Muscarinic activation of Na\(^{+}\)-dependent ion transporters and modulation by bicarbonate in rat submandibular gland acinus

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Submitted 8 September 2004; accepted in final form 3 November 2004

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The application of a fluid secretory stimulus such as acetylcholine (ACh) or substance P to the salivary acinar cells leads to a rise in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) that, in turn, causes the opening of Ca\(^{2+}\)-activated Cl\(^{-}\) and K\(^{+}\) channels in the apical and basolateral membranes, respectively. This increase in K\(^{+}\) and Cl\(^{-}\) conductance allows KCl to flow out of the cell, resulting in an accumulation of Cl\(^{-}\) and their associated negative electrical charge in the acinar lumen. Na\(^{+}\) then follows Cl\(^{-}\) paracellularly to preserve electroneutrality, and the resulting osmotic gradient for NaCl causes a transepithelial movement of water from the interstitium to lumen (2, 17, 30). The exit of Cl\(^{-}\) through the apical membrane requires the compensatory uptake of Cl\(^{-}\) in the basolateral membrane. The Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC) and the combined operation of the Na\(^{+}/H^{+}\) exchanger (NHE) and the Cl\(^{-}/HCO_{3}^{-}\) exchanger are widely proposed mechanisms for Cl\(^{-}\) uptake in salivary gland acini (2, 17, 30), and their activities are positively regulated by secretory agonists such as ACh (6, 7, 14, 20).

The activation of various ion channels during the secretion of primary saliva is reflected in the membrane voltage (V\(_{m}\)) of the acinar cells, which can be further analyzed using voltage-clamp methods. The most prominent changes in V\(_{m}\) during stimulation with ACh are the initial depolarization and subsequent repolarization that reflect the activation of apical Cl\(^{-}\) channels and basolateral K\(^{+}\) channels, respectively. The sequential activation of Cl\(^{-}\) and K\(^{+}\) channels is also in accordance with the wavelike increase in [Ca\(^{2+}\)]\(_{i}\) (Ca\(^{2+}\) wave) from the apical pole to the basolateral cytoplasm in salivary acinar cells stimulated with ACh (9). In addition, in a study that analyzed the ACh-induced V\(_{m}\) changes, it was found that a delayed hyperpolarization follows the initial transient changes in V\(_{m}\) in mouse parotid acini (25). This delayed hyperpolarization was blocked by ouabain, which indicates the activation of electrogenic Na\(^{+}/K^{+}\)-ATPase. Because the activity of Na\(^{+}/K^{+}\)-ATPase is sensitive regulated by the intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{i}\)) (29), the influx of Na\(^{+}\) coupled with a Cl\(^{-}\) uptake mechanism was suggested to be responsible for the delayed activation of the Na\(^{+}\) pump in mouse parotid gland (17, 25).

The role of Na\(^{+}\)-coupled transporters in the regulation of [Na\(^{+}\)]\(_{i}\) during the muscarinic stimulation has been investigated in various salivary glands. For this purpose, sodium-binding benzofuran isophthalate (SBFI) is the most widely used fluorescent probe that allows for the noninvasive monitoring of [Na\(^{+}\)]\(_{i}\) in an exocrine gland. However, SBFI cannot be used together with several agents, such as the amiloride-derived NHE inhibitor ethylisopropylamiloride (EIPA), because of their excitation in the UV range of the light spectrum. Although a new type of dye, such as sodium green, which is excited by visible wavelengths, can circumvent such disadvantages of SBFI, sodium green does not show a spectral shift, and so it cannot be used for ratiometry. Thus the availability of other types of in vivo signals reflecting the changes in [Na\(^{+}\)]\(_{i}\) could be helpful for a real-time monitoring of Na\(^{+}\)-coupled transporters when combined with specific blockers.

According to the classic measurement of fluid secretion in the perfused rat and rabbit submandibular glands, basolateral Cl\(^{-}\) uptake via bumetanide-sensitive cotransporters (NKCC) plays a major role in ACh-induced salivary secretion (30). However, measurements of [Na\(^{+}\)]\(_{i}\) in salivary acinar cells...
showed that NHE, rather than NKCC, is the more crucial pathway for the ACh-induced Na\(^+\) influx (4, 26). Thus the relative contribution of NKCC and NHE to the fluid and electrolyte secretion remains to be elucidated for salivary glands. In a pilot study, using the isolated acini from rat submandibular gland (RSMGA), we observed a strong delayed hyperpolarization after muscarinic stimulation (Vh,ACh). Because the Vh,ACh was totally blocked by a Na\(^+\)/K\(^+\)-ATPase inhibitor, the intriguing possibility was raised that the Vh,ACh could be used to monitor the contributions of various Na\(^+\)-dependent transporters. Because the driving force for basolateral Cl\(^-\) reuptake is ultimately generated from the activity of Na\(^+\) pumps, it was tempting to investigate the relative contribution of NHE and NKCC in terms of their effectiveness on the Na\(^+\)-dependent regulation of Na\(^+\)-pump activity. Because the activity of NHE and anion exchangers can be greatly influenced by the pH buffering conditions, we compared the effects of blockers of NHE and NKCC in both the presence and absence of HCO\(_3\^-\). We found that the inclusion of a physiological level of HCO\(_3\^-\) (24 meq/l) not only enhances the NHE activity but also inhibits the ACh-induced facilitation of NKCC.

