Changes in pH_i associated with activation of ion secretion in avian nasal salt gland cells

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Shuttleworth, Trevor J., and Chris M. Wood. Changes in pH_i associated with activation of ion secretion in avian nasal salt gland cells. *Am. J. Physiol.* 262 (*Cell Physiol.* 31): C221–C228, 1992.—The fluorescent pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to determine changes in intracellular pH (pH_i) associated with activation of secretion in isolated cells from the salt-secreting avian nasal gland. A correction procedure overcoming artifacts due to BCECF leakage is described. Resting pH_i averaged 7.15 ± 0.03 and was unaffected by the nominal removal of medium HCO_3^- or by the addition of the anion-exchange inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) but was significantly reduced by amiloride (7.07 ± 0.02). Muscarinic activation of secretion resulted in a rapid intracellular acidification that was compensated by mechanisms which raised pH_i to restore approximately resting levels within 5 min. The principal mechanism involved was amiloride-sensitive and independent of any sustained intracellular Ca^{2+} concentration change. Recovery of pH_i was also aided by HCO_3^--dependent and DIDS-sensitive mechanisms not seen in the resting cell. The direction of the latter was pH_i-dependent, with DIDS further decreasing pH_i in acidified cells and increasing pH_i in alkalinized cells. This suggests that the DIDS-sensitive pathways are activated under conditions where pH_i has been shifted away from resting levels in either direction and act primarily to restore resting pH_i.

2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; amiloride; 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; sodium-hydrogen exchange; anion exchange; intracellular calcium concentration; exocrine secretion; muscarinic receptors

CLASSIC TISSUES for the study of agonist-induced fluid and electrolyte (NaCl) secretion by exocrine cells are the salivary glands, lacrimal glands, and pancreatic acini. Considerable evidence has accumulated supporting a model for the mechanism of primary secretion in such tissues involving a secondary active transport of chloride ions that enter the cell via a Na^+-$2Cl^-$-K^+ cotransporter, driven by gradients developed by the activity of a basolateral Na^+-K^+ pump, and exit via apical Cl^- channels. The initiation of secretory activity is believed to involve the opening of these apical Cl^- channels, together with corresponding K^+ channels in the basolateral membrane, by appropriate intracellular messengers such as changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) or adenosine 3′,5′-cyclic monophophate (see Ref. 23).

Stimulation of secretion in such cells is associated with marked increases in metabolic activity, largely as a result of the increased turnover of the Na^+-K^+ pump, with corresponding implications for the intracellular pH (pH_i) of the cells. In addition, changes in cell volume may accompany the initiation of secretion as the chloride ions and potassium ions exit via their respective apical and basolateral channels. Data from a variety of other cells suggest that the responses to such cell volume changes are likely to involve the transfer of acid-base equivalents via Na^+-H^+ and Cl^--HCO_3^- exchanges (see Ref. 6 for review). Furthermore, agonist-induced changes in pH_i may directly regulate some components of the secretory mechanism such as ion channels or may be involved in the control or integration of other intracellular signals such as changes in $[Ca^{2+}]_i$ (30). Thus changes in pH_i, or changes in the mechanisms responsible for regulating pH_i, may have a significant role in the overall secretory response. However, studies on the pH_i changes associated with the receptor-mediated activation of secretion per se in many such tissues (e.g., salivary glands) are complicated by their known ability to secrete significant quantities of HCO_3^- (3, 4, 13, 18).

The basic mechanism of ion and fluid secretion as described above is also responsible for the elimination of excess salt by the avian extrarenal salt-secreting nasal gland (5, 10, 14, 28). The advantage of this tissue is that it is a highly active, purely NaCl-secreting tissue with a very homogeneous cell population. We have recently shown that isolated dispersed cells of the duck nasal salt gland provide an excellent model system for the study of muscarinic receptor-activated ion secretion in exocrine cells. Intracellular signaling pathways appear essentially identical to those found in the more "classic" tissues (29). In the study described here, we have used such isolated dispersed cell preparations from the avian nasal gland to investigate the changes in pH_i after the activation of secretion via muscarinic receptors and the mechanisms involved in its control. The pH-sensitive fluorescent dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (25) has been employed to provide a continuous record of the temporal changes in pH_i after receptor activation.

METHODS

Cell isolation and loading with BCECF. Suspensions of dispersed isolated cells were obtained from the supraorbital nasal salt glands of the duck (*Anas platyrhynchos*) as previously described (29). Cells were loaded with BCECF by incubation in saline in the presence of the acetoxymethyl ester [BCECF/AM, 2 μM + 0.2% dimethyl sulfoxide (DMSO)] for 30 min at 38°C. The composition of the saline was as follows (in mM): 110.4 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 22.8 $NaHCO_3$, 6.0 glucose, 8.4 Na-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 6.6 HEPES-free acid (pH 7.4 when equilibrated with 5% CO_2 in air). It should be noted that HEPES was included in the normal HCO_3^--containing medium for consistency of comparison with the nominally HCO_3^--free medium (see below), where the use of 15 mM HEPES was mandatory to control extracellular pH. Preliminary experiments showed that the presence of 15 mM HEPES had no effect on the response observed in normal HCO_3^--containing medium (data not shown). The nominally HCO_3^--

free saline used in some experiments was the same as above with the substitution of equimolar NaCl for the $NaHCO_3$ and was equilibrated with air. After loading with BCECF, cells were washed twice by centrifugation at 60 *g* for 10 min, resuspended in fresh saline plus 0.1% BSA, and then stored at 38°C until used. Immediately before the experiment, each aliquot of cells was washed and centrifuged three times (80 *g* for 2 min each) and resuspended in the appropriate saline including 0.1% bovine serum albumin (BSA). The 0.1% BSA was employed to greatly reduce the rate of leakage of BCECF from the cells (see RESULTS).

