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pH Regulatory Na/H Exchange By *Amphiuma* Red Blood Cells

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ABSTRACT In *Amphiuma* red blood cells, the Na/H exchanger has been shown to play a central role in the regulation of cell volume following cell shrinkage (Cala, P. M. 1980. *Journal of General Physiology.* 76:683–708.) The present study was designed to evaluate the existence of pH regulatory Na/H exchange in the *Amphiuma* red blood cell. The data illustrate that when the intracellular pH_i was decreased below the normal value of 7.00, Na/H exchange was activated in proportion to the degree of acidification. Once activated, net Na/H exchange flux persisted until normal intracellular pH (6.9–7.0) was restored, with a half time of ≈ 5 min. These observations established a pH_i set point of 7.00 for the pH-activated Na/H exchange of *Amphiuma* red blood cell. This is in contrast to the behavior of osmotically shrunken *Amphiuma* red blood cells in which no pH_i set point could be demonstrated. That is, when activated by cell shrinkage the Na/H exchange mediated net Na flux persisted until normal volume was restored regardless of pH_i. In contrast, when activated by cell acidification, the Na/H exchanger functioned until pH_i was restored to normal and cell volume appeared to have no effect on pH-activated Na/H exchange. Studies evaluating the kinetic and inferentially, the molecular equivalence of the volume and pH_i-induced *Amphiuma* erythrocyte Na/H exchanger(s), indicated that the apparent Na affinity of the pH activated cells is four times greater than that of shrunken cells. The apparent V_{max} is also higher (two times) in the pH activated cells, suggesting the involvement of two distinct populations of the transporter in pH and volume regulation. However, when analyzed in terms of a bisubstrate model, the same data are consistent with the conclusion that both pH and volume regulatory functions are mediated by the same transport protein. Taken together, these data support the conclusion that volume and pH are regulated by the same effector (Na/H exchanger) under the control of as yet unidentified, distinct and cross inhibitory volume and pH sensing mechanisms.

Dedicated to the memory of John C. Parker, always a source of inspiration and enthusiasm.

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INTRODUCTION

The Na/H exchanger fulfills what has come to be known as a "housekeeping" role in many cells, functioning in a volume and/or pH regulatory capacity (Thomas, 1977; Cala, 1980; Grinstein, Clark, and Rothstein, 1983; Grinstein, Cohen, and Rothstein, 1984a; Grinstein, Goetz, Cohen, Furuya, Rothstein, and Gelfand, 1985*b;* Parker and Castranova, 1984; Piwnica-Worms Jacob, Horres, and Lieberman, 1985; Siebens and Kregenow, 1985; Cala, Anderson, and Cragoe, 1988; Frelin, Vigne, Ladoux, and Lazdunski, 1988; Weissenberg, Little, Cragoe, and Bobik, 1988; Breyer and Jacobson, 1989; Hoffmann and Simonsen, 1989; Weintraub and Machen, 1989). As a volume regulatory effector mechanism, the Na/H exchanger is activated as cell volume is decreased below normal levels and mediates net solute and osmotically obliged water uptake. In the ideal case, net Na/H exchange flux decreases to zero as cell volume is returned to normal. However, when the Na/H exchanger of the human lymphocyte (Grinstein et al., 1983) and many other cell types is activated by osmotic shrinkage, net Na/H exchange is deactivated before restoration of normal control volume. Thus, in these cells the Na/H exchanger is volume activated but not volume regulatory since cell volume regulation is incomplete (Rotin and Grinstein, 1989). In contrast, when lymphocyte pH_i is decreased below normal, the Na/H exchanger, functioning in a pH regulatory capacity, is activated and mediates net Na uptake and H loss until normal pH_i is restored, at which point (the pH_i set point) the Na/H exchanger is deactivated. Thus, the lymphocyte Na/H exchanger is pH but not volume regulatory. It has been suggested that, in cells where Na/H exchange is volume activated yet not volume regulatory, failure to restore normal volume is a reflection of the conflicting demands dictated by the pH regulatory function of the Na/H exchanger (Grinstein et al., 1983; Grinstein et al., 1985*a, b;* Rotin and Grinstein, 1989). That is, the net H efflux which is a consequence of Na uptake results in an increase in cell pH until the new volume-induced pH set point is reached, resulting in pH-dependent deactivation of volume-activated Na/H exchange. Implicit in this explanation for incomplete cell volume regulation is the notion that there are separate mechanisms responsible for pH and volume dependent activation yet deactivation appears to be solely determined by pH_i.

In contrast to cells such as the human lymphocyte in which cell volume regulation is incomplete, at all but the most modest levels of cell shrinkage, the *Amphiuma* erythrocyte demonstrates volume regulatory capacity over a wide range of media osmolarity (Cala, 1980; Cala, 1983*b;* Siebens and Kregenow, 1985; Cala, 1986*a;* Cala, Mandel, and Murphy, 1986). This behavior could reflect more effective H buffering (little or no change in pH_i occurs during volume regulation), or alternatively that the *Amphiuma* red blood cell Na/H exchanger is not subject to pH_i-dependent control. In this regard, our first report describing the mechanisms of *Amphiuma* red blood cell volume regulation suggested that volume-dependent changes in pH_i may serve as a transducer for changes in cell volume and thereby play a role in the activation of the volume regulatory ion flux pathways (Cala, 1980). In light of the *Amphiuma* red blood cell's high fixed (protein) buffer capacity, together with dynamic buffering via the Cl/HCO_3 exchanger it was reasoned that Na/H exchange-dependent pH_i regulation by these cells was superfluous if not impossible. The above notwithstanding, studies

suggesting a pH regulatory role for the *Amphiuma* red cell K/H exchanger (Cala, 1985*a*) together with the well documented presence of Na/H exchange dependent pH regulation in virtually all cells prompted us to evaluate the pH regulatory capacity of the *Amphiuma* red cell Na/H exchanger.

This manuscript describes studies designed to investigate the existence of pH regulatory behavior and a pH "set point" for the *Amphiuma* red cell Na/H exchanger. The data are consistent with the interpretation that the Na/H exchanger is capable of pH regulation, because when pH_i is displaced (acid direction) from the normal value of 7.00, the Na/H exchanger is activated and mediates net Na uptake in exchange for H loss until normal pH_i is restored, regardless of the consequences with respect to cell volume. In contrast, when cell volume is decreased below normal, the Na/H exchanger mediates net Na/H exchange flux until normal volume is restored even though Na/H exchange-dependent regulation of volume results in significant alkaline shifts in pH_i. These observations suggest that when activated by cell shrinkage, the *Amphiuma* red cell Na/H exchanger is not deactivated by pH_i even when pH_i is increased by nearly one unit above the normal pH set point (7.00). Thus, the pH and volume set points for deactivation are distinct and cross-inhibitory. Finally, kinetic analyses of volume and pH-activated Na/H exchange, based solely upon differences in apparent V_{max} and K_m for Na, are consistent with the interpretation that volume and pH are regulated by different Na/H exchange processes. However, a more thorough analysis of the data, based upon bisubstrate enzyme kinetics, (Segel, 1975) demonstrates behavior consistent with that of a single Na/H exchanger with Na transport kinetics which are a variable function of [H] at sub saturating concentrations of hydrogen ion.

