20 s before changing solutions. The relative currents are expressed as a percentage of the anion current induced by CCh alone under either the conventional whole-cell patch or the gramicidin-perforated patch configurations (n = 3).
Representative trace showing the effect of 4 μM gramicidin-perforated patch configuration. CCh (500 nM), NPPB on the CCh-induced anion current recorded under the configuration. (B) Representative trace showing the effect of increased because of enhanced NHE activity (Evans et al., 1999; Evans et al., 2000; Park et al., 2001a). In contrast with the previous reports in which the Cl− uptake was estimated by monitoring intracellular pH (Pirani et al., 1987; Evans et al., 1999; Park et al., 2001a), our data directly revealed the contribution of transporters to both Cl− uptake and Cl− secretion under the gramicidin-perforated patch configuration. As revealed by the effects of bumetanide and methazolamide in the present study, Cl− uptake was mainly (>85%) mediated by NKCC activity. Interestingly, submandibular acinar cells express at least 10-fold more NKCC protein than do parotid acinar cells (Zeng et al., 1997), which may suggest that the predominant role in submandibular gland cells is accounted for by the higher expression level. HCO3− uptake by Na+/HCO3− cotransporters has been proposed in submandibular acinar cells (Luo et al., 2001). However, the contribution of Na+/HCO3− cotransporter activity to the anion currents observed in the present study would seem to be minor, since the elimination of external HCO3− had little effect on the CCh-induced anion current under the gramicidin-perforated patch configuration, and the current was remarkably inhibited by bumetanide.

Thus, maintenance of [Cl−]i above electrochemical equilibrium appears to be mediated mainly by NKCC, which provides the driving force for Cl− to exit the cell via a Cl− channel presumably on the apical membrane. Although the current oscillations appear to reflect the opening and closing of the Cl− channels tightly regulated by [Ca2+]i oscillations, which activity, the Ca2+-activated Cl− channel or the cotransporter, predominantly determines the amplitude of the oscillatory Cl− currents? To address this issue, first, we investigated the effects of genistein. We found that genistein enhanced the activity of the cotransporter, but was without effect on the activity of the Ca2+-activated Cl− channel. Nevertheless, genistein stimulated the Cl− currents in the gramicidin-perforated patch configuration. This result therefore suggests that the activity of the cotransporter is substantially involved in determining the amplitude of the oscillatory Cl− currents. A similar conclusion was reached in experiments that manipulated the activity of the channel. Thus, partial inhibition of the channel activity by NPPB had much less effect on the CCh-induced Cl− current under the gramicidin-perforated patch configuration than it did on the Cl− currents measured in the normal whole-cell configuration. The lower sensitivity implies that the channel activity may not prevailingly determine the rate of current flow in the gramicidin-perforated patch configuration. Finally, our results suggest that the NKCC activity modulated the duration of a decreasing phase of the current spikes. Ca2+-activated Cl− channels are reported to be regulated by [Ca2+]i, and membrane potential (Arreola et al., 1996; Ishikawa, 1996; Park et al., 2001b). Since the current was recorded
under the voltage-clamp condition, the magnitude of the current reflects the combined influences of [Ca\(^{2+}\)], and Cl\(^{-}\)-driving force. The rate of decay of the Cl\(^{-}\)-current following a Ca\(^{2+}\) oscillation will reflect the rate of decline of [Ca\(^{2+}\)], modified by any changes in Cl\(^{-}\)-driving force. The observed modulation of the rate of decay of the Cl\(^{-}\)-current by bumetanide implies that the rate of Cl\(^{-}\)-influx via the cotransporter was involved in determining the amplitude of oscillatory Cl\(^{-}\}-currents under this condition as well. Taken together, these results suggest that basolateral NKCC, rather than luminal Ca\(^{2+}\)-activated Cl\(^{-}\)-channels, may play a prevailing role in restricting the transcellular Cl\(^{-}\}-movement induced by CCh in the gramicidin-perforated patch configuration, while the opening and a part of the closing of Ca\(^{2+}\)-activated Cl\(^{-}\}-channels may participate in restricting the repetitive spikes of Cl\(^{-}\}-efflux. The molecular aspects underlying NKCC regulation in exocrine acinar cells remain to be determined (Kurihara et al., 2002). However, it was reported that a reduction of [Cl\(^{-}\)], resulting from Cl\(^{-}\}-loss through the activated Cl\(^{-}\}-channels, reduces [Cl\(^{-}\)]-mediated inhibition of NKCC (Foskett, 1990; Robertson and Foskett, 1994), presumably by permitting an increase in phosphorylation of NKCC by a Cl\(^{-}\)-dependent kinase, and plays a key role in leading to the activation or up-regulation of NKCC (Lytle and Forbush, 1992; Haas et al., 1995; Lytle, 1997; Haas and Forbush, 2000; Flemmer et al., 2002; Dowd and Forbush, 2003). Thus, NKCC appears to be regulated by intracellular Cl\(^{-}\), allowing the transporter to adjust to the rate imposed by the Cl\(^{-}\)-channel. The coordinated activation and inactivation of these transporters may efficiently elicit the oscillatory Cl\(^{-}\)-secretion, depending upon [Ca\(^{2+}\)] and [Cl\(^{-}\)]. Therefore, it is likely that the manipulation of the NKCC activity has therapeutic value for xerostomia as considered previously (Turner and Sugiyama, 2002), since the transcellular Cl\(^{-}\)-movement may play an essential role in fluid secretion. Along these lines, genistein might be also be of benefit, particularly in cystic fibrosis by means of up-regulation of NKCC in addition to enhancing the channel activity of the disease-associated CFTR mutants (Wang et al., 1998).