Measurement of pH_i. Fluorescence measurements were made on cell suspensions in a spectrofluorimeter (Perkin-Elmer LS5B) fitted with a thermostatically controlled (38°C), magnetically stirred cuvette holder. In the experiments using HCO_3^--containing medium, all salines were preequilibrated with 5% CO_2 in air and the cuvette was fitted with a cap containing gas lines that permitted the atmosphere above the fluid in the cuvette to be continually gassed with water-saturated 5% CO_2 in air throughout the measurements. BCECF fluorescence was continuously recorded using excitation and emission wavelengths of 490 and 530 nm, respectively. Output from the spectrofluorimeter was directed to a chart recorder and simultaneously to a computer, where data was collected at a rate of two readings per second. At the beginning and end of each experiment and occasionally at intermediate times, measurements of fluorescence were made with an excitation wavelength of 440 nm, at which BCECF is insensitive to pH. Over the course of an experiment the fluorescence at this excitation wavelength never changed by >4%. From the assumption that any such change was linear, the fluorescence at an excitation wavelength of 440 nm could be determined for each time point during the experiment, and from these data, the ratio of the fluorescence at the two excitation wavelengths could be calculated. Measurement of autofluorescence of unloaded cells at the two wavelengths proved negligible.

Calibration was carried out by two separate means as follows: *1*) an intracellular calibration of the dye by the method of Thomas et al. (32) in which the cells were suspended in high-K^+ medium (same as the low-HCO_3^- medium described above with equimolar substitution of KCl for the NaCl) plus nigericin (10 μg/ml), thereby equilibrating pH_i and extracellular pH (pH_o); pH was then adjusted over the range 6.5–7.5 with small amounts of HCl and NaOH; and *2*) an extracellular calibration protocol involving lysis of the cells with digitonin (10 μM) followed by similar adjustment of the pH over the range 6.5–7.5. Bovine carbonic anhydrase (4 mg/ml) was added to ensure rapid equilibration of pH in HCO_3^--buffered medium. At each point, the pH was measured in the cuvette with a needle electrode (MI-411, Microelectrodes), the fluorescence was recorded with an emission wavelength of 530 nm and excitation alternating between 490 and 440 nm, and the fluorescence ratio was calculated. The two techniques yielded parallel calibration curves in which pH was a linear function of fluorescence ratio, but the extracellular calibration (digitonin-lysis procedure) resulted in a calculated pH_i that was 0.132 ± 0.013 (n = 9) lower than that obtained with the intracellular technique (high K^+-nigericin). Similar differences in the calibration curves obtained by the two procedures have been reported by other workers (13, 22, 25). Although the KCl-nigericin method offered the advantage of true intracellular calibration, it required the resuspension of the cells in a medium different from that used in the experiment. The digitonin-lysis method offered the advantages of convenience and calibration with every sample being made under the exact conditions of each experiment, an important consideration in view of effects of certain of the drugs used (see below). Consequently, the digitonin-lysis protocol was used routinely, and appropriate correction for intracellular to extracellular calibration curve displacement made to the data obtained.

Of the various drugs used, it was found that amiloride (100 μM) quenched the fluorescence of BCECF and caused a mean shift in the calibration curve of −0.057 units. A similar effect has previously been noted by Paradiso et al. (22). On the other hand, the stilbene 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, 100 μM) consistently enhanced the fluorescence, producing a +0.029-unit shift. The two drugs in combination shifted the curve by −0.028 pH unit. All these effects on the determined pH_i were corrected as appropriate. None of the other drugs used were found to significantly affect the fluorescence recorded or the calibration curve obtained.

Experimental protocol and presentation of data. In view of the time-dependent leakage of BCECF from the cells (see RESULTS), a strict time protocol was followed in each experiment. Where appropriate, cells were incubated with inhibitors (e.g., amiloride, DIDS) for 20 min before transfer to the spectrofluorimeter. Such inhibitors were included in the washing and final resuspension salines and were present throughout the experimental period. In the case of low-Ca^{2+} treatment, the low-Ca^{2+} saline was introduced only at the start of the experimental period as the final resuspension medium. Continuous fluorescence measurements were made on suspended cells for 3 min before addition of agonist (carbachol, 500 μM) and then for a further 15 min, after which the experiment was terminated by the addition of 10 μM digitonin and calibration was performed in the experimental medium.

In practice, the fluorescence readings (2/s) stored on the computer were fed into a program that incorporated a series of equations to transform the raw data to pH_i. The program first calculated the fluorescence ratio at each time point and then subtracted the portion of the signal due to extracellular BCECF based on measured rates of BCECF leakage (see RESULTS), the known pH_o, and the known extracellular calibration curve. Finally, true pH_i was calculated based on the known relationship between intracellular and extracellular calibration curves in the particular test medium (see above). To best display mean responses to the various experimental treatments without the complication of minor variations in absolute pH_i among preparations, the preagonist control pH_i was subtracted from each run before averaging. Therefore, the traces presented in Figs. 2–7 represent the mean ΔpH_i usually from four to nine separate experiments performed on different cell preparations.