METHODS

Amphiuma red blood cells were obtained by cardiac puncture with a heparinized syringe containing modified Ringer solution. The cells were separated from plasma by gentle centrifugation (1,000 *g*) in a Clay, Adams "Dynac" centrifuge (Clay, Adams, Parsippany, NJ) and washed three times in 10–15 vol of isotonic (control) medium matched to the plasma osmolarity of the animal (220–250 mOsm) from which blood was obtained. The isotonic medium contained (mM) 90–110, NaCl; 3, KCl; 1, $MgCl_2$; .5, $CaCl_2$; 18, HEPES; 5, glucose adjusted to pH 7.65 at 23°C. The cells were then suspended at a hematocrit of 10% and preincubated with gentle shaking for 1.5 h before experimental treatment. Alterations in the osmolarity of the experimental medium were accomplished by varying [NaCl] unless otherwise specified. All media contained 1 mM ouabain. When the anion exchange inhibitor 4,4'diisothiocyanostilbene-2,2'disulfonate (DIDS) (Calbiochem-Behring Corp., San Diego, CA) was used, it was prepared immediately before addition to cell suspensions. To initiate an experiment, cells were separated from suspension media by gentle centrifugation and experimental medium was added to the cell pellet to yield a hematocrit of 10%. Samples (400 μl) were removed at appropriate intervals and transferred to preweighed 400 μl polyethylene tubes (Stockwell Scientific, Monterey Park, CA) and centrifuged for 4 min at 12,000 *g* (model 3200, Brinkman Instruments, Inc., Westburg, NY). After centrifugation, the supernatant was separated from the cell pellet and both were stored for analysis. The weight of the cell pellet (wet) was determined gravimetrically and then 230 μl of double distilled H_2O containing 4 mM $MgSO_4$ (cofactor for endogenous nuclease to prevent DNA/hemoglobin gel formation upon cell disruption) was added to the tube containing the pellet. Following disruption of the cell

pellet by mechanical agitation, 20 μl of 0.5 M $ZnSO_4$ was added to the lysate in order to precipitate proteins. The lysate was then centrifuged for 4 min at 12,000 *g* in order to separate the solid insoluble pellet from the clear supernatant. The supernatant was then sampled and analyzed for Na and K by flame photometry (Instrumentation Laboratories, model 443; Boston, MA) and Cl by potentiometric titration with silver ions (Buchler Chloridometer, Searle Diagnostics, Inc., Fort Lee, NJ) as previously described, (Cala, 1980; Cala and Hoffmann, 1989). Subsequently, the water-insoluble pellet was dried to constant weight at a temperature of 80°C for 24 h. Cell H_2O content was determined by the difference in wet and dry weight and corrected for trapped extracellular H_2O by an empirically determined factor (Adorante and Cala, 1987; Cala and Hoffmann, 1989).

Unidirectional ^{22}Na Flux Measurements

Unidirectional Na influx was measured by suspending cells (10% hematocrit) in experimental media containing ^{22}Na (2 μCi/ml) and removing 100 μl aliquots at pre determined time intervals (every minute; 10–15 samples/condition). The aliquots were placed in a 1.5 ml centrifuge tube and the cells were separated from the supernatant by centrifugation (5 s at 14,000 *g*) through 500 μl of isotope-free flux medium and 400 μl of dibutylpthalate. The supernatant was removed along with a portion of the dibutylpthalate and the tube was then cut just above the cell pellet (to minimize background radioactivity) and the tip of the centrifuge tube containing the cell pellet was counted for ^{22}Na using a Packard solid scintillation counter (Packard Instrument Co., Downers Grove, IL). Parallel samples were taken for determination of dry cell weight. The rate of Na uptake was calculated from linear regression ($r > .97$) analysis and flux was expressed as mmol Na/kg dcs × min. During the sampling interval the specific activity of the medium was always at least three orders of magnitude greater than that of the intracellular compartment in order to minimize back flux.

Experiments Involving Changes in Intracellular pH

To obtain measurements of intracellular pH, cells were first frozen rapidly in liquid nitrogen then thawed (Funder and Wieth, 1966) and the lysate pH was immediately measured using a radiometer Copenhagen micro pH electrode (model G297/G2, Copenhagen, DK) and an Orion Research (model 601A, Cambridge, MA) pH meter. The intracellular pH was acidified by exposing cells to the weak acid propionate or by loading nystatin permeabilized cells with NH_4 followed by resealing and media NH_4 removal (see below) (Boron and De Weer, 1976).

Propionate Induced Acidification of pH_i

The cell interior can be acidified in a predictable fashion by suspension of cells in well buffered (MES and/or HEPES) propionate medium at the desired pH (de Hemptinne, Marrannes, and Vanheel, 1983; Grinstein et al., 1984*a*). Briefly, cells were suspended in medium with Cl replaced by propionate ($pH_o = 7.65$) which causes cells to lose Cl and gain HCO_3 via the Cl/HCO_3 exchanger. Subsequently cells were transferred to propionate media at pH 6.2–6.9, with the result that propionic acid (pK = 4.87) enters the cells. The undissociated propionic acid subsequently dissociates titrating intracellular buffers and acidifying the cell interior until pH_i approximates pH_o. Because the *Amphiuma* red blood cell has a buffer capacity of 180–200 mM H/kg dry cell solid × unit ΔpH_i, propionate-induced acidification is accompanied by significant entry of propionic acid and osmotically obliged H_2O (cell swelling). In studies designed to evaluate effects of pH_i, independent of changes in cell volume, the medium osmolarity was altered by an empirically determined factor in order to maintain isotonicity. In the *Amphiuma* red blood cell the osmolarity required to maintain isotonicity in propionate media over the range of pH_i from 6 to 7.65 is given by the empirically derived, linear

relationship:

$$\text{isotonic osmolarity} = 943\text{-}99.4\ pH_o.$$

All propionate media were Cl free and contained the following: 60 mM Na propionate, 3 mM K, 1 mM Mg, 0.5 mM Ca, 30–50 mM buffer and 5 mM glucose with [Na gluconate] as needed to maintain isotonicity (see above). The buffer was either MES (for pH 6.0–6.5), HEPES (for pH > 7.0) or a combination of the two (for pH 6.6–7.0). It is necessary to titrate the solutions to 0.5 mM Ca in gluconate-containing media because gluconate is a Ca chelator (Kenyon and Gibbons, 1977). To adjust [Ca], a Ca specific electrode (Ionetics, Inc., Costa Mesa, Ca.) was employed. It was necessary to add 5.66 mmol Ca to each liter of 150 mM Na gluconate solution in order to obtain free [Ca] of .5 mM. Media [Na] was altered at fixed osmolarity by replacing Na with freshly purified tetramethyl ammonium (TMA) or *N*-methyl-D-glucamine (NMDG).

