J. Membrane Biol. 129, 311–321 (1992)

The Journal of
Membrane Biology

Regulation of Cytoplasmic pH in Rat Sublingual Mucous Acini at Rest and During Muscarinic Stimulation

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Summary. The regulation of intracellular pH (pH_i) in rat sublingual mucous acini was monitored using dual-wavelength microfluorometry of the pH-sensitive dye BCECF (2',7'-biscarboxyethyl-5(6)-carboxyfluorescein). Acini attached to coverslips and continuously superfused with HCO_3^--containing medium (25 mM $NaHCO_3$/5% CO_2; pH 7.4) have a steady-state pH_i of 7.25 ± 0.02. Acid loading of acinar cells using the NH_4^+/NH_3 prepulse technique resulted in a Na^+-dependent, MIBA-inhibitable (5-(N-methyl-N-isobutyl) amiloride, $K_i \sim 0.42$ μM) pH_i recovery, the kinetics of which were not influenced by the absence of extracellular Cl^-. The rate and magnitude of the pH_i recovery were dependent on the extracellular Na^+ concentration, indicating that Na^+/H^+ exchange plays a critical role in maintaining pH_i above the pH predicted for electrochemical equilibrium. When the NH_4^+/NH_3 concentration was varied, the rate of pH_i recovery was enhanced as the extent of the intracellular acidification increased, demonstrating that the activity of the Na^+/H^+ exchanger is regulated by the concentration of intracellular protons. Switching BCECF-loaded acini to a Cl^--free medium did not significantly alter resting pH_i, suggesting the absence of Cl^-/HCO_3^- exchange activity. Muscarinic stimulation resulted in a rapid and sustained cytosolic acidification ($t_{1/2} < 30$ sec; 0.16 ± 0.02 pH unit), the magnitude of which was amplified greater than two-fold in the presence of MIBA (0.37 ± 0.05 pH unit) or in the absence of extracellular Na^+ (0.34 ± 0.03 pH unit). The agonist-induced intracellular acidification was blunted in HCO_3^--free media and was inhibited by DPC (diphenylamine-2-carboxylate), an anion channel blocker. In contrast, the acidification was not influenced by removal of extracellular Cl^-. The Ca^{2+} ionophore, ionomycin, mimicked the effects of stimulation, whereas preloading acini with BAPTA (bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid) to chelate intracellular Ca^{2+} blocked the agonist-induced cytoplasmic acidification. The above results indicate that during muscarinic stimulation an intracellular acidification occurs which: (i) is partially buffered by increased Na^+/H^+ exchange activity; (ii) is most likely mediated by HCO_3^- efflux via an anion channel; and (iii) requires an increase in cytosolic free $[Ca^{2+}]$.

Key Words Na^+/H^+ exchange · salivary gland · muscarinic stimulation · anion channel · fluid secretion

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Introduction

The regulation of intracellular pH (pH_i) is critical for many normal cell functions such as fluid and electrolyte absorption and secretion, enzyme activity, and cell growth and proliferation (Aronson, 1985; Boron, 1986; Moolenaar, 1986). Current evidence suggests that several ion transport pathways are involved in pH_i regulation, including Cl^-/HCO_3^- exchangers and Cl^--independent Na^+/H^+ exchangers (Sardet, Franchi & Pouyssegur, 1989; Kopito, 1990), as well as Cl^--dependent Na^+/H^+ exchange and Na^+/HCO_3^- cotransport mechanisms, which appear to have a more limited distribution (Boyarsky, et al., 1988; Reinertsen et al., 1988; Geibel, Giebisch & Boron, 1990; Hart & Nord, 1991). Several recent reports indicate that salivary glands possess Cl^-/HCO_3^- and Na^+/H^+ exchangers that act in parallel with $Na^+/K^+/2Cl^-$ cotransport to increase the intracellular $[Cl^-]$ above its electrochemical gradient (Novak & Young, 1986; Turner & George, 1988). The current fluid-secretion model, first proposed for the shark rectal gland (Silva et al., 1977), predicts that the transepithelial movement of Cl^- is the driving force for fluid and electrolyte secretion. During stimulated fluid secretion an increase in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) activates anion channels located in the apical membrane, resulting in the efflux of Cl^- down its electrochemical gradient into the lumen (Iwatsuki et al., 1985; Martinez & Cassity, 1986; Nauntofte & Poulsen, 1986; Ambudkar, Melvin & Baum, 1988; Cook et al., 1988). Stimulated Cl^- efflux is accompanied by an initial transient acidification due to HCO_3^- efflux, presumably mediated by the same apical anion channel (Melvin, Moran & Turner, 1988; Lau, Elliot & Brown, 1989). Activation of the Na^+/H^+ exchanger located in the basolateral membrane rapidly buffers the intracellular acidification by extruding protons in exchange for

extracellular Na^+ (Melvin et al., 1988; Lau et al., 1989; Soltoff et al., 1989; Stewart, Seo & Case, 1989). Initial changes in acinar cell volume (Foskett et al., 1989; Foskett & Melvin, 1989) may be involved in the rapid activation of a Na^+/H^+ exchanger (Manganel & Turner, 1991). During prolonged stimulation, Na^+/H^+ exchange activity is enhanced by a Ca^{2+}-dependent process, apparently by increasing the transport capacity and by shifting the pH responsiveness of the Na^+/H^+ exchanger to a more alkaline pH (Manganel & Turner, 1989, 1990). Activation of the Na^+/H^+ exchanger results in an intracellular alkalinization of ~0.1 pH unit above resting pH_i (Pirani et al., 1987; Melvin et al., 1988; Soltoff et al., 1989; Stewart et al., 1989). As the pH_i rises, the activity of the Cl^-/HCO_3^- exchanger increases, thereby accelerating the uptake of interstitial Cl^- in exchange for intracellular HCO_3^- (Melvin et al., 1988; Melvin & Turner, 1992). In salivary glands, paired exchangers therefore participate in maintaining the driving force for anion-dependent fluid secretion as well as regulating pH_i.