**MATERIALS AND METHODS**

**Preparation of RSMGA.** Sprague-Dawley Rats (150–200 g) were killed by exposure to 100% CO\(_2\), and the submandibular glands were quickly removed. The tissues were cut into small pieces (1–2 mm\(^3\)) in the phosphate-buffered Ringer solution (PBS) that contained (in mM) 145 NaCl, 1.6 K\(_2\)HPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 1.3 CaCl\(_2\), 10 Na-acetate, 5 d-glucose, 2 glycine, and 1 \(\alpha\)-ketoglutarate, gassed with 95% O\(_2\)-5% CO\(_2\). For digestion, collagenase IV (0.8 mg/ml; Sigma, Heidelberg, Germany) and trypsin inhibitor (0.5 mg/ml; Sigma, St. Louis, MO) were added to the preparation solution. The tissue was incubated for 12 min at 37°C and gently agitated using a fire-polished wide-bore (1–2 mm) Pasteur pipette. After the mixture was allowed to settle, the acini were washed twice with enzyme-free solution and finally filtered through a nylon mesh (pore size 150 \(\mu\)m). After filtering, the suspension was centrifuged at 800 rpm for 2 min, and the supernatant was removed. The isolated acini were kept at 4°C for up to 3 h in the phosphate-buffered Ringer solution (PBS) that contained (in mM) 145 NaCl, 1.6 K\(_2\)HPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 1.3 CaCl\(_2\), 1 MgCl\(_2\), and 5 d-glucose, pH 7.4.

**Patch-clamp methods.** The isolated acini were transferred into a bath chamber mounted on the stage of an inverted microscope (Olympus IX-70; Tokyo, Japan). The bath, with a volume of \(\sim\)0.3 ml, was

**Fig. 1.** ACh-induced changes in intracellular ion concentrations ([Ca\(^{2+}\)], [Na\(^{+}\)], and [Cl\(^-\)]) and membrane voltage (V\(_m\)) of rat submandibular gland acini (RSMGA). A: original traces of fura-2 fluorescence ratio (F-fura340/380, solid line), sodium-binding benzofuran isophthalate (SBFI) fluorescence ratio (F-SBFI340/380, shaded line), and 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ) fluorescence intensity (F-SPQ, dotted line). ACh (5 \(\mu\)M) was commonly applied for 20 s as indicated (horizontal bar). Each measurement was performed in a single acinus as marked by the box in photo at right of representative RSMGA. Note that F-SPQ is plotted upside down because the [Cl\(^-\)]\(_c\) is inversely related with F-SPQ. **B**: representative trace of V\(_m\) and the effect of ACh are plotted on the same time scale of A. C: summary of V\(_m\) measured at different phases as indicated in B: 1, control; 2, initial depolarization; 3, repolarization; 4, transient depolarization; and 5, delayed hyperpolarization. D: current-voltage (I-V) curves obtained by ramp-depolarizing pulse at different phases of V\(_m\) change (see phases 1–5 in B). Because I-V curves at the control (1) and delayed hyperpolarization (5) phases are very similar, they are plotted separately (right) with expanded scales. Note that the I-V curve at phase 5 (shaded line) does not cross the abscissa. E: means of I-V curves obtained by digital subtraction of control I-V from that measured at delayed hyperpolarization (n = 6). F: V\(_m\) changes induced by substance P (0.2 \(\mu\)M, 20 s). G: effects of ACh (5 \(\mu\)M) on V\(_m\) in response to pretreatment with isoprenaline (5 \(\mu\)M). The V\(_m\) trace of recovery from delayed hyperpolarization was truncated.

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superfused at 10 ml/min at room temperature (25°C). The bath solution was either PBS solution or bicarbonate-buffered Ringer solution (BBS) that contained (in mM) 120 NaCl, 0.4 KH2PO4, 1.6 K2HPO4, 1 MgCl2, 25 NaHCO3, 1.3 CaCl2, and 5 D-glucose, gassed with 95% O2-5% CO2, pH 7.4. For whole cell recordings, the patch pipettes (tip resistance 2 MΩ) were filled with the following solution (in mM): 95 K-gluconate, 30 KCl, 1.2 NaH2PO4, 4.8 Na2HPO4, 0.4 CaCl2, 3 MgCl2, 1 EGTA, 5 D-glucose, and 3 ATP, pH 7.2, pCa 7. The Vm was recorded under the zero-current clamp mode of the whole cell patch-clamp condition. All the chemicals used were of the highest grade of purity available and were obtained from Sigma or RBI (Natick, MA).