NH_4-induced Acidification of pH_i

The NH_4 prepulse method of cell acidification (Boron and De Weer, 1976) has been used in a variety of cell types. Briefly, NH_4-dependent cell acidification by the prepulse method relies upon entry of NH_4 (via NH_4 conductance and/or Na-K pump) and exit of NH_3 gas, upon removal of medium NH_4. Because of the low conductive permeability of the *Amphiuma* red blood cell and correspondingly low pump flux rate (Cala, 1980; Cala, 1983*b*; Cala, 1985*a*), the simple NH_4 prepulse method of (isotonic) cell acidification will not work. Consequently, it was necessary to increase the cell cation conductance with nystatin, (Cass and Dalmark, 1973) then isotonically load the cells in media at the desired [NH_4]. After NH_4 loading and resealing (see below), resuspension of the cells in NH_4 free medium causes both NH_3 and NH_4 to exit the cell as NH_3 since the membrane is most permeable to the gas. Consequently, the H which entered as NH_4 is trapped in the cell interior, resulting in decreased pH_i in proportion to the NH_4 ion entry and as an inverse function of cell buffer capacity. It should be pointed out that it is possible to acidify cells having a fairly active Cl/HCO_3 (Cl/OH) exchanger even in the absence of significant NH_4 permeability. That is, such cells will gain NH_3 which will combine with intracellular H which at fixed P_{CO_2} will cause $[HCO_3]_i$ to increase and exit the cell in exchange for Cl_o via the Cl/HCO_3 exchanger. This process will however produce changes in cell volume as well as pH_i which are difficult to control and predict. Because the present studies represent attempts to isolate the variables of volume and pH this approach was rejected in favor of the nystatin method.

Nystatin Loading of Amphiuma Red Blood Cells

Amphiuma red blood cells were permeabilized by suspension at a 2% hematocrit in media containing nystatin (N3503 sigma 30 mg/ml), for 30–45 min at 4°C (Cass and Dalmark, 1973; Haas, Schmidt and McManus, 1982). During permeabilization, cell volume was maintained by balancing the colloid osmotic forces due to intracellular impermeants with extracellular gluconate 63 mM and 30 mM HEPES, in a media chosen to maintain normal $[K]_i$ and $[Na]_i$ and yield the desired intracellular [NH_4]. (Note: the Donnan potential of permeabilized cells is −6.4 mV; Cala et al., 1986, thus, $[Na]_i$ and $[K]_i$ are ~1.29 times $[Na]_o$ and $[K]_o$.) Because the buffer capacity of the *Amphiuma* red cell is high (170–200 mM H/kg dcs/unit ΔpH_i) it was necessary to load cells to a final [NH_4] as high as 150 mM. In this extreme case, the loading ringer osmolarity was twice normal. While nystatin increases the membranes permeability to small cations, the reflection coefficients are not zero. Consequently the best results (cell viability) were obtained if [NH_4], and therefore medium osmolarity were changed progressively in 4–5 steps. This procedure permitted us to increase media NH_4 and osmolarity isotonically. The cells were exposed to each medium for 10–15 min for a total of 60 min exposure to

nystatin. Upon completion of the loading procedure cells were transferred to medium identical to the last loading solution yet free of nystatin and containing 1% albumin (fraction V BSA, A4503, Sigma Chemical Co., St. Louis, MO) in order to remove nystatin and restore normal membrane permeability (resealing). The resealing procedure consisted of five washes at 2% hematocrit. The cells were then suspended at a hematocrit of 10% in albumin free, DIDS (10 μM) containing medium for 30 min. The DIDS exposure is necessary in order to inhibit the Cl/HCO_3 exchanger and thus prevent the pathway from altering pH_i upon removal of NH_4 from the suspension medium. In all NH_4 washout experiments Cl_i was measured in order to assure DIDS inhibition of the anion exchanger. Those experiments where the anion exchanger was not completely blocked were not used. While the data are not shown, control studies were routinely performed in which the nystatin loaded and resealed cells were suspended in isotonic Na free (TMA or NMDG) media in order to assure that loading, resealing, and complete DIDS inhibition of the anion exchanger were successful.

NH_4 Wash Out

Just as NH_4 loading was performed in steps in order to prevent large shifts in cell volume, the washout procedure was performed in five steps in which $[NH_4]$ and osmolarity were progressively reduced thereby maintaining isotonicity. Also, in order to prevent Na-dependent changes in pH_i during the wash period, all Na was replaced by the impermeant cation TMA or NMDG in the wash media.

RESULTS

H-activated Na Uptake: Studies in Propionate Media

To investigate pH_i-dependent activation of the *Amphiuma* red blood cell Na/H exchanger, cells were suspended in iso-osmotic media at pH 6.4, 6.5, or 6.7 in which Cl was replaced by propionate. As a result of the entry of undissociated propionic acid and subsequent intracellular dissociation, pH_i is shifted in the acid direction and approaches that of the extracellular medium (de Hemptinne et al., 1983; Grinstein et al., 1984*a*, *b*). As illustrated in Fig. 1 the rate and magnitude of Na uptake by *Amphiuma* red blood cells, treated as described above, is proportional to the degree of cell acidification.

Because the buffer capacity of the *Amphiuma* red blood cell is on the order of 170–200 mmol H/kg dcs/unit change in pH_i, the cells from which the data in Fig. 1 were obtained were swollen in proportion to the degree of cell acidification resulting from net propionate entry (the propionate media are iso-osmotic yet not isotonic). Specifically, normal cell H_2O content is on the order of 1.8 l/kg dcs yet the water content of the cells used for Fig. 1 varied from 1.9 l/kg dcs for the least acidic (lowest rate of Na uptake) to 3.1 l/kg dcs for the most acid (highest rate of Na uptake). Because the volume-induced Na/H exchanger is inactive at cell volumes in excess of 1.8 l/kg dcs, (Cala, 1980; Cala, 1985*a*) it is likely that the Na uptake depicted in Fig. 1 is a result of $[H]_i$ and not volume-dependent activation.

The pH_i set point for Na/H exchange activation. If the propionate-induced Na uptake is reflective of pH regulatory function it is expected that Na flux should be a graded function of pH_i, as pH_i is decreased below the normal value of 7.00. The data in Fig. 2 depict the net Na flux in the first 15 min after isotonic acidification of the cell interior (in propionate media) to the indicated value. Note that these data

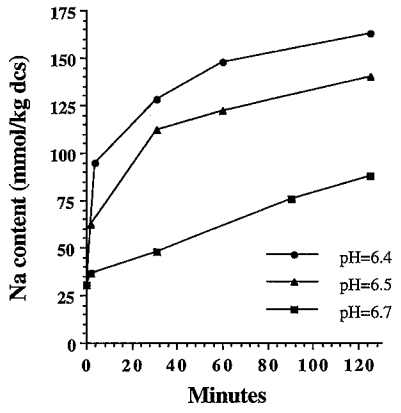

FIGURE 1. Net Na/H exchange flux in *Amphiuma* red blood cells acidified by suspension in Na propionate-containing media at pH = 6.4, 6.5, and 6.7. The media are iso-osmotic yet not isotonic because cells are swollen due to propionic acid entry. Cell swelling is inversely proportional to pH_o. Thus, the highest net Na/H exchange rate and magnitude is associated with the cells at pH 6.4 which are also the most swollen. These data are typical of eight similar experiments performed upon cells with pH_i varied from pH_i = 6 to 6.9. Within any group of cells net Na uptake is inversely proportional to pH_i. Between groups however, the absolute magnitude of flux may vary.

represent the net Na uptake in 15 min and are not a measure of initial rate because, as shown in Fig. 1, Na uptake is not linear during this time. Nevertheless, these data illustrate that acid-induced net Na uptake is an inverse function (exponential) of pH_i which approaches zero as cell pH approaches the normal value of 7.00. These data define the pH set point for activation of the *Amphiuma* red blood cell Na/H exchanger.