The above studies of pH_i regulation in exocrine glands have focused on serous glands. It is unknown if the same transport mechanisms are functionally important in the sublingual mucous gland. We recently observed that sublingual mucous acini, in regard to its net ion movements, initially responds in a similar fashion to other salivary glands during stimulation. The rate and magnitude of the initial net efflux of Cl^- and of K^+ (Melvin, Koek & Zhang, 1991) are quantitatively comparable to the Cl^- loss (Martinez & Cassity, 1986; Nauntofte & Poulsen, 1986; Melvin et al., 1987; Ambudkar et al., 1988) and K^+ loss (Nauntofte & Dissing, 1988; Melvin & Turner, 1992) seen in the other major salivary glands. This dramatic loss of KCl is followed by a net re-uptake of K^+ and Cl^- within 5 min in parotid and submandibular glands (Martinez & Cassity, 1986; Nauntofte & Poulsen, 1986; Melvin et al., 1987; Melvin & Turner, 1992). In contrast, no re-uptake of K^+ or Cl^- is observed in sublingual acini during prolonged stimulation (Melvin et al., 1991). Cl^- re-uptake can be induced in sublingual acini when Ca^{2+} entry is prevented by stimulating in a Ca^{2+}-free medium. As $[Ca^{2+}]_i$ is depleted, fluid secretion ceases (Melvin et al., 1991). Under these conditions, Cl^- re-uptake is totally blocked by bumetanide, a specific $Na^+/K^+/2Cl^-$ cotransport inhibitor, whereas, DIDS, an inhibitor of Cl^-/HCO_3^- exchange, had no effect (Melvin et al., 1991). These results suggest the absence of physiologically significant levels of Cl^-/HCO_3^- exchange activity in rat sublingual acini. Re-uptake of Cl^- via the $Na^+/K^+/2Cl^-$ cotransporter is driven by the inward-directed Na^+ chemical gradient. The rate and the magnitude of the agonist-induced increase in Na^+ content of sublingual acini (Melvin et al., 1991) is less than the response observed in parotid acini (Dissing & Nauntofte, 1990).

It is unclear whether the distinct functional characteristics of sublingual mucous acinar cells are due to fundamental differences in the types and/or numbers of ion transporters present or to differences in regulation (Melvin et al., 1991). Since cytoplasmic pH_i has been suggested to be important for driving anion-dependent fluid secretion (*see* Turner, 1992), the present study was conducted in an attempt to identify the ion transport mechanism(s) involved in maintaining steady-state pH_i and for regulating pH_i during stimulation. Our results reveal that sublingual mucous acini possess a Cl^--independent Na^+/H^+ exchanger, the activity of which increases when the pH_i is acidified. In comparison with other exocrine glands, the sublingual mucous gland has little or no Cl^-/HCO_3^- exchange activity. We also found that during muscarinic stimulation, a sustained and dramatic acidification occurs which is dependent on an increase in $[Ca^{2+}]_i$. This agonist-induced acidification is independent of extracellular Cl^- and is DPC inhibitable, suggesting the involvement of an anion channel.

Materials and Methods

Materials

Male, 150–250 g Wistar strain rats (Charles River, Kingston facility, NY) were used in all experiments. BCECF (2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein), BAPTA (bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid), and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) were from Molecular Probes (Eugene, OR). MIBA (5-(N-Methyl-N-isobutyl) amiloride) was synthesized as previously described (Cragoe et al., 1967). Nigericin, hyaluronidase (type 1S), atropine sulfate, HEPES (4-(2-hydroxyethyl)-1-piperazine ethansulfonate), BSA (type V; bovine serum albumin), and carbachol were obtained from Sigma (St. Louis, MO). Collagenase (type CLSPA) was from Worthington Biochemical (Freehold, NJ). BME (basal medium Eagle) was from Gibco (Grand Island, NY). Methazolamide was a gift from American Cyanamid, (Pearl River, NY). DPC (diphenylamine-2-carboxylate) was from Fluka (Switzerland). All other chemicals used were of the highest grade available.

The digestion medium consisted of BME medium, 1% BSA, 50 U/ml collagenase and 0.02 mg/ml hyaluronidase. Unless otherwise stated the experiments were carried out in a Modified Earle's-Hank's solution (MEH) containing (in mM): 110 NaCl, 25 $NaHCO_3$, 20 HEPES, 10 glucose, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, and 0.33 NaH_2PO_4 adjusted to pH 7.4 at 37°C with NaOH after bubbling with 95% O_2/5% CO_2. In Cl^--free MEH, NaCl, $CaCl_2$ and KCl were replaced with the appropriate gluconate salts. The calcium concentration was increased to 5.87 mM with Ca^{2+}-gluconate to compensate for Ca^{2+} chelation by gluconate (Star, Burg & Knepper, 1985). In HCO_3^--free MEH, $NaHCO_3$

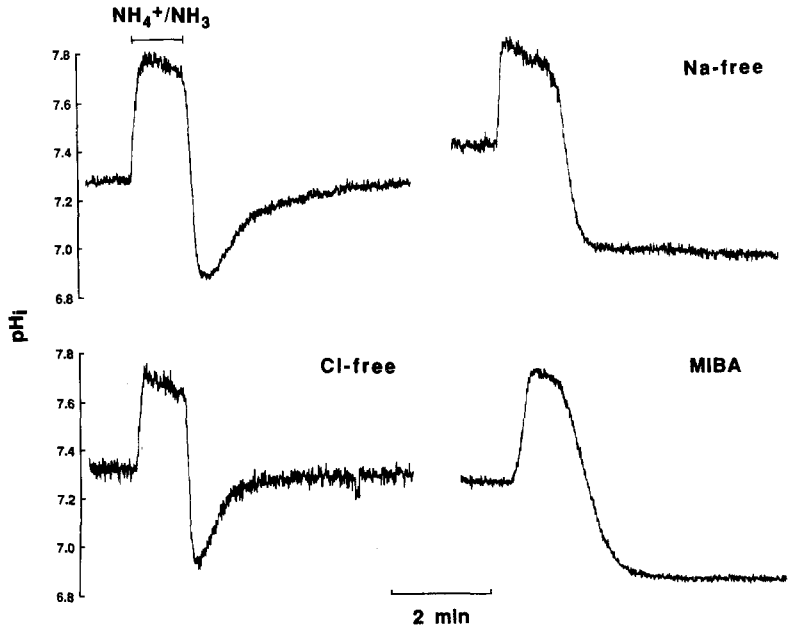

Fig. 1. The effects of Na^+-free or Cl^--free media and MIBA on the pH_i recovery from an acute acid load in rat sublingual mucous acini. An acid load was induced in BCECF-loaded cells by using a 60-sec 60-mM NH_4^+/NH_3 prepulse technique (*see* Materials and Methods). In the MIBA studies, 25 μM of the inhibitor was present during the entire trace. See Materials and Methods for the composition of the Na^+- and Cl^--free solutions. Each trace is a representative response (control, n = 13; Na-free, n = 11; Cl-free, n = 5; MIBA, n = 4).

was replaced with sodium gluconate, and the $CaCl_2$ concentration was increased to 2.2 mM. For the Na^+-free MEH solution, Na^+ salts were replaced with N-methyl-D-glucamine. In experiments in which cells were treated with NH_4Cl or $(NH_4)_2SO_4$, NaCl was replaced with equimolar concentrations of the appropriate compound.