An Ag-AgCl reference electrode was used, and the liquid junction voltage was nullified using the circuit of an amplifier. The pipette capacitance was not cancelled. pCLAMP software (v. 7.0) and Digi-data-1200A (both from Axon Instruments, Forster City, CA) were used for data acquisition and to apply the command pulses. The voltage and current data were low-pass filtered (5 kHz) and plotted using a thermal pen recorder (Graphtec, Yokohama, Japan). In parallel, the data were sampled at 10 kHz and stored in a computer for analysis using Clampex (v. 6.0; Axon Instruments) and Origin (v. 6.1; Microcal Software, Northampton, MA).

Measurement of intracellular ion concentrations. Isolated acini were loaded with the Ca2+-sensitive fluorescent indicator fura-2 (2 μM, 15 min), the Na+-binding SBFI (10 μM, 1 h), sodium green (5 μM, 1 h), the pH-sensitive indicator 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; 2 μM, 12 min), or 6-methoxy-N-(3-sulphopropyl)quinolinium (SPQ; 10 mM, 12 min) by incubation of the acini in the presence of the membrane-permeable acetoxymethyl ester forms of each probe (except SPQ) in PBS solution at room temperature, and then the unloaded indicators were washed out with fresh solution. The recordings of [Ca2+]c, [Na+]c, [H+]c (intracellular pH, pH), and [Cl−]c were performed with a microfluorometric system consisting of an inverted fluorescence microscope (Olympus IX-70) with a dry-type fluorescence objective lens (×40, NA 0.65), a photo multiplier tube (type R 1527; Photonics, Hamamatsu, Japan), and a Deltascan illuminator (Photon Technology International, Lawrenceville, NJ). Light was provided by a 75-W xenon lamp (Ushino, Tokyo, Japan). A chopper wheel alternated the light path to monochromators filtering specific wavelengths of light: 345 and 380 nm for fura-2 and SBFI, 345 nm for SPQ, 488 nm for sodium green, and 488 and 440 nm for BCECF. As a measure of [Ca2+]c and [Na+]c, the ratio of the fluorescence emissions at 345- and 380-nm excitation is presented. For pH, the ratio of fluorescence emissions at 488- and 440-nm excitation is presented.

NKCC activity. As previously reported in rat parotid acini (7), the initial rate of pH recovery from an NH4Cl-induced acute alkaline load was used as a measure of NKCC activity. In the control condition and after various experimental treatments, RSMGA were subjected to a 20 mM NH4Cl challenge by substituting 20 mM NaCl in the bath solution.

Fig. 2. Activation of Na+/K+-ATPases and ACh-induced delayed hyperpolarization (Vm,ACh). A: representative original trace of Vm. After the ACh-induced changes of Vm were confirmed, the RSMGA was pretreated with ouabain (0.5 mM, open horizontal bar), during which time ACh (5 μM, 20 s) was applied again. B: summary of Vm (means ± SE, n = 7) measured at different phases (1–5) marked in A. Vm,ACS after washout of ouabain (5) was not different from that of the control (2) (P = 0.14, n = 7). C: effects of charybdotoxin (ChTx, 50 nM) on ACh-induced changes in Vm. D: summary of Vm (n = 3) measured at different phases (1–4) marked in C. Note that the Vm,ACS persists in response to pretreatment with ChTx, whereas the initial repolarization was largely abolished (downward arrowhead in C).
perfusion, during which time the acini were rapidly alkalinized and then recovered toward their resting pH or below. The initial part (0–30 s) of this recovery phase was linearly fitted using the program Origin 6.0. In the experiment with BCECF fluorescence measurement, 10 mM HEPES was added to both PBS and BBS (pH 7.4).

Data presentation. The data are presented as original recordings, current-voltage relationships (I-V curves), and bar graphs showing means ± SE (for n acini). Student’s t-test for paired or unpaired samples was applied, and the probability values (P) <0.05 were regarded as significant.