The gramicidin-perforated patch technique is a useful tool for deducing and clarifying the cellular mechanisms underlying ion transport by the concerted effect of the anion efflux channels and the anion influx transporters at a single cell level. Of note, the gramicidin-perforated patch configuration enables the time-resolved determination of the activities of the electroneutral transporters in the cellular systems of ion transport. In the present study, using the CCh-stimulated acinar cells under this configuration, we deduced and characterized the oscillatory nature of secretory Cl\(^{-}\)-movements, governed by the Cl\(^{-}\)-efflux through the Ca\(^{2+}\)-activated Cl\(^{-}\)-channels and the Cl\(^{-}\)-influx through the electro-neutral NKCC, and steeply controlled by [Ca\(^{2+}\)] and [Cl\(^{-}\)]. In intact acinar cells, the oscillatory Cl\(^{-}\}-movements may effectively drive the oscillatory secretion of fluid and electrolyte, while cooperating with and being regulated by the activities of the Na\(^{+}\)-K\(^{+}\)-ATPase and Ca\(^{2+}\)-activated K\(^{+}\)-channels.

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References

Allen, T.W., O.S. Andersen, and B. Roux. 2003. Structure of gramicidin A in a lipid bilayer environment determined using molecular dynamics simulations and solid-state NMR data. J. Am. Chem. Soc. 125:9868–9877.

Andersen, O.S., and R.E. Koepppe. 1992. Molecular determinants of channel function. Physiol. Rev. 72:S89–S158.

Arreola, J., J.E. Melvin, and T. Begenisch. 1996. Activation of calcium-dependent chloride channels in rat parotid acinar cells. J. Gen. Physiol. 108:35–47.

Burkhart, B.M., N. Li, D.A. Langs, W.A. Pangborn, and W.L. Duax. 1998. The conducting form of gramicidin A is a right-handed double-stranded double helix. Proc. Natl. Acad. Sci. USA. 95:12950–12955.

Dowd, B.F., and B. Forbush. 2003. PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). J. Biol. Chem. 278:27347–27353.

Ebihara, S., K. Shirato, N. Harata, and N. Akaike. 1995. Gramicidin-perforated patch recording: GABA response in mammalian neurons with intact intracellular chloride. J. Physiol. 484:77–86.

Evans, R.L., and R.J. Turner. 1997. Upregulation of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\)-cotransporter activity in rat parotid acinar cells by muscarinic stimulation. J. Physiol. 499:351–359.

Evans, R.L., S.M. Bell, P.J. Schultheis, G.E. Shull, and J.E. Melvin. 1999. Targeted disruption of the Nhe1 gene prevents muscarinic agonist-induced up-regulation of Na\(^{+}\)/H\(^{+}\)-exchange in mouse parotid acinar cells. J. Biol. Chem. 274:29025–29030.

Evans, R.L., K. Park, R.J. Turner, G.E. Watson, H-V. Nguyen, M.R. Dennet, A.R. Hand, M. Flagella, G.E. Shull, and J.E. Melvin. 2000. Severe impairment of salivation in Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\}-cotransporter (NKCC1)-deficient mice. J. Biol. Chem. 275:26720–26726.

Flemmer, A.W., I. Gimenez, B.F. Dowd, R.B. Darman, and B. Forbush. 2002. Activation of the Na-K-Cl cotransporter NKCC1 detected with a phospho-specific antibody. J. Biol. Chem. 277:37551–37558.

Foskett, J.K. 1990. [Ca\(^{2+}\)] modulation of Cl\(^{-}\}-content controls cell volume in single salivary acinar cells during fluid secretion. Am. J. Physiol. 259:C998–C1004.

Foskett, J.K., and J.E. Melvin. 1989. Activation of salivary secretion cell coupling of cell volume and [Ca\(^{2+}\)], in single cells. Science. 244:1582–1585.

Gray, P.T.A. 1988. Oscillations of free cytosolic calcium evoked by cholinergic and catecholaminergic agonists in rat parotid acinar cells. J. Physiol. 406:35–53.

