pH Regulation in Spread Cells and Round Cells*

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The aim of this work was to characterize the changes in pH regulation that lead to increased intracellular pH (pHi) in well-spread cells on tissue culture plastic relative to cells on a nonadhesive surface. Bicarbonate was not required for maintenance of a control steady state pHi, or of the difference in pHi, between round and spread cells. In the absence of bicarbonate, lowering the sodium content of the medium led to decreased pHi, and elimination of the difference between round and spread cells. In the presence or absence of bicarbonate, adding ethylisopropyl amiloride lowered pH, and eliminated the difference between round and spread cells. Measurements of recovery from acidification in the absence of bicarbonate confirmed that Na⁺/H⁺ exchange was enhanced in spread cells. However, recovery from both acidification and alkalinization, the presence of bicarbonate showed that bicarbonate in-dependent recovery in both directions, most likely due to sodium-dependent and -independent HCO₃⁻/Cl⁻ exchangers, was also stimulated in spread cells. We conclude that Na⁺/H⁺ exchange has a primary role in determining steady state pHi in 3T3 cells in serum and is responsible for the lower pHi in round cells. Bicarbonate-dependent pH regulatory mechanisms are also inhibited in round cells.

It has recently been reported that cytoplasmic pH (pHi) is altered by attachment and spreading of cells on solid surfaces (1, 2). Normal, anchorage-dependent cells were 0.15-0.3 pH units more alkaline when attached to plastic coated with fibronectin or with their endogenous fibronectin-rich extracellular matrix when cells were examined also had a Na⁺/H⁺ exchange, which brings HCO₃⁻ into the cell and therefore alkalinizes the cytoplasm, and a Na⁺-independent HCO₃⁻/Cl⁻ exchanger, which primarily excretes HCO₃⁻ and acidifies the cells (5-7).

The primary aim of this study was to determine which of the pH regulatory systems was responsible for the effects of spreading on pHi. Because spreading on extracellular matrix proteins is slow compared with, for example, the action of growth factors on pHi, understanding how spreading alters pHi required an analysis of which transporters determine steady state pHi. Our results show that Na⁺/H⁺ exchange is of primary importance in maintaining steady state pHi, and is responsible for the higher pHi in well-spread cells, and that the activity of the Na⁺/H⁺ and both HCO₃⁻/Cl⁻ exchangers are increased in spread cells.

MATERIALS AND METHODS

Chemicals—5-(N-Ethyl-N-isopropyl)amiloride (EIPA) was synthesized as described (8). Culture medium was purchased from Medi-tech (Herndon, VA) and serum from Hazelton (Lenexa, KS). BCECF-AM was from Molecular Probes (Eugene, OR). Other chemicals were of the highest grade commercially available.

Cells—BALB/c 3T3 cells (2) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, in 5% CO₂/air. For pH experiments, log phase cells were plated in 35-mm dishes at approximately 20% of confluence 24 h before. Dishes were coated with poly(HEMA) as described previously (9).

For experiments in which sodium was varied, medium contained choline chloride or sodium chloride at a total concentration of 135 mM, 20 mM HEPES (pH 7.4), or 25 mM NaHCO₃, 10 mM glucose, 4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂ (6). Medium with bicarbonate was equilibrated with 5% CO₂ to bring the pH to 7.4.

pH Measurements—Cells in 35-mm dishes were loaded with 1.5 μM BCECF-AM in dimethyl sulfoxide for 10-30 min before each experiment. The concentration of dimethyl sulfoxide did not exceed 0.1% and had no effect on pHi. Dishes were then transferred to the microscope stage where they were kept at 37 °C in a humidified atmosphere of either air or 5% CO₂/air. The apparatus described previously (2) has been automated, and all operations are controlled by a Digicom IBM-compatible XT computer. For each measurement, a single cell was excited with light of 450 nm for 1 s, and the emitted light of 520 nm (0.3-s delay), the cell illuminated, and the emitted light recorded as before. To decrease phototoxicity, neutral density filters were used to reduce the excitation light to the minimum level required for good signal-to-noise; generally between 1 and 10% of the maximal intensity was used, depending on the shape of the cells and the extent of dye spreading in. The costs of publication of this article were defrayed in part by the payment of publication charges. This work was supported by a grant from the Lucille P. Markey Foundation (to C. P. L.). The costs of publication of this article were defrayed in part by the payment of publication charges. The costs of publication of this article were defrayed in part by the payment of page charges. This work was supported by a grant from the Lucille P. Markey Foundation (to C. P. L.). The costs of publication of this article were defrayed in part by the payment of page charges.

