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pH_i controls cytoplasmic calcium in rat parotid cells

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The goal of this investigation was to determine if cytoplasmic pH (pH_i) modulated the basal level of the concentration of calcium ions in the cytoplasm (Ca_i) in rat parotid cells. We investigated the effects of various experimental manipulations on both pH_i and Ca_i as measured with BCECF and the calcium photoprotein aequorin, respectively. We found that various experimental manipulations that increased pH_i, such as exposure of the cells to NH_4Cl, a decrease of the partial pressure of CO_2 or an increase in extracellular pH in the presence of nigericin invariably increased Ca_i. Moreover, experimental manipulations which lowered Ca_i, such as a reduction of extracellular [$NaHCO_3$] or the removal of loaded NH_4 invariably decreased Ca_i. Thus pH_i and Ca_i are directly related in parotid cells. Since recent studies have shown that Ca_i directly influences pH_i, we suggest that Ca_i-handling and pH_i-handling are tightly linked in parotid cells.

Introduction

The importance of the production of an alkaline saliva for good oral health has prompted several investigators to determine how salivary acinar cells handle hydrogen ions (H^+). In acinar cells, H^+ ions are transported across the inner membrane of the mitochondria as the result of metabolic activity. Many of the H^+ ions reenter the matrix of the mitochondria through the ATP synthase/hydrogen ion symport to make ATP. A small fraction of the surplus H^+ ions are removed from the cytoplasm by the Na^+/H^+ exchanger on the basolateral membrane [1–3,6]. The rest are neutralized by HCO_3^- which result from the action of carbonic anhydrase on carbonic acid or enters the cytoplasm by the Cl^-/HCO_3^- exchanger in the basolateral membrane [1,4–6]. All of the above transporters work together to maintain the cytoplasmic pH_i at a slightly acidic pH compared to the extracellular space.

Recent evidence suggests that the regulation of pH_i is at least in part controlled by the cytoplasmic concentration of calcium ions (Ca_i). For example, Pirani et al. showed that acetylcholine, which increased Ca_i, also decreased cytoplasmic pH (pH_i) by releasing HCO_3^- into the acinar lumen [1]. Melvin et al. [6] demonstrated that HCO_3^- is lost from the cytoplasm by the Ca_i-regulated anion channel in the apical membrane. Manganel and Turner [2] and Melvin et al. [6] showed that the activity of the Na^+/H^+ exchanger was increased by Ca_i-agonists. Therefore Ca_i alters pH_i by controlling the activities of the Na^+/H^+ exchanger and the anionic channel.

Whether pH_i might influence Ca_i in rat parotid cells is unknown. The only related data concerning pH_i and Ca_i in acinar cells are from pancreatic acinar cell studies and those results are not in agreement with one another. Tsunoda used fura-2 to measure Ca_i and BCECF to measure the pH_i of single pancreatic cells [8]. He found that acidic conditions increased Ca_i while alkaline conditions decreased it [8]. Muallen et al., using a population of dispersed pancreatic acinar cells and the same dyes found the opposite to be true [9]. In both cases, the finding that Ca_i measurements by fura-2 was sensitive to the type of pH buffer used in the experiment [10], necessitates a study to determine

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Abbreviations: H^+, hydrogen ions; pH_i, intracellular pH; pH_o, extracellular pH; Ca_i, the cytoplasmic concentration of calcium; HCO_3^-, bicarbonate anion; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate, BCECF-AM, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester; BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; DPC, diphenylamine 2-carboxylate (*N*-phenylanthranilic acid); BAPTA, 1,2-bis(2-aminophenooxylethane-*N,N,N',N'*-tetraacetic acid.

if there is some linkage between Ca_i and pH_i in salivary acinar cells using an alternative method to measure Ca_i.

We decided to investigate this relationship in rat parotid acinar cells using BCECF to measure pH_i and aequorin to measure Ca_i [11,12]. Using a number of biochemical tests we found that alkalization of the cytoplasm increased Ca_i and acidification decreased it. This suggested to us that one or more of the calcium transporters of the parotid gland was sensitive to pH_i. Because Ca_i appears to control pH_i [1,2,6], we suggest that there is a tight link between Ca_i and pH_i in rat parotid cells at least in the quiescent state.