RESULTS

Effects of ACh on V_m and intracellular ion concentration. The overall changes in [Ca^{2+}]_c, [Cl^-]_c, and [Na^+]_c induced by the muscarinic stimulation of RSMGA were monitored using fura-2, SPQ, and SBFI, respectively (Fig. 1A). Because of the overlapped wavelengths for the excitation of the ion-sensitive dyes used, each of the measurements was performed in a different acinus. For reliable comparison of the ACh-induced changes in ion concentrations, the measurements were made on the same experimental date, and care was taken to keep the same flow rate for the bath perfusion with PBS and the same duration for applying ACh (20 s). Figure 1A demonstrates the representative results when an application of ACh (5 μM, 20 s) evoked a fast increase in [Ca^{2+}]_c, which was followed by a decrease in [Cl^-]_c and an increase in [Na^+]_c. After the washout of ACh, the recovery of [Ca^{2+}]_c was faster than the recoveries of [Cl^-]_c and [Na^+]_c.

In the same bath-flow conditions, the application of ACh induced dramatic changes in V_m, which were composed of an initial depolarization, a repolarization, a transient depolarization, and, finally, a strong delayed hyperpolarization (V_h,ACh), which took some 3–4 min to reach steady-state recovery (Fig. 1B). The mean values of V_m in each phase are summarized in Fig. 1C. Notably, the mean of V_h,ACh was increased by 10.220.33.1 on March 21, 2017 http://ajpgi.physiology.org/ Downloaded from

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Fig. 1. Effects of ACh on V_m changes. The recording of V_m was performed in either phosphate-buffered Ringer solution (PBS; A and C) or bicarbonate-buffered Ringer solution (BBS; B and D). A: representative original trace of V_m recorded in PBS (HCO_3^- free). After ACh-induced changes of V_m were confirmed, the RSMGA was pretreated with bumetanide (50 μM, open horizontal bar), during which time ACh (5 μM, 20 s) was applied again. B: representative original trace of V_m recorded in BBS (HCO_3^-/CO_2). The protocol for drug application was the same as that in A. C: summary of V_m (means ± SE, n = 16) measured at different phases (1–4) marked in A. The V_h,ACh was blocked by bumetanide in PBS (P < 0.01). D: summary of V_m (n = 3) measured at different phases (1–4) marked in B. The V_h,ACh was not affected by bumetanide in BBS (P = 0.22).
−95 ± 1.8 mV (n = 60), and this was more negative than the equilibrium potential for the potassium ion. The recorded RSMGA were intermittently voltage clamped, and ramplike depolarizations (from −90 to 80 mV) were applied to obtain a brief I-V curve. The I-V curves show a huge increase in membrane conductance during the initial transient changes in \( V_m \) (Fig. 1D). In contrast, the I-V curve obtained at the maximal peak of \( V_{h, ACh} \) is similar to the control I-V curve. However, upon closer observation, the I-V curve obtained during the \( V_{h, ACh} \) is shifted slightly upward (Fig. 1D, right), and the digitally subtracted I-V curve (i.e., the difference between the control and \( V_{h, ACh} \) I-V curves) shows a standing outward current that did not reverse its polarity (Fig. 1E). Such a physiological property is in accordance with the voltage-independent activity of the electrogenic \( Na^+ / K^+ -ATPase \) (Na\(^+\) pump).

This phenomenon of delayed hyperpolarization was induced not only by ACh but also by substance P (0.2 \( \mu \)M; Fig. 1F), for which the maximum delayed hyperpolarization was −95 ± 2.4 mV (n = 3). In contrast, the application of isoprenaline, a \( \beta \)-adrenoceptor agonist, did not induce the delayed hyperpolarization but only induced a sustained depolarization from −55 ± 3.8 mV to −44 ± 4.7 mV (P < 0.05, n = 5; Fig. 1G). Despite the depolarizing action, the pretreatment with isoprenaline did not affect the pattern of \( V_m \) changes evoked by ACh, and the \( V_{h, ACh} \) in the presence of isoprenaline was −92.5 ± 0.5 mV (n = 2, Fig. 1G).

In the presence of ouabain, the \( V_{h, ACh} \) was almost completely blocked, and it appeared only after the washout of ouabain (Fig. 2). The prominent depolarization by the treatment with ouabain alone indicated the substantial contribution from the electrogenic \( Na^+ / K^+ -ATPase \) to the resting membrane potential of RSMGA. Although the reason is not yet clear, the level of the initial repolarization upon muscarinic stimulation became more negative in the presence of ouabain (P < 0.01, n = 12; Fig. 2A, compare downward arrows). In contrast to the effects of ouabain, the blockade of \( K^+ \) channels by pretreatment with charybdotoxin (50 nM) did not block the \( V_{h, ACh} \), whereas the initial repolarization was largely abolished (Fig. 2, C and D).