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To determine pH, the values were corrected for background intensity from a nearby cell-free area, and the 490:450 ratio was calculated. The absolute pH was determined by comparison with a calibration curve, prepared by equilibrating cells with high K+ medium of known pH using the ionophore nigericin (10). All data collection and statistical analyses were performed automatically by the computer. Values for average pH represent averages from at least 15 individual cells.

RESULTS

The Effect of Bicarbonate

We first determined whether bicarbonate-dependent transporters were needed to maintain the difference in pH, between spread cells on tissue culture plastic and round cells on polyHEMA. Cells were plated in dishes coated with sufficient concentrations of the nonadhesive polymer, polyHEMA, that they remained entirely round, although they attached weakly. In order that round and spread cells could be compared in the same dish, small areas of polyHEMA in each dish were scraped with a plastic pipette tip to expose the underlying plastic.

The medium was changed from the usual bicarbonate-buffered DMEM under 5% CO2 to HEPES-buffered medium at the same pH, without CO2, pH was followed in single cells before and after transfer (Fig. 1A). As previously observed (6, 11), pH underwent a rapid increase, followed by a slower return to starting values. pH returned to approximately the same value after 5-10 min and remained level. Measurements of average pH in spread and round cells made before and after transfer (Fig. 1A). The difference between the two, ΔpH, was approximately 0.15 pI units before and after changing the medium. Similar results were obtained in five out of five such experiments. Using medium that had been degassed to remove the small amount of CO2 in equilibrium with the air or medium that contained 0.1 mM DIDS to block HCO3/Cr exchange had no effect on pH (not shown). We conclude that with 3T3 cells in serum, bicarbonate is not required to maintain steady state pH.

The Effect of Sodium

To test the function of the Na+/H+ exchanger, cells in medium without bicarbonate were transferred to medium containing variable concentrations of sodium, with choline added to keep ionic strength constant. Fig. 2A shows the time course for a typical spread cell after changing to medium without sodium. pH decreased after transfer, until a new steady state pH was reached after approximately 15 min. Measurements of average pH in spread cells made 1 h after changing medium showed that pH decreased strongly on sodium (Fig. 2B). Cells in 0 Na+ were very acidic, but regained nearly control pH, in only 14 mM Na+ (half-maximal effect at 5 mM). pH in 0 sodium was an average of 6.81 ± 0.12 (10 experiments). When pH was measured in rounded cells and spread cells, the difference, ΔpH, depended on sodium over the same concentration range (Fig. 2C). In medium without sodium, ΔpH was an average of 0.02 ± 0.02 (5 experiments) and was identical to control in 14 mM sodium. Two control experiments showed that the elimination of ΔpH was due to absence of sodium and not to the low pH, per se. First, in sodium-free medium of pH 8.0, cells had a pH of approximately 6.7, but ΔpH was still 0. Second, in medium with normal sodium but with pH 6.5, spread cells had a low pH (approximately 6.7), but ΔpH was identical to control conditions (data not shown).

These experiments lead to two conclusions. First, in bicarbonate-free medium, maintaining pH in the normal range requires sodium, but relatively low concentrations of sodium are sufficient. Second, the difference in pH, between spread and round cells changes over the same range of sodium concentrations. Previous work (6) has shown that the extracellular binding site on the Na+/H+ exchanger has an affinity for sodium of 15–50 mM. Thus, the results would suggest that the Na+/H+ exchanger is required for maintenance of pH, and that it is responsible for the effect of spreading on pH.

The Effect of EIPA

To confirm that the Na+/H+ exchanger was responsible for the effect of spreading on pH, the drug EIPA was used. EIPA has a high affinity and, when used at low doses, is relatively specific for the Na+/H+ exchanger (12). In initial experiments, cells in normal (bicarbonate-free) medium showed changes in pH, only at rather high concentrations of EIPA (>60 μM). To avoid potential toxicity associated with high concentrations of EIPA, we adopted the approach of L’Allemain et al. (13). Cells in medium containing 15 mM Na+ were treated with EIPA; under these conditions EIPA is effective at lower doses.
because competition with extracellular Na⁺ is decreased. A sodium concentration of 15 mM was chosen because it is the lowest concentration that gave a normal pH, (Fig. 1B).

