BIOLOGY OF REPRODUCTION **59,** 1483–1490 (1998)

Regulation of Intracellular pH in Hamster Preimplantation Embryos by the Sodium Hydrogen (Na^+/H^+) Antiporter[1]

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ABSTRACT

This study was an investigation of the mechanisms for the regulation of intracellular pH (pH_i) by hamster preimplantation embryos. The resting pH values of hamster embryos were similar at the 1-cell (7.19 ± 0.34), 2-cell (7.21 ± 0.21), and 8-cell (7.22 ± 0.41) stages. Cleavage-stage hamster embryos alleviated intracellular acidosis by activity of the Na^+/H^+ antiporter. The rate of recovery from acidosis was similar for embryos at 1-cell, 2-cell, and 8-cell stages. When Na^+/H^+ antiporter activity was inhibited by either incubation in Na^+-free medium or the presence of an inhibitor, pH_i was unable to recover to initial levels. Instead, pH_i remained acidic. The Na^+/H^+ antiporter was also found to contribute to baseline pH regulation, as incubation in Na^+-free medium resulted in an immediate intracellular acidification. The set point for Na^+/H^+ antiporter was pH 7.14. There was no evidence at any developmental stage for activity of either Na^+-dependent HCO_3^-/Cl^- exchanger or H^+-ATPase in the regulation of pH_i. Inhibition of the Na^+/H^+ antiporter by an amiloride derivative significantly reduced the ability of 2-cell embryos to develop in culture when challenged with acidosis, indicating that the Na^+/H^+ antiporter is an essential regulator of pH_i.

INTRODUCTION

Intracellular pH (pH_i) regulates many processes important for cell growth and differentiation—such as enzyme activity, cell division, membrane transport, protein synthesis, cell-cell communication, and differentiation [1]—as well as for egg activation in the sea urchin [2, 3], *Xenopus* [4–6], and surf clam [7]. Cellular regulation of pH_i occurs via a number of transporters in the cell membrane [8, 9]. Most mammalian cells utilize the Na^+/H^+ antiporter for alleviation of intracellular acidosis. The Na^+/H^+ antiporter utilizes the normal cellular transmembrane sodium gradient to export protons out of the cell with a stoichiometry of 1:1. However, activity of this antiporter is absent in a small number of nucleated cell types, such as rabbit pulmonary macrophages [10] and undifferentiated chick somites [11]. Other mechanisms can be used by cells to alleviate intracellular acidosis, such as the Na^+-dependent HCO_3^-/Cl^- exchanger, which imports Na^+ and HCO_3^- into the cell with the coupled export of Cl^- [12], or H^+-ATPases, which utilize ATP to export H^+ ions from the cell [10, 13]. Many cells have both the Na^+/H^+ antiporter and the other exchangers, and most cells that lack the Na^+/H^+ antiporter have one of these alternative mechanisms to alleviate intracellular acidosis. Studies by Baltz et al. [14, 15] demonstrated that BDF 2-cell mouse embryos lack any mechanism for the alleviation of acidosis. Instead the mouse 2-cell embryo recovers from an acid load by the passive movement of H^+ out of the cell until the electrochemical gradient is lost and an equilibrium is reestablished [14, 15]. In contrast, a recent report on 2-cell embryos from a different strain of mouse, the Quackenbush, showed that the Na^+/H^+ antiporter is involved in the alleviation of acidosis [16], indicating that there may be strain differences in the regulation of pH_i in mouse embryos.

While there is considerable knowledge about the role of the Na^+/H^+ antiporter in the development of the early embryo of sea urchin and *Xenopus*, our understanding of pH_i regulation in mammalian embryos is restricted to the early mouse embryo, where there are apparent strain differences. Considerable differences also exist between the physiology of the mouse embryo and that of preimplantation embryos from other species [17]. Therefore, the aim of this study was to determine the mechanism for the regulation of pH_i and the alleviation of acid loads by the preimplantation hamster embryo.

MATERIALS AND METHODS

Media

The culture medium used in this study was hamster embryo culture medium-3 [18], modified to contain 1.0 mM lactate and 0.5 mM taurine; this medium was bicarbonate free and buffered by Hepes, pH 7.35, and designated bfHH3t (Table 1). In some experiments the medium formulation was altered to be sodium free, with Na^+ being replaced by choline (sfH3t), or both bicarbonate and sodi-

TABLE 1. Composition (mM) of media used in measurement of pH_i.

Component	H3t	sfH3t[a]	bfHH3t[b]	sbfHH3t[a]
NaCl	113.8	—	113.8	—
KCl	3.0	3.0	3.0	3.0
$MgCl_2 \cdot 6H_2O$	0.5	0.5	0.5	0.5
$CaCl_2 \cdot 2H_2O$	2.0	2.0	2.0	2.0
$NaHCO_3$	25.0	—	—	—
$KHCO_3$	—	25.0	—	—
Sodium lactate[#]	1.0	—	1.0	—
Calcium lactate[#]	—	1.0	—	1.0
Glutamine	0.5	0.5	0.5	0.5
Taurine	0.5	0.5	0.5	0.5
Choline chloride	—	113.8	—	113.8
Hepes	—	—	25.0	25.0

[a] Media were adjusted to pH 7.35 with KOH.
[b] Medium was adjusted to pH 7.35 with NaOH.
[#] Concentration of L-isomer (sodium lactate was added as 50% D/L isomers).

Accepted August 6, 1998.
Received June 3, 1998.
[1]This research was supported by National Cooperative Program on Non-Human In Vitro Fertilization and Preimplantation Embryo Development by the National Institute of Child Health and Human Development (NICHD) through Grant HD22023. J.M.B. is a Medical Research Council of Canada Scholar.
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um free (sbfHH3t), or bicarbonate buffered (H3t) (Table 1). The medium for long-term embryo culture, hamster embryo culture medium (HECM)-10 [19], was prepared fresh from stocks the day before each experiment. To some media, 5,5-dimethyl-2,4-oxazolidinedione (DMO; Sigma Chemical Co., St. Louis, MO) was added at 10 mM.