The nature of pH-activated Na uptake. As is the case with volume-induced Na uptake, acid-induced Na uptake by *Amphiuma* red blood cells is inhibited by amiloride (Cala, 1980; Siebens and Kregenow, 1985) and its 5 min substituted analogs (not shown). While this observation is consistent with the interpretation that both volume and pH-induced Na uptake are via Na/H exchange, it is not unique to this interpretation. As previously discussed, a more direct, less ambiguous demonstration of alkali metal/H exchange can be achieved based upon the correspondence between net alkali metal ion flux and the thermodynamic force to which flux is coupled (Cala, 1983*a*; Cala, 1985*a*, *b*). In the case of Na/H exchange, the force driving net transport is the difference in the chemical potential differences for Na

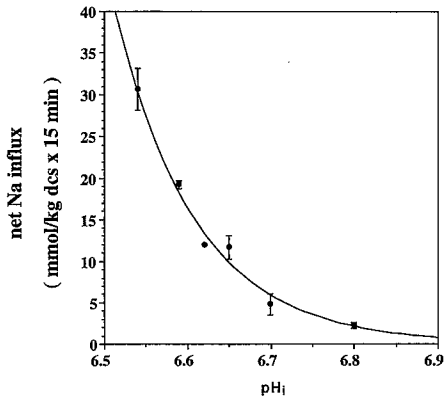

FIGURE 2. Net Na flux rate in isotonic (see Methods) media as a function of pH_i of cells acidified due to propionic acid entry. Net Na flux approaches zero as pH_i approaches the normal intracellular value of 6.9–7.1. These results are presented as mean ± SEM for five individual samples.

and H ($\Delta\mu_{Na} - \Delta\mu_H$). The data in Fig. 3 illustrate the relationship between net Na flux by cells acidified by suspension in isotonic propionate medium and the force driving net Na/H exchange. The fact that force and flux change sign at the origin identifies the pathway as Na/H exchange.

NH_4 Pre-pulse: pH Regulation

The pH_i set point for Na/H exchange deactivation. While the above data illustrate that acid pH_i can induce net Na/H exchange flux, they do not address pH regulatory capacity, since cells in propionate media are unable to regulate pH. That is, as H is removed from the cell by net Na/H exchange, the equilibrium between propionate and propionic acid is disrupted causing more of the undissociated acid to enter the cell, clamping pH_i at the value of pH_o. Thus, the data obtained from propionate-acidified cells, demonstrate that Na/H exchange is activated as pH_i is decreased below the normal value of 7.00 yet the data do not demonstrate pH regulation. To evaluate Na/H exchange-dependent pH_i regulatory function and determine the pH_i at which Na/H exchange deactivates, it is necessary to acidify the cell interior with a

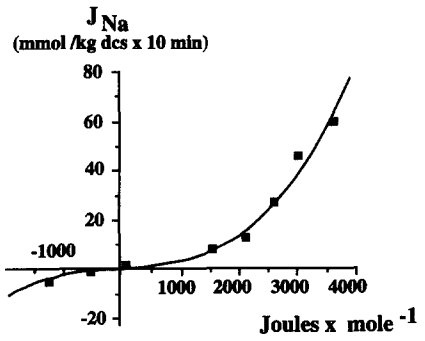

FIGURE 3. The correspondence between acid-induced net Na flux in propionate acidified (pH_i = pH_o = 6.2; isotonic media) cells and the force driving net Na/H exchange ($\Delta\mu_{Na} - \Delta\mu_H$). As force approaches zero so too does net Na flux and as force changes sign net flux changes direction. The upper right quadrant depicts net Na uptake while the lower left quadrant depicts efflux. These results are typical of four experiments.

discrete acid pulse. A commonly employed means of acidifying the cytosol is the NH_4 pre pulse method first described by Boron and De Weer (1976). The data depicted in Fig. 4, illustrate that in response to acid shifts in pH_i, the *Amphiuma* red cell Na/H exchanger is: (*a*) activated in proportion to the change in pH_i (compare left hand ordinates), and (*b*) deactivated upon restoration of normal intracellular pH (6.9–7.1). While the studies in propionate define the pH_i set point for activation, the data obtained following NH_4 washout illustrate pH regulatory Na/H exchange in the *Amphiuma* red blood cell and demonstrate that the pH_i for activation and deactivation is the same ($pH_i \approx 7.00$).

The pH and Volume Set Points of the Na/H Exchanger

The data in Figs. 2 and 4 illustrate that the set point for pH regulatory Na/H exchange is the normal pH_i of the *Amphiuma* red cell (pH 7.0). Implicit in these data is the notion that pH-dependent activation of Na/H exchange is able to overcome volume-dependent deactivation of the flux pathway. That is, the cells from which the data in Fig. 1 were obtained were swollen, (due to propionic acid and H_2O entry)

beyond the volume at which shrinkage-activated Na/H exchange is deactivated. Also, the cells used to obtain the data in Fig. 4 were swollen beyond control volume as a result of the pH regulatory, net Na/H exchange flux and consequent H_2O uptake. Clearly then, swelling-dependent deactivation is unable to override pH-dependent activation. This observation calls into question the effect(s) of pH_i on the volume-induced Na/H exchange. That is, if the pH set point for the Na/H exchanger is the normal intracellular pH (7.00), how is it that the shrinkage activated Na/H exchange is not deactivated by the cell alkalization resulting from volume regulatory Na/H exchange? One possibility is that, as described by Grinstein et al. (1984*a*) there is a volume-induced shift in the pH set point.

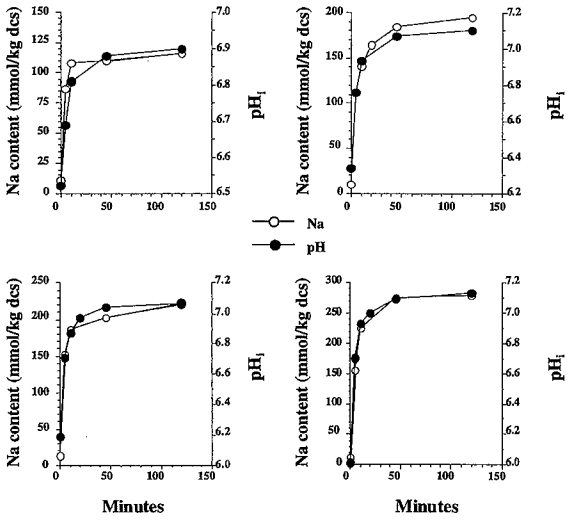

FIGURE 4. Na content (○) and intracellular pH (●) as a function of time following intracellular acidification by the NH_4 prepulse method. Clockwise from upper left cells were acidified to pH_i = 6.45, 6.3, 6.1, and 6.0. Cells were acidified by isotonically loading nystatin permeabilized cells with 60, 80, 100, and 120 mM NH_4 followed by resealing and NH_4 washout. The results were obtained from a single experiment using subsamples of cells obtained from the same animal and are representative of seven similar experiments. The variability between experiments (animal to animal) reflects variability in the degree to which cells are acidified. As demonstrated above, the response to acidification is always net Na uptake and H loss until pH_i = 6.9–7.1. Note that the net Na uptake is equal to the calculated amount of intracellular NH_4 before wash out. The amount of intracellular NH_4 is equal to the product of the Donnan ratio (1.29) and $[NH_4]_o$ times cell H_2O content which is $\approx$1.9 l/kg dcs.