Isolation of Sublingual Acini

After CO_2 anesthesia, rats were killed by exsanguination and the glands treated as previously described (Melvin et al, 1991). Briefly, the sublingual glands were removed and injected with ice-cold digestion medium (0.2 ml/gland). Glands were dissected free of connective tissue and minced in ice-cold digestion medium. The minced glands were incubated at 37°C with continuous gassing (95% O_2/5% CO_2) and shaking. The incubated glands were dispersed by gently pipetting 10 times with a 10-ml pipette at 15-min intervals. After 45 min of digestion, the medium was changed by centrifuging (400 × g) for 15 sec and replacing the supernatant with fresh digestion medium. After digesting the gland mince for a total of 1.5 hr, the well-dispersed sublingual acini were washed three times with MEH containing 0.01% BSA at room temperature. The resulting acini were separated into aliquots consisting of small (3–15 acini/clump) and large aggregates by centrifugation at 400 × g for 15 sec. The supernatant was recentrifuged at 400 × g for 1 min to obtain the smaller aggregates which were used for the pH_i determinations.

Intracellular pH Measurement

Isolated sublingual acini were loaded with BCECF/AM (2 μM) at room temperature for 30 min as previously described (Melvin et al., 1988). Stock solutions of 2 mM BCECF/AM were made in dimethyl sulfoxide (DMSO) so that the final concentration of DMSO during loading was 0.1%. After loading, the acini were washed and centrifuged at 400 × g for 1 min and then resuspended in MEH containing 0.01% BSA. The BCECF-loaded acini were continuously agitated and bubbled with 95% O_2/5% CO_2 at room temperature until use.

An aliquot of BCECF-loaded acini was centrifuged at 400 × g for 15 sec and the supernatant decanted. Acini were resuspended in BSA-free MEH to promote attachment (Foskett et al., 1989) and added to a coverslip mounted to the bottom of a perfusion chamber. Using this method, the acini could be superfused sequentially with a series of solutions. The perfusion system consisted of reservoirs connected via polyethylene tubing to the perfusion chamber. Solutions were bubbled continuously with 95% O_2/5% CO_2 (100% O_2 for HCO_3^--free MEH). The perfusion rate was ~4 ml/min, turning over the solution in the perfusion chamber in less than 5 sec. The perfusion chamber was located on the stage of a Nikon inverted microscope interfaced to a SPEX AR CM fluorometer (Edison, NJ). BCECF fluorescence was continuously monitored by alternating the excitation wavelength between 500 and 440 nm at 0.5-sec intervals and measuring emitted fluorescence at 530 nm. Measured values of R, the ratio of the 500/440 nm excitation wavelengths, were converted to pH using an *in situ* calibration curve. During the measurements, dye leakage was determined by monitoring BCECF fluorescence at the 440-nm excitation wavelength, the pH-insensitive isosbestic point. In 10 min there was no detectable decrease in the BCECF fluorescence at 440 nm, suggesting that dye leakage was minimal.

Intracellular pH Calibration

At the end of an experiment, a calibration curve for BCECF was obtained by the high K^+-nigericin technique as previously described (Thomas et al., 1979). In brief, acini were superfused with a HEPES-buffered high K^+ solution containing 5 μM nigericin, 120 mM KCl, 20 mM NaCl, 0.8 mM $MgCl_2$ and 20 mM HEPES at different pH's. In the presence of nigericin, $[K]_{intracellular} = [K]_{extracellular}$, and therefore, $pH_i = pH_e$. In the pH range of 6.6–7.8, the fluorescence ratio was linearly related to the pH_i.

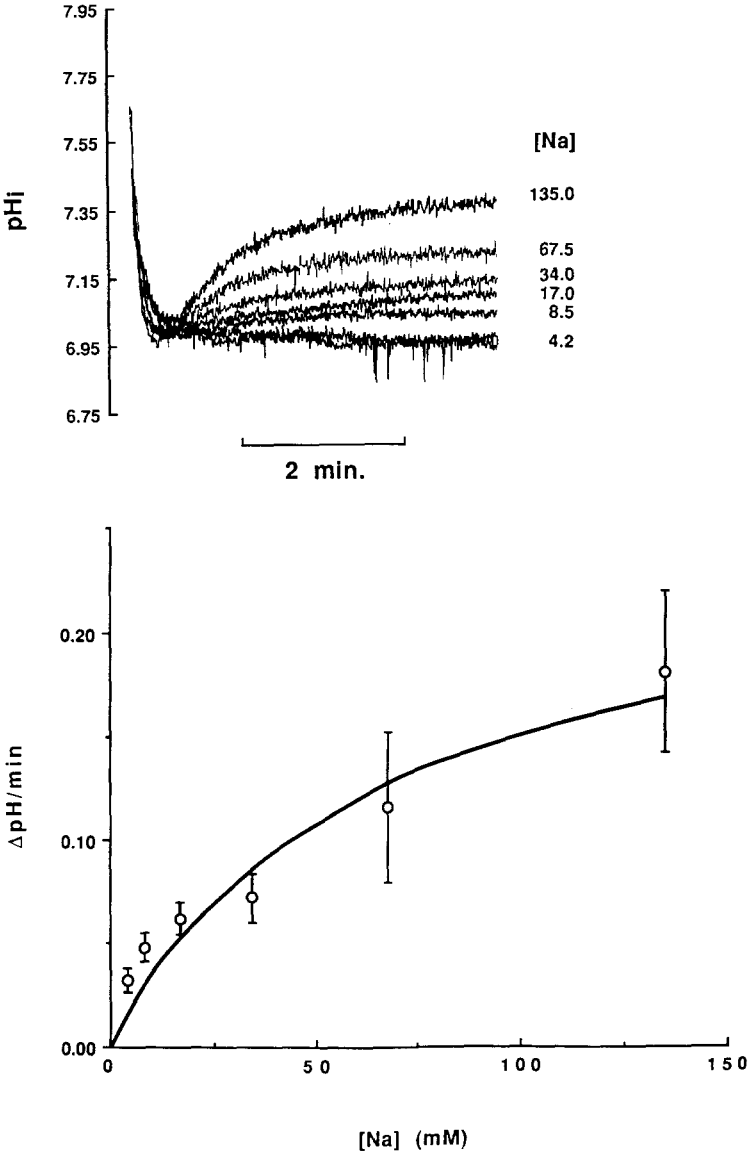

Fig. 2. The dependence of pH_i recovery from an acute acid load on the extracellular Na^+ concentration. An acid load was induced in BCECF-loaded cells using a 60-sec 60-mM NH_4^+/NH_3 prepulse technique. (*Upper panel*) Representative traces are shown for each concentration of Na^+ that was present during recovery (135, 67.5, 34, 17, 8.5, and 4.2 mM). The zero Na^+ and the 4.2-mM traces are similar; thus for clarity the zero Na^+ trace is not labeled. The magnitude of alkalinization and the subsequent acidification were equivalent under all conditions. (*Lower panel*) Each point represents the initial rate of recovery during the first 30 sec when the rate was linear. Results represent the mean ± SEM of four separate experiments. The line was fit to the data using a nonlinear regression analysis program (k·cat® by Biometallics, Princeton, NJ) that gave values for K_m of 66 mM for extracellular Na^+ and V_{max} of 0.26 pH U/min.