Cells in low sodium underwent a slow decrease in pH, after addition of EIPA, which stabilized after 10–15 min. Fig. 3A shows a typical time course for a single spread cell. Measurements of average pH, after 30–60 min showed that EIPA was maximally effective at 40 μM (Fig. 3B), which is the approximate concentration at which EIPA completely inhibited Na⁺/H⁺ exchange in previous work (12). Average pH, in 40–60 μM EIPA was 6.97 ± 0.11 (11 experiments). Measurements of pH, in spread and round cells using this protocol showed that ΔpH₃ was eliminated by EIPA over the same concentration range (Fig. 3C). The average ΔpH₃ was 0.00 ± 0.02 (5 experiments). We conclude that, in the absence of bicarbonate, Na⁺/H⁺ exchange contributes significantly to steady state pH, and is required for the effect of spreading on pH₃.

To analyze the role of the Na⁺/H⁺ exchanger under conditions where HCO₃⁻/Cl⁻ exchangers also operate, cells in medium with bicarbonate and CO₂ were treated with EIPA. The medium had 25 mM sodium, with choline added to keep ionic strength constant as before. Transfer of cells from normal DMEM to this medium had no effect on pH, (not shown). Addition of EIPA to spread cells resulted in decreased pH,
(Fig. 4A). The average pH of cells in 40–60 μM EIPA was 7.06 ± 0.05 (10 experiments). When round and spread cells were compared, ΔpH was reduced over the same concentrations of EIPA (Fig. 4B). Average ΔpH in 40–60 μM EIPA was 0.02 ± 0.02 (4 experiments). These results show that Na+/H+ exchange maintains pH at a level that is higher than HCO3-/Cl- exchangers alone and that the Na+/H+ exchanger is solely responsible for the effect of spreading on steady state pH.

pHi Recovery

Na+/H+ Exchange—To characterize further the changes in proton transport caused by cell spreading, the recovery from acute changes in pH was examined in round and spread cells. First, the Na+/H+ exchanger was studied by measuring the recovery of cells from acidification in the absence of bicarbonate. Cells were acidified by equilibrating in medium with 7 mM NH4Cl for 10–15 min and then quickly switching to medium without NH4Cl (14). Cells were rapidly acidified by approximately 0.5 pH units and then recovered slowly toward their initial pH. Time course measurements were made on single cells before and up to 15 min after acidification.

Typical recovery curves are shown for spread and round cells in Fig. 5A. Recovery of the round cell was significantly slower, and the cell reached a lower steady state pH. Recovery from acidification under these conditions appears to be largely dependent on Na+/H+ exchange, since it was blocked by carrying out the experiment in medium without sodium or by adding 50 μM EIPA (not shown, but see Fig. 5B). Data for a number of recovery experiments were quantitated by fitting the recovery curves to a single exponential as described under "Materials and Methods." The half-time for each recovery was calculated from the fitted constants. Average values (Table I, Half-time) showed that the half-time was 59% longer for round cells. Recovery experiments of this type have also been analyzed by comparing the rate of recovery, dPH/dt, at a given pH, for cells in different states (6, 15). Therefore, dPH/dt was calculated at PH = 6.7 from the best-fit curves.

Fig. 4. Role of the Na+/H+ exchanger with bicarbonate. A, effect of EIPA on pH. EIPA was added to cells in medium with 25 mM sodium and 25 mM bicarbonate, under 5% CO2. Cells and measurements were otherwise as in Fig. 3. B, effect of EIPA on ΔpH, pH was determined in spread cells and round cells as in A, and the difference, ΔpH, was calculated.