Methods

Isolation of cells

Rat parotid acini were harvested by enzymatic digestion which was modified from Merrit and Rink [13]. Briefly, rat parotid glands were removed from Sprague-Dawley rats (300 g of either sex), minced and incubated in 10 ml of solution A at 37°C containing 0.5 mg trypsin and rotated at 130 rpm in the Orbital Bath (Lab-Line Laboratories Melrose IL) for 15 min. Solution A consisted of 107 mM NaCl,4 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 20 mM Hepes (pH = 7.4), 11.5 mM glucose and 0.5% bovine serum albumin (fraction 5). The cells were then washed and incubated for 5 min in 10 ml of solution B with 10 mg of trypsin inhibitor. Solution B was identical to solution A with the inclusion of 2 mM EDTA and the exclusion of 1.2 mM $MgSO_4$ and 1 mM $CaCl_2$. The cells were then incubated in solution A containing 3.3 mg of collagenase (275 IU/mg Worthington Biochemical Co.) and rotated at 250 rpm. The suspension was then passed through a nylon sieve and washed with solution A. It was then carefully layered over 10 ml of solution A containing 5% BSA in a centrifuge tube and centrifuged at 300 × g for 5 min. This removed most of the erythrocytes from the cell suspension. The cells were then stored in solution A containing 0.1% BSA at 37°C, rotated at 130 rpm and gassed with 100% O_2 until used.

pH_i-measurements using BCECF (Rink et al. [26])

BCECF was loaded into the parotid cells by incubating 10 ml of cell suspension in 2 μM. After incubation for 20 min the cells were washed twice with solution A containing 0.1% BSA, and then incubated in the same solution at 37°C and gassed with 100% O_2. All of the experiments were done within 2 h of loading.

The measurements of pH_i were made using a Perkin-Elmer Spectrofluorometer Model MPF-4) with excitation at 500 nm, 14 nm slit width and emission at 530 nm, 18 nm slit width. All measurements were done using the ratio mode. The cells were kept in suspension with a magnetic stirring bar driven by a stirrer positioned above the cuvette. The experiments were carried out at room temperature (approx. 22°C).

Ca_i-measurements with aequorin

Rat parotid cells were 'bulk' loaded with aequorin [14,15]. The cells were washed in Chelex-treated loading solution containing 140 mM NaCl and 10 mM Hepes (pH = 7.4). Chelex-treated means that this solution had been passed through a column of Chelex-100 resin (2 cm i.d. × 40 cm height) to reduce the contamination of divalent cations to below 0.1 μM. After the last wash, the cells were resuspended in 0.2 ml of the same solution containing 20–40 μg of aequorin. The cell slurry was passed through a pipette tip (1 mm i.d.) 5 to 10 times. The cells were then centrifuged, the supernatant was discarded or used in other experiments and the cells were resuspended in calcium-free Krebs-Henseleit bicarbonate buffer solution (KHB). KHB contained: 120 mM NaCl, 24 mM $NaHCO_3$, 4 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1.3 mM $CaCl_2$ and 5 mM dextrose. When gassed with 95% O_2/5% CO_2, the solution attained a pH = 7.4 at room temperature. Small aliquots of 1.3 M $CaCl_2$ solution were added over 1/2 h incubation in the orbital bath. We found that a gradual increase in extracellular calcium increases the number of viable cells.

The measurement of luminescence from the aequorin-loaded cells was accomplished with our homemade photometer [16]. The aequorin-loaded cells were entrapped within an agarose thread which was kept within the bottom 1 cm of a glass cuvette of the photometer with a plug of glass wool. KHB was pumped into the bottom of the cuvette at 1 ml/min and removed from the top of the cuvette without disturbing the position of the cells. The dead volume between the reservoir and cuvette was 1 ml.

The cuvette was positioned approximately 10 cm above the photocathode of a 9635 QA photomultiplier tube (Thorn EMI Gencom, UK) surrounded by two hemielliptical mirrors. These mirrors reflected the photons emitted from the aequorin in the cytoplasm of the cells in the cuvette, to the photocathode of the photomultiplier tube. The light signal was measured with a photon counter (Model C-10, Thorn EMI Gencom, UK) which was connected to a stripchart recorder (Recordall, Fisher Scientific, Pittsburgh, PA) and an Apple 2e Computer (Apple, Cupertino, CA) containing an ADALAB Analog-Digital Converter (Interactive Microware, State College, PA).

