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Intracellular-pH dependence of Na–H exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells

(growth factors/acid–base metabolism)

GREGORY BOYARSKY*, MICHAEL B. GANZ†‡, E. J. CRAGOE, JR.§, AND WALTER F. BORON*¶

*Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510; †Department of Medicine, Yale University School of Medicine, and ‡The West Haven Veterans Administration Medical Center, West Haven, CT 06516; and §2211 Oak Terrace Drive, Lansdale, PA 19446

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ABSTRACT **We studied intracellular pH (pH_i) regulation in the absence of HCO_3^- in single mesangial cells (MCs) with the pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5(and -6)carboxyfluorescein. Our approach was to acid load the cells by an NH_4^+ prepulse and to monitor the subsequent pH_i recovery. Previous work on MCs and other cells has shown that the recovery is prevented by adding ethylisopropyl amiloride (EIPA) or removing Na^+ before the recovery begins, suggesting that at low pH_i only Na–H exchange contributes to the recovery. This conclusion is often extrapolated to the entire pH_i range. To test this, we interrupted the recovery with EIPA at various pH_i values, finding that EIPA unmasked a background acidification that was negligible at pH_i less than ≈6.7 but increased steeply at higher pH_i values. Correcting the total recovery rate for this EIPA-insensitive component, we found that the EIPA-sensitive (Na–H exchange) rate fell steeply with increasing pH_i between 6.3 and 6.7 but was relatively pH_i insensitive between 6.7 and 7.2. Thus, the recovery halts as pH_i approaches ≈7.2 not so much because Na–H exchange slows, but because acid loading accelerates. Applying the mitogen arginine vasopressin (AVP; 100 nM) caused a rapid pH_i decrease of ≈0.4, followed by a slower increase to a level ≈0.15 higher than the initial pH_i. Coincident with this biphasic change in pH_i was a biphasic change in Na–H exchange kinetics. In the early phase (i.e., pH_i recovery commencing ≈8 min after AVP addition), AVP linearized the pH_i dependence of the exchanger; its rate was unaffected by AVP at pH_i less than ≈6.7 but was progressively inhibited at higher pH_i values. In the later phase (i.e., pH_i recovery commencing ≈14 min after AVP addition), AVP shifted this linear pH_i dependence in the alkaline direction; the exchanger was stimulated at pH_i <6.9 but was modestly inhibited at higher pH_i values (i.e., in the physiological range). At all times, AVP greatly inhibited background acid loading. Thus, AVP raises steady-state pH_i not because Na–H exchange is stimulated but because, although the exchanger is inhibited, acid loading is inhibited even more.**

The Na–H exchanger is an acid-extrusion mechanism located in the plasma membrane of nearly all mammalian cells. It plays a key role in the regulation of intracellular pH (pH_i) (1) and, in some cells, the regulation of cell volume (2). Furthermore, it is generally accepted that the intracellular alkalinization elicited by growth factors applied in the absence of CO_2/HCO_3^- is caused by stimulation of Na–H exchange (3–6). One of the hallmarks of the Na–H exchanger is thought to be its pH_i dependence: an acid-extrusion rate (J_{Na-H}) that is zero at pH_i values above a threshold pH_i, and that increases more or less linearly at lower pH_i values. The kinetic basis of growth factor-induced stimulation of Na–H exchange is thought to be an increase (i.e., alkaline shift) in the pH_i threshold (3) and/or an increased J_{Na-H}–pH_i slope (7).

pH_i threshold behavior was first demonstrated for another acid-extrusion mechanism, the Na^+-dependent Cl–HCO_3^- exchanger (8). The approach was to block the exchanger with a stilbene derivative at several points during the recovery of pH_i from an acid load and to compute the stilbene-sensitive component of the pH_i recovery over a wide pH_i range. A comparable approach has been applied to the Na–H exchanger in only a few cases. However, in these studies the Na–H exchanger clearly exhibited a threshold (ref. 9; G.B., N. Rosenthal, E. Barrett, and W.F.B., unpublished results). In the present experiments, we examine the pH_i dependence of the Na–H exchanger in rat mesangial cells (MCs), smooth muscle-like cells from the glomerulus of the kidney. Ethylisopropyl amiloride (EIPA), a potent inhibitor of Na–H exchange, was used to interrupt the recovery of pH_i from an acid load in the nominal absence of CO_2/HCO_3^- (11). We found that the Na–H exchanger of quiescent MCs lacks a pH_i threshold but that the growth factor arginine vasopressin (AVP) causes the appearance of a threshold and also leads to other time-dependent changes in the pH_i dependence of the exchanger.