Fig. 5. Recovery from acute changes in pH. A, Na+/H+ exchange. Cells in bicarbonate-free medium were incubated with 7 mM NH4Cl for 10–15 min and then switched to medium without NH4Cl. Time course measurements were made on single cells. Open circles, a spread cell in normal DMEM. Filled circles, a round cell in normal DMEM. Squares, a spread cell in sodium-free medium. B, Na+-dependent HCO3-/Cl- exchange. Time course measurements were made on single cells after acidification as in A. The medium for experimental conditions contained 25 mM sodium and 25 mM bicarbonate as described under "Materials and Methods," and 50 μM EIPA. Buffers with bicarbonate were kept under 5% CO2. Open circles, a spread cell. Filled circles, a round cell. Open squares, control with a spread cell in medium lacking bicarbonate and CO2. Filled squares, control with a spread cell in medium without sodium. C, Na+-independent HCO3-/Cl- exchange. Cells in normal DMEM with bicarbonate under 5% CO2 were rapidly switched to bicarbonate-free medium under air and pH monitored in single cells. Filled circles, a spread cell. Open circles, a round cell. Squares, +0.1 mM DIDS.
been attributed to a sodium-independent HCO₃⁻/H⁺ exchanger (7, 16). Round and spread cells were observed after changing to bicarbonate-free medium. Typical recovery curves are shown in Fig. 5C. It is apparent that recovery of the round cell was significantly slower than the spread cell. Recovery was strongly inhibited by pretreatment of cells with 0.1 mM DIDS for 10-15 min (Fig. 5C), but was independent of external sodium (not shown). Analysis of data for a number of experiments showed that recovery was stimulated in spread cells to about the same extent as was Na⁺-dependent HCO₃⁻/Cl⁻ exchange (Table I).

### DISCUSSION

Our results lead to three main conclusions. First, the difference in steady state pH between spread cells and round cells is due to changes in the activity of the Na⁺/H⁺ exchanger. This conclusion holds for cells in medium with or without bicarbonate. It is based on studies with low sodium and with EIPA and is supported by measurements of recovery from low pH. It should be emphasized that round cells still have substantial capacity to recover from acid loads. They differ in that the rates are somewhat diminished relative to spread cells, and the pH levels off at a lower value. These differences are similar to the type of changes observed after serum stimulation of quiescent attached cells (17). Note that Margolis et al. (1) reported that the change in pH upon spreading was due to the Na⁺/H⁺ exchanger and that its activity was also necessary for cell spreading. However, both of these conclusions were apparently based on experiments that used sodium-free medium that lowered pH to well below the physiological range and that also would be expected to have effects on cells other than blocking Na⁺/H⁺ exchange. Our data (Ref. 2, and Footnote 2) have consistently shown that changes in pH in the physiological range, or specific blockade of the Na⁺/H⁺ exchanger, do not affect cell shape or cell spreading to an appreciable extent.

Second, Na⁺/H⁺ exchange does make a significant contribution to steady state pH in the presence of absence of bicarbonate. The role of Na⁺/H⁺ exchange in setting steady state pH, however, has been the subject of some controversy in the literature. Evidence in favor of the idea that Na⁺/H⁺ exchange determines pH, came in part from experiments showing that cells in medium without bicarbonate were stimulated with serum or growth factors, pH rapidly increased due to activation of the Na⁺/H⁺ exchanger (for reviews, see Refs. 17, 18). More recently, it has been shown that the effect of serum on pH is abolished by adding bicarbonate to the medium (11). Bicarbonate raises the pH of serum-starved cells to about the same extent as was Na⁺-dependent HCO₃⁻/Cl⁻ exchange, recovery of the round cell was significantly slower than the spread cell. Recovery was strongly inhibited by pretreatment of cells with 0.1 mM DIDS for 10-15 min (Fig. 5C), but was independent of external sodium (not shown). Analysis of data for a number of experiments showed that recovery was stimulated in spread cells to about the same extent as was Na⁺-dependent HCO₃⁻/Cl⁻ exchange (Table I). The buffering capacity of cells, βp, can be determined from the magnitude of the pH change after washout of NH₄Cl, where βp = concentration of NH₄Cl/pH change (6). When the data for individual recovery experiments were analyzed in this manner, it was found that βp was 19.0 ± 5.0 mM/pH for spread cells and 18.3 ± 3.5 mM/pH for round cells. Thus, the buffering capacity for spread and round cells under these conditions does not differ significantly. We conclude that the differences in recovery rates reflect differences in proton flux, so that the Na⁺/H⁺ exchanger appears to be activated in spread cells relative to round cells.