The calibration method of Allen and Blinks has been previously described [17]. Briefly, the aequorin-loaded cells were destroyed at the end of the experiment using 140 mM KCl, 10 mM Hepes (pH = 7.0), 1 mM $MgSO_4$, 10 mM $CaCl_2$ and containing 0.4% Triton X-100 (Sigma Chemical) and the resulting light was

integrated. The integral was multiplied by an empirically derived ratio expressing the peak of light transient divided by its integral resulting from the rapid mixing of aequorin and an identical solution under identical experimental conditions. The product (called the L_{max}) represented the total amount of aequorin present during the experiment. The observed light intensity (L_o) was divided by the L_{max} to normalize the magnitude of the signal with respect to the amount of loaded aequorin. L_o/L_{max} was interpolated on a calibration curve derived under identical experimental conditions using known concentrations of free calcium. We have assumed a cytoplasmic concentration of 1 mM Mg^{2+} for these estimates of Ca_i.

Statistics

Student's *t*-test ($P < 0.05$) was used to test for statistical significance.

Results

In order to do these experiments in a straight forward manner, it was first necessary to determine if aequorin was sensitive to pH as suggested by the experiments of Moisescu et al. [18]. We placed 10 μg of aequorin in 2 ml of a solution containing 140 mM KCl, 5 mM Hepes (pH = 7.3), 2 mM EGTA and 0.8 mM $CaCl_2$. The free calcium of this solution was approx. 0.1 μM using the 'mixed' binding constants given by the method of Bers [19]. After measuring the initial light intensity with the photometer, we added 5-μl aliquots of 0.5 M KOH to the cuvette and measured the intensity of the luminescence after each addition. We repeated this experiment with the same solutions and measured the pH with a pH-electrode (Chemcadet, Cole Parmer, Barrington, IL). In Fig. 1, the light intensity (closed circles), expressed as a percentage of the initial intensity was plotted against the pH. The calculated decrease in free calcium (open circles) was also plotted in Fig. 1 and these two sets of data are obviously different. When we plotted the decrease in the cubic root of free calcium at each pH (three calcium ions bind to aequorin to cause one photo-emission [20]) we found excellent agreement between light intensity and free calcium at all pH values examined. The correlation coefficient describing the calculated estimates and the value of the cube root of light (when expressed as a percentage of the initial value) was greater than 0.99 (Fig. 1, inset). We concluded that the fall in light intensity by an increase in pH was due entirely to the enhanced ability of EGTA to bind calcium ions. This observation confirmed the results of Blinks [11] and Fabaito [12] that aequorin was not sensitive to pH when measured under physiological conditions.

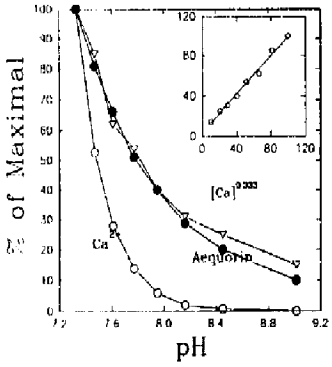

Fig. 1. The effect of pH on the intensity of the aequorin signal in a calcium-EGTA buffer system. Closed circles: decrease in the luminescence intensity expressed as a % of the initial intensity. Open circles: the calculated changes in the concentration of free calcium expressed as a % of the initial concentration. Inverted triangle: the cubic root of the calculated free calcium concentration (i.e., as aequorin would detect it). Inset: the plot of the cubic root of the change in free calcium versus aequorin light signal intensity. The regression coefficient (r^2) = 0.99 showing that all of the decrease in light intensity was due to the enhanced ability of EGTA to bind calcium ions and not due to a direct effect of pH on aequorin.

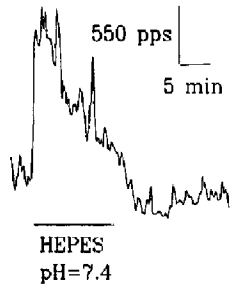

Fig. 2. The light signal from aequorin-loaded rat parotid cells was increased transiently when the buffer component of the perfusate was changed from CO_2/HCO_3^- to Hepes. A solid line was drawn to mark the basal level of aequorin intensity. This example represents the most dramatic of five experiments.