NH_4^+/NH_3 Prepulse Technique

BCECF-loaded acini were exposed to an acute acid load induced by the NH_4^+/NH_3 prepulse technique (Roos & Boron, 1981). During the prepulse, NH_3 rapidly enters cells passively until $[NH_3]_i = [NH_3]_o$, resulting in a rise in pH_i due to intracellular NH_4^+ formation ($NH_3 + H^+ \Rightarrow NH_4^+$). Removing extracellular NH_4^+/NH_3 reverses the process, rapidly dropping pH_i as NH_3 effluxes, to an pH_i which is less than the original steady-state pH_i. The magnitude of the acidification below the original steady-state pH_i is dependent on the exposure time and the concentration of the NH_4^+/NH_3 prepulse. NH_4^+ influx is thought to be responsible for this pH_i undershoot phenomenon (Roos & Boron, 1981).

Statistics

All results are presented as means ± SEM. Comparisons were made between different treatments using the unpaired Student's *t* test.

Results and Discussion

It has been suggested that fluid and electrolyte secretion in salivary glands is modulated by pH_i (Novak & Young, 1986; Melvin et al., 1988; Turner & George, 1988; Manganel & Turner, 1989; Melvin & Turner, 1992). The present study examined for the first time the ion transport pathways that regulate pH_i in rat sublingual mucous acini. Our results indicate that the ion transport pathways present in sublingual mucous acini differ in two respects from those previously reported in the other major salivary glands. First, the pH_i response of sublingual mucous acini during muscarinic stimulation is distinct; stimulation results in a sustained rather than a transient intracellular acidification. Second, little if any functional Cl^-/HCO_3^- exchange activity is present in rat sublin-

gual acini, consistent with our previous Cl^- flux studies (Melvin et al., 1991).

Intracellular pH Regulation under Resting Conditions

We found steady-state pH_i in HCO_3^--containing medium (pH 7.4) to be 7.25 ± 0.02, considerably higher than the value of 6.6–6.7 predicted for electrochemical equilibrium in cells with a membrane potential of ~ −59 mV (Nauntofte & Dissing, 1988). To examine the ability of sublingual mucous cells to recover from an intracellular acid challenge, BCECF-loaded acini were exposed to an acute acid load induced by the NH_4^+/NH_3 prepulse technique (Roos & Boron, 1981). When rat sublingual mucous acini were acid loaded with 60 mM NH_4^+/NH_3, pH_i recovered rapidly from the intracellular acidification to the original steady-state pH_i (Fig. 1; $t_{1/2} < 1$ min). Figure 1 also demonstrates that this recovery is entirely dependent on the presence of extracellular Na^+, is MIBA inhibitable (*see below*), and is insensitive to the removal of Cl^- (*see* Figs. 1 and 4), suggesting that a Cl^- independent Na^+/H^+ exchange mechanism is involved in pH_i recovery. The dependence of the recovery from an acid load on the concentration of extracellular Na^+ is shown in Fig. 2. The characteristics of the Na^+ dependence indicate that the steady-state pH_i is directly associated with the extracellular $[Na^+]$ via the Na^+/H^+ exchanger. As the concentration of Na^+ present during recovery was decreased, not only was the initial rate of recovery attenuated but the final magnitude of the recovery was reduced. A similar phenomenon was observed with increasing concentrations of MIBA in the recovery solution (*see below;* Fig. 3). Diminishing the size of the pH_i recovery by either reducing the Na^+ chemical gradient or by inhibition with MIBA indicates that Na^+/H^+ exchange plays a critical role in establishing the steady-state pH_i above the predicted electrochemical equilibrium and that the Na^+ chemical gradient provides the driving force for the exchange in sublingual acinar cells.

Recovery from an acid load was blocked by MIBA (Figs. 1 and 3). This compound and other potent amiloride derivatives specifically inhibit the Na^+/H^+ exchanger (Kleyman & Cragoe, 1988). Inhibition of pH_i recovery by MIBA is therefore consistent with the involvement of Na^+/H^+ exchange in the recovery process. The Na^+/HCO_3^- cotransport mechanism is insensitive to amiloride derivatives (Jentsch et al., 1986; Townsley & Machen, 1989; Geibel et al., 1990). This fact, as well as the lack of inhibition of pH_i recovery when HCO_3^- was removed (*data not shown*), suggests that the pH_i recovery was not due to Na^+/HCO_3^- cotransport. Using a