The medium for calibration of pH consisted of 100 mM KCl, 25 mM NaCl, 21 mM Hepes, and 75 mM sucrose and was adjusted to either pH 6.7, 7.0, 7.4, or 7.8. Immediately prior to use, nigericin (10 μg/ml) and valinomycin (5 μg/ml) were added to the calibration media from concentrated stocks (1000-strength).

Inhibitors

Amiloride (Sigma) and 5-(*N*-ethyl-*N*-isopropyl)-amiloride, hydrochloride (EIPA; Molecular Probes, Eugene, OR) were used to inhibit Na^+/H^+ antiporter activity. The stilbene derivative 4,4′-diisothiocyanatostilbene-2,2′-di-sulfonic acid (DIDS; Sigma) was added to culture media to inhibit HCO_3^-/Cl^- transport; antimycin A (Sigma) and *N*,*N*′-dicyclohexylcarbodimide (DCCD; Sigma) were used to inhibit H^+-ATPase activity. Amiloride, EIPA, DIDS, and antimycin A were dissolved in DMSO as 1000-strength stock solutions, and DCCD was dissolved as a 100-strength stock solution in ethanol. All inhibitors were added to the culture medium immediately prior to pH analysis.

Embryos

Embryos were collected from 3- to 4-mo-old golden hamsters. Multiple ovulations were induced by an i.p. injection of 20–25 IU eCG (Pregnyl; Diosythn, Chicago, IL) given on the morning of postestrous discharge (Day 1). On Day 4, females were placed with fertile males for subsequent collection of embryos. One-cell embryos were collected at 10 h post-egg activation (PEA), 2-cell embryos at 32 h PEA, and 8-cell embryos at 52 h PEA. Embryos were flushed from the oviduct with either medium bfHH3t or H3t equilibrated at 5% O_2:10% CO_2:85% N_2 at 37°C [18].

Measurement of pH_i

Intracellular pH was assessed using the pH-sensitive probe 2′,7′-*bis*(2-carboxyethyl)-5-(and -6)-carboxy fluorescein (BCECF; Molecular Probes). Embryos were loaded with BCECF using 1.2 μM acetoxymethyl ester (BCECF-AM) at 37°C for 15 min in the same medium in which baseline pH_i was to be measured. Embryos were washed twice in medium without the probe, and embryos were placed in 1.5 ml of medium in a temperature-controlled chamber (Biophysica, Baltimore, MD). To change the medium, the chamber was flushed with 20 ml of fresh medium and the excess medium removed using a syringe-pump. With this method, dynamic changes in the pH_i of the same group of embryos could be observed throughout the incubation period as the surrounding medium was altered and intracellular acidosis induced. Measurement of pH was achieved using a Nikon (Garden City, NY) Diaphot inverted microscope connected by a Nikon Dual Optical Path Tube to a Photometrics PXL cooled camera (Huntington Beach, CA) for high-resolution recording of epifluorescent BCECF images. Image analysis of fluorescent images was performed using Metamorph/Metafluor hardware and software (Universal Imaging Corporation, West Chester, PA). Emission wavelength was set to 535 nm, and the ratio of fluorescence intensities of excitation wavelengths 500 (pH sensitive) to 440 nm (pH insensitive) was obtained for each embryo. The ratio of fluorescence intensities was linearly proportional to pH. Fluorescent ratios were calibrated in situ using the nigericin/high K^+ method with the pH calibration solutions at four pHs: 6.7, 7.0, 7.4, and 7.8 [20].

To ensure that the observed changes in embryo pH_i were not due to temporal changes in pH_i, control experiments were conducted in which embryos were exposed to either the control medium bfHH3t or H3t for 60 min and pH_i was measured every 30 sec. There was no change in pH_i when embryos were incubated in medium bfHH3t or H3t for 60 min.

Induction of Intracellular Acidosis

After the baseline pH_i measurement, intracellular acidosis was induced by an NH_4Cl pulse in which the embryos were exposed to 25 mM NH_4Cl for 10 min. This produces an immediate alkalization due to the rapid equilibration of NH_3 across the membrane, followed by a slower partial reacidification as a result of slower influx of the less permeable NH_4^+. Upon flushing of external NH_4Cl from the chamber, NH_3 rapidly exits the cell, leaving behind the H^+ that had entered as NH_4^+, thereby causing a net acidification of the cell [8, 14, 21].

The rate of recovery from the induced acidification was from the initial points of the recovery from the lowest pH_i after the NH_4^+ was flushed from the chamber (usually 4–6 readings). Rate of recovery was calculated from the gradient (dpH/dt) of the initial points and expressed as pH units/min.

Embryo Culture and Morphology

Two-cell embryos were cultured in groups of 10 in 35-μl drops of HECM-10 under mineral oil (Sigma). Embryos were cultured for 48 h at 37°C in either 10% CO_2:5% O_2:85% N_2 or 16% CO_2:5% O_2:79% N_2. Embryo development to the morula and blastocyst stages was assessed after 48 h of culture.

Statistical Analysis

Differences in resting pH, buffering capacity, and recovery from acid loading between different stages of development and between different treatments were determined by ANOVA followed by Bonferoni procedure for multiple comparisons [22]. Analysis of embryo development was performed with use of linear-logistic regression in which the error distribution was assumed to be binomial [23]. The null hypothesis of no treatment effect against a treatment effect was tested using the log-likelihood ratio statistic. The GLIM statistical package (Numerical Algorithms Group Ltd., Oxford, UK) was used for analysis.