The intensity of light emitted from aequorin-loaded cells depended on the type of buffer used to control the pH of the medium (Fig. 2). When the CO_2/HCO_3^- buffer system of the KHB was replaced with 10 mM Hepes-100% O_2 there was a brisk rise in intracellular calcium which took approx. 20 min to return to baseline. As shown in Fig. 2, return back to the CO_2/HCO_3^- buffer system caused a transient decrease in the aequorin signal which lasted approx. 40 min. Since aequorin luminescence was not pH_i sensitive (Fig. 1) and extracellular pH (pH_o) was maintained at 7.4, we hypothesized the following to explain the data: When the aequorin-loaded cells were exposed to the Hepes-100%

O_2 solution, the cells immediately lost CO_2 which increased pH_i and somehow increased Ca_i. The cytoplasmic concentration of HCO_3^- fell as the anion moved down its concentration gradient which decreased pH_i and therefore decreased Ca_i. The return of the $5\%CO_2/HCO_3^-$ buffer caused an inflow of CO_2, which decreased pH_i. This was followed by a slower influx of HCO_3^-, which eventually restored the normal pH_i. Again, Ca_i appeared to follow the changes in pH_i. We concluded that the Ca_i of rat parotid cells was influenced by changes in pH_i.

Changes in pH_i

The results of at least six similar experiments suggested that there may be some interrelationship between pH_i and Ca_i. To determine if pH_i modulated Ca_i, we first measured the effects of several experimental manipulations on pH_i as indicated with BCECF.

Recent studies from a number of laboratories have shown that BCECF may rapidly leak or be actively secreted out of cells and distribute into subcellular regions where it becomes insensitive to pHi [29–31]. To reduce the influences of these possible artifacts we limited the duration of the loading of BCECF-AM to 20 min. Moreover, we shortened the duration of each experiment to less than 3 min to avoid imprecision due to leakage. Also, we included a calibration regimen involving nigericin, the proton-potassium ionophore [27], with every population of cells to reduce the possible differences in loading and distribution.

In designing our experiments we took advantage of the fact that the intensity of the fluorescence of BCECF increased with pH_i but saturated when $pH_i > 10$. Above pH = 10, the observed intensity is proportional to the amount of BCECF present. Therefore, we standardized the data with respect to differences in the amount of BCECF between determinations. To do this, BCECF-loaded cells were resuspended in a desired medium gassed with either 100% O_2 or 95% O_2/5% CO_2 and transferred to the cuvette which was immediately sealed with parafilm to maintain the partial pressure of the gasses in the solution. The cuvette was quickly inserted into the spectrofluorometer and the basal signal was measured (Fig. 3, left trace). Then the parafilm was opened and 40 μl of 1 M NaOH was added followed by digitonin (final concentration 50 μM) and the new fluorescence intensity was measured. The initial reading was then divided by the latter to obtain a ratio of fluorescence intensities that expressed the pH_i that was independent of the amount of loaded BCECF. It was determined within a few minutes of washing so that leakage of loaded BCECF had minimal impact on the precision of the assay.

The pH_i was calculated by determining the relationship between the ratios derived in our experiments and pH_i. To do this a separate aliquot of the same population of loaded cells was bathed in a solution containing 140 mM KCl, 10 mM Hepes and 10 μM nigericin. The initial pH_o was incremented by additions of 1 M NaOH and the fluorescence intensity was measured after each addition (Fig. 3, right trace). The total volume of 1 N NaOH added was less than 3% of the initial volume. The effect of these additions on pH_o on a fresh aliquot of the same solution was determined with a pH electrode in a separate experiment. Each fluorescence intensity was divided by that intensity observed at a pH greater than 10 which was taken as the saturated signal. This ratio changed linearly with pH within the pH range of 6.5 and 8 and this function was used to calculate pH_i from the fluorescence ratios obtained at different pH_o.

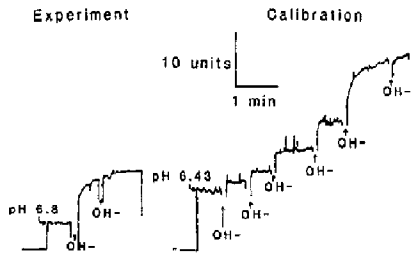

Fig. 3. (a, left) The fluorescence intensity from BCECF-loaded parotid cells equilibrated at $pH_o = 6.8$. Aliquots of 40 μl of 1 M NaOH were added as indicated by OH^-. Digitonin (final concentration 50 μM) was added with the addition of the last aliquot of alkali. The initial fluorescence intensity was divided by the maximal intensity to obtain a ratio that normalizes the measured pH with respect to the amount of loaded BCECF. (b, right) The calibration procedure in which BCECF-loaded cells were resuspended in a solution containing 140 mM KCl, 10 mM Hepes and 10 μM nigericin. Small aliquots of 1 M NaOH were added as indicated by the OH^- and digitonin was added to the last aliquot. The fluorescence intensity obtained after each addition was divided by the maximal intensity and this ratio was plotted against the resulting pH. The pH was measured in separate experiments with a pH electrode using identical solutions. The ratio-pH relationship was used to convert the experimental ratio to pH_i.