![Fig. 4. Effects of ethylisopropylamiloride (EIPA) on ACh-induced \( V_m \) changes.](http://ajpgi.physiology.org/)

A: representative original trace of \( V_m \) recorded in PBS. After ACh-induced changes of \( V_m \) were confirmed, the RSMGA was pretreated with EIPA (50 \( \mu \)M, open horizontal bar), during which time ACh (5 \( \mu \)M, 20 s) was applied again. B: representative original trace of \( V_m \) recorded in BBS. The protocol for drug application was the same as that in A. C: summary of \( V_m \) (means ± SE, n = 4) measured at different phases (1–4) marked in A. The \( V_{h, ACh} \) was not affected by EIPA in PBS (P = 0.90). D: summary of \( V_m \) (n = 4) measured at different phases (1–4) marked in B. The \( V_{h, ACh} \) was decreased by EIPA in BBS (P = 0.038).
Because the $V_{h, ACh}$ was preceded by an increase in $[Na^+]_c$, a representative condition stimulating Na$^+$/K$^+$-ATPases (Fig. 1), we wondered whether the inhibition of Na$^+$ influx could also block the $V_{h, ACh}$. In this study, NKCC and NHE were regarded as the main pathways of Na$^+$ uptake into the RSMGA. Because the relative contribution of the NHE over the NKCC could be changed depending on the pH buffering system, the following experiments were performed in both the absence (using PBS) and presence of 24 meq HCO$_3^-$/5% CO$_2$ (using BBS) in the bath perfusate.

Effects of inhibitors of NKCC and NHE on $V_{h, ACh}$ and ACh-induced Δ$[Na^+]_c$. In PBS, the application of bumetanide (50 μM) alone consistently depolarized the $V_m$ of RSMGA by 21 ± 2.9 mV ($n = 16$). In the presence of bumetanide, the $V_{h, ACh}$ was almost completely abolished, and it reappeared after the washout of bumetanide (Fig. 3, A and C). In BBS, however, the pretreatment with bumetanide had no significant effect on the $V_{h, ACh}$, indicating that another Na$^+$-transporting mechanism (e.g., NHE) was recruited (Fig. 3, B and D). In a manner opposite to the effects of bumetanide, pretreatment with the NHE inhibitor EIPA (15 μM) did not affect the $V_{h, ACh}$ in PBS (Fig. 4, A and C). In contrast, under perfusion with BBS, the extent of $V_{h, ACh}$ was significantly reduced by pretreatment with EIPA (Fig. 4, B and D).

These results suggested that the predominant pathway of ACh-induced Na$^+$ influx switches from NKCC to NHE under HCO$_3^-$-buffered conditions. This assumption was examined more directly by using the Na$^+$-sensitive fluorescent dyes SBFI and sodium green. In RSMGA, repetitive application of ACh induced a reversible and consistent increase in the ratio of SBFI fluorescence ($\Delta F_{SBFI, ACh}$) or in the fluorescence intensity of sodium green ($\Delta F_{SG, ACh}$), reflecting the ACh-induced increase in Na$^+$ concentration ($\Delta [Na^+]_{c, ACh}$; Fig. 5A). Application of ouabain completely blocked the recovery of $\Delta [Na^+]_{c, ACh}$ (Fig. 5B). To compare the $\Delta [Na^+]_{c, ACh}$ with the results of $V_m$ recording, we applied the same protocols of drug application. In PBS, pretreatment with bumetanide suppressed the $\Delta F_{SBFI, ACh}$, to 32.5 ± 12.5% ($n = 8$) of the control response, whereas pretreatment with EIPA had no significant effect on the $\Delta F_{SG, ACh}$ ($n = 3$; Fig. 5C). In BBS, pretreatment with bumetanide or EIPA decreased the $\Delta F_{SBFI, ACh}$ and $\Delta F_{SG, ACh}$ to 56.4 ± 5.7% ($n = 10$) or 47.7 ± 3.8% ($n = 6$) of the control response, respectively (Fig. 5, C and D).

![Fig. 5. ACh-induced changes in $[Na^+]_c$, and the effects of bumetanide and EIPA. A: application of ACh (5 μM, 20 s) increased $F_{SBFI, 340/380}$ and the fluorescence intensity of sodium green ($F_{SG, ACh}$), and both responses were reversed by washout of ACh. B: recovery of ACh-induced increase in $F_{SBFI, 340/380}$ was completely blocked by pretreatment with ouabain (0.5 mM). C and D: summary of the effects of bumetanide and EIPA on ACh-induced changes in $[Na^+]_c$ measured by SBFI ($\Delta F_{SBFI, ACh}$) or sodium green ($\Delta F_{SG, ACh}$) in PBS (C) or BBS (D). In each case, ACh was applied twice, and the second response to ACh was obtained after pretreatment with bumetanide (50 μM) or EIPA (10 μM). Amplitudes of the second $\Delta F_{SBFI, ACh}$ and $\Delta F_{SG, ACh}$ measurement were normalized to the initial control changes of $\Delta F_{SBFI, ACh}$ and $\Delta F_{SG, ACh}$, respectively. The inhibition of Na$^+$ influx by bumetanide was not different from the effect of EIPA in BBS. *P < 0.05, difference between 2 groups (unpaired Student’s t-test).]
ACh-induced acidification and activation of NKCC. In the next experiment, the activity of NHE was monitored by recording the EIPA-induced changes in pHi. In BBS, the application of ACh induced a sharp decrease in the BCFE fluorescence ratio, indicating cytosolic acidification (ΔpHi,ACh). The ΔpHi,ACh recovered slowly during the application of ACh. Pretreatment with EIPA (10 μM) markedly increased the ΔpHi,ACh and the recovery was blocked (Fig. 6, A and B). Similarly, under sustained treatment with ACh in BBS, the addition of EIPA strongly acidified the RSMGA (Fig. 6C). In PBS, the application of ACh first induced a transient acidification and then a rebound alkalinization above the resting pHi. Addition of EIPA reversed the alkalinized pHi, but no significant acidification was observed (Fig. 6C).