RESULTS

Baseline pH_i and Intracellular Buffering Capacity of In Vivo-Developed Hamster Embryos

Initial experiments were performed to determine the resting pH_i of hamster embryos at the 1-cell, 2-cell, and 8-cell stages immediately after collection. The resting pH_i of 1-cell embryos was 7.19 ± 0.34 and remained constant for development to the 2-cell (7.21 ± 0.21) and 8-cell (7.22 ± 0.41) stages (n = at least 200 embryos per stage of development). The buffering capacity of embryos, which is a measure of the ability of the cytoplasm of the cell to buffer

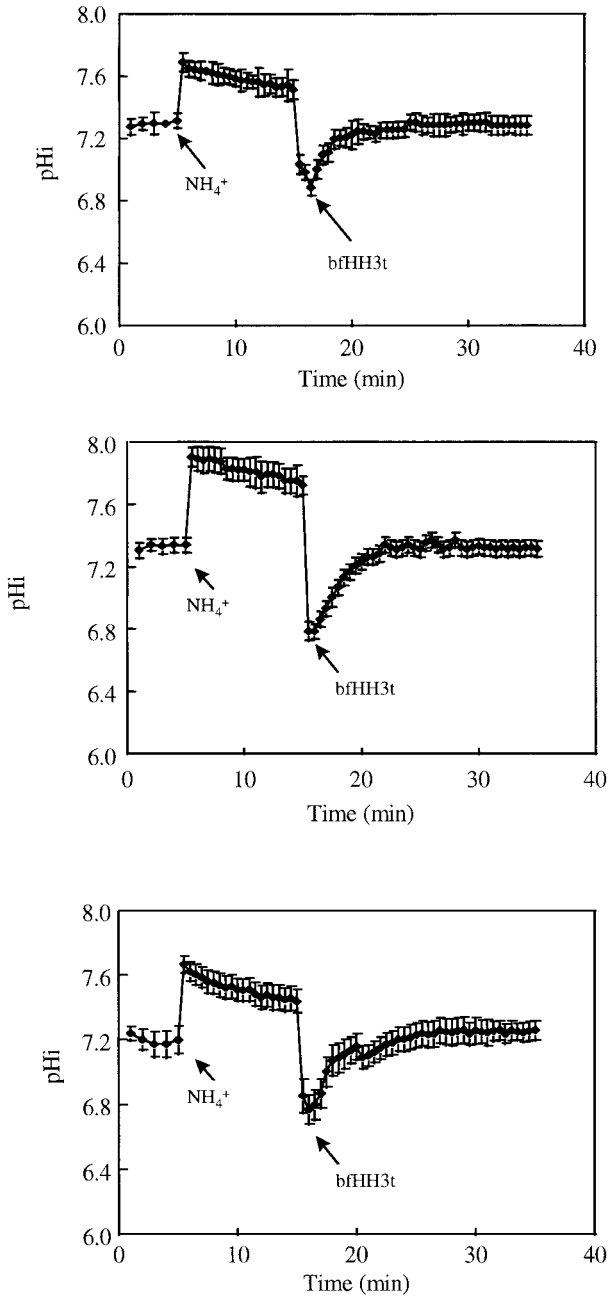

FIG. 1. Recovery from intracellular acidosis by hamster embryos. Baseline pH_i was determined in bfHH3t for 5 min, followed by a 10-min exposure to 25 mM NH_4Cl. Recovery was assessed in bfHH3t for 20 min. Values indicate means ± SEM for one replicate of 10 embryos. **Top**) 1-cell embryos; **middle**) 2-cell embryos; **bottom**) 8-cell embryos.

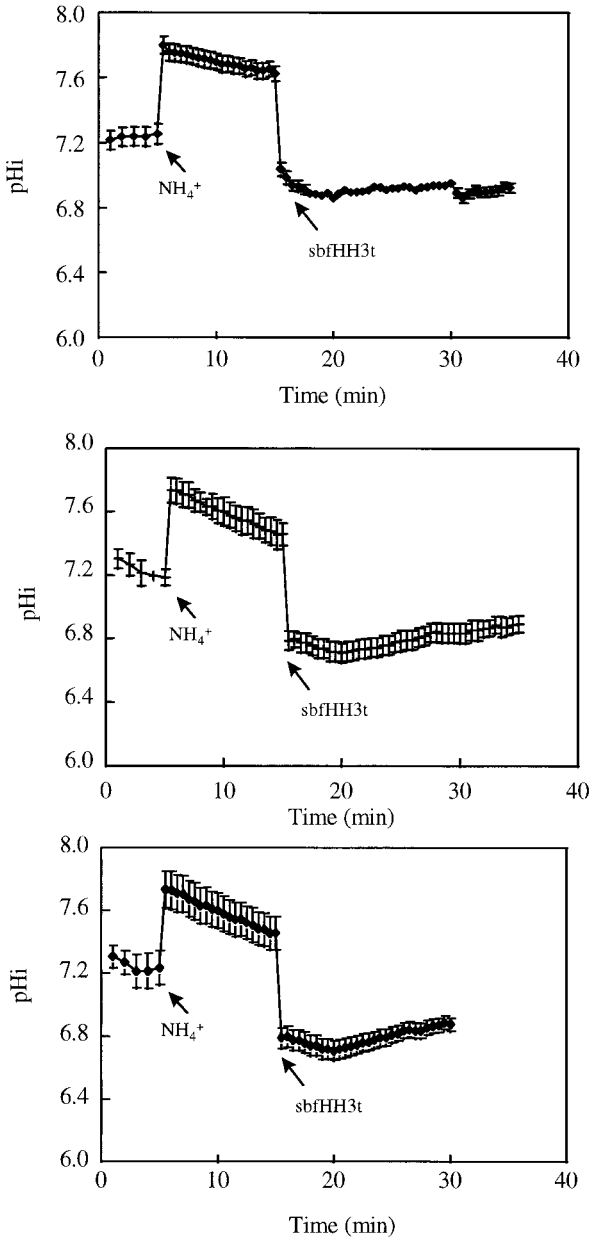

FIG. 2. Dependence on Na^+ of the recovery from acidosis by hamster embryos. Baseline pH_i was determined in bfHH3t for 5 min, followed by a 10-min exposure to 25 mM NH_4Cl. Recovery was assessed in sbfHH3t for 20 min. Values indicate means ± SEM for one replicate of 10 embryos. **Top**) 1-cell embryos; **middle**) 2-cell embryos; **bottom**) 8-cell embryos.