pH-dependent Inactivation of Volume-induced Na/H Exchange

To assess the existence of a pH_i set point for volume-induced Na/H exchange, studies were performed upon cells shrunken (Fig. 5) in medium 1.65 times normal osmolarity (400 mOsm). This treatment results in a $\approx$40% reduction in volume and activates robust net Na/H exchange. Because for this degree of shrinkage, the cells must gain more than 200 mmol of solute/Kg dcs in order to restore normal volume, they must lose large amounts of H ion. Due to the presence of the Cl/HCO_3 exchanger, however, much of the H lost through the Na/H exchanger is buffered by a parallel loss of HCO_3 (Fig. 5*A*, see also Cala, 1980). Due to the large volume-induced net Na/H exchange flux the Cl/HCO_3 exchanger is able to limit but not prevent changes in pH_i. In contrast, inhibition of the anion exchanger with DIDS

causes pH_i of a subsample of cells from the same experiment to increase to about 8.0 during volume regulation (Fig. 5 *B*). It should be noticed that while cells depicted in Fig. 5 *A* regulate volume back to normal levels, those in Fig. 5 *B* were not able to regulate volume because as a result of increased intracellular $[Na]_i$ and pH_i, the force driving the Na/H exchanger has dissipated before volume recovery. As such, a value of $pH_i \approx 8.0$ does not represent a new pH_i set point.

Priority for pH versus volume regulation. Given that the mechanisms for volume and pH-dependent deactivation appear to be separate and distinct, the connection between volume and pH-dependent activation mechanisms was investigated. We attempted to determine whether or not the volume and pH signals are prioritized.

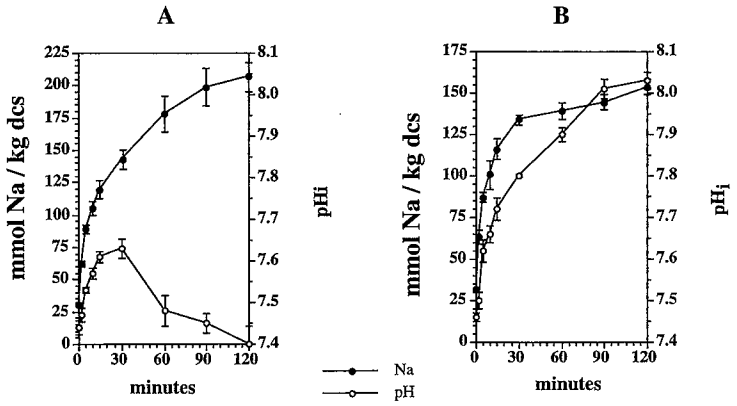

FIGURE 5. Intracellular Na (●) content (mmol/kg dcs) and pH (○) as a function of time following osmotic shrinkage (media osmolarity is 1.65 × control) in the absence (*a*) and presence (*b*) of the anion exchange inhibitor DIDS (15 μM). These data were obtained from cells suspended in media containing 60 mM HEPES; twice the normal concentration. Because these experiments are performed in media with high HEPES the [Cl] has been correspondingly reduced resulting in an initial net loss of cell Cl and gain in cell HCO_3 through the anion exchanger. It is this phenomenon that is responsible for the elevated initial values of pH_i. The results are presented as mean ± SEM for four experiments performed under identical conditions.

Accordingly, cells were either shrunken before acidification (by NH_4 washout) or acidified before osmotic shrinkage. The changes in cell volume resulting from the above treatments are depicted in Fig. 6. Control volume for the cells used in this experiment was ~2 liters/kg dcs. These data illustrate that the *Amphiuma* red cell Na/H exchanger responds to the first stimulus, be it a change in cell volume or pH. That is, cells which are simply shrunken (*solid triangles*) or shrunken before cell acidification (*open circles*) regulate volume back to control levels. In contrast, if cells are acidified before osmotic shrinkage, (*solid squares*) the Na/H exchanger is activated and extrudes H (causing the cells to gain Na and therefore H_2O) until pH_i is regulated and then deactivates even though cell volume is abnormal (in the present

case volume is .25 liter/kg dcs below normal). If however, cells are acidified in isotonic medium (*solid circles*) the Na/H exchanger is activated and extrudes H (causing the cells to gain Na and therefore H_2O), until pH_i is regulated, which in the present case results in a volume increase to almost 1 liter/kg dcs above normal. Clearly, the cell is somehow prioritizing signals for Na/H exchange-dependent volume and pH regulation. Thus, it appears that the set point for deactivation of net Na/H exchange is determined by the first stimulus, be it decreased cell volume or pH. It is noteworthy that the initial rate of water gain (net Na/H exchange transport rate) is higher in all cells in which pH_i has been decreased. This is a reflection of the affinity of the Na/H exchanger for $[H]_i$ (see below).

Identity of Volume and pH Induced Na/H Exchangers

Based upon the above data, if the volume and pH-induced responses are mediated by the same Na/H exchanger, then it must be under the control of separate and

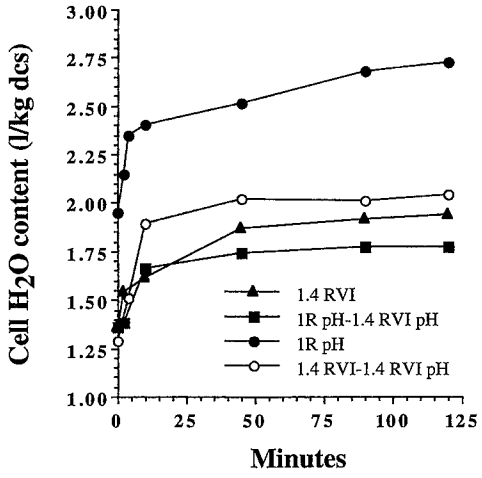

FIGURE 6. Cell water content as a function of time following osmotic shrinkage, 1.4 RVI, (▲); osmotic shrinkage followed by intracellular acidification to pH_i 6.4, 1.4 RVI–1.4 RVI pH, (○); cell acidification to pH 6.4 at control volume (2 liters/kg dcs), 1R pH, (●); and intracellular acidification to 6.4 before cell shrinkage, 1R pH–1.4 R, (■). Cells were acidified to pH 6.4 by isotonic loading with NH_4 as described in Methods. These results are representative of four such studies.