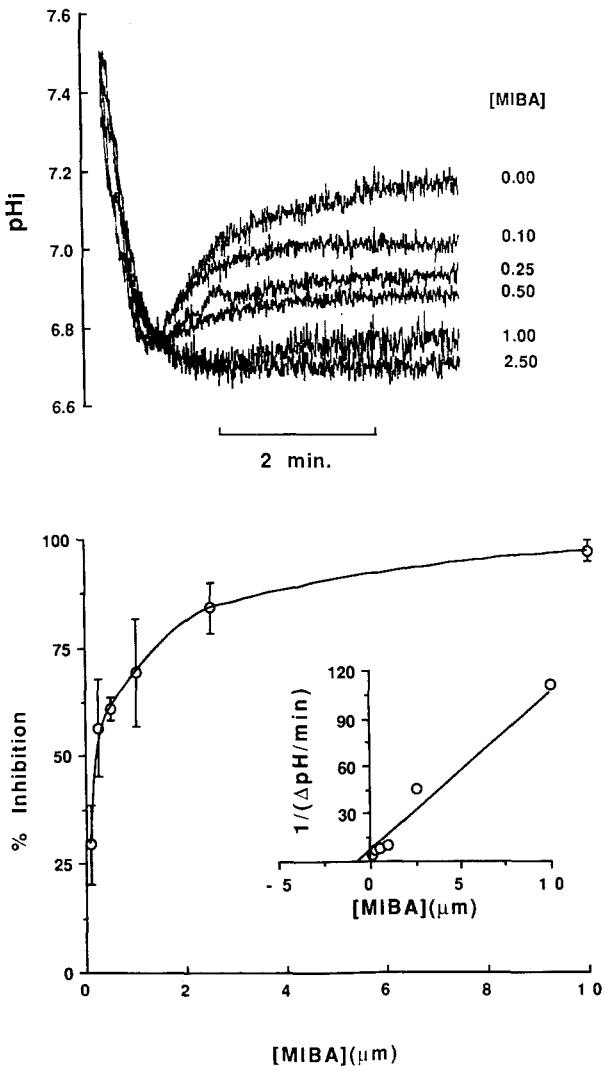

Fig. 3. Inhibition of the pH_i recovery from an acute acid load as a function of the concentration of MIBA. An acid load was induced in BCECF-loaded cells using a 60-sec 60-mM NH_4^+/NH_3 prepulse technique. (*Upper panel*) Representative traces are shown for the following concentrations of MIBA (in μM) present during pH_i recovery: 2.5, 1, 0.5, 0.25, 0.1. Because of overlap with the 2.5-μM trace, the 10-μM MIBA trace is not shown. (*Lower panel*) Each point for the % inhibition of recovery represents the mean ± SEM of five separate experiments. The line was fit to the data using a nonlinear regression analysis program. The inset is a Dixon plot (1/*V versus* [MIBA]) of the [MIBA] dependence of pH recovery (apparent K_i = 0.42 μM).

Dixon plot, the apparent K_i for MIBA inhibition was estimated to be ~0.42 μM (*see* inset, Fig. 3).

When the extent of the initial acidification was controlled by varying the concentration of NH_4Cl (5–60 mM) during the NH_4^+/NH_3 prepulse, the rate of the pH_i recovery was enhanced as the extent of the acidification increased (Fig. 4). The rate of recovery increased linearly with decreasing pH_i,

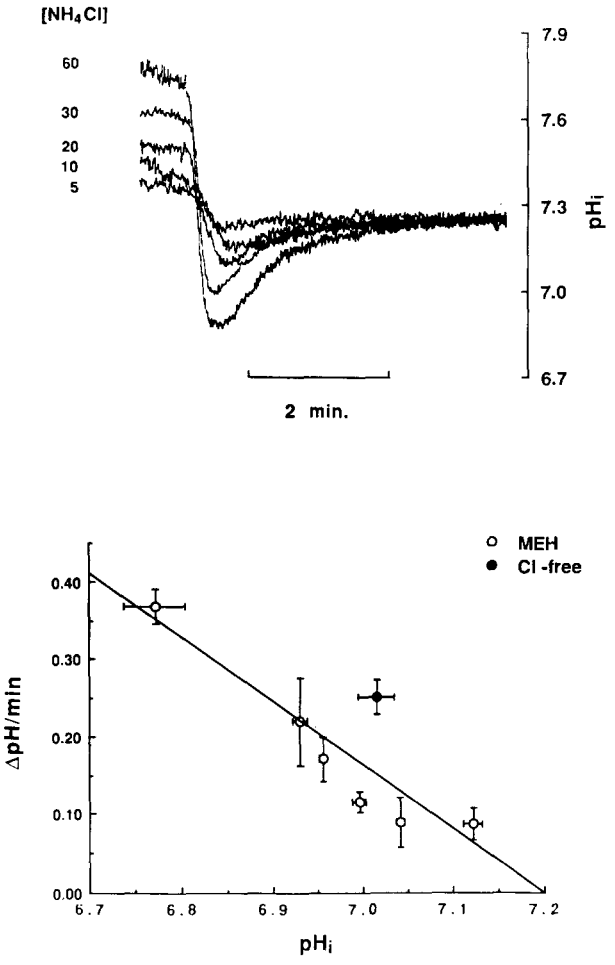

Fig. 4. The dependence of pH recovery on intracellular pH. An acid load was induced in BCECF-loaded cells using a 60-sec prepulse technique with various concentrations of NH_4^+/NH_3. (*Upper panel*) Representative traces are shown for each concentration of NH_4^+/NH_3 present during pH recovery (60, 30, 20, 10, and 5 mM). (*Lower panel*) Each point represents the initial rate of recovery during the first 30 sec when the rate was linear. Open circles represent the mean ± SEM of five separate determinations to which a straight line was fit by least-squares analysis. The filled circle labeled 'Cl-free' was not included in the least-squares fit (n = 5).

suggesting that the activity of the Na^+/H^+ exchanger in sublingual mucous acini increases as the pH_i drops. The pH_i sensitivity of the Na^+/H^+ exchanger has been previously documented in several tissues including parotid salivary glands (Manganel & Turner, 1989) and is due to an internal proton modifier site (Aronson, 1985; Grinstein & Rothstein, 1986; Moolenaar, 1986; Sardet et al., 1989). Figure 4 also shows that the rate of recovery from an acid load is not reduced in the absence of extracellular Cl^-, excluding the possibility that the pH_i recovery is mediated by a Cl^--dependent Na^+/H^+ exchange mechanism. The rate of pH_i recovery in a Cl^--free solution was 0.24 ± 0.02 pH U/min (starting pH_i = 7.02 ± 0.03) compared to the rate of 0.16 pH U/min predicted for pH_i 7.02 by the least-squares analysis of the data shown in Fig. 4. Taken together, the above results reveal that recovery from an acid load occurs by an exchange of intracellular H^+ for external Na^+ via a Cl^--independent Na^+/H^+ exchange mechanism.

In many tissues, including salivary glands (Novak & Young, 1986; Turner & George, 1988; Lau et al., 1989), Na^+/H^+ exchange is paired with Cl^-/HCO_3^- exchange. To determine if anion exchange activity is present in rat sublingual mucous acinar cells, we examined the pH_i response of BCECF-loaded acini to the removal of extracellular Cl^-. When the outwardly directed Cl^- chemical gradient is maximized by removing extracellular Cl^-, the Cl^-/HCO_3^- exchanger will rapidly exchange intracellular Cl^- for extracellular HCO_3^-, thereby increasing pH_i. Removal of extracellular Cl^- induced an extremely limited intracellular alkalinization in 4 out of 19 acini studied (the other 15 acini showed no response). The magnitude of the pH_i increase in sublingual acini that responded to Cl^- removal was always < 0.1 pH unit, considerably less than that observed in parotid acini (~0.4 pH unit; Melvin & Turner, 1992). The reason for the difference in the magnitude of the response to Cl^- removal between these glands is most likely related to one of two possibilities. First, the activity and/or the number of anion exchangers is apparently less in sublingual acini, suggesting that the outwardly directed Cl^- gradient produced by removing extracellular Cl^- primarily dissipates via other ion transport pathways, e.g., the $Na^+/K^+/2Cl^-$ cotransporter or Cl^- channel. This explanation seems most likely for the following reasons: (i) extracellular Cl^- removal (or addition of DIDS, an inhibitor of Cl^-/HCO_3^- exchange; *data not shown*) did not inhibit the pH_i recovery observed in BCECF-loaded sublingual acini that were alkaline loaded using a Na^+ propionate prepulse technique (or by removal of HCO_3^-/CO_2; *data not shown*), suggesting that another mechanism besides Cl^-/HCO_3^- exchange is involved in this pH_i recovery (control, pH_i recovery = 0.027 ± 0.002 U/min; Cl^--free, pH_i recovery = 0.028 ± 0.003 U/min; n = 4); and (ii) in order to totally block net Cl^- re-uptake in parotid acini during stimulation, inhibitors of both $Na^+/K^+/2Cl^-$ cotransport and Cl^-/HCO_3^- exchange must be present (Melvin & Turner, 1992), whereas, the $Na^+/K^+/2Cl^-$ inhibitor, bumetanide, is sufficient to eliminate Cl^- re-uptake in sublingual acini, while DIDS had no effect on Cl^- re-uptake (Melvin et al., 1991). A second, although less likely possibility, is that the efflux of Cl^-/cell via the anion exchanger