changes in acids and bases, was estimated from the change in pH_i after exposure to 25 mM NH_4^+ [8]. There was no difference in the apparent buffering capacity of hamster embryos among the 1-cell (24.5 ± 5.4 mM/pH), 2-cell (31.5 ± 11.2 mM/pH), and 8-cell (36.5 ± 9.1 mM/pH) stages ($p = 0.12$; n = at least 200 embryos per stage of development).

Contribution of Na^+/H^+ Antiporter Activity to Recovery from Acidosis

The rates of recovery from acidosis induced by a 10-min exposure to NH_4^+ were assessed in medium free of bicarbonate (bfHH3t), to eliminate any possible activity of

TABLE 2. Rate of recovery from acidosis of hamster embryos.[a]

Stage of development	Rate of recovery[b] (pH units/min)	Half-time of recovery[b] (min)
1-cell	0.187 ± 0.026	2.408 ± 0.167
2-cell	0.292 ± 0.074	2.077 ± 0.244
8-cell	0.195 ± 0.023	2.343 ± 0.125

[a] n, At least 6 replicates examined at each stage of development.
[b] A mean rate of recovery and half-time recovery from acidosis was established for each replicate (8–10 embryos), and the means ± SEM for the 6 replicates are reported.

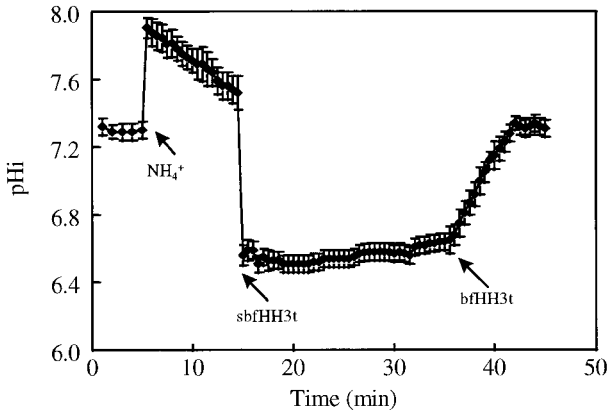

FIG. 3. Dependence on Na^+ of the recovery from acidosis by 2-cell hamster embryos. Baseline pH_i was determined in bfHH3t for 5 min, followed by a 10-min exposure to 25 mM NH_4Cl. Recovery was assessed in sbfHH3t for 20 min. After 20 min, embryos were incubated in bfHH3t (with Na^+), and pH_i was assessed for a further 10 min. Values indicate means ± SEM for one replicate of 10 embryos.

HCO_3^-/Cl^- exchange, for 1-cell, 2-cell, and 8-cell embryos. A short incubation in NH_4^+ produced an acidification of the blastomeres. In embryos at all stages, pH_i recovered rapidly (Fig. 1) back to the initial resting pH_i (Table 2). The rate of recovery from acidosis was found to be similar for embryos at each stage of development (Table 2). Similarly, the calculated half-time of recovery to resting pH_i was similar for 1-, 2-, and 8-cell embryos (Table 2).

To establish whether this recovery from acidosis was dependent on the presence of Na^+, the experiments were repeated in a medium free of Na^+ and bicarbonate (sbfHH3t) during the recovery phase. In embryos at each stage of development, the ability to reestablish pH_i after induction of acidosis in medium sbfHH3t was lost (Fig. 2). Intracellular pH did not recover to the initial levels (Fig. 2) even after 40 min in Na^+-free medium (data not shown). The rates of recovery in the absence of Na^+ were similar at all stages of development, and the pooled recovery rate of 0.015 ± 0.004 pH units/min (n = at least 40 embryos at each stage of development) was significantly reduced compared to the pooled recovery rates in the presence of Na^+ (0.253 ± 0.041 pH units/min; $p < 0.01$, Fig. 1, Table 2). However, when Na^+ was restored after 20 min, embryos immediately began to recover pH_i, and initial pH_i levels were restored after 7–8 min (Fig. 3).

The addition of either 1 mM amiloride or 50 μM EIPA (which inhibit Na^+/H^+ antiporter activity) to medium bfHH3t after induction of acidosis prevented recovery from acid loading in embryos at all stages of development (Fig. 4). The rates of recovery from acidosis in the presence of either amiloride or EIPA were similar for all stages of development. The pooled rate of recovery from acidosis in the presence of amiloride (0.015 ± 0.007 pH units/min; n = at least 40 embryos at each stage of development) or EIPA (0.011 ± 0.008 pH units/min; n = at least 40 embryos at each stage of development) was significantly reduced compared to the pooled recovery rates in bfHH3t (0.253 ± 0.041 pH units/min; $p < 0.01$, Fig. 1, Table 2). Resting pH_i could not be restored in the presence of EIPA even after 40 min. This inhibition was reversible, since washing out the drugs after 20 min enabled pH_i to recover from acidosis. This indicates that the failure to recover from acidosis was not due to toxicity of the inhibitors (Fig. 5).