RESULTS

BCECF leakage and its correction. In early experiments using salines without BSA, the fluorescence ratio, and therefore apparent pH_i, increased markedly over the 15-min experimental period in nonstimulated cells. Preliminary experiments, in which loaded cells were removed by centrifugation after various time intervals in the experimental saline and the fluorescence of the supernatant was measured at the pH-insensitive excitation wavelength of 440 nm, revealed that this apparent increase in pH_i was due to a significant leakage or secretion of BCECF into the external medium where pH_o was higher than pH_i. Such experiments demonstrated that only about half of the total BCECF was intracellular at the start of the experiment, of which almost 4%/min was lost to the external medium over the following 15 min. Incorporation of 0.1% BSA into the saline greatly reduced this leakage to a rate less than that reported for many other preparations but one that still significantly affected the fluorescence readings and resulted in an

apparent progressive increase in pH_i in resting cells. The starting intracellular BCECF at 3 min after suspension in saline containing 0.1% BSA was 83.7 ± 1.6% (n = 10), and the subsequent leakage followed a simple exponential with time, with a rate constant of −0.0181 ± 0.0021 min^{-1} (n = 10).

An important point apparently overlooked in many previous studies is that such leakage will not cause a constant error in apparent pH_i but one that increases both with time and absolute difference between the pH_o and the true pH_i. Fortunately, the phenomenon can be easily modeled, as illustrated in Fig. 1, based on the leakage rates (with 0.1% BSA) and mean intracellular and extracellular calibration curves measured in the particular cell preparation under the relevant experimental conditions. For example, at a pH_o of 7.4 and a true pH_i of 7.3, the initial overestimation of pH_i amounts to 0.035 unit, rising to 0.078 unit after 15 min. However, at a pH_o of 7.4 and a true pH_i of 6.7, pH_i is initially overestimated by 0.139 unit, increasing to 0.302 unit after 15 min.

To correct for this error, an equation based on the mean starting BCECF distribution (83.7% intracellular) and leakage time constant (−0.0181/min), together with the relevant intracellular and extracellular calibration curves for each experimental run, was incorporated into the program for calculating pH_i. The validity of this correction protocol was then tested by applying it to seven different nonstimulated cell preparations varying in starting pH_i from 6.95 to 7.43. In the absence of correction, the overestimation of pH_i was 0.062 ± 0.010 unit at the start and increased to 0.135 ± 0.016 unit at 15 min. The increase became significant after only 3 min. The lower the pH_i, the larger were the discrepancies. However, after correction, pH_i remained constant in each run throughout the 15-min experimental period (mean change = −0.026 ± 0.023, n = 7), thereby confirming the efficacy of the corrective procedure.

Resting pH_i. Resting pH_i averaged 7.15 ± 0.03 (n = 11) in the normal HCO_3^--containing saline. Suspension of cells in nominally HCO_3^--free saline did not result in any significant sustained change in pH_i (mean 7.17 ± 0.01, n = 17; or 7.13 ± 0.03 vs. 7.15 ± 0.02 in paired experiments, n = 9). Similarly, addition of the stilbene DIDS (100 μM) also failed to change resting values of pH_i (mean 7.17 ± 0.03, n = 6). Addition of amiloride (100 μM) to cells suspended in the nominally HCO_3^--free saline did, however, result in a significant reduction in resting pH_i of ~0.1 pH unit (from 7.18 ± 0.03 to 7.07 ± 0.02, n = 5), a response that was unaffected by the simultaneous addition of DIDS (7.06 ± 0.02, n = 5).

Effect of receptor activation. The stimulation of secretion in the avian nasal gland is known to be coupled to the activation of muscarinic cholinergic receptors (10, 14, 28). Addition of carbachol (500 μM) to activate these receptors produced a clear biphasic change in pH_i. Initially there was rapid fall in pH_i of ~0.05 unit within the first 30–50 s. This was followed by a much slower increase in pH_i, so that values similar to resting levels were restored ~5 min after the initial stimulation (Fig. 2). The observed gradual restoration of pH_i after the stimulation of secretory activity was dependent on medium $[HCO_3^-]$. In cells suspended in nominally HCO_3^--free saline, addition of carbachol produced a significantly greater initial decline in pH_i (~0.1 vs. 0.05 pH unit in control cells) and a very much reduced recovery (Fig. 2). In the nominal absence of medium HCO_3^-, pH_i in carbachol-stimulated cells stabilized at a value ~0.07 pH unit below that seen in the unstimulated cells.