Next, the activity of NKCC was measured from the BCFE fluorescence ratio (see MATERIALS AND METHODS). In PBS, the pHi decay during the NH4+ pulse period was greatly accelerated by a transient application of ACh (Fig. 7, A and C). The acceleration of the pHi decay rate by ACh was mostly blocked by pretreatment with bumetanide (100 μM), confirming the involvement of NKCC. In contrast, when in BBS, the bumetanide-sensitive acceleration of pHi decay was negligible compared with the responses obtained in PBS (Fig. 7, B and D).

Considering a crucial role of [Cl\(^-\)]c in the regulation of NKCC activity (10, 26), we tested whether the decrease in the [Cl\(^-\)]c of RSMGA by ACh is affected by the presence of 24 meq HCO3-. As already shown in Fig. 1A, the stimulation of RSMGA with ACh increased the fluorescence of SPQ (ΔFSPQ) loaded into the cell, indicating a net decrease in [Cl\(^-\)]c. Compared with the control response in PBS, the ACh-induced ΔFSPQ was not significantly changed in BBS (P = 0.54, n = 6; Fig. 8, A and B).

The more prominent ΔpHi,ACh in BBS (Fig. 6) suggested that the acidification by ACh might somehow inhibit the activation of NKCC in BBS condition. To test this possibility, we applied 20 mM Na-acetate along with ACh in the PBS condition, which induced a transient acidification similar to the ΔpHi,ACh in BBS. However, the facilitation of NKCC activity was not affected by the preceding acidification (P = 0.98, n = 6; Fig. 8, C and D).

**DISCUSSION**

In the present study, ACh-induced changes in Vm and the cytosolic concentrations of major ions were measured in an attempt to obtain a comprehensive understanding of electrolyte secretion in salivary acini. An interesting feature of RSMGA is that the increase in [Na\(^+\)]c due to the activation of Na\(^+\)-coupled transporters is effectively reflected as Vh,ACh via stimulation of Na\(^+\)-pump activity. Also, the presence of HCO3- greatly affects the mode of Na\(^+\)-coupled ion transporters recruited in the process of electrolyte secretion in salivary glands (RSMGA). In particular, the facilitation of NKCC by ACh is strongly suppressed in the presence of physiological HCO3-.

The Vm of RSMGA consistently showed discrete phases of response to a transient application of ACh: initial depolarization, repolarization, slow depolarization, and delayed hyperpolarization (Vh,ACh). From previous reports describing the Ca\(^2+\)-dependent activation of Cl\(^-\) and K\(^+\) channels in salivary gland acini, it could be easily deduced that the initial depolarization and repolarization are caused by sequential activation of the luminal Cl\(^-\) channels and basolateral K\(^+\) channels (2, 17, 30). Although the molecular nature of the Ca\(^2+\)-activated Cl\(^-\) channel is still unclear, there are two types of Ca\(^2+\)-activated K\(^+\) channels, namely, the “intermediate” and “maxi-K” classes, in salivary acinar cells (18). Compared with the other phases of the ACh-induced Vm changes, the nature of the third phase (slow depolarization) is still unclear. We can speculate that the underlying mechanism of the slow depolarization is that the Na\(^+\)-coupled influx of Cl\(^-\) would increase the [Cl\(^-\)]c and [Na\(^+\)]c, which simultaneously shift the equilibrium potentials for Cl\(^-\) and Na\(^+\) toward a depolarizing direction.

Both the sensitive blockade by ouabain and the standing outward nature of the I-V curve indicates that the Vh,ACh is caused by the increased Na\(^+\)/K\(^+\)-ATPase activity. A delayed hyperpolarization after an ACh application has been initially reported in rat parotid gland by investigators using conventional intracellular microelectrode techniques (25). In comparison, the Vh,ACh of RSMGA is much stronger than the delayed hyperpolarization in the rat parotid acini.