induced by Cl^- removal may be considerably less in sublingual cells, therefore reducing the uptake of HCO_3^- via the Cl^-/HCO_3^- exchanger. $[Cl^-]_i$ is concentrated ~5 times above its electrochemical gradient in parotid acinar cells (Foskett, 1990). The $[Cl^-]_i$ in sublingual cells is not known (we estimate ~55 mM; G.H. Zhang & J.E. Melvin, *unpublished observations*), but the Cl^- content/DNA (Melvin et al., 1991) is comparable to that in parotid acinar cells (Ambudkar et al., 1988; Melvin & Turner, 1992). Therefore, unless sublingual acinar cells have considerably larger cell volumes, the $[Cl^-]_i$ should also be similar. Taken together, the above results suggest that anion exchange activity is physiologically insignificant in sublingual acini under these experimental conditions.

Muscarinic-Induced Intracellular Acidification

Previous studies with salivary acinar cells have demonstrated that muscarinic stimulation induces a transient acidification followed by a sustained alkalinization (Pirani et al., 1987; Melvin et al., 1988; Soltoff et al., 1989; Stewart et al., 1989). The sustained alkalinization is involved in the production of salivary secretions by employing two mechanisms which are distinct from that driven by the $Na^+/K^+/2Cl^-$ cotransporter (Novak & Young, 1986; Melvin et al., 1987; Turner & George, 1988). First, Cl^- uptake via the Cl^-/HCO_3^- exchanger is enhanced (Pirani et al., 1987; Melvin et al., 1988; Turner & George, 1988; Melvin & Turner, 1992), thereby raising $[Cl^-]_i$ and maintaining the driving force for Cl^- exit and Cl^--mediated fluid secretion. Second, the increased pH_i results in a higher concentration of intracellular HCO_3^-, thus driving the HCO_3^--dependent component of fluid secretion (Turner, 1992). In the present study, we examined the effects of muscarinic stimulation on pH_i in rat sublingual mucous cells. The acidification noted in the other salivary glands has been attributed to HCO_3^- secretion mediated by the apical anion channel (Melvin et al., 1988; Lau et al., 1989). As in the other major salivary glands, we observed an intracellular acidification during muscarinic stimulation (Fig. 5; $t_{1/2} < 30$ sec; 0.16 ± 0.02 pH units) which could be inhibited with atropine, a muscarinic antagonist (Fig. 5). The duration of this acidification, however, is markedly different from that previously reported in the other major salivary glands. The acidification in sublingual acinar cells was sustained, while only a transient acidification (<2 min) was noted in the parotid and submandibular glands (Melvin et al., 1988; Lau et al., 1989; Soltoff et al., 1989; Stewart et al., 1989). When carbachol

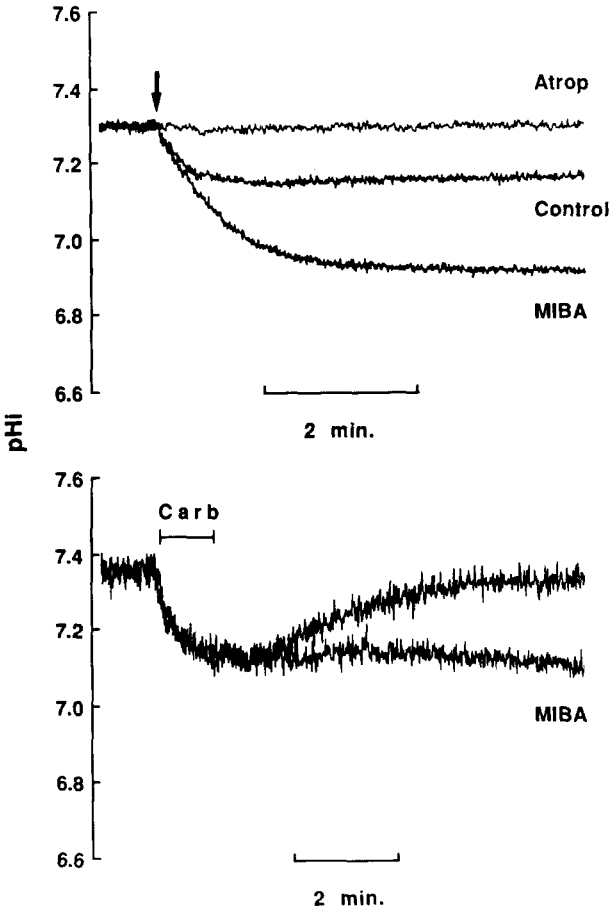

Fig. 5. The response of pH_i to muscarinic stimulation and the effect of inhibition of Na^+/H^+ exchange. BCECF-loaded sublingual acinar cells were stimulated with 10 μM carbachol when indicated by the arrow or by the 60-sec bar. (*Upper panel*) Either 10 μM atropine or 25 μM MIBA was present during muscarinic stimulation (control, $n = 16$; atropine, $n = 4$; MIBA, $n = 4$). (*Lower panel*) Cells were stimulated with 10 μM carbachol for 60 sec and then switched to carbachol-free media ± 25 μM MIBA (carbachol, $n = 4$; carbachol + MIBA, $n = 4$).