Effect of inhibition of metabolic response. It seems likely that the observed changes in pH_i after stimulation could result simply from the increased metabolic activity of the cells and/or from the activation of specific acid-base regulatory processes. To discriminate between these two contributions, cells were stimulated with carbachol after suspension in a low-Ca^{2+} medium [0.04 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$)]. Previous studies have shown that, in such a medium, changes in $[Ca^{2+}]_i$ after receptor activation are only transient (29): there is no sustained increase in $[Ca^{2+}]_i$, and as a result, no sustained secretory response or elevated metabolic activity is ob-

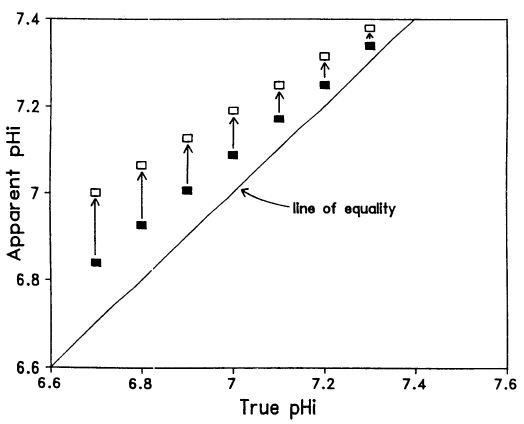

Fig. 1. Graph illustrating effect of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) leakage on apparent intracellular pH (pH_i) when extracellular pH (pH_o) is 7.4. Values were calculated for apparent pH_i at *time 0* (■) and after 15 min (□) at each value of true pH_i. Note that extent of error increases with both difference between true pH_i and pH_o and with time. Of total BCECF measured 83.7% was assumed to be intracellular at *time 0* and rate of leakage was assumed to have a rate constant of −0.0181/min (see text).

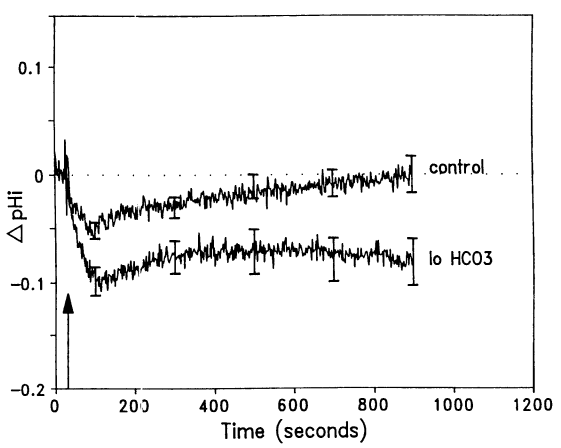

Fig. 2. Effect of muscarinic receptor activation on pH_i in cells incubated in normal saline (control) and in nominally HCO_3^--free saline (lo HCO_3^-). Data are presented as change in pH_i (ΔpH_i) from prestimulation values. Carbachol (500 μM) was added as indicated by arrow, and pH_i was monitored continuously as described. Traces represent means obtained from 8 separate experiments on different cell preparations, with ±SE illustrated at 200-s intervals.

served (29, 31). Figure 3 shows that, under such conditions, the transient acidification and subsequent recovery seen in normal medium was transformed into a marked, and sustained, increase in pH_i in the low-Ca^{2+} medium. Thus, in the low-Ca^{2+} medium, addition of carbachol produced a small and variable initial reduction in pH_i followed by a progressive alkalinization that reached a maximum value ~0.1 pH unit above resting levels. This effect of medium $[Ca^{2+}]$ on the changes in pH_i observed was further emphasized in experiments where $[Ca^{2+}]_o$ was restored to normal values (1.3 mM) during the initial stages of the pH_i response after receptor activation. Figure 3 (*inset*) shows that restoration of extracellular Ca^{2+} immediately converted the onset of the alkalinization seen at low $[Ca^{2+}]_o$ to the typical response seen in the normal medium.

In contrast to its marked effects in the normal Ca^{2+} medium (Fig. 2), nominal removal of HCO_3^- failed to have any significant effect on the response observed in the low-Ca^{2+} medium (Fig. 3). This indicates that in the absence of any secretory response and the associated increase in metabolic activity, nominal removal of medium HCO_3^- was without influence on the changes in pH_i observed.

As noted above, stimulation of cells incubated in a low-Ca^{2+} medium will block both the sustained elevation in $[Ca^{2+}]_i$ after receptor activation and the associated increase in metabolic activity. To distinguish between the contribution of these two components, the changes in pH_i after receptor activation in cells in the low-Ca^{2+} medium were compared with those seen in cells incubated in the presence of the loop diuretic bumetanide. Because of their inhibitory actions on Na^+-$2Cl^-$-K^+ cotransport, loop diuretics such as bumetanide block secretory activity and result in a very much reduced stimulation of oxygen consumption after addition of carbachol (10, 28, 29). However, increases in $[Ca^{2+}]_i$ are unaffected. To maximize the initial changes in pH_i observed, these experiments were performed in nominally HCO_3^--free medium. Figure 4 shows that, in the presence of bumetanide (10 μM), addition of carbachol resulted in a transient decrease in pH_i which was similar to that seen in cells in the absence of bumetanide and significantly larger than that recorded in the low-Ca^{2+} medium. Subsequently, pH_i increased in both bumetanide-treated cells and cells in the low-Ca^{2+} medium to reach values significantly higher than those seen in either resting cells or in stimulated cells incubated in the normal medium. The responses remained identical when the bumetanide concentration was increased to 100 μM. Bumetanide, at either concentration, was without effect on resting pH_i (7.18 ± 0.08 vs. 7.16 ± 0.03 in paired experiments, n = 5). These results suggest that although the initial acidification may be associated in some way with the transient mobilization of $[Ca^{2+}]_i$, the more sustained acidification which was clearly seen in nominally HCO_3^--free medium (Fig. 2) and was blocked by bumetanide (Fig. 4), most likely resulted from metabolic activation.