To determine the set point for Na^+/H^+ antiporter activity

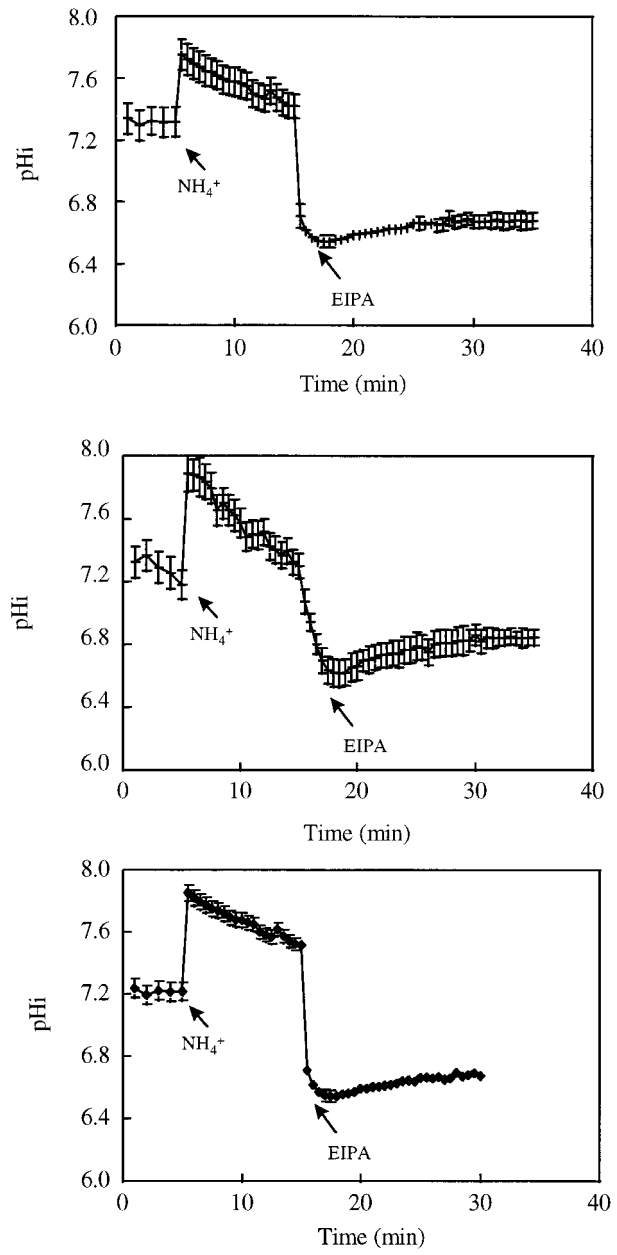

FIG. 4. Recovery from acidosis by hamster embryos in the presence of 50 μM EIPA. Baseline pH_i was determined in bfHH3t for 5 min, followed by a 10-min exposure to 25 mM NH_4Cl. Recovery was assessed in bfHH3t with 50 μM EIPA for 20 min. Values indicate means ± SEM for one replicate of 10 embryos. **Top**) 1-cell embryos; **middle**) 2-cell embryos; **bottom**) 8-cell embryos.

in hamster embryos, the rates of recovery from acidosis were determined at specific pH_i values for each trace. The calculated rates of recovery were subsequently plotted at the various pH_i (Fig. 6). Rates of recovery were increased at pH_i values of less than 6.9. At pH 6.9 and higher, rates of recovery decreased, and at a pH of 7.1 the activity of the antiporter was close to zero. Using a Line-Weaver Burk plot it was established that the there was a steeper dependence of antiporter activity on pH_i than could be attributed to Michaelis-Menten kinetics. The set point for activation of the Na^+/H^+ antiporter in hamster embryos was 7.14.

Contribution of Na^+/H^+ Antiporter Activity to Resting pH_i

Incubation in Na^+-free medium resulted in an immediate decrease in pH_i in embryos collected at the 1-cell, 2-cell,

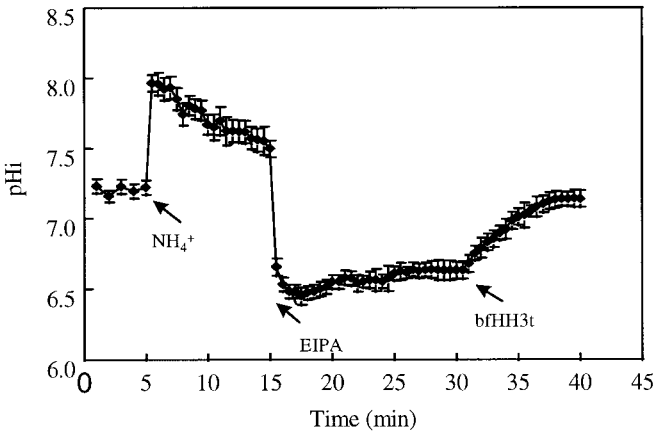

FIG. 5. Recovery from acidosis by 2-cell hamster embryos in the presence of 50 μM EIPA. Baseline pH_i was determined in bfHH3t for 5 min, followed by a 10-min exposure to 25 mM NH_4Cl. Recovery was assessed in bfHH3t with 50 μM EIPA for 15 min. After 15 min, EIPA was removed and pH_i assessed for a further 10 min. Values indicate means ± SEM for one replicate of 10 embryos.