METHODS

Rat MCs from passages 2–5 were grown on glass coverslips to <50% confluence and rendered quiescent by reducing fetal calf serum to 0.5% for at least 24 hr (12). The standard Hepes-buffered solution, titrated to pH 7.4, contained 145 mM Na^+, 5 mM K^+, 1.2 mM Mg^{2+}, 1 mM Ca^{2+}, 110 mM Cl^-, 1.2 mM, SO_4^{2-}, 2 mM phosphate, 17.8 mM Hepes (anionic form), 14.4 mM Hepes (neutral form), and 10.5 mM glucose. NH_3/NH_4^+-containing solutions were prepared by replacing 20 mM NaCl by 20 mM NH_4Cl. AVP was obtained from Sigma. EIPA was prepared as a 50 mM stock in dimethyl sulfoxide and was diluted 1:1000 into EIPA-containing solutions.

Our approach for measuring pH_i in single MCs has been described (11). pH_i was measured in single MCs by using a microscope-based fluorometer and the fluorescent pH-sensitive indicator 2′,7′-bis(2-carboxyethyl)-5(and -6)carboxyfluorescein (13), introduced as its acetoxymethyl ester derivative (Molecular Probes). Intracellular dye was alternately excited at 440 and 490 nm as the emitted fluorescence intensity (I) was monitored at 530 nm. The pH_i calibration of the I_{490}/I_{440} ratio was obtained by the high-K^+/nigericin method (14). We computed the net acid–base flux (J_{net}) as the product of the previously measured intrinsic buffering power (11), which varies with pH_i, and the rate of change of pH_i

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Abbreviations: pH_i; intracellular pH; AVP, arginine vasopressin; EIPA, ethylisopropyl amiloride; MC, mesangial cell.

¶To whom reprint requests should be addressed.

(dpH$_i$/dt). To obtain an unbiased estimate of dpH$_i$/dt for the pH$_i$ recovery from an acid load, we grouped each data point with its two neighbors, determined the coefficients for a quadratic equation, and evaluated the derivative of this equation at that center data point. The acid-loading rate (J_{acid}) was computed from the EIPA-induced acidification that followed the pH$_i$ recovery. We fitted a polynomial (second to fourth order) to all data points obtained after application of EIPA and evaluated the derivative of the polynomial at the same discrete pH$_i$ values sampled during the pH$_i$ recovery portion of the experiment. For each experiment, the EIPA-sensitive acid-extrusion rate (J_{Na-H}) was determined as the difference between J_{net} and J_{acid} for each discrete pH$_i$ sampled during the pH$_i$ recovery. To determine this difference at pH$_i$ values below the lowest pH$_i$ attained during the EIPA-induced acidification, we assumed that J_{acid} had the same low value as that prevailing at the lowest pH$_i$. These assumed J_{acid} values are not plotted in Figs. 1*C*, 2*B*, and 3*B*.

RESULTS

Quiescent MCs. *pH$_i$ dependence of uncorrected acid extrusion and background acid loading.* Fig. 1*A* illustrates an experiment in which a single MC was acid loaded in a HCO_3^--free solution by briefly applying and withdrawing 20 mM NH_4^+. pH$_i$ rapidly recovered to its initial value. From this pH$_i$ recovery, and from those of 11 other experiments, we determined (see *Methods*) the pH$_i$ dependence of the net acid–base flux (J_{net}). As illustrated in Fig. 1*B*, J_{net} varies linearly with pH$_i$, intercepting the abscissa at an apparent pH$_i$ threshold of ≈7.2.