### Sodium-dependent HCO₃⁻/Cl⁻ Exchange

To analyze bicarbonate-dependent pH regulation, the recovery of cells from acidification or alkalization, and the half-time for each recovery curve was calculated as described under "Materials and Methods." Shown are the average values ± S.E.

| Exchange mechanism | Spread cells | Round cells | Increase % |
|--------------------|--------------|-------------|------------|
| Na⁺/H⁺ exchange    | 0.16 ± 0.02  | 0.26 ± 0.03 | 65*        |
| Na⁺-dependent      | 0.13 ± 0.02  | 0.21 ± 0.06 | 55*        |
| HCO₃⁻/Cl⁻ exchange | 0.21 ± 0.04  | 0.32 ± 0.06 | 50*        |

**Half-times.** Time course measurements were made on single cells after acidification or alkalization, and the half-time for each recovery curve was calculated as described under "Materials and Methods." Shown are the average values ± S.E.

**Statistically significant at p < 0.05, according to Student's t test.**

Recovery rate. The rate of recovery at a given pH was calculated for each experiment. For Na⁺/H⁺ exchange and Na⁺-dependent HCO₃⁻/Cl⁻ exchange, recovery at pH 6.7 is shown. For Na⁺-independent HCO₃⁻/Cl⁻ exchange, recovery at pH 7.6 is shown.

### Sodium-independent HCO₃⁻/Cl⁻ Exchange

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after addition of serum cannot be extrapolated to the entire cell cycle.

Experiments with amiloride or its derivatives have also generated disagreement concerning the importance of Na+/H+ exchange in setting pH. One study found that these drugs only altered steady state pH, at very high concentrations, where they appeared to act by nonspecific means (20). They concluded that Na+/H+ exchange was only activated by low pH, and did not function under normal conditions. Although we are in agreement with the data from the latter experiments, we do not support their conclusions. It appears that Na+/H+ exchange can be partially inhibited without substantially altering steady state pH. We found, however, that complete inhibition, either by removal of sodium from the medium or by adding EIPA to low sodium medium, resulted in a significant decrease in pH. Other workers (6) have also shown an effect of amiloride derivatives on steady state pH. Thus, at steady state, Na+/H+ exchange does appear to operate at a level that makes a significant contribution to pH.

Our third conclusion is that pH recovery due to HCO3-/Cl- exchange is increased in spread cells. Our cells alkalized via a mechanism that has the characteristics of the previously described sodium-dependent HCO3-/Cl- exchanger and acidified via what appears to be the sodium-independent HCO3-/Cl- exchanger. Both of these were inhibited in round cells to about the same extent (Table I). Ganz et al. (16) recently showed that stimulation of mesangial cells with vasopressin triggered an increase in Na+/H+ exchange, in sodium-dependent HCO3-/Cl- exchange, and in sodium-independent HCO3-/Cl- exchange. The net effect in their system was acidification, because the largest increase was in the rate of HCO3 efflux due to sodium-independent exchange. In our system, there was no difference in steady state pH between round and spread cells when only HCO3-dependent mechanisms were active. This may be because the changes in uptake and efflux of HCO3, balance, an idea that is consistent with the result that alkalization due to Na+-dependent HCO3-/Cl- exchange, and acidification due to Na+-independent HCO3-/Cl- exchange decreased to about the same extent in round cells. Alternatively, it may be that the HCO3-/Cl- exchangers are inactive at resting pH, or that other factors such as acid production are involved.

Our results are consistent with the general view that normal cells deprived of an extracellular matrix on which to spread are in a state similar to that of cells deprived of growth factors. In both cases, the absence of as yet unknown cytoplasmic signals leads to a general decrease in the activity of the transport proteins responsible for pH regulation. Other factors such as altered geometry could conceivably also play a role in the decreased rate at which round cells recover from acute changes in pH, but this need not be the case. Round cells develop a highly convoluted surface, so that no net loss of membrane surface area need occur (21). Diffusion of protons and other ions from the interior of the cell to the plasma membrane might also be slower in round cells. The diffusion times, however, are orders of magnitude faster than the observed recovery times and should therefore not be rate-limiting.

The next stage in this work will be to identify the cytoplasmic signaling pathways that are activated by ECM and that lead to altered pH regulation.

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