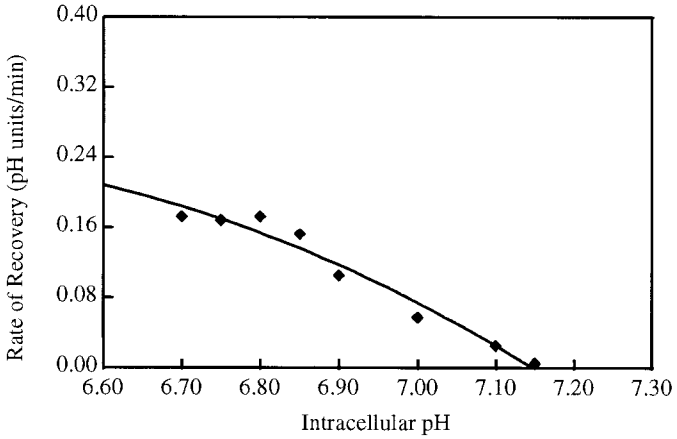

FIG. 6. Set point of activation of the Na^+/H^+ antiporter in hamster 2-cell embryos. The line indicates a line of best fit. The set point of Na^+/H^+ antiporter activity was determined to be 7.14.

or 8-cell stage; the embryos did not recover from this decrease even after 60 min. The decrease in pH when embryos were incubated in Na^+-free medium was similar at each stage of development: 1-cell (0.20 ± 0.03 pH units), 2-cell (0.21 ± 0.03 pH units), and 8-cell (0.18 ± 0.03 pH units) (n = at least 50 embryos at each stage of development). When Na^+ was returned to the medium after 10 min, pH_i returned to initial baseline levels within 5 min (Fig. 7).

Contribution of Na^+-Dependent HCO_3^-/Cl^- Transporter to Recovery from Acidosis

Recovery from acidosis was again determined for embryos, this time in the presence of bicarbonate (H3t) at 10% CO_2:5% O_2:85% N_2. The rates of recovery in the presence of bicarbonate and CO_2 were not significantly different from those observed in the absence of bicarbonate in embryos at either the 1-cell, 2-cell, or 8-cell stage (Fig. 8). Similarly, the addition of 100 μM DIDS (inhibits both Na^+-dependent and -independent HCO_3^-/Cl^- exchange [15]) to the medium also did not affect the recovery from acidosis as compared to that in medium H3t at all stages of development (Fig. 8). However, addition of EIPA to the medium significantly reduced acid recovery in 1-cell, 2-cell, and 8-cell embryos compared to that in medium H3t alone ($p > 0.05$). There was a small degree of initial recovery at the 2-cell stage in the presence of EIPA (0.038 pH units/min); however, pH_i did not return to initial levels. Addition of both EIPA and DIDS to medium H3t prevented any recovery from acid loading at all stages of embryo development (Fig. 8). However, there was no difference in the recovery rates between treatment with EIPA and treatment with EIPA and DIDS.

Contribution of H^+-ATPase to Acid Recovery

The rate of recovery from NH_4^+-induced acidosis of embryos at all stages of development was examined in bfHH3t in the absence and presence of inhibitors of H^+-ATPase, either DCCD or antimycin A (n = at least 40 embryos at each stage of development for each treatment). The rates of recovery of 1-cell (0.241 ± 0.05 pH units/min), 2-cell (0.232 ± 0.07 pH units/min), and 8-cell (0.219 ± 0.06 pH units/min) embryos were not different from the recovery rates in the presence of either DCCD (0.202 ± 0.06 pH units/min, 0.218 ± 0.07 pH units/min, and 0.204 ± 0.08 pH units/min, respectively) or antimycin A (0.198 ± 0.07 pH units/min, 0.221 ± 0.08 pH units/min, and 0.199 ± 0.11 pH units/min, respectively).

Titration of EIPA Concentration on Inhibition of Na^+/H^+ Antiporter Activity

The sensitivity of Na^+/H^+ antiporter activity to EIPA was examined in 2-cell embryos. Na^+/H^+ antiporter activity was assessed by recovery from acid loading induced by a 10-min exposure to NH_4^+ in the presence of 0.02 (below K_m value reported for inhibition of Na^+/H^+ antiporter isoform 1; [24]), 0.1 (below K_m value reported for inhibition of Na^+/H^+ antiporter isoform 2; [24]), 3.13, 6.25, 12.5, 25, or 50 μM EIPA. Recovery from acidosis by 2-cell embryos was completely inhibited by EIPA concentrations of 3.13–50 μM (Fig. 9). A concentration of 0.1 μM EIPA partially reduced recovery of 2-cell embryos from acidosis (Fig. 9). The IC_{50} of EIPA inhibition of 2-cell hamster embryo recovery from acidosis was calculated as 1.03 μM.

Effect of Inhibition of Na^+/H^+ Antiporter Activity on Embryo Development in Culture

The importance of the Na^+/H^+ antiporter activity to embryo development in culture was examined by the addition

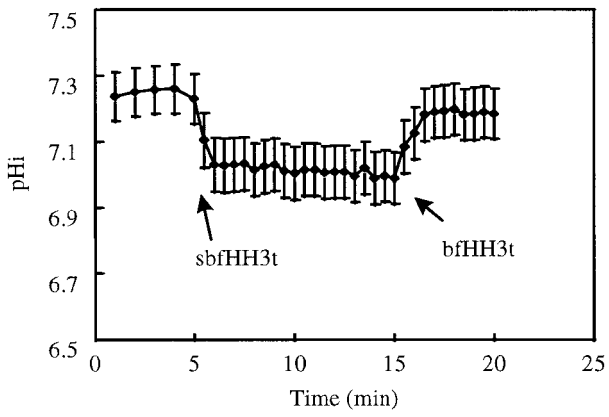

FIG. 7. Effect of incubation in Na^+-free medium on pH_i of 2-cell hamster embryos. Baseline pH_i was determined in bfHH3t for 5 min, followed by incubation in sbfHH3t for 10 min and then again in bfHH3t for 5 min. Values indicate means ± SEM for one replicate of 10 embryos.