Haas, M., and B. Forbush. 2000. The Na-K-Cl cotransporter of secretory epithelia. Annu. Rev. Physiol. 62:515–534.
Haas, M., D. McBrayerm, and C. Lyte. 1995. [Cl⁻]-dependent phosphorylation of the Na-K-Cl cotransport protein of dog tracheal epithelial cells. J. Biol. Chem. 270:28955–28961.

He, X., C.M. Tse, M. Donowitz, S.L. Alper, S.E. Gabriel, and B.J. Baum. 1997. Polarization of key membrane transport proteins in the rat submandibular gland. Pflugers Arch. 433:260–268.

Hirono, C., M. Sugita, K. Furuya, S. Yamagishi, and Y. Shiba. 1998. Potentiation by isoproterenol on carbachol-induced K⁺ and Cl⁻ currents and fluid secretion in rat parotid. J. Membr. Biol. 164:197–203.

Hirono, C., T. Nakamoto, M. Sugita, Y. Iwasa, Y. Akagawa, and Y. Shiba. 2001. Gramicidin-perforated patch analysis on HCO₃⁻ secretion through a forskolin-activated anion channel in rat parotid intralobular duct cells. J. Membr. Biol. 180:11–19.

Ishikawa, T. 1996. A bicarbonate- and weak acid-permeable chloride conductance controlled by cytosolic Ca²⁺ and ATP in rat submandibular acinar cells. J. Membr. Biol. 153:147–159.

Kovacs, F., J. Quine, and T.A. Cross. 1999. Validation of the single-stranded channel conformation of gramicidin A by solid-state NMR. Proc. Natl. Acad. Sci. USA. 96:7910–7915.

Kurihara, K., N. Nakashima, M.L. Moore-Hoon, and R.J. Turner. 1992. Phosphorylation of the salivary Na⁺–K⁺–2Cl⁻ cotransporter. Am. J. Physiol. Cell Physiol. 282:C817–C823.

Lau, K.R., A.C. Elliot, and P.D. Brown. 1989. Acetylcholine-induced intracellular acidosis in rabbit salivary gland acinar cells. Am. J. Physiol. 256:C288–C295.

Lau, K.R., A.J. Howorth, and R.M. Case. 1990. The effects of bumetanide, amiloride, and Ba²⁺ on fluid and electrolyte secretion in rabbit salivary gland. J. Physiol. 425:407–427.

Lee, M.G., X. Xu, W. Zeng, J. Diaz, T.H. Kuo, F. Wuytack, L. Racymaekers, and S. Muallem. 1997a. Polarized expression of Ca²⁺ pumps in pancreatic and salivary gland cells. Role in initiation and propagation of [Ca²⁺], waves. J. Biol. Chem. 272:15771–15776.

Lee, M.G., X. Xu, W. Zeng, J. Diaz, R.J. Wojcikiewicz, T.H. Kuo, F. Wuytack, L. Racymaekers, and S. Muallem. 1997b. Polarized expression of Ca²⁺ channels in pancreatic and salivary gland cells. Correlation with initiation and propagation of [Ca²⁺], waves. J. Biol. Chem. 272:15765–15770.

Luo, X., J.Y. Choi, S.B.H. Ko, A. Pushkin, I. Kurzt, W. Ahn, M.G. Lee, and S. Muallem. 2001. HCO₃⁻ salvage mechanisms in the submandibular gland acinar and duct cells. J. Biol. Chem. 276:9808–9816.

Lytte, C. 1997. Activation of the avian erythrocyte Na-K-Cl cotransport protein by cell shrinkage, cAMP, fluoride, and calycin-A involves phosphorylation at common sites. J. Biol. Chem. 272:15069–15077.

Lytte, C., and B. Forbush. 1992. The Na-K-Cl cotransport protein of shark rectal gland. II. Regulation by direct phosphorylation. J. Biol. Chem. 267:25438–25443.

Manganel, M., and R.J. Turner. 1991. Rapid secretagogue-induced activation of Na⁺/H⁺ exchange in rat parotid acinar cells. J. Biol. Chem. 266:10182–10188.

Martinez, J.R., and N. Cassity. 1985. Cl⁻ requirement for saliva secretion in the isolated, perfused rabbit submandibular gland. J. Am. Physiol. 249:C464–C469.

Melvin, J.E., A. Moran, and R.J. Turner. 1988. The role of HCO₃⁻ and Na⁺/H⁺ exchange in the response of rat parotid acinar cells to muscarinic stimulation. J. Biol. Chem. 263:19564–19569.

Moore, M.L., J.N. George, and R.J. Turner. 1995. Anion dependence of bumetanide binding and ion transport by the rabbit parotid Na⁺–K⁺–2Cl⁻ co-transporter: evidence for an intracellular anion modifier site. Biochem. J. 309:637–642.