mutually exclusive activation/deactivation mechanisms. Alternatively, the volume and pH-induced Na/H exchange functions may reflect separate populations of Na/H exchangers. To address this issue, kinetic studies were performed to evaluate the correspondence of the kinetic constants of volume and pH-activated Na/H exchange. Fig. 7 *A* shows that the unidirectional Na uptake rate is a saturable function of the extracellular Na concentration. A double reciprocal (Lineweaver-Burk) plot of these data is presented in Fig. 7 *B*. In the volume-activated cells the apparent K_m and V_{max} for Na were 44.9 mM and 4.74 mmol/kg dcs/min, respectively, whereas for the pH-activated cells a K_m of 9.3 mM and a V_{max} of 9.26 mmol/kg dcs/min were obtained. These results indicate that the higher rate of Na (and therefore water) uptake observed in the pH-activated cells (Fig. 6), results from a higher apparent affinity for Na and an increased V_{max}. At first glance, the difference in the K_m and V_{max} for Na suggests that Na/H exchange in volume and pH-activated cells is the result of different Na/H exchange proteins. If however, as shown in Fig. 8 the Na/H

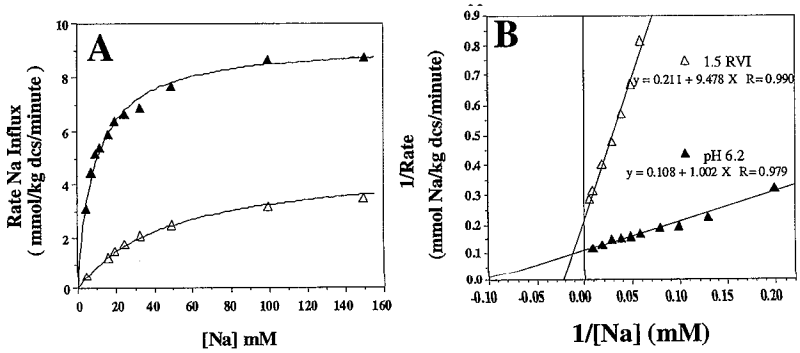

FIGURE 7. (*A*) Unidirectional Na uptake rate as a function of $[Na]_o$ by cells osmotically shrunken in media of osmolarity 1.5 times iso-osmotic (Δ) or by cells acidified (pH_i = 6.2; ▲) by ammonium pre pulse. 7 *B* is a double reciprocal plot of the data in *A*. These data are typical of three similar experiments. The apparent V_{max} for volume-activated cells is 4.74 mmol/kg dcs × min and the apparent k_m for Na is 44.94 mM. In contrast the apparent V_{max} of pH-activated cells is 9.26 mmol/kg dcs × min while the apparent k_m for Na is 9.28 mM.

exchanger is activated over an increasing range of pH_i from 6 to 6.5 the apparent Na affinity of the pH-activated cells decreases, approaching that of volume-activated cells, as pH_i is progressively increased. This behavior is precisely that expected of a bireactant enzyme as one substrate (Na) is varied at subsaturating concentrations of the second (H) (Segel, 1975). In simple terms, the K_m for Na cannot be evaluated if the reaction rate is limited by [H]. Consequently, if $[H]_i$ is much lower than that required for saturation, the apparent K_m for Na will be an overestimate of the true K_m. The data are consistent with the notion that both cell volume and pH regulation are mediated by the same transport protein which exhibits different apparent Na affinities, reflecting differences in $[H]_i$, during volume and pH regulation.

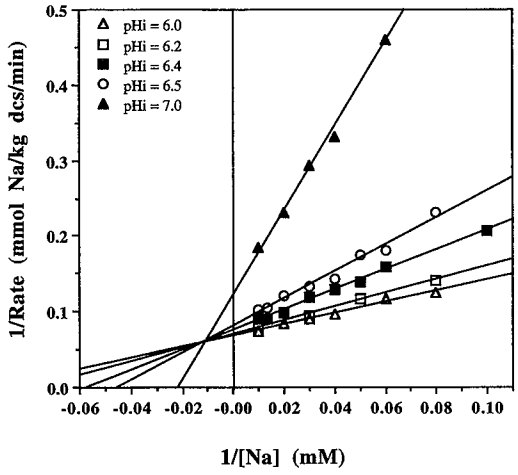

FIGURE 8. Double reciprocal plot of unidirectional Na uptake vs $[Na]_o$ for cells acidified (pH_i = 6.0 to 6.5), isotonically by ammonium prepulse or osmotically shrunken in media 1.5 times that of iso-osmotic medium (pH_i = 7.0). Note that as pH_i is decreased the apparent km for Na is increased. See text for detailed explanation. These data are representative of the relationship between cells acidified to pH_i < 6.5 as compared to cells at normal pH_i (≈7.00) in medium 1.5 times normal osmolarity as seen in three similar experiments.

DISCUSSION

Acid-induced Na Uptake

The pH-induced Na uptake by *Amphiuma* red blood cells is Cl-independent in that it occurs in media where all Cl is replaced by propionate (Figs. 1–3). The acid-activated Na uptake is also DIDS insensitive as demonstrated by the data depicted in Figs. 4, 6, 7, and 8. In addition, the Na uptake occurs in nominally HCO_3 free media ($[HCO_3]_o$ = .24 mM at pH_o, 7.65) because all media are prepared using non-HCO_3 buffers. Finally, the data in Fig. 3 (obtained using cells in Cl free media at $[HCO_3]_o$ = 0.008 mM) demonstrate that the net Na flux is in close correspondence with the magnitude and more important the direction (sign) of the force ($\Delta\mu_{Na} - \Delta\mu_H$) driving Na/H exchangers. While not shown, the driving force for conductive Na flux ($\Delta\mu_{Na} + zFE_m$) equals 4824 Joules/mol when the pH-activated net Na flux equals zero (see also Cala, 1985*a;* Cala 1986*b*). In addition, all $[H]_i$-induced Na gain is inhibited by the Na/H exchange inhibitor ethylisopropyl amiloride (data not shown). Taken together the above data eliminate conductive as well as Cl and or HCO_3 coupled Na-dependent hydrogen extrusion mechanisms and strongly favor Na/H exchange as the means of pH_i-induced Na uptake and acid extrusion.

In previous studies of osmotically challenged *Amphiuma* red blood cells, it was established that net Na uptake following osmotic shrinkage is due to Na/H exchange. Further, this and other laboratories (Cala, 1980; Siebens and Kregenow, 1985) have demonstrated that the *Amphiuma* red cell Na/H exchanger is not only volume-sensitive but volume-regulatory. This conclusion is based on the observations that net Na/H exchange flux is: (*a*) activated as volume is reduced below control levels; (*b*) a graded function of cell shrinkage; and (*c*) active until normal cell volume is restored. The present studies, employing the same criteria, establish that the *Amphiuma* red cell Na/H exchanger will function in a pH regulatory role. That is, *Amphiuma* red cell Na/H exchange flux is: (*a*) activated as cell pH is decreased below normal; (*b*) proportional in rate and magnitude to the degree to which the cell interior is acidified (Figs. 1 and 2); and most important, (*c*) able to restore pH_i to normal (Fig. 4). To address points *a* and *b* above, cells were acidified by suspension in Na propionate media, titrated to the desired pH over the range of 6.2 to 7.00 (Fig. 2). The data illustrate that the magnitude of net Na/H exchange is an inverse function of pH_i and further, that at normal pH_i (7.00) net Na/H exchange flux approaches zero. This last point is significant as it relates to the pH_i at which Na/H exchange is activated: the pH_i "set point" for activation is the normal intracellular pH. The studies in propionate media do not however, address pH regulatory function or the set point for deactivation, because in propionate media, the cells cannot regulate pH. That is, net H efflux via Na/H exchange shifts the propionic acid equilibrium toward the dissociated form thereby favoring entry of more of the undissociated acid. Thus, cells in propionate media are effectively pH clamped.