Effect of amiloride. Stimulation of cells with carbachol (500 μM) in the presence of amiloride (100 μM) resulted in a marked and sustained fall in pH_i (Fig. 5). The initial fall in pH_i after addition of carbachol followed that seen in control cells. Control cells showed a progressive recovery of pH_i after ~60 s. However, in the presence of amiloride, pH_i in stimulated cells continued to decline reaching a minimum of some 0.18 pH unit below resting levels after ~200 s of stimulation. Despite a subsequent gradual increase, pH_i still remained markedly below resting levels in the amiloride-treated cells even 15 min after addition of carbachol. A similar though somewhat attenuated effect of amiloride was seen in cells stimulated with carbachol in the the low $[Ca^{2+}]_o$ medium. Under these conditions, amiloride significantly reduced the increase in pH_i seen in cells after addition of carbachol.

Effect of DIDS. As noted above, suspension of the cells in the presence of DIDS (100 μM) had no significant effect on the resting pH_i. However, after the addition of

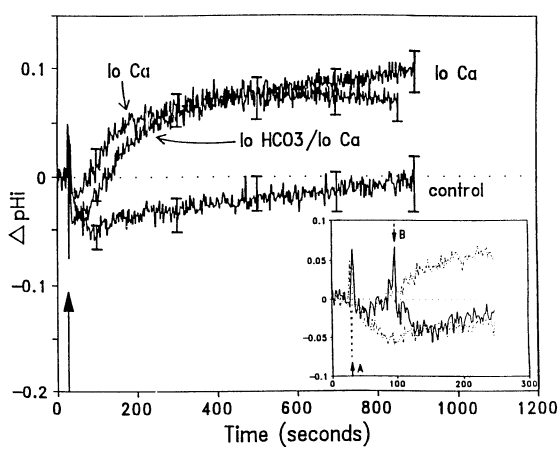

Fig. 3. Effect of low extracellular $[Ca^{2+}]$ on pH_i in cells after muscarinic receptor activation. Results are shown for cells in normal saline (control), in low-Ca^{2+} saline (lo Ca; $[Ca^{2+}]$ = 40 μM), and in nominally HCO_3^--free low-Ca^{2+} saline (lo HCO_3^-/lo Ca). Traces are mean ΔpH_i of 5 separate cell preparations. Other details as for Fig. 2. *Inset*: cells incubated in the low-Ca^{2+} medium were stimulated with carbachol (500 μM, arrow A); 1 min later (arrow B), extracellular $[Ca^{2+}]$ was raised to 1.3 mM. Trace (continuous line) represents mean ΔpH_i of 2 experiments on different cell preparations and is compared with corresponding traces obtained in normal saline and in low-Ca^{2+} saline as in main part of figure (broken lines).

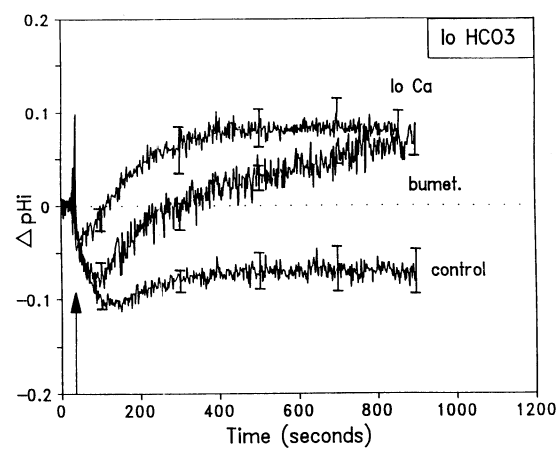

Fig. 4. Comparison of effect of bumetanide and low-Ca^{2+} saline on carbachol-induced changes in pH_i. Cells were incubated in presence of bumetanide (10 μM) or in low-Ca^{2+} saline before being stimulated with carbachol (500 μM) as indicated (arrow). All experiments were performed in nominal absence of medium HCO_3^-. Traces are mean ΔpH_i of either 5 or 6 separate experiments on different cell preparations. Other details as for Fig. 2.

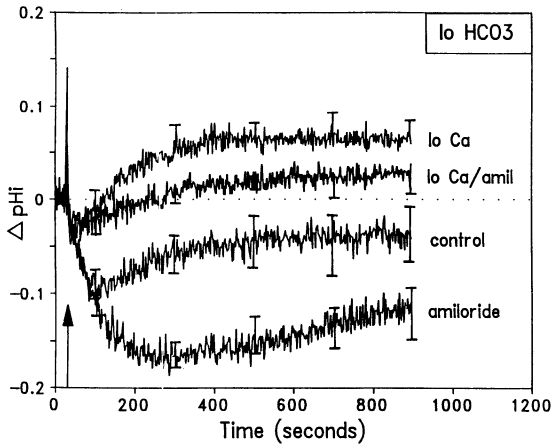

Fig. 5. Effect of amiloride on responses in normal saline and low-Ca^{2+} saline. Cells were incubated either in absence or presence of amiloride (100 μM), in normal saline or low-Ca^{2+} saline before addition of carbachol (500 μM) as indicated (arrow). All experiments were performed in nominal absence of medium HCO_3^-. Traces are mean ΔpH_i from 5 separate experiments on different cell preparations. Other details as for Fig. 2.