The data from four separate populations of cells were plotted as shown in Fig. 4. Each population was indicated by a separate symbol. The pH_o was altered by changes in the concentration of $NaHCO_3$. The line ($pH_i = 1.88 + 0.7 \cdot pH_o$) was drawn by linear regression and had the correlation coefficient (r^2) of 0.87. Solving for normal $pH_o = 7.4$, we calculate that pH_i was 7.06 which was within the range of estimates of pH_i in other experiments [1,2,4–6]. We concluded that in the range of $pH_o = 6.75$ to 7.5 pH_i and pH_o were linearly related when the latter was manipulated by changes in the concentration of $NaCHO_3$.

Another method of changing pH_i that was employed was to maintain the $NaHCO_3$ and replace the 95% O_2/5% CO_2 with 100% O_2. In this case,

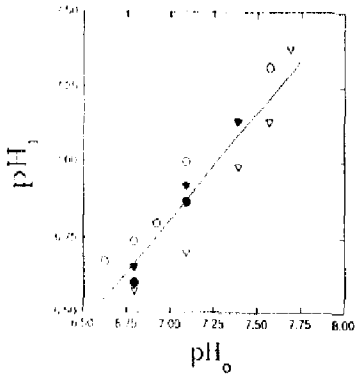

Fig. 4. The effect of pH_o on pH_i. The data were taken from four different populations of cells with each population identified by a separate signal. In each experiment an aliquot of cells was resuspended in KHB in which the $NaHCO_3$ was changed so that the pH_o was set as indicated on the abscissa. The concentration of NaCl was varied in each solution to maintain isotonicity. The pH_i was determined as described in the legend of Fig. 3. The line $pH_i = 1.88 + 0.7 \cdot pH_o$ was drawn by linear regression.

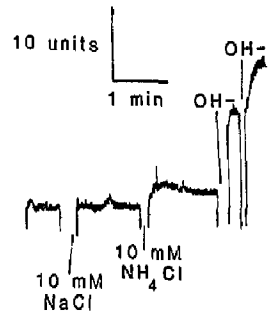

Fig. 5. The effect of 10 mM NaCl and 10 mM NH_4Cl on the fluorescence signal from cells loaded with BCECF.

BCECF-loaded cells were resuspended in normal KHB saturated with 100% O_2 ($pH_o = 8$). The cell suspension was transferred to a cuvette, sealed with parafilm and then measured with the spectrofluorometer. To the cuvette 40 μl 1N NaOH and digitonin were added to obtain the maximal signal. The ratio of the observed intensity divided by the maximal possible intensity value was compared to the calibration curve described above. Basal pH_i increased to 7.44 ± 0.01 (5) (mean $\pm$ standard error (number of observations) $P < 0.05$) compared to basal pH_i measured in the same population of cells.

A third method of changing pH_i was to expose the cells to 10 or 20 mM NH_4Cl. We resuspended the BCECF-loaded cells in normal KHB and transferred the cells to the cuvette which was then sealed with parafilm. After the initial reading was made, a small perforation was placed into the parafilm and then we added 30 μl of 1 M NaCl. We observed no change in the fluorescence signal (Fig. 5). We then added 30 μl of 1 M NH_4Cl and there was a transient increase in the fluorescence signal. As before, we added NaOH and digitonin to obtain the maximal signal to normalize for the amount of BCECF present. NH_4Cl (10 mM) increased from a basal pH_i increased from 7.02 ± 0.02 (6) to 7.25 ± 0.03 (6), $P < 0.05$.

pH_o evoked changes in Ca_i

When aequorin-loaded cells were exposed to KHB (that was vigorously gassed with 100% O_2), there was a rise in aequorin luminescence (Fig. 6). Low CO_2 levels ($pH_o = 8$) caused a gradual increase in Ca_i which took 2–5 min to establish a plateau that continued as long as the cells were exposed to 100% O_2. If the pH_o was maintained at pH = 7.4 with a Hepes buffer, 100% O_2 caused a larger but transient rise in Ca_i. From this type of experiment we concluded that an increase of pH_i is sufficient to increase Ca_i.