The issue of pH regulatory function of the *Amphiuma* red cell Na/H exchanger was addressed by nystatin loading cells in NH_4 (40–120 mM) containing media and subsequently suspending the cells in NH_4 free medium. These data illustrate that net Na/H exchange flux is: (*a*) activated in response to decreases in pH_i below the

normal value of 7.00; (*b*) inversely proportional to $pH_i < 7.00$; and (*c*) that net Na/H exchange flux ceases when pH_i is returned to normal, establishing the existence of a pH_i set point for deactivation and its equivalence with the set point for activation. Thus, the *Amphiuma* red blood cell Na/H exchanger is capable of both volume and pH regulation. Further, as demonstrated by studies of osmotically shrunken cells in the absence and presence of the Cl/HCO_3 exchange inhibitor DIDS (Fig. 5), the *Amphiuma* Na/H exchanger will regulate volume and disrupt pH_i. In contrast, the data obtained from cells acidified at control volume and cells acidified first and then osmotically shrunken (Fig. 6) illustrate that pH_i is regulated at the expense of cell volume disturbance (see also Figs. 1, 2, and 4). That is, the *Amphiuma* red cell Na/H exchanger does not deactivate at normal cell volume when activated by pH_i, nor does it deactivate at normal pH_i when activated by volume (Fig. 5). In this regard the data in Fig. 5 illustrate that volume-dependent activation of net Na/H exchange causes what appears to be a shift in the pH set point to a value higher than the normal pH_i (7.00). In Fig. 5 *B* the Na/H exchanger of cells treated with the anion exchange inhibitor DIDS causes Na_i to increase and the cells to alkalinize to a pH of ≈ 8.0 at which point further net Na uptake and H extrusion cease. Because the DIDS treated cells (Fig. 5 *B*) gain less Na and Cl than those in the absence of DIDS (Fig. 5 *A*) H_2O uptake is reduced and volume regulation is incomplete. Such behavior is consistent with pH_i-dependent deactivation of net Na/H exchange and is often cited as an example of a shift in the pH_i set point. In the present case however, net Na/H exchange and H_2O uptake cease not as a consequence of decreased $[H]_i$ (reflecting the pH_i set point for Na/H exchange deactivation) but because the thermodynamic driving force for net Na/H exchange has dissipated. If then, volume perturbation causes the pH_i set point to shift, it is nominally 1 pH unit higher than normal, yet the precise value of the pH_i set point of volume activated Na/H exchange cannot be ascertained from studies of net flux due to thermodynamic constraints.

The above behavior is in contrast to that described for human lymphocytes where pH appears to override volume as a stimulus/repressor of Na/H exchange function. A series of careful studies performed by Grinstein and coworkers (Grinstein et al., 1983; Grinstein et al., 1985*a, b*) have shown that following osmotic shrinkage lymphocyte volume is regulated by a Na/H exchanger that functions only if pH_i is less than ~ 7.4. The pH_i sensitivity of the lymphocyte volume-activated Na/H exchanger is thought, at least in part, to explain the incomplete nature of volume regulation by these cells. That is, the lymphocyte, as is the case with many other cell types, exhibits incomplete volume regulation in true hyper-osmotic medium. If however, the cells are shrunken by suspension in media of normal osmolarity following cell swelling and volume regulation in hypo-osmotic media, then cell volume regulation is more complete (Grinstein et al., 1984*c*). This behavior is a consequence of the fact that the volume of osmotically obliged water which accompanies each mmol of net solute flux is an inverse function of medium osmolarity. As such, cells shrunken in more dilute medium gain more H_2O with each millimole of Na/H exchange-mediated net Na uptake than do cells in more concentrated media. Consequently, the change in pH_i (Na/H exchange-dependent net H flux) associated with a given volume of osmotically obliged H_2O transport will be directly proportional to medium osmolarity. Thus, volume regulation which is less complete as medium

osmolarity is increased may be diagnostic of pH-dependent deactivation of volume-activated Na/H exchange. Consistent with this view, the data of Grinstein et al. (1984*a*) demonstrate that: (*a*) the pH set point of the lymphocyte Na/H is shifted by ~0.2 pH U in the alkaline direction when cells are osmotically shrunken; and (*b*) that volume-activated net Na/H exchange flux is deactivated at the new pH_i set point regardless of whether or not cell volume has been restored. Taken together, these observations suggest that the human lymphocyte Na/H exchanger serves only a pH regulatory role, since pH_i is able to override and prevent complete regulation of cell volume.

Na/H Exchange-dependent Volume and pH Regulation: Conflicting Demands

Na/H exchanger in poorly buffered cells: pH_i change is accompanied by little change in volume. The above discussion points out the conflicting demands of Na/H exchange-dependent volume and pH regulatory functions. That is, in order for the Na/H exchanger to produce changes in cell volume it is necessary for hydrogen ion to be effectively buffered. In the absence of H buffering, net Na and water uptake will be limited because the force driving net Na/H exchange will approach zero as $[H]_i/[H]_o$ approaches $[Na]_i/[Na]_o$ (see Fig. 5 *B*). To illustrate this point, a cell with a water content of 2 liters/kg, a buffer capacity of zero, $[Na]_i = 23$, $[Na]_o = 150$, pH_i and pH_o of 7.0 and 7.4, respectively, can gain only 1.88×10^{-7} mol of Na/kg dcs at which point, as a result of changes in pH_i, the force driving net Na/H exchange is dissipated. Thus, in a cell having no buffer capacity, the Na/H exchanger would be an ideal pH regulatory mechanism since, it could change pH without producing measurable changes in Na_i and therefore cell volume, yet it would be ineffective in a volume regulatory capacity.

Na/H exchange in well buffered cells: volume change with little change in pH_i. Since most cells are well buffered by protein (fixed) and/or dynamic buffers (i.e., the Cl/HCO_3 exchanger) it is possible for the Na/H exchanger to function in a volume regulatory capacity, without dissipating the force which drives it. That is, in the case of cells with both fixed protein buffers and a dynamic buffer such as the anion exchanger, volume can be regulated over a relatively large range with only modest alteration in pH_i (and therefore in the force driving Na/H exchange, see Fig. 5 *A*). In addition to its role as a dynamic buffer, the anion exchanger increases the volume regulatory efficiency of the Na/H exchanger. That is, when functioning in parallel with a Cl/HCO_3 exchange pathway, each time the Na/H exchanger cycles the cell gains a Na ion and some fraction of a Cl ion (depending on the stoichiometry of the functional coupling between the Na/H and Cl/HCO_3 exchangers; which is in turn a function of cell and medium fixed buffer capacity). Thus, the number of osmotically active particles and therefore the volume of osmotically obliged water transported are increased as a result of the parallel operation of Na/H and Cl/HCO_3 exchangers, while changes in pH_i are minimized; ideal for volume regulation yet suboptimal for the regulation of pH_i.