pH$_i$ dependence of background acid loading. It commonly has been assumed that the Na–H exchanger is the only process contributing to the pH$_i$ recovery, and that the linear profile in Fig. 1*B* represents the pH$_i$ dependence of the exchanger. If this were true, then blockade of the Na–H exchanger with 50 μM EIPA would reduce the pH$_i$ recovery rate (dpH$_i$/dt) to zero at all pH$_i$ values. Indeed, we have previously shown that this is true at a pH$_i$ of ≈6.6 (11). However, Fig. 1*A* shows that at a pH$_i$ of ≈7.2, EIPA causes a substantial decline in pH$_i$. A similar pH$_i$ decline is observed without any prior exposure to NH_4^+ (11). Thus, at a pH$_i$ of ≈7.2, the normal steady-state pH$_i$ in the absence of HCO_3^-, the Na–H exchanger must balance a substantial background acid-loading process that is unmasked by EIPA. We also applied EIPA at various times during the pH$_i$ recovery (i.e., between pH$_i$ ≈6.6 and ≈7.2). At a given pH$_i$, dpH$_i$/dt was similar regardless of whether EIPA had been applied at that pH$_i$ or at a higher pH$_i$. Thus, it appears that the EIPA-unmasked acidification is uniquely related to pH$_i$, rather than to the history of the cell. The equivalent flux responsible for this acidification (J_{acid}) can be estimated from the rate of pH$_i$ decline (see *Methods*). As summarized in Fig. 1*C*, J_{acid} is insignificant at pH$_i$ values below ≈6.7, but it steeply increases at higher pH$_i$ values.

pH$_i$ dependence of Na–H exchange. At any pH$_i$, we can determine the EIPA-sensitive acid extrusion rate, presumably J_{Na-H}, by subtracting J_{acid} from J_{net} for that cell, as described in *Methods*. Fig. 1*D*, a plot of the pH$_i$ dependence of the average $J_{net}-J_{acid}$ difference, shows that the Na–H exchanger has a pH$_i$ profile quite different than previously assumed. Although J_{Na-H} falls steeply with increasing pH$_i$ between 6.3 and ≈6.7, it is relatively pH$_i$ insensitive between ≈6.7 and 7.2. For the low-pH$_i$ data, the best-fit slope is −96 ± 6 (SD) μM·sec^{-1} per pH unit, which is nearly 6-fold higher than the slope for the high-pH$_i$ data, −16 ± 4 μM·sec^{-1} per pH unit.

MCs Exposed to AVP. *Early effects of AVP.* We previously found that application of 100 nM AVP, a potent mitogen for MCs, causes a biphasic pH$_i$ change in the absence of CO_2/HCO_3^- (12). As shown in Figs. 2*A* and 3*A*, within 2 min pH$_i$ falls by ≈0.40, and then over the next 10–15 min it increases to a value as much as 0.15 greater than the initial one. We therefore examined the effect of AVP on J_{Na-H} and J_{acid} both early during the exposure to AVP (soon after pH$_i$ reached its minimum) and later during the AVP exposure (after pH$_i$ had peaked). Fig. 2*A* illustrates an experiment in which pH$_i$ recovery from the acid load began ≈5 min (i.e., early) after AVP addition. Fig. 2*B* summarizes the pH$_i$ dependence of J_{Na-H} and J_{acid} for five such experiments (mean time to initiation of pH$_i$ recovery, 7.6 ± 1.5 min). For comparison, curves of best fit from the control cells (Fig. 1 *C* and *D*) are plotted as dashed lines. At these early times after AVP addition, the pH$_i$ dependence of J_{Na-H} (solid circles) became linear, with a threshold pH$_i$ of ≈7.1 and a slope of −87 ± 10 μM·sec^{-1} per pH unit. Na–H exchange was unaffected by AVP at pH$_i$ values below ≈6.7 but was inhibited to progressively greater extents at higher pH$_i$ values, including those in the physiological range. EIPA-insensitive acid loading (solid