Other aequorin-loaded cells were exposed to a solution containing 140 mM KCl, 10 mM Hepes (pH_o set at either pH = 7.0 or pH = 7.5) and 10 μM nigericin. Under these conditions pH_o was equal to pH_i. We found that Ca_i was 0.27 ± 0.03 μM (5) in the pH 7.0 solution and 0.45 ± 0.06 μM (5) in the 7.5 solution. Again, it appeared that cytoplasmic alkalization increased Ca_i.

Introduction of small quantities of NH_4Cl (1–20 mM) caused a transient rise in cytoplasmic pH and the removal of NH_4Cl caused a transient decrease in cytoplasmic pH. Some NaCl was removed from the perfusate to compensate for the added NH_4Cl to maintain iso-osmotic conditions. These changes occurred with-

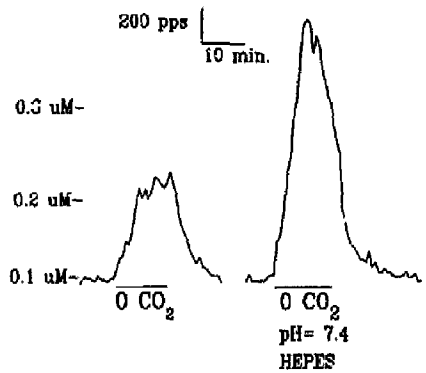

Fig. 6. The effect of lowering pCO_2 on Ca_i. (Left) The result of the removal of pCO_2 on cytoplasmic calcium. (Right) The same with the extracellular buffer fortified with Hepes so that the extracellular pH was maintained at 7.4. All of these responses were fully reversible.

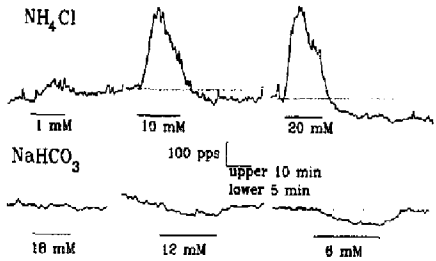

Fig. 7. The effect of NH_4Cl or $NaHCO_3$ on Ca_i. Calibration bar: upper traces: horizontal 10 min; vertical 100 pps; lower traces: horizontal 5 min; vertical 100 pps. Traces were taken from one experiment in which NH_4Cl was increased to 1, 10 or 20 mM or $NaHCO_3$ decreased to 18, 12 or 6 mM was introduced and removed as indicated by the straight line under the trace. Each experiment was separated by at least 30 min. A dashed line was drawn on some of the traces to indicate the basal signal.

out a change in extracellular pH [21]. As shown in Fig. 7, NH_4Cl promoted a similar change in Ca_i, i.e., an initial, transient increase was followed by a decrease when NH_4Cl was removed.

When exposed to solutions gassed with 95% O_2/5% CO_2 containing 1/2 or 1/4 the normal concentration of $NaHCO_3$ (pH_o = 7.1 or 6.8 respectively), there was a fall in Ca_i which accompanied the decrease in pH_i (Fig. 4). The fall was not transient and was fully reversible as was the changes in pH_i. Lower concentrations of $NaHCO_3$ (i.e., less than 6 mM) invariably caused an irreversible change in Ca_i presumably because extreme acidotic conditions damaged the cells. The result suggested that a fall in intracellular pH caused a decrease in Ca_i.

The results of these experiments were presented in Table I. We have assumed an intracellular concentration for magnesium of 1 mM for these estimates of free calcium. The assumption of a lower free magnesium concentration would reduce our estimates of Ca_i. Both 10 and 20 mM NH_4Cl significantly increased Ca_i by about 63 to 84% and there was a tendency for a decrease in Ca_i (8 to 11%) when NH_4Cl was removed. Reducing HCO_3^- to 6 mM with CO_2 maintained at 5% (pH_o = 6.8) caused a 24% decrease in Ca_i while the reduction of the partial pressure of CO_2 increased Ca_i more than two-fold. The results are therefore consistent and significant: acidosis causes a decrease in Ca_i whereas alkalization increases Ca_i.