Mueller, P., and D.O. Rudin. 1967. Development of K⁺-Na⁺ discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. Biochem. Biophys. Res. Commun. 26:398–404.

Nisato, N., Y. Ito, and Y. Marunaka. 1999. Activation of Cl⁻ channel and Na⁺/K⁺/2Cl⁻ cotransporter in renal epithelial A6 cells by flavonoids: genistein, daidzein, and apigenin. Biochem. Biophys. Res. Commun. 254:368–371.

Park, K., R.L. Evans, G.E. Watson, K. Nehrke, L. Richardson, S.M. Bell, P.J. Schultheis, A.R. Hand, G.E. Shull, and J.E. Melvin. 2001a. Defective fluid secretion and NaCl absorption in the parotid glands of Na⁺/H⁺ exchanger-deficient mice. J. Biol. Chem. 276:27442–27450.

Park, M.K., R.B. Lomax, A.V. Tepikin, and O.H. Petersen. 2001b. Local uncaging of caged Ca²⁺ reveals distribution of Ca²⁺-activated Cl⁻ channels in pancreatic acinar cells. Proc. Natl. Acad. Sci. USA. 98:10948–10953.

Paulais, M., and R.J. Turner. 1992. β-Adrenergic upregulation of the Na⁺-K⁺-2Cl⁻ cotransporter in rat parotid acinar cells. J. Clin. Invest. 89:1142–1147.

Petersen, O.H. 1992. Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. J. Physiol. 448:1–51.

Pirani, D., L.A.R. Evans, D.I. Cook, and J.A. Young. 1987. Intracellular pH in the rat mandibular salivary gland: the role of Na-H and G-HCO₃ antipots in secretion. Pflugers Arch. 408:178–184.

Poronnik, P., M.C. Ward, and D.I. Cook. 1992. Intracellular Ca²⁺ release by fluorfenamic acid and other blockers of the non-selective cation channel. FEBS Lett. 296:245–248.

Putney, L.K., C.R.T. Tibat, and M.E. O’Donnell. 1999. Intracellular Cl regulates Na-K-Cl cotransport activity in human trabecular meshwork cells. Am. J. Physiol. 277:C373–C383.

Robertson, M.A., and J.K. Foskett. 1994. Na⁺ transport pathways in secretory acinar cells: membrane cross talk mediated by [Cl⁻]. Am. J. Physiol. 267:C146–C156.

Robertson, M.A., M. Woodside, J.K. Foskett, J. Orlovski, and S. Grinstein. 1997. Muscarinic agonists induce phosphorylation-independent activation of the NHE-1 isoform of the Na⁺/H⁺ antiporter in salivary acinar cells. J. Biol. Chem. 272:287–294.

Russell, J.M. 2000. Sodium-potassium-chloride cotransport. Physiol. Rev. 80:211–276.

Smith, P.M., and D.V. Gallacher. 1992. Acetylcholine and caffeine-evoked repetitive transient Ca²⁺-activated K⁺ and Cl⁻ currents in mouse submandibular cells. J. Physiol. 449:109–120.

Soltott, S.P., M.K. McMillian, L.C. Cantley, E.J. Cragoe Jr., and B.R. Tamalo. 1989. Effects of muscarinic, alpha-adrenergic, and substance P agonists and ionomycin on ion transport mechanisms in the rat parotid acinar cell. The dependence of ion transport on intracellular calcium. J. Gen. Physiol. 93:285–319.

Sugita, M., C. Hirono, S. Tanaka, T. Nakahari, Y. Imai, Y. Kanno, and Y. Shiba. 2000. Visualization of the secretory process involved in Ca²⁺-activated fluid secretion from rat submandibular glands using the fluorescent dye, calcine. Eur. J. Cell Biol. 79:182–191.

Turner, R.J. 1993. Mechanisms of fluid secretion by salivary glands. Ann. N. Y. Acad. Sci. 694:24–35.

Turner, R.J., and J.N. George. 1988. Ionic dependence of bumetanide binding to the rabbit parotid Na/K/Cl cotransporter. J. Membr. Biol. 102:71–77.

Turner, R.J., and H. Sugiyama. 2002. Understanding salivary fluid and protein secretion. Oral Dis. 8:3–11.

Wang, F., S. Zeltwanger, I.C-H. Yang, A.C. Nairn, and T-C. Hung. 1998. Actions of genistein on cystic fibrosis transmembrane conductance regulator chloride channel gating. J. Gen. Physiol. 111:477–490.

Zeng, W., M.G. Lee, and S. Muallem. 1997. Membrane-specific regulation of Cl⁻ channels by purinergic receptors in rat submandibular gland acinar and duct cells. J. Biol. Chem. 272:32956–32965.