The effects of bumetanide and EIPA on Vh,ACh and Δ[Na\(^+\)]c,ACh in PBS clearly indicate that an increase in [Na\(^+\)]c is the critical factor of Vh,ACh, namely, the Na\(^+\)/K\(^+\)-ATPase activation. The dependence of Na\(^+\)/K\(^+\)-ATPase activity on the [Na\(^+\)]c seems to be saturated above a certain level. For exam-

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**Fig. 6.** ACh-induced changes in intracellular pH (pHi) and the effects of EIPA. A: the fluorescence ratio of 2’,7’-bis(2-carboxethyl)-5(6)-carboxyfluorescein (FBCF,488/440) was measured in BBS. ACh (5 μM) was applied in either the absence (solid line) or presence of EIPA (10 μM, dotted line). B: summary of maximum changes in FBCF,488/440 induced by ACh (n = 6), *P < 0.01, significant difference. C: effects of sustained stimulation with ACh (5 μM) and EIPA (10 μM) on pHi of RSMGA. Representative traces of FBCF,488/440 obtained in PBS (HCO3- free) or BBS (HCO3-/CO2) are plotted. Time break indicated represents 30 s.
ple, in BBS, the application of bumetanide, which inhibits the \( [\text{Na}^+]_c,\text{ACh} \) by \( \sim 40\% \), had no significant effect on \( V_{\text{h,ACh}} \) (compare Figs. 3 and 5). The general understanding of the mechanism of \( [\text{Na}^+]_c,\text{ACh} \) in salivary gland is that the paired exchangers (NHE and anion exchanger, AE) and NKCC act in parallel to provide a \( \text{Cl}^- \) entry pathway that is accompanied by \( \text{Na}^+ \) influx across the basolateral membrane (17, 30, 31). In addition to NKCC and NHE, \( \text{Na}^+ \)-bicarbonate cotransporters (NBC) are operating in some exocrine glands (30). In RSMGA, however, the functional contribution of the NBC seemed negligible, because the \( p\text{Hi} \) recovery after acidification was largely blocked by EIPA. The absence of a significant contribution by NBC also has been demonstrated in mouse sublingual gland acini (11, 19).

In an early study of rat submandibular gland, it was estimated that the fluxes related to NHE are responsible for \( \sim 25\% \) of the total fluid secretion induced by ACh, with the remaining \( 75\% \) arising from NKCC (23). In rat parotid acinar cells, Robertson and Foskett (26) reported that a decrease in \( [\text{Cl}^-]_c \) itself was required to activate \( \text{Na}^+ \) entry pathways wherein \( \sim 30\% \) of the carbachol-induced \( \text{Na}^+ \) influx was sensitive to bumetanide (NKCC activity). Thus, according to these reports, the relative contribution of NKCC and NHE to the total \( \text{Na}^+ \) influx in salivary acini was found to be quite variable, which might have been due to different pH buffering conditions as well as the different types of tissues tested. In our present study, we have demonstrated that the presence of \( \text{HCO}_3^- \) greatly affects the mode of recruiting \( \text{Na}^+ \)-coupled ion transporters. In particular, the facilitation of NKCC by ACh was suppressed in the presence of physiological \( \text{HCO}_3^- \).

The NKCCs play a vital role in salt and water movement across a number of secretory and absorptive epithelia (10). Experimental results from NKCC1-deficient mice have demonstrated that NKCC1 is the major \( \text{Cl}^- \) uptake mechanism that is critical for the saliva secretion (6). The activity of the rat parotid NKCC is increased by a variety of physiological stimuli (7, 8, 22, 28), which would serve to increase the transepithelial \( \text{Cl}^- \) transport during stimulation. In general, a decrease in \( [\text{Cl}^-]_c \) or decreased cell volume has been suggested to mediate the phosphorylation and activation of NKCC1 by the secretagogue (10). More specifically, a proline-alanine-rich STE20-related kinase (PASK) was recently demonstrated as a
linker between low [Cl\(^{-}\)], and phosphorylation of the regulatory NH\(_2\) terminus of NKCC1 (5). However, in rat parotid acini, it was reported that the Ca\(^{2+}\)-mediated upregulation of NKCC is mediated by the products of cytochrome P-450, and not by decreased Cl\(^{-}\} activity (7). Unfortunately, our present study does not provide direct evidence to explain the secretagogue-induced activation of NKCC.