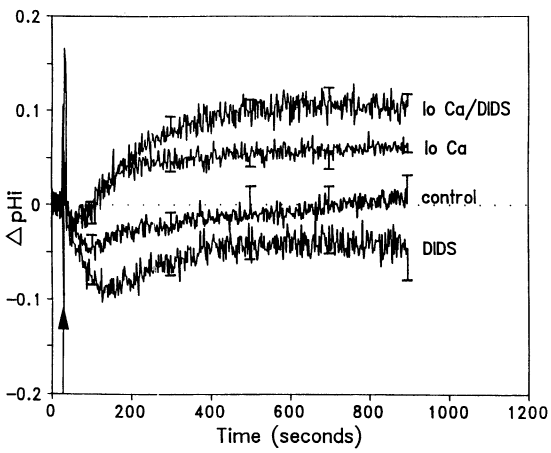

Fig. 6. Effect of DIDS on responses in normal saline and low-Ca^{2+} saline. Cells were incubated either in absence or presence of DIDS (100 μM), in normal saline or low-Ca^{2+} saline before addition of carbachol (500 μM) as indicated (arrow). Traces are mean ΔpH_i of either 4 (normal saline) or 5 (low-Ca^{2+}) separate experiments on different cell preparations. Other details as for Fig. 2.

carbachol, a somewhat larger and more sustained decline in pH_i was seen in the the DIDS-treated cells compared to control cells (Fig. 6). In the presence of DIDS, the maximum fall in pH_i amounted to ~0.1 pH unit compared with 0.05 in the control cells, and the subsequent recovery in pH_i was somewhat attenuated compared with control cells, with pH_i remaining ~0.05 pH unit below resting levels even 15 min after stimulation with carbachol. It should be emphasized, however, that the observed effects were small. In cells stimulated in the low-$[Ca^{2+}]_o$ medium, the presence of DIDS resulted in a somewhat greater increase in pH_i compared with control cells (Fig. 6), so that the steady-state pH_i of the DIDS-treated cells after stimulation was ~0.04 pH unit higher than that seen in the control cells. An important point to note is that this effect of DIDS in the low-Ca^{2+} medium was opposite to that seen with DIDS in the normal medium. This contrasts with the response seen with amiloride, where incubation with the drug resulted in lower values of pH_i after stimulation in either normal Ca^{2+} or low-Ca^{2+} medium (Fig. 5).

Effect of amiloride plus DIDS. The above experiments indicated a role for amiloride-sensitive, DIDS-sensitive, and HCO_3^--dependent processes in the restoration of pH_i after activation of muscarinic receptors. To reveal the underlying changes in pH_i that would follow receptor activation in the absence of these compensating mechanisms, experiments were performed in which the effects of carbachol (500 μM) addition were examined in cells incubated in the nominally HCO_3^--free medium in the presence of amiloride (100 μM) and DIDS (100 μM) (Fig. 7). Under these conditions, receptor activation resulted in a profound and sustained decrease in pH_i of at least 0.2 pH unit. No recovery of pH_i occurred over at least 15 min after stimulation. Furthermore, it appears that the effects of HCO_3^- removal, amiloride, and DIDS were all additive in their inhibition of the restoration of pH_i. The data presented in Fig. 7 also clarify the effect of DIDS on pH_i recovery. In the experiments discussed above, where DIDS was added alone (Fig. 6), the observed effect on pH_i recovery was relatively small. However, when added in combination with amiloride, DIDS had a very clear effect on pH_i recovery. It should also be noted that this effect of DIDS was apparent despite the nominal absence of medium HCO_3^- in these experiments.

DISCUSSION

BCECF leakage. The model calculations of Fig. 1 indicate that the leakage (or secretion) of BCECF to the higher pH of the extracellular medium can potentially give rise to significant errors in the calculation of pH_i. Many studies appear to overlook such problems, whereas others monitor and tolerate it without correction when the rate constant is low (ca. −0.006/min; e.g., Refs. 3, 9, 13). Alternatively, attempts to minimize the problem have involved continual replacement of the external medium (e.g., Refs. 7, 24) or reduced experimental temperatures (e.g., Refs. 22, 27). In the present study, we elected to maintain physiological conditions where possible and

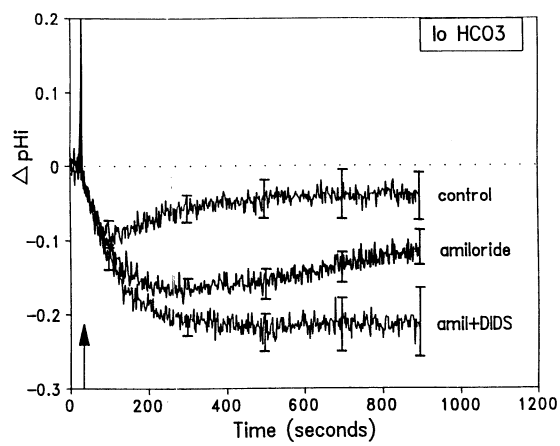

Fig. 7. Combined effects on muscarinic receptor-induced changes in pH_i of amiloride and DIDS in nominal absence of medium HCO_3^-. Cells were incubated in nominally HCO_3^--free saline alone (control), with 100 μM amiloride, or with 100 μM amiloride + 100 μM DIDS. Carbachol (500 μM) was added as indicated (arrow). Traces are mean ΔpH_i of 5 separate experiments on different cell preparations. Other details as for Fig. 2.

were able to reduce the leakage to a constant level (−0.0181/min) by the inclusion of 0.1% BSA in the saline. This leakage rate was then corrected for mathematically, a procedure that proved valid in independent tests on resting cells. This rate may be compared with −0.0176 for rat hepatocytes (7), −0.0277 for rat parotid acinar cells (18), and −0.0346 for rat hepatocytes (24). Our approach appears similar to that of Melvin et al. (18), although that study employed 0.01% BSA and no computational details were given. It should be noted that a key assumption underlying the method is that the leakage rate does not change with the various experimental treatments.