Discussion

Aequorin is an excellent indicator for Ca_i because: (1) its low cytoplasmic concentration and relatively low affinity for calcium ensure that it does not influence the rate of change of Ca_i; (2) the bulk loading procedures we have developed have been thoroughly tested by more than 40 parameters including rates of protein secretion, influences on cytoplasmic concentrations of ions and cyclic AMP, rates of ionic transport, distribution of calcium ions, rates of metabolism and cell growth [15], and finally (3) aequorin can monitor Ca_i for > 3 days if necessary [15,16]. In contrast to fluorescence probes, aequorin requires no expensive equipment, it can be calibrated without assumptions pertaining to its binding constant, it does not alter cell metabolism, appears to be biologically inert and does not require the application of intense light [28]. Although aequorin fails to provide information about a single cell our concern in the present study is how pH_i affects the Ca_i of a population of cells.

The utility of aequorin for the measurement of the effect of pH on Ca_i has been demonstrated with the experiments of Blinks et al. [11], Fabiato [12] and again (by a different experimental paradigm) in this paper.

TABLE I

The effect of experimental conditions on the pH_i and cytoplasmic calcium of rat parotid cells

Data were expressed in μM as mean ± S.E. (number of observations) assuming a free cytoplasmic magnesium of 1 mM; n.a., not applicable; n.d., not determined; * $P < 0.05$ compared to control pH; ** $P < 0.05$ compared to basal calcium.

Experimental conditions	pH_i	Basal Ca_i	Maximal rise	Maximal fall [a]
Control	7.02 ± 0.02 (6)	0.25 ± 0.02 (5)		
NH_4Cl (10 mM)	7.25 ± 0.03 (6) *	0.27 ± 0.02 (6)	0.44 ± 0.04 (6) **	0.24 ± 0.01 (6)
NH_4Cl (20 mM)	n.d.	0.25 ± 0.02 (6)	0.46 ± 0.01 (6) **	0.23 ± 0.01 (6)
$NaHCO_3$ (12 mM)	6.87 ± 0.05 (4) *	0.33 ± 0.03 (4)	n.a.	0.25 ± 0.03 (4) **
$NaHCO_3$ (6 mM)	6.64 ± 0.03 (4) *	0.33 ± 0.02 (4)	n.a.	0.24 ± 0.02 (4) **
pCO_2	7.44 ± 0.01 (5) *	0.26 ± 0.02 (6)	0.57 ± 0.07 (12) **	n.a.
Nigericin (pH_i = 7.0)		0.27 ± 0.03 (5)		
Nigericin (pH_i = 7.5)		0.45 ± 0.06 (5)		

[a] Refers to the fall in Ca_i after the removal of NH_4Cl.

These results do not necessarily contradict those of Moisescu et al., who reported a 0.2 pCa shift for a change in pH from 6.8 to 7.1 [13]. Their experiments were done under hypotonic conditions which may have made the tertiary structure of aequorin unstable. Indeed, Moisescu et al. stated that the apparent pH sensitivity of aequorin is substantially reduced when the experiment is repeated under more physiological conditions. Therefore, we conclude that aequorin is a reasonable probe to use to measure the effect of pH_i on Ca_i.

In parotid cells we found that experimental conditions that change pH_i also change Ca_i. As described by Boron and Roos [21], the introduction of NH_4Cl caused a transient alkalosis (by 0.2 pH unit) and a transient acidosis by 0.2 pH unit when it was removed. NH_4^+ dissociates to $NH_3 + H^+$. NH_3 enters the cytoplasm through the membrane because it is uncharged [21]. Once inside, NH_3 binds to H^+ to form NH_4^+ causing an alkalinization until a new equilibrium is established. The cell readjusts its pH_i to its former value by action of the H^+ transporters of the surface membrane. During removal of the NH_4Cl, cytoplasmic NH_4^+ dissociates, releasing an H^+ ion which causes a transient acidification that continues until all of the loaded NH_3^+ leaves the cell. These changes occur in the absence of changes in pH_o. The time course of the aequorin signal follows the changes in pH_i quite well again suggesting that Ca_i and pH_i are interdependent.

The relationship between pH_i, pCO_2 and HCO_3^- are described by the bicarbonate chemical equilibrium:

$$pCO_2 \rightleftharpoons CO_2(l) + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

5% 1.2 mM 55 M 10 μM 40 nM 24 mM

The reduction of the extracellular HCO_3^- without a change in pCO_2 caused a rightward shift of the bicarbonate chemical equilibrium due to mass action. The rightward shift lead to a rise in H^+ at the expense of a very small amounts of H_2O and CO_2. We calculated that the pH_o was 7.1 and 6.8, respectively, using 0.8 μM as the dissociation constant for the bicarbonate equilibrium. Using our empirically derived equation for pH_i and pH_o of rat parotid cells:

$$pH_i = 1.88 + 0.7 \cdot pH_o$$

we calculated that pH_i should be 6.85 in the presence of 12 mM and 6.63 in the presence of 6 mM HCO_3^-, respectively. These values agree well with the pH_i of 6.87 and 6.64 measured with BCECF under these experimental conditions.