Although saliva secretion in rat submandibular gland can be observed in the absence of HCO\(_3^{-}\) (15), the strong acidification of RSMGA by ACh in the bicarbonate-buffered condition supports the model that a significant net efflux of HCO\(_3^{-}\) arises via anion channels activated by Ca\(^{2+}\) under physiological conditions (30). According to this model, the loss of HCO\(_3^{-}\) should be compensated by the operation of carbonic anhydrase, and the released H\(^{+}\) ions are eliminated from the cytosol by using NHE. In BBS, the influx of Na\(^{+}\) via NHE and the subsequent increase in [Na\(^{+}\)], is likely to dissipate, at least partially, the driving force for the operation of NKCC. Such speculation is in accordance with the insignificant effects of bumetanide on \(V_{\text{Na,LACH}}\) under the bicarbonate-buffered condition. Also, a reduced driving force for NKCC by the parallel operation of NHE might explain the reduced facilitation of NKCC in BBS. However, in our experimental protocol, the measurement of NKCC activity was performed 60–90 s after the transient (20 s) application of ACh, during which time the major portion of the \(\Delta[\text{Na}^{+}]_{\text{C,ACH}}\) was reversed to the initial control level. In addition, the ACh-induced decrease in [Cl\(^{-}\}], a key intracellular signal for NKCC activation (10), was not significantly different in the absence and presence of HCO\(_3^{-}\). Also, an artificially induced acidification did not block the facilitation of NKCC (Fig. 8, C and D). Taking these results together, it could be argued that the physiological level of HCO\(_3^{-}\) itself might be the experimental condition primarily responsible for affecting the regulation of NKCC.

Not only NKCC but also the NHE activity is regulated by various stimuli, including the secretory hormones (3, 12, 24, 27), and the activation of NHE by muscarinic stimulation has been demonstrated in rat parotid gland (13, 16). In our present study, the slow increase of pH\(_{i}\) above its resting level by a sustained stimulation with ACh (Fig. 6C) might indicate that a similar direct activation of NHE occurs in RSMGA. However, the role of NHE as the Na\(^{+}\) influx pathway appears to be negligible in PBS, and this is most likely because the agonist-induced acidification is weak. In addition to the uptake of NaCl by a paired operation with AE, the more crucial role of NHE is suggested as preventing the acidification of pH\(_{i}\) resulting from the influx of HCO\(_3^{-}\) (21, 30). Because the dependence of NHE activity on pH\(_{i}\) is quite steep near the physiological level of pH\(_{i}\) (1), the greater role of NHE in the presence of bicarbonate is highly suspected. In the RSMGA superfused with BBS, the extent of ACh-induced acidification was greatly amplified by EIPA treatment, indicating that the immediate compensation by NHE largely prevents the strong acidification under the muscarinic stimulation (Fig. 6).

In summary, the phenomenon of \(V_{\text{Na,LACH}}\) in RSMGA enabled us to monitor the facilitation of Na\(^{+}\)-coupled ion transporters by ACh. Both the electrical measurements and the signals from fluorescent ion-sensitive dyes indicated that the relative contribution of NKCC and NHE changes significantly depending on the presence of physiological HCO\(_3^{-}\). The NKCCs in RSMGA are sensitively activated by a prior transient stimulation with ACh in PBS but not in BBS. In BBS, the facilitation of NHE by an intracellular acidification accounts for the major part of Na\(^{+}\) influx after muscarinic stimulation. Although the precise mechanism is still unclear, the suppression of NKCC facilitation and the activation of NHE might be beneficial for the maximum operation of NHE to attenuate the strong acidification.

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**Fig. 8.** ACh-induced decrease in [Cl\(^{-}\)]\(_{c}\) and intracellular acidification do not explain the different regulation of NKCC in PBS and BBS. **A:** original trace of FSPQ. Repetitive applications of ACh (5 \(\mu\)M, 20 s) induced transient increases in FSPQ, indicating a net decrease in [Cl\(^{-}\)]. In the middle of the experiment, the bath perfusate was changed from PBS (HCO\(_3^{-}\) free) to BBS (HCO\(_3^{-}\)/CO\(_2\)). **B:** summary of ACh-induced changes in FSPQ (\(\Delta F_{\text{SPQ}}\), \(n = 6\)). The \(\Delta F_{\text{SPQ}}\) measured in BBS (HCO\(_3^{-}\)/CO\(_2\)) was normalized to that measured in PBS (HCO\(_3^{-}\) free). C: during measurement of the pH\(_{i}\) in PBS, the application of ACh was accompanied by 20 mM Na-acetate. After washout of both ACh and Na-acetate, the application of NH\(_4\)Cl (20 mM) induced a transient alkalinization, followed by a sharp acidification. **D:** decay slopes of FBCECF-488/440 (\(\Delta R_{\text{atio}}/s\)) was measured, and the summarized results (filled bar) are compared with the controls measured without Na-acetate pulse (open bars). Note that the open bars are replicates of Fig. 7C.
fication by HCO₃⁻ efflux and to support the generation of HCO₃⁻ by carbonic anhydrases.

GRANTS

This research was supported by Korea Science & Engineering Foundation Grant R01-2001-000-00208-0.

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