Resting pH_i. In the unstimulated condition, the steady-state pH_i in the isolated cells of the avian nasal gland is independent of the presence of medium HCO_3^- and is insensitive to the anion-exchange inhibitor DIDS. However, in the nominal absence of medium HCO_3^-, an amiloride-sensitive acid-extruding mechanism contributes to the maintenance of resting pH_i. In other exocrine cells an amiloride-sensitive Na^+-H^+ exchange plays a major role in the regulation of pH_i, both in parotid cells (18) and pancreatic acini (20), although in the latter this was only true in the nominal absence of HCO_3^-. In the presence of HCO_3^-, additional DIDS-sensitive mechanisms (Cl^--HCO_3^- exchange and Na^+-HCO_3^- cotransport) were also involved and steady-state pH_i was shifted to a lower value (20).

Effect of stimulation. In normal (HCO_3^--containing) saline, the activation of secretion via muscarinic receptors resulted in a transient acidification of pH_i followed by a gradual recovery to resting levels. The origin of the initial acidification is not entirely clear, but similar responses to a variety of Ca^{2+}-mobilizing agonists have been reported in various tissues (3, 13, 21). Our data indicate that neither amiloride nor DIDS reduced the rate of this initial acidification, so that it appears unlikely to be mediated by any of the specific acid-base transporting mechanisms discussed below. In parotid and mandibular acinar cells, intracellular acidification after receptor activation results from an enhanced loss of HCO_3^- from the cell via anion channels (3, 13, 18). These authors report that incubation in the nominal absence of medium HCO_3^- decreased this receptor-activated acidification (an effect ascribed to the corresponding depletion of intracellular HCO_3^- concentrations). However, the data presented here for the avian nasal gland cells show that this treatment actually increased the underlying acidification rather than decreased it. This effect could result from the blockade of a HCO_3^--dependent regulatory mechanism and/or a reduced intracellular buffering capacity in cells incubated in the nominally HCO_3^--free medium (see below). The initial acidification may simply result from an increase in metabolism in the cells on activation of secretion, an effect shown to be critically dependent on the sustained increase in $[Ca^{2+}]_i$ (29). This could account for the attenuated initial acidification response seen in the low-Ca^{2+} medium. However, addition of carbachol to cells in the presence of bumetanide resulted in an initial drop in pH_i that was almost as large as in normal medium and much larger than seen in the low-Ca^{2+} medium. The presence of bumetanide should block the increase in metabolism, but $[Ca^{2+}]_i$ will rise as normal. This suggests that the initial acidification is related in some way to changes in $[Ca^{2+}]_i$ rather than just an increase in metabolism. Activation of muscarinic receptors in these cells produces a rapid increase in $[Ca^{2+}]_i$ from resting levels of ~100 nM to values in excess of 450 nM within 3–5 s. $[Ca^{2+}]_i$ then declines to a new stable, but still elevated, level of ~300–350 nM after some 60–90 s (29). Clearly, the onset of the pH_i effect is very much slower than that of the $[Ca^{2+}]_i$ response. Furthermore, the significant attenuation of intracellular acidification in the low-Ca^{2+} medium, where the rapid initial increase in $[Ca^{2+}]_i$ still occurs but the sustained increase does not, indicates that the initial acidification seen on stimulation is unlikely to be directly related to the changes in $[Ca^{2+}]_i$. One possibility is that the observed fall in pH_i results from increases in the activity of membrane Ca^{2+}-adenosinetriphosphatases mediating H^+ entry into the cytosol, as reported for vascular smooth muscle cells (9).

After this initial transient acidification, the subsequent recovery of pH_i observed in normal medium could involve passive buffering processes and/or the activation of specific acid-base regulatory mechanisms. When HCO_3^--dependent, amiloride-sensitive, and DIDS-sensitive recovery processes are all blocked, carbachol induces a profound and sustained fall in pH_i of some 0.2 unit (Fig. 7). In the absence of any sustained metabolic activation (i.e., in low-Ca^{2+} medium or in the presence of bumetanide), the normal recovery response is converted into a sustained elevation in pH_i. These data suggest that the stimulation of secretory activity is associated with an underlying intracellular acidification which, unlike the initial acidification discussed above, is probably due to the greatly enhanced metabolic activity shown by actively secreting cells. Under normal conditions, this underlying acidification is compensated for by the activation of appropriate alkalinizing mechanisms.

Nature of recovery of pH_i after stimulation. Part of the overall process responsible for the recovery of pH_i is dependent on the presence of HCO_3^-. However, this HCO_3^--dependent component does not contribute to pH_i regulation in resting cells and could not be detected in the absence of a sustained metabolic activation (e.g., in the low-Ca^{2+} medium), suggesting that the activation of this process in stimulated cells is not immediately coupled to receptor occupation. The precise nature of this HCO_3^--dependent component is not clear. It may be related to the DIDS-sensitive mechanisms discussed below, although the greater intracellular buffer capacity of cells in a HCO_3^- medium may be an additional factor. Whatever the exact explanation, the marked effects of HCO_3^- removal on the changes in pH_i observed after receptor activation, despite the lack of any corresponding effect on pH_i in resting cells, serve to illustrate the dangers of performing measurements of pH_i under HCO_3^--free conditions (33).