Thus, the ability of the Na/H exchanger to function effectively as a volume and/or pH regulatory pathway will be highly influenced by both fixed and dynamic buffers. This point is further illustrated by experiments of the type shown in Fig. 5, in which cells were osmotically shrunken in the absence or presence of the Cl/HCO_3 exchange inhibitor DIDS. The cells in the absence of DIDS (Fig. 5 *A*) are shrunken from a

normal volume of 1.8 liters/kg dcs to a volume of 1.23 liters/kg dcs and gain Na, Cl and H_2O until the normal cell volume of 1.81 liters/kg dcs is restored. Thus, the decrease in net Na uptake with time coincides with the restoration of cell volume. In contrast, cells exposed to DIDS, (Fig. 5 *B*) while shrunken to the same degree, achieve a volume of 1.38 liters/kg dcs during the time of the experiment. The difference in H_2O gain in the presence and absence of DIDS is attributable to the fact that the DIDS free cells gained 163 mmol of Cl while the DIDS treated cells gained <9 mmol of Cl. In addition, the Na uptake by the DIDS treated cells ceases, not because cell volume is restored but because the force driving net Na/H exchange decreases from 5660 Joules/mol at the start of the experiment to between 0 and 200 Joules/mol at the experiment's conclusion (the implications of this point are demonstrated in Fig. 3).

In summary, volume regulatory Na/H exchange in a well buffered cell will lead to increased Na and H_2O content with minor changes in pH. While pH changes secondary to volume regulatory Na/H exchange flux can be buffered, increased Na and H_2O content resulting from pH regulatory Na/H flux are imposed upon the cell. Thus, to the extent that the cell is well buffered and Na/H exchange is responsible for both volume and pH regulation, then regulation of one parameter will lead to disturbance of the other; this is particularly true with respect to disruption of cell volume which occurs as a consequence of pH regulation.

The Identity of the Volume and pH Regulatory Na/H Exchanger

Implicit in the above discussion is the notion that a single effector, the Na/H exchanger, is under the control of separate and distinct sensing mechanisms for volume and pH. Evidence for the molecular equivalence of volume and pH regulatory Na/H exchangers is based largely on: (*a*) sensitivity to amiloride and its analogs (with the compounds having a more lipophilic group replacing the amino group in the 5 position on the pyrazine ring being the most potent) or as in the case of the human lymphocyte; and (*b*) the observation that both volume and pH-activated Na/H exchange are deactivated by alkaline pH_i, (Grinstein et al., 1984*a*, *c*, and Rotin and Grinstein, 1989). The data presented in Fig. 8 are relevant to the issue of the molecular equivalence of volume and pH regulatory Na/H exchangers of the *Amphiuma* red blood cell and studies of Na/H exchange in general. In this regard, the prevailing view is that there are two main types of Na/H exchanger: that in the apical membrane of epithelia involved in net fluid and electrolyte transport and that in non epithelial cells and in the basolateral membranes of epithelia (for review see Clark and Limbird, 1991).

The apical membrane Na/H exchanger is characterized by relatively high apparent Na affinity ($K_m^{Na} \approx 10$ mM), low amiloride sensitivity, and until recently was thought to function constitutively and mediate net Na and therefore fluid absorption. In contrast, pH and volume regulatory functions of the Na/H exchanger (housekeeper) found in the basolateral membranes of epithelia and the membranes of non epithelial cells, are characterized by a relatively low apparent Na affinity ($K_m^{Na} \geq 40$ mM), and relatively high amiloride sensitivity. It is interesting to note that kinetic studies of the apical membrane Na/H exchanger are typically performed on vesicles in low medium [Na] with Na flux driven by a steep transvesicle pH gradient (pH_i close to 6 and $pH_o = 8$). In contrast, the housekeeping studies, particularly those

involving volume regulation, are performed using intact cells where pH_i is 7.0 to 7.5. In studies of pH regulation by intact cells, the pH_i, while reduced below normal, is rarely less than 6.8. Consequently, a single Na/H exchange with K_m^{Na} of 2.5×10^{-7} M (pH_i 6.6) will appear to have very different values of apparent K_m^{Na} as pH is varied between 6.00 and 7.4 (Segel, 1975). Therefore, attempts to evaluate Na/H exchangers based upon the kinetic constants for Na must be performed at the same pH or ideally where pH on the H binding aspect of the membrane is saturating. As in the present case where the Na/H exchanger is both volume and pH-activated this approach is not practical because, in order to obtain an unambiguous result, it is necessary to isolate the variables of cell volume and pH.

A further complication in this type of analysis is the fact that the Na/H exchange is an inducible system where the population of active carriers changes in proportion to the magnitude of the activating stimulus (Maldonado and Cala, 1992). Consequently, we have chosen to perform kinetic studies on pH-activated cells over a range of pH_i from 6.1 to 6.5 and on volume-activated cells at $pH_i = 7.0$. These data illustrate that as pH_i of the pH-activated cells approaches that of the volume-activated cells, K_m^{Na} approaches that of the volume-activated cells (Fig. 7 *B*). More convincing, is the fact that solution of the forward rate equation at a pH of 7.00 yields an apparent K_m^{Na} for pH-activated cells, equivalent to that obtained in studies of volume-activated cells (Maldonado and Cala, 1993). These observations are consistent with the notion that both volume and pH regulation in *Amphiuma* red blood cells are mediated by the same Na/H exchange protein. Alternatively, but less likely, these same data can be explained by a model featuring two separate populations of Na/H exchangers identical with regard to transport kinetics yet differing with respect to the basis for their control. In either case the model must be consistent with the prioritized nature of *Amphiuma* red blood cell volume and pH regulatory behavior. That is, the volume and pH regulatory control mechanisms appear to be distinct and cross inhibitory: pH-activation of the Na/H exchanger precludes volume regulation and vise versa.

The Regulation of Volume and pH by a Single Na/H Exchange: Physiological Implications

The pH regulatory behavior of the Na/H exchanger, while interesting as it relates to the *Amphiuma* red blood cell is, to the best of our knowledge, without functional significance. That is, due to the robust Cl/HCO_3 exchanger of *Amphiuma* red blood cells, pH regulatory Na/H exchange would be terribly inefficient at all but the lowest [HCO_3]. pH_i regulatory Na/H exchange is however functionally significant, in the context of hypoxic and ischemic cell damage. In this regard (Cala et al., 1988; Anderson, Murphy, Steenbergen, London, and Cala, 1990), we have demonstrated that most if not all of the myocyte $[Ca^{2+}]_i$ increase resulting from hypoxia, is a direct result of the pH (metabolic H) activated Na/H exchanger. That is, during hypoxia, pH-activated Na/H exchange results in increased $[Na]_i$, reducing the driving force for Ca efflux via the Na/Ca exchanger, with the result that $[Ca^{2+}]_i$ increases. This $[Na]_i$-dependent rise in intracellular Ca^{2+} is believed to be directly responsible for a variety of changes associated with hypoxia-induced cell damage. Within this context, we are currently examining the interplay between volume and pH in studies of the effects of hypertonic exposure on pH-induced Na/H exchange flux during hypoxic and ischemic episodes (Ho, Anderson, Holcroft, and Cala, 1991).

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