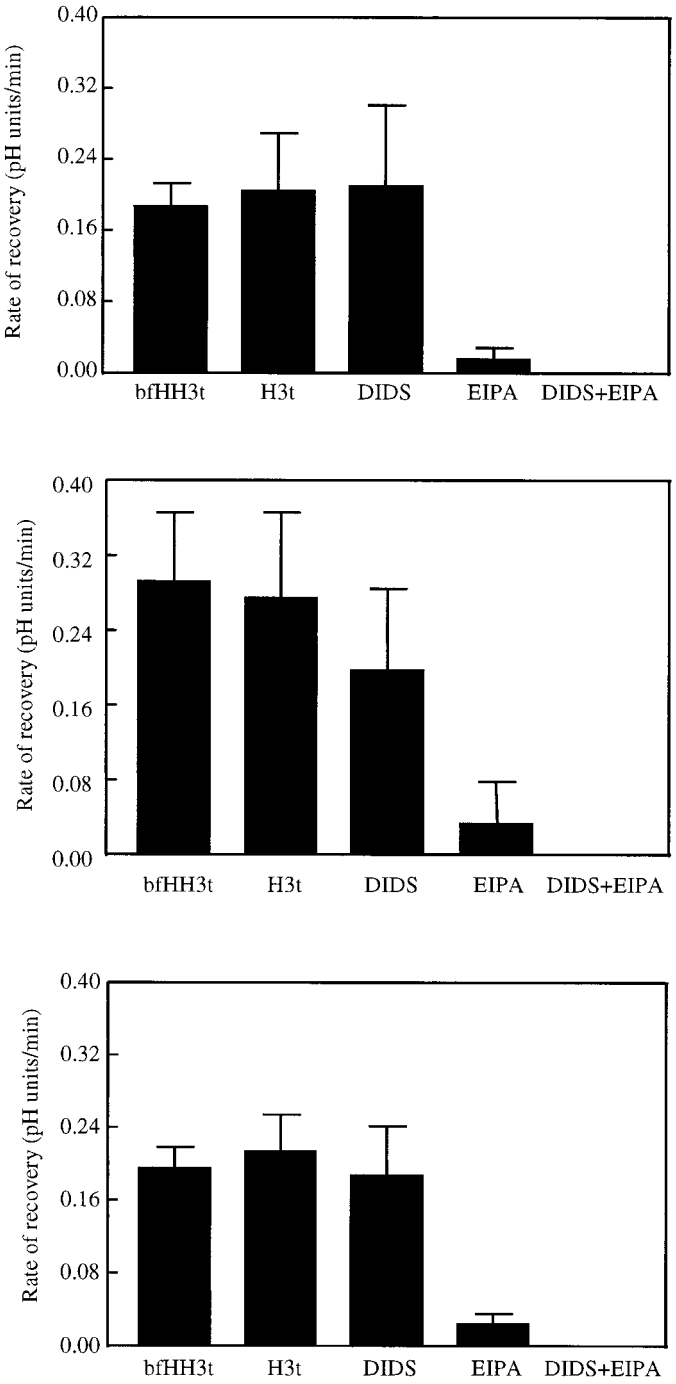

FIG. 8. Effect of EIPA and DIDS on the rate of recovery after acidosis by hamster embryos in media supplemented with bicarbonate. Recovery from acidosis by 2-cell embryos was assessed in either medium bfHH3t, H3t, H3t with 100 μM DIDS, H3t with 50 μM EIPA, or H3t with 100 μM DIDS and 50 μM EIPA. Recovery was assessed for 20 min and the rate of recovery determined. No recovery was observed in embryos incubated with both EIPA and DIDS. Values are mean ± SEM (n = at least 40 embryos per treatment). **Top**) 1-cell embryos; **middle**) 2-cell embryos; **bottom**) 8-cell embryos.

of weak acid DMO or CO_2 in the presence or absence of the Na^+/H^+ antiporter inhibitor EIPA.

In initial experiments the effect of EIPA concentrations on 2-cell embryo development in vitro was examined. Two-cell embryos were cultured with EIPA at concentrations of 0, 1.51, 3.13, 6.25, 12.5, 25, or 50 μM, and development to the morula/blastocyst stage was assessed after 48 h of

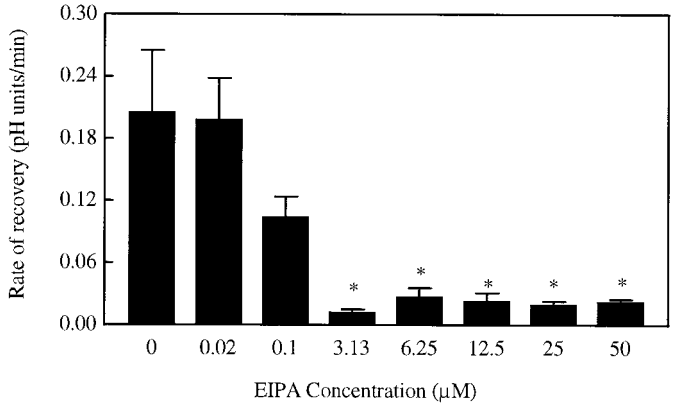

FIG. 9. Effect of EIPA concentrations on the recovery from acidosis by hamster 2-cell embryos; n = at least 30 embryos per EIPA concentration. $R^2 = 0.768$ for EIPA concentrations of 0-3.13 μM. *Rate of recovery is significantly different from control recovery of 0.213 pH units/min ($p < 0.05$). The calculated IC_{50} of inhibition by EIPA was 1.03 μM.

culture. Development of morulae/blastocysts was not affected by culture with EIPA concentration of 1.5 to 12.5 μM (Fig. 10). However, a concentration of 25 or 50 μM reduced development to the morula/blastocyst stage compared to that in the control medium HECM-10 ($p < 0.01$) (Fig. 10).