The decrease of the pCO_2 by bubbling the perfusion solution containing 24 mM $NaHCO_3$ with 100% O_2 caused a left shift of the bicarbonate equilibrium. pH_o increased to 8.0 as HCO_3^- bound up H^+ to form

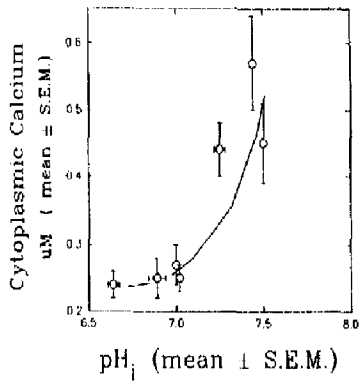

Fig. 8. Summary of the data showing the relationship between pH_i and Ca_i. The line is drawn by eye. Data from Table I. Missing error bars are obscured by the symbol.

H_2O and CO_2. Again using our empirically derived equation we calculated that the resulting pH_i would be 7.48 which agrees well with the value of 7.44 we measured using BCECF. Therefore the experimental manipulations we used in these experiments caused predicted changes in pH_i.

When the data from Table I were plotted as Ca_i versus pH_i, Fig. 8, it was apparent that alkaline pH_i was associated with a strong increase in Ca_i whereas acidity was associated with a much weaker decrease in Ca_i. The relative feebleness of low pH_i to decrease Ca_i is not due to an effect of pH_o on pH_i which is linear within this region (Fig. 4). Instead the inability of pH_i to lower Ca_i may be due to the non-linearity of aequorin which becomes rapidly less sensitive to Ca_i below 0.1 μM. On the other hand, exposure to $pH_o <$ 6.8 causes the surface membrane to become leaky to calcium ions (Snowdowne, unpublished) which could overwhelm the mechanism responsible for lowering Ca_i. Clearly, the use of a probe that is linear for Ca_i in the 0.1 μM range, e.g. fura-2, would provide important information about how acidic pH_i influences Ca_i.

It is possible pH_i and Ca_i could be linked by a simple chemical reaction. However, if H^+ ions were simply displacing Ca^{2+} from binding sites within the cells, a rise in H^+ should increase Ca_i. Therefore, a mechanism involving simple antagonism between Ca^{2+} and H^+ of a common binding site is doubtful. Instead one might speculate that some calcium transporter might be sensitive to physiological changes. Indeed, the calcium pump of the surface membrane is sensitive to pH between 6.8 to 7.5 [22]. On the other hand, we have shown that the Na^+/Ca^{2+} exchanger of the parotid membrane is also sensitive to pH_i [23]. Finally Ca^{2+} influx appears to likewise be under the control of pH_i [9]. Thus pH_i might influence a number of Ca_i-transporters.

One can speculate on the importance of the control of pH_i on Ca_i. Melvin et al. [7] demonstrated that carbachol caused a dramatic acidification of the cytoplasm in the presence of the Na^+/H^+ exchanger blocker, amiloride. Acidification of the cytoplasm was reduced by blocking the carbachol-evoked rise in Ca_i with the combination of the intracellular and extracellular calcium chelators BAPTA and EGTA or the Cl^- channel blocker DPC. Since Ca_i controls the opening of the Cl^- channel [2,7,24], and HCO_3^- traverses the apical membrane through the Cl^- channel [2,6,25], it is likely that muscarinic stimulation of parotid cells increases Ca_i which then opens the Cl^- channel to permit the efflux of HCO_3^- and the resulting decrease in pH_i.

If at the same time, a fall in pH_i inhibits the increase in Ca_i by altering the transport of calcium ions into the cytoplasmic space, then our observations could indicate a potentially important negative feedback loop that could limit the loss of HCO_3^- from the cells. Theoretically, the inhibition of the loss of HCO_3^- would occur during a time when they are needed to thwart the fall in pH_i owing to an enhancement of the rate of metabolism. Thus, in transporting epithelium, the activator of transport, Ca_i, may be limited in its action by the disappearance of its product, i.e., HCO_3^-.

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