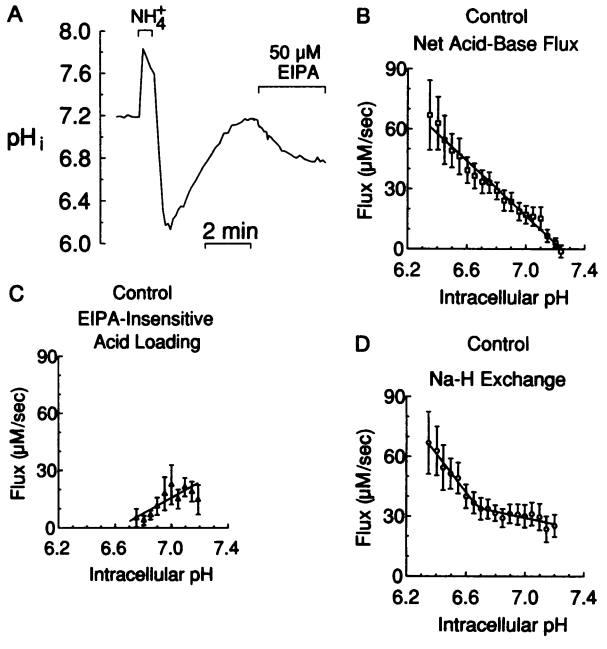

FIG. 1. pH$_i$ regulation in single quiescent MCs. (*A*) pH$_i$ recovery from an acid load induced by an NH_4^+ pulse. The cell was exposed to a solution containing 20 mM NH_4^+ during the indicated period. EIPA (50 μM) was applied after the pH$_i$ recovery was complete. (*B*) pH$_i$ dependence of mean net acid–base flux, computed from recovery of pH$_i$ from an NH_4^+-induced acid load. □, Mean flux for 12 pH$_i$ recoveries, plotted at intervals of ≈0.05 pH units. The vertical bars represent SEM of the flux; the SEMs of the mean pH$_i$ values were smaller than the size of the box. The solid line is the line of best fit, computed over all individual data points; it has an intercept of 496 ± 18 (SD) μM·sec^{-1} and a slope of −68 ± 3 μM·sec^{-1} per pH unit. (*C*) pH$_i$ dependence of mean EIPA-insensitive acid-loading rate (expressed as a flux), computed from relaxation of pH$_i$ after application of EIPA. For the sake of simplicity, we have plotted this apparent flux as a positive number, although the effect of acid loading on pH$_i$ is opposite that of acid extrusion (e.g., Fig. 1*B*). △, Acid-loading rate for 12 pH$_i$ relaxations. The solid line is the line of best fit, computed over all individual data points; it has an intercept of −270 ± 6 μM·sec^{-1} and a slope of +41 ± 8 μM·sec^{-1} per pH unit. (*D*) pH$_i$ dependence of mean Na–H exchange rate (i.e., EIPA-sensitive acid-extrusion rate). ○, Mean difference between the net acid–base flux and the EIPA-induced acid-loading rate, computed for each of 12 cells. The solid line between pH$_i$ 6.3 and 6.7 is the best fit to the data in this pH$_i$ range and has an intercept of 674 ± 36 μM·sec^{-1} and a slope of −96 ± 6 μM·sec^{-1} per pH unit. The solid line between pH$_i$ 6.7 and 7.2 is the best fit to the data in this higher pH$_i$ range and has an intercept of 138 ± 25 μM·sec^{-1} and a slope of −16 ± 4 μM·sec^{-1} per pH unit.

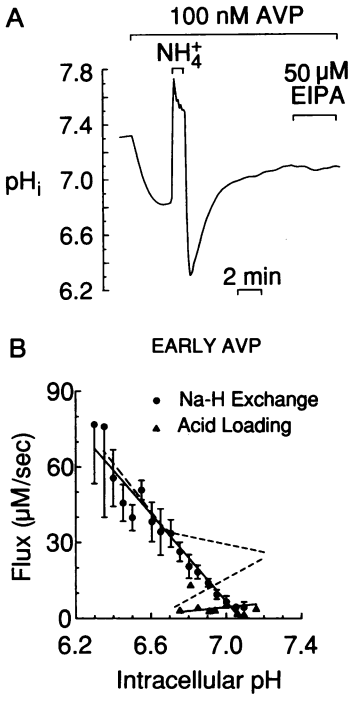

FIG. 2. pH_i regulation in single MCs early after application of AVP. (*A*) pH_i recovery from an acid load imposed by an NH_4^+ pulse soon after application of 100 nM AVP. The approach was similar to that of Fig. 1*A*. (*B*) pH_i dependence of mean rates of Na–H exchange (●) and acid loading (▲) soon after application of AVP. In five experiments similar to that of *A*, we monitored pH_i recoveries that began <10 min after application of 100 nM AVP. EIPA-insensitive and -sensitive fluxes were computed as described in Fig. 1 *C* and *D*. The solid line of best fit through the circles has an intercept of 615 ± 64 $\mu M \cdot sec^{-1}$ and a slope of −87 ± 10 $\mu M \cdot sec^{-1}$ per pH unit. The solid line of best fit through the triangles has an intercept of −13 ± 5 $\mu M \cdot sec^{-1}$ and a slope of +1.4 ± 7.0 $\mu M \cdot sec^{-1}$ per pH unit. The dashed lines reproduce the comparable fitted lines for quiescent cells from Fig. 1 *C* and *D*.