A major component of the pH_i recovery process is clearly amiloride sensitive and acts to increase pH_i. As noted above, this component also contributes to the regulation of pH_i in the resting cell. Activation of this amiloride-sensitive component after muscarinic stimu-

lation occurs even in the absence of any sustained increase in secretion-related metabolism (i.e., in low-Ca^{2+} medium) and can still be detected when pH_i is actually elevated above normal levels (Fig. 5). This indicates that the activation of this acid-extruding component is not coupled to changes in pH_i per se but rather is closely coupled to muscarinic receptor occupancy. The observed amiloride-sensitive component is consistent with the presence of a Na^+-H^+ exchange process as found in several other cell types including exocrine cells (15, 16, 20). Definitive identification of such a mechanism by the usual protocols involving removal of extracellular sodium was, however, precluded by the demonstration of profound effects on the carbachol-induced changes in pH_i after inhibition of the Na^+-$2Cl^-$-K^+ cotransporter with bumetanide (Fig. 4). In parotid acinar cells, the activation of Na^+-H^+ exchange is dependent on agonist-induced increases in $[Ca^{2+}]_i$ (16). However, in the present study, the activation of the amiloride-sensitive component was not dependent on any sustained elevation in $[Ca^{2+}]_i$, perhaps indicating a protein kinase C-mediated activation such as has been reported for several other cell types (19).

Finally, there is also a DIDS-sensitive component in the overall pH_i response. Under normal conditions, this plays only a small role in the restoration of pH_i after stimulation of secretory activity, and DIDS was without effect on pH_i in resting cells. Nevertheless, like the amiloride-sensitive response, an effect of DIDS on pH_i could be detected in stimulated cells in both the normal medium and in the low-Ca^{2+} medium (i.e., in the absence of any secretion-related increase in metabolism). However, in contrast to the amiloride-sensitive component, the DIDS component exhibited an interesting pH_i-dependent directionality. Addition of DIDS resulted in a further acidification in those cells showing a reduced pH_i, and a further alkalinization in those cells whose pH_i was already elevated. Therefore, the DIDS-sensitive component appears to be activated whenever pH_i is displaced from its "normal" value. It is not clear whether this effect reflects the activity of two separate processes acting antagonistically or is a single mechanism that can operate in either direction.

The DIDS-sensitive mechanism, at least in the normal Ca^{2+} medium, is not affected by the nominal removal of medium HCO_3^- (Fig. 7). This observation does not conclusively exclude an anion-exchange process because these may still be active at the low HCO_3^- concentrations (200 μM) present in nominally HCO_3^--free medium (26). At least part of the HCO_3^--dependent mechanism and the DIDS-sensitive mechanism seen in normal $[Ca^{2+}]_o$ medium could in fact be the same. For example, several cell types possess a DIDS-sensitive Na^+-dependent Cl^--HCO_3^- exchange mechanism that normally operates in such a way as to produce an increase in pH_i (2, 12). A Na^+-$(HCO_3^-)_n$ cotransporter such as reported in kidney (1), pancreatic acini (20), and parietal cells (34) could also be responsible for the observed effects. Like the anion exchanger discussed above, such a Na^+-$(HCO_3^-)_n$ cotransporter could still function despite the nominal absence of medium HCO_3^- (11).

However, such DIDS-sensitive "acid-extruding" mechanisms cannot explain the observed effects on pH_i in cells stimulated in the low-Ca^{2+} medium (Fig. 6), where the DIDS-sensitive component appeared to be acting as a base extruder. In other cell types, including exocrine secretory cells (20), the most commonly seen DIDS-sensitive base-extruding mechanism is a Na^+-independent Cl^--HCO_3^- exchanger. The reported regulation of such an exchanger by intracellular HCO_3^- (2, 8, 17) could then explain its activation when pH_i is elevated. Once again, the demonstrated profound effects of inhibition of the Na^+-$2Cl^-$-K^+ cotransporter on carbachol-induced changes in pH_i precluded attempts to confirm the presence of this exchanger by experiments involving removal of extracellular Cl^-. Alternative explanations for the observed response include HCO_3^- exit via a DIDS-sensitive anion channel or the Na^+-$(HCO_3^-)_n$ cotransporter operating in such a way as to result in HCO_3^- efflux (7, 20, 21). Whatever the precise nature of these DIDS-sensitive components, they clearly play a role in limiting pH_i fluctuations associated with metabolic activation in avian nasal salt gland cells.

The finding that the activation of secretion via muscarinic receptors in the avian nasal gland cells results in no sustained change in the steady-state pH_i under normal conditions (Fig. 2) has certain important implications. First, it suggests that pH_i is unlikely to be a significant signal in the control of secretory activity or in the regulation of the ion transport pathways responsible for secretion (e.g., ion channels, carriers, pumps). Second, although the steady-state pH_i of stimulated cells does not differ from that in unstimulated cells, this constancy in fact results from the increased activity of a variety of pH_i-regulatory mechanisms. The coordinated activity of these processes may well be important for protecting the cell against adverse changes in pH_i during periods of increased secretory activity. These processes may also play a role in other functions including cell volume regulation (6, 26), cell proliferation (19), and cellular differentiation in response to prolonged stimulation by agonists.

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