was added in the presence of MIBA (Fig. 5), a specific inhibitor of Na^+/H^+ exchange, or in the absence of extracellular Na^+, the magnitude of the acidification was increased >two-fold (0.37 ± 0.05 and 0.34 ± 0.03 pH unit, respectively), suggesting that the extent of the agonist-induced intracellular acidification was significantly blunted by Na^+/H^+ exchange activity. Since the removal of extracellular Na^+ did not inhibit the acidification, the agonist-induced drop in pH_i was not due to a Na^+/HCO_3^- cotransport mechanism. Further evidence that the activity of the Na^+/H^+ exchanger increases during the stimulation-induced acidification is presented in Fig. 5. Switching to a carbachol-free superfusate after 60-sec stimulation resulted in a rapid recovery

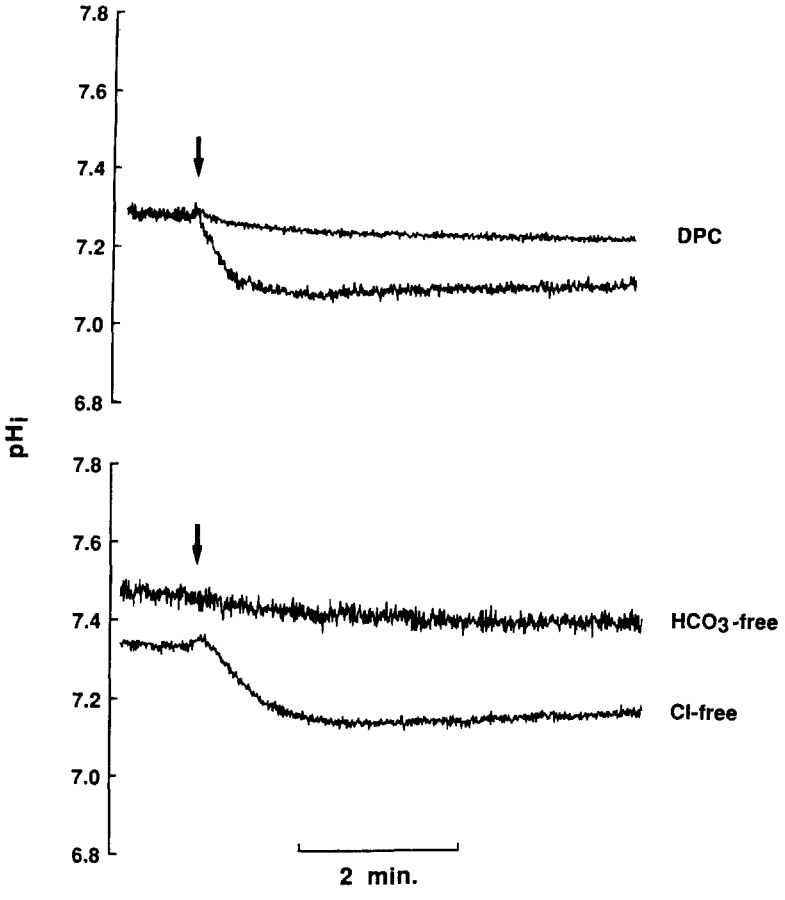

Fig. 6. The effects of Cl^--free or HCO_3^--free media and DPC on the muscarinic-induced acidification. BCECF-loaded sublingual acinar cells were stimulated with 10 μM carbachol as indicated by arrow in either physiological, Cl^--free or HCO_3^--free media, or in the presence of 1 mM DPC (physiological salt solution, n = 16; Cl^--free, n = 7; HCO_3^--free, n = 5; DPC containing, n = 4).

to the prestimulatory steady-state pH_i. MIBA addition (Fig. 5) or Na^+ removal (*data not shown*) completely blocked this recovery. Since pH_i recovery was totally prevented by MIBA addition or Na^+ removal, these results suggest that, like the recovery observed in cells prepulsed with NH_4^+/NH_3 (Fig. 1), the recovery observed after an agonist-induced acidification was mediated by a Na^+/H^+ exchanger. The extent of the muscarinic-induced acidification is thus limited by the buffering action of the Na^+/H^+ exchanger; however, recovery of pH_i and an intracellular alkalinization was not seen in sublingual acini as in the other major salivary glands (Melvin et al., 1988; Lau et al., 1989; Soltoff et al., 1989; Stewart et al., 1989). This most likely suggests that the Na^+/H^+ exchanger in sublingual acini: (i) does not experience a shift in its pH responsiveness as noted in rat parotid acini (Manganel & Turner, 1989); (ii) the rate of acidification via the anion channel is larger in these cells in relationship to Na^+/H^+ exchange activity; or (iii) the number of copies or the transport capacity of the Na^+/H^+ exchanger in sublingual acinar cells is less.

In sublingual cells, removal of extracellular Cl^- did not alter the rate or magnitude of the drop in pH_i caused by muscarinic stimulation (Fig. 6). In contrast, HCO_3^- replacement dramatically inhibited by >90% the agonist-induced acidification (Fig. 6), and preincubation in the presence of methazolamide, a carbonic anhydrase inhibitor, totally blocked the residual stimulation-induced acidification in HCO_3^--free medium (*data not shown*), suggesting that the acidification is dependent on HCO_3^- but not Cl^-. The Cl^- independence of the muscarinic-induced acidification indicates the lack of involvement of an anion exchange mechanism and is consistent with a DIDS-insensitive Cl^- uptake in this tissue (Melvin et al., 1991). DPC, a Cl^- channel blocker in salivary glands (Melvin et al., 1987; Lee & Turner, 1991) inhibited the agonist-induced acidification (Fig. 6). Inhibition of the muscarinic-induced acidification by HCO_3^- removal, but not by Cl^- removal, as well as the inhibition observed in the presence of DPC, strongly support the hypothesis that the carbachol-induced acidification in sublingual acini is primarily mediated by a DPC-sensitive anion channel (Melvin et al., 1987, 1988; Lee & Turner, 1991).