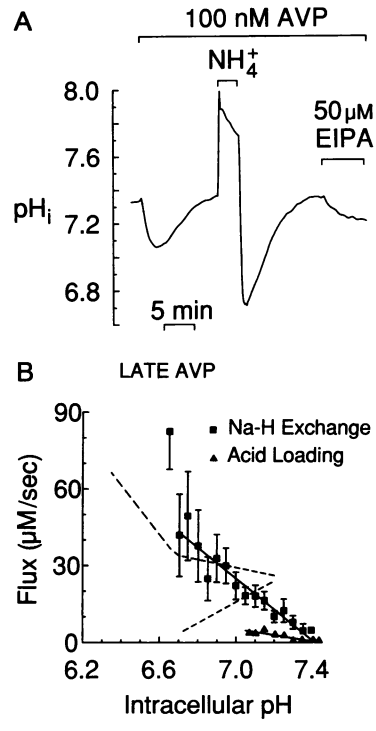

FIG. 3. pH_i regulation in single MCs late after application of AVP. (*A*) pH_i recovery from an acid load imposed by an NH_4^+ pulse after a longer exposure to 100 nM AVP. The approach was similar to that of Fig. 2*A*, except that the pH_i recovery began >10 min after the introduction of AVP. (*B*) pH_i dependence of mean rates of Na–H exchange (▪) and acid loading (▲) after a longer exposure to AVP. In seven experiments similar to *A*, we monitored pH_i recoveries that began >10 min after application of AVP. EIPA-insensitive and -sensitive fluxes were computed as described in Fig. 2*B*. The solid line of best fit through the squares has an intercept of 448 ± 60 $\mu M \cdot sec^{-1}$ and a slope of −60 ± 9 $\mu M \cdot sec^{-1}$ per pH unit. The solid line of best fit through the triangles has an intercept of 90 ± 43 $\mu M \cdot sec^{-1}$ and a slope of −12 ± 6 $\mu M \cdot sec^{-1}$ per pH unit. The dashed lines reproduce the comparable fitted lines for quiescent cells from Fig. 1 *C* and *D*.

triangles) was even more profoundly inhibited by AVP than was Na–H exchange, with little activity being observed up to a pH_i of ≈7.2.

Late effects of AVP. Fig. 3*A* illustrates an experiment in which the pH_i recovery from an acid load began ≈16 min (i.e., late) after application of AVP. Fig. 3*B* is a plot of J_{Na-H} and J_{acid} for seven such experiments (mean time to initiation of pH_i recovery, 14.2 ± 0.8 min). The fitted control curves are again superimposed for ease of comparison. The data show that at these later times after AVP addition, the J_{Na-H}–pH_i relationship (solid squares) remained approximately linear, with a slope (−60 ± 9 $\mu M \cdot sec^{-1}$ per pH unit) only slightly less than at earlier times. However, the curve was shifted ≈0.3 pH unit toward more alkaline pH_i values. Thus, at later times, Na–H exchange is stimulated at all pH_i values less than ≈6.9 but is inhibited at higher pH_i values, including those in the physiological range. As was the case for the early times, EIPA-insensitive acid loading (solid triangles) was profoundly inhibited by AVP in the physiological pH_i range.

DISCUSSION

Validity of the Assay. In principle, our data concerning the effect of EIPA on the time course of pH_i recovery from intracellular acid loads should permit us to extract the pH_i dependence of both Na–H exchange (i.e., the EIPA-sensitive component) and background acid loading (i.e., the EIPA-insensitive component). However, our analysis requires two assumptions: (*i*) that the only pH_i-related effect of EIPA is to block Na–H exchange completely, and (*ii*) that the degree of blockade is neither pH_i nor AVP sensitive. Support for the first assumption is that, at relatively low pH_i values in the absence of HCO_3^-, Na^+ removal is no more effective than EIPA at inhibiting the pH_i recovery from acid loads (11). We cannot rule out the possibility that, at higher pH_i values, EIPA becomes less effective at inhibiting Na–H exchange. However, if this were the case, the true J_{Na-H} at physiological pH_i would be even higher than our estimate.

pH_i Dependence of Na–H Exchange Under Control Conditions. We find that, in control MCs, the Na–H exchange rate is steeply pH_i dependent only for pH_i values below ≈6.7. Above this pH_i, and extending to nearly 7.2, the Na–H exchanger is substantially less sensitive to changes in pH_i. Thus, the Na–H exchanger in MCs has no true pH_i threshold in the physiological pH_i range. M. B. Sjaastad, E. Wenzel, and T. Machen (personal communication) recently have made a similar observation in IEC-6 cells. These observations raise the question of why the recovery of pH_i from an intracellular acid load becomes progressively slower as pH_i increases toward the normal range. For the MC, the answer appears to lie in the pH_i dependence of the background acid-loading process that is unmasked by EIPA. The rate of acid loading due to this process is insignificant below ≈6.7, but it increases rather steeply at higher pH_i values. Thus, to some extent the pH_i recovery from an acid load in control MCs slows because Na–H exchange is mildly decreased at higher pH_i values (i.e., J_{Na-H} falls 16 $\mu M \cdot sec^{-1}$ per pH unit;