Ca^{2+} Dependence of the Muscarinic-Induced Acidification

It is well established that several of the ion transport pathways associated with stimulated fluid secretion in salivary glands are activated by Ca^{2+}, e.g., K^+ channels (Putney, 1976; Maruyama, Gallacher & Petersen, 1983; Nauntofte & Dissing, 1988), Cl^- channels (Iwatsuki et al., 1985; Nauntofte & Poulsen, 1986; Ambudkar et al., 1988; Cook et al., 1988), and the Na^+/H^+ exchanger (Manganel & Turner, 1989, 1990). Muscarinic stimulation in salivary glands (Merritt & Rink, 1987; Nauntofte & Dissing, 1987; Ambudkar et al., 1988; Foskett et al., 1989; Foskett & Melvin, 1989), including the rat sublingual mucous gland (Melvin et al., 1991), is associated with a rapid and sustained increase in $[Ca^{2+}]_i$. However, at the single cell level, the $[Ca^{2+}]_i$ in parotid acinar cells has been shown to oscillate with a period of ~5 min when stimulated with 10 μM carbachol (Foskett & Melvin, 1989). To determine if the muscarinic-induced acidification with 10 μM carbachol in sublingual mucous acini is dependent on an increase in $[Ca^{2+}]_i$, BCECF-loaded cells were stimulated in a Ca^{2+}-free perfusate. In the absence of extracellular Ca^{2+}, pH_i rapidly acidified ($t_{1/2} < 30$ sec; 0.13 ± 0.03 pH units) in a similar fashion to that seen in the presence of extracellular Ca^{2+}. However, in contrast to the results observed in a Ca^{2+}-containing medium, the pH_i dropped only transiently, returning to the resting steady-state pH_i in ~5 min (Fig. 7). The time course for the pH_i recovery in a Ca^{2+}-free medium shown in Fig. 7 is comparable to that previously observed for $[Ca^{2+}]_i$ in fura-2-loaded sublingual acini stimulated under similar conditions (Melvin et al., 1991); i.e., the initial muscarinic-induced rise in $[Ca^{2+}]_i$ was not altered, while the increase in $[Ca^{2+}]_i$ during prolonged stimulation returns to resting levels in <5 min in a Ca^{2+}-free medium. The initial increase in $[Ca^{2+}]_i$ is thought to be due to mobilization of an intracellular pool of Ca^{2+}, whereas the sustained increase is due to Ca^{2+} entry (Merritt & Rink, 1987; Nauntofte & Dissing, 1987; Foskett et al., 1989; Foskett & Melvin, 1989; Melvin et al., 1991). If the sublingual acini were first loaded with the Ca^{2+} chelator, BAPTA, the stimulation-induced acidification (Fig. 7) and the rise in $[Ca^{2+}]_i$ (*data not shown*) were totally inhibited in a Ca^{2+}-free medium. It thus appears that the initial increase in $[Ca^{2+}]_i$ is sufficient and necessary to produce the drop in pH_i, while the sustained acidification requires the uptake of extracellular Ca^{2+}. To further test the hypothesis that the acidification in sublingual acini is induced by an increase in $[Ca^{2+}]_i$, ionomycin, a Ca^{2+} ionophore, was used to increase directly $[Ca^{2+}]_i$ and by-pass the muscarinic receptor.

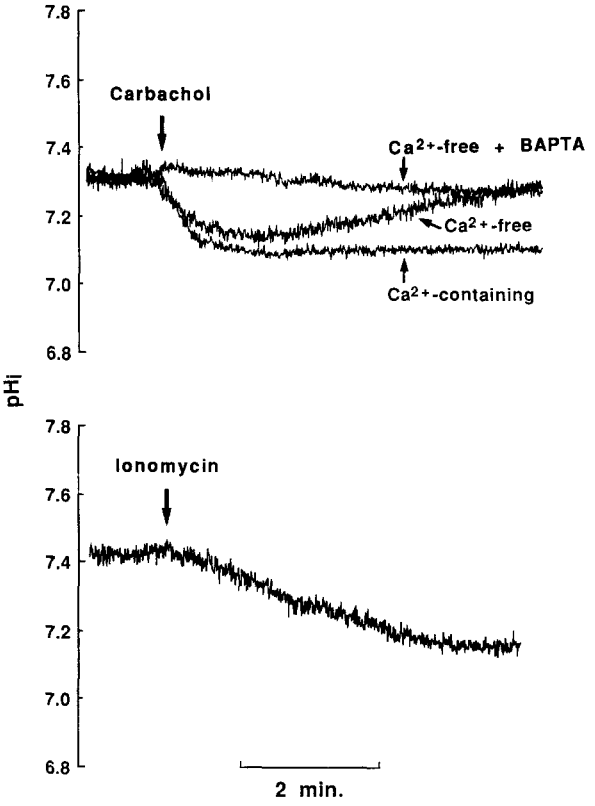

Fig. 7. The role of extracellular and intracellular Ca^{2+} on the muscarinic-induced acidification in sublingual mucous acini. (*Upper panel*) BCECF-loaded sublingual acinar cells were stimulated with 10 μM carbachol when indicated by the arrow in either Ca^{2+}-containing or Ca^{2+}-free media. To examine the effect of intracellular Ca^{2+} chelation, acini were loaded with BAPTA by incubating BCECF-loaded cells for 1 hr with 25 μM BAPTA/AM. (*Lower panel*) BCECF-loaded sublingual acinar cells were stimulated with 0.5 μM ionomycin at the time indicated by the arrow (physiological salt solution, $n = 16$; Ca^{2+}-free, $n = 6$; BAPTA-loaded, Ca^{2+}-free medium, $n = 4$; ionomycin, $n = 8$).

Ionomycin decreased pH_i in a similar fashion to that seen during carbachol stimulation (0.23 ± 0.04 pH unit), consistent with a rise in $[Ca^{2+}]_i$ being required to induce an intracellular acidification (Fig. 7). The mechanism by which Ca^{2+} decreases the pH_i in sublingual acini remains to be determined. Our results, however, are consistent with the current fluid secretion model (Petersen & Gallacher, 1988; Turner, 1992) which predicts that Ca^{2+} activates the putative apical anion channel (Iwatsuki et al., 1985; Cook et al., 1988), resulting in the efflux of HCO_3^-. Additionally, since the acidification was dependent on HCO_3^- efflux (Fig. 6), it appears that little of the acidification is mediated by Ca^{2+}-activated generation of metabolic acid.

In summary, rat sublingual mucous acini main-

tain steady-state pH_i well above the predicted pH for electrochemical equilibrium by primarily employing a Cl^--independent Na^+/H^+ exchange mechanism (*see* Figs. 2 and 3). In contrast to the rat parotid gland (Melvin & Turner, 1992), Cl^-/HCO_3^- exchange appears to play no physiological role in regulating pH_i or in driving Cl^- re-uptake and Cl^- efflux during stimulation. Muscarinic stimulation results in a dramatic and sustained acidification, the magnitude of which is blunted by increased activity of a Na^+/H^+ exchanger. The agonist-induced acidification is precipitated by the efflux of HCO_3^-, most likely mediated by a Ca^{2+}-activated, DPC-sensitive anion channel. The role of the sustained acidification in mucous cells during stimulation is unknown, but it may be related to the secretory function of these cells. Further studies are necessary to examine whether acidification regulates the discharge of mucins and/or is involved in modulating the volume of fluid produced by these glands.

The authors would like to thank Drs. P.A. Knauf and L.A. Tabak for their helpful comments during the preparation of this manuscript and L. Koek for technical assistance. This work was supported in part by grants from the National Institutes of Health, R01 DE08921 and P50 DE07003 (JEM).

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Received 5 February 1992; revised 14 April 1992