Fig. 1*D*). However, the major reason for the slowing of the pH_i recovery is that J_{acid} is strongly increased at higher pH_i values (i.e., J_{acid} increases 41 $\mu M \cdot sec^{-1}$ per pH unit; Fig. 1*C*). A steady-state pH_i is achieved when the steeply pH_i-sensitive J_{acid} comes into balance with the relatively pH_i-insensitive J_{Na-H}. We have no information on the identity of the acid-loading process unmasked by EIPA. It could represent metabolic generation of H^+, a mechanism for the influx of H^+, and/or Cl-base exchange.

Effect of AVP on Na–H Exchange. As previously reported for other cells (4, 16), we found that application of a mitogen (i.e., AVP) in the absence of HCO_3^- leads to a biphasic pH_i change: a rapid but transient acidification, followed by a slower but sustained alkalinization. However, we also found that the biphasic pH_i change elicited by AVP is roughly paralleled by a biphasic change in the pH_i dependence of the Na–H exchanger. At early times after the application of AVP, the pH_i dependence of the Na–H exchanger became approximately linear, with a true pH_i threshold at ≈7.1, and a slope that was indistinguishable from that of the control exchanger at pH_i values below ≈6.7. Thus, early on, AVP has virtually no effect on the Na–H exchanger at pH_i values below ≈6.7, but it progressively inhibits the exchanger at higher pH_i values. Later, AVP alkaline shifts this linear relationship by ≈0.3, with only an ≈30% reduction in the slope. Thus, the effect of AVP on Na–H exchange is complex, depending on both time and pH_i. The importance of time is illustrated by the exchanger's activity at pH_i 6.6, where AVP has little effect at early times but is stimulatory later. An example of the importance of pH_i is seen at later times after AVP addition, where the mitogen stimulates at pH_i values below ≈6.9 but inhibits at higher pH_i values (i.e., in the physiological pH_i range).

Effect of AVP on Acid Loading. It is generally assumed that application of a growth factor stimulates chronic metabolic production of acid. Indeed, previous work on MCs examined in the presence of HCO_3^- suggested that, at pH_i of ≈6.6, AVP enhances a chronic acid-loading process that is insensitive to both EIPA and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (10). However, the present data, obtained in the absence of HCO_3^-, demonstrate that the AVP inhibits chronic acid loading at pH_i values above ≈6.7. The mechanism(s) responsible for this acid loading, as well as its inhibition by AVP, is unknown.

Mechanism of the AVP-Induced pH_i Changes in the Absence of HCO_3^-. The early effect of AVP on pH_i is an abrupt acidification. This could be due to an acute (i.e., transient) release or production of acid (15, 16). By itself, such an acute acid load would have no effect on steady-state pH_i: the Na–H exchanger would merely return pH_i to its initial level. A second early effect of AVP is to severely inhibit Na–H exchange in the physiological pH_i range. This could not only contribute to the abrupt acidification but could also slow the subsequent pH_i recovery. This slowness is somewhat mitigated by the near-total blockade by AVP of chronic acid loading. If AVP had only these early effects on Na–H exchange and chronic acid loading, then the recovery of pH_i would halt at ≈7.0, the pH_i coordinate of the intersection of the J_{Na-H} and J_{acid} lines at early times (Fig. 2*B*). The reason that the pH_i recovery continues to ≈7.4 is not because of a change in chronic acid loading (which remains almost fully inhibited) but probably because AVP gradually shifts the J_{Na-H} line to more alkaline pH_i values. Thus, the recovery of pH_i would mirror the gradual shift of the exchanger's pH_i threshold. Eventually, pH_i reaches a value (≈7.4) that is even more alkaline than the initial pre-AVP level (≈7.2). However, it should be emphasized that in the steady state prevailing after AVP addition, both Na–H exchange and acid loading are inhibited relative to the pre-AVP condition. The reason that AVP causes steady-state pH_i to increase from ≈7.2 to ≈7.4 in MCs is that AVP inhibits acid loading more profoundly than it inhibits Na–H exchange.

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