Initially the acidification resulting from incubation with 10 mM DMO was assessed in 2-cell hamster embryos. Addition of 10 mM DMO to the culture medium caused an initial decrease in pH_i from a resting level of 7.23 ± 0.15 to 6.91 ± 0.03 ($p < 0.05$). Intracellular pH was able to recover to 7.25 ± 0.11 after 4 h of incubation with the DMO. Addition of 6.25 μM EIPA did not affect pH_i (7.24 ± 0.12). However, use of 6.25 μM EIPA in addition to the DMO prevented any recovery, and after 4 h the pH_i was 6.92 ± 0.04.

The effect of these treatments on the development of 2-cell embryos was subsequently assessed. Two-cell embryos were cultured in medium HECM-10, HECM-10 with 6.25 μM EIPA (a concentration that inhibits Na^+/H^+ antiporter activity), HECM-10 with 10 mM DMO, HECM-10 with 10 mM DMO and 6.25 μM EIPA, HECM-10 at 16% CO_2, or HECM-10 at 16% CO_2 with 6.25 μM EIPA for 48 h. Culture with 6.25 μM EIPA did not affect development to the morula/blastocyst or blastocyst stage compared to that for embryos cultured in HECM-10 (Fig. 11). Culture with 10

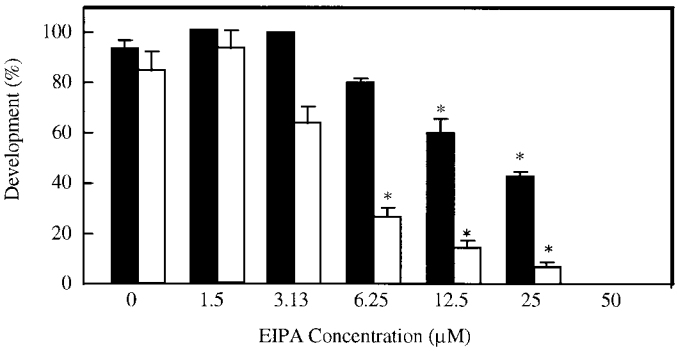

FIG. 10. Effect of EIPA concentration on the development of hamster 2-cell embryos in culture. Percentage morula/blastocyst development is represented by the solid bars and blastocyst development by the open bars; n = 40 embryos cultured per treatment. *Significantly different from values in control medium HECM-10 ($p < 0.05$).

mM DMO alone also did not affect development. Similarly, culture with increased CO_2 (which combines with intracellular H_2O to produce the weak acid H_2CO_3) did not alter development compared to that in HECM-10. However, when embryos were exposed to a small acidosis by the presence of either DMO or CO_2, exposure to EIPA severely impaired development to either the morula/blastocyst ($p < 0.05$) or blastocyst stage ($p < 0.05$) as compared to that in the control (Fig. 11).

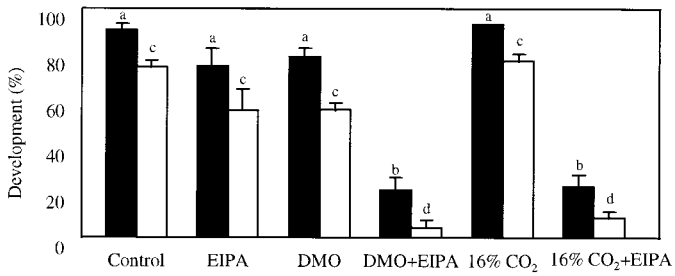

FIG. 11. Development of hamster embryos challenged with acidosis in the presence or absence of EIPA. Percentage morula/blastocyst development is represented by the solid bars and blastocyst development by the open bars; n = 80 embryos per treatment. [a–d] Different superscripts indicate significant differences ($p < 0.05$).

DISCUSSION

This study investigated the mechanism for the regulation of pH_i in the preimplantation hamster embryo. The pH_i of hamster embryos measured immediately after collection at either the 1-, 2-, or 8-cell stage were around 7.2. These values of pH_i are similar to that reported for the CF1 mouse embryo from the 1-cell to the blastocyst of about 7.1 [25, 26]; to 7.3 for the 2-cell Quackenbush mouse embryo [16]; and to 7.13 [27], 7.25 [28], and 7.1 [29] for mouse oocytes. The intracellular buffering power of cells, or the relative ability of the cytoplasm to regulate changes in internal acid-base balance, falls within a large range of 9–118 mM/pH [8] where higher values represent an increased ability of the cell to buffer changes in acids or bases. Intracellular buffering power of hamster embryos was similar at all stages of development, and the values obtained between 24.5 and 36.5 mM/pH were similar to the 25.3 mM/pH reported for the 2-cell CF1 mouse embryo [15] and slightly higher than the 18 mM/pH reported for the *Xenopus* embryo. Therefore, it appears that the resting pH_i and buffering capacity of embryos are similar across different species.

After acidification of pH_i to below 6.9 by incubation with NH_4^+, hamster cleavage-stage embryos recovered pH_i quickly so that within 5–8 min the pH_i had returned to that observed before acidosis. This recovery was Na^+ dependent, HCO_3^- independent, DIDS insensitive, and amiloride sensitive. There was no evidence for either Na^+-dependent HCO_3^-/Cl^- exchanger or H^+-ATPase activity in embryos at any stage of development. Therefore the sole mechanism for recovery from acidosis by hamster embryos is activity of the Na^+/H^+ antiporter. Na^+/H^+ antiporter activity was also involved in the maintenance of resting pH_i of hamster embryos, as incubation in Na^+-free medium resulted in an immediate decrease in pH_i consistent with the reverse activity of the Na^+/H^+ antiporter. The activity of the Na^+/H^+ antiporter in hamster embryos was dependent on pH_i. At low pH_i, antiporter activity is increased, while at physiological pH_i the antiporter activity is very low. Antiporter activity in hamster embryos had an increased dependence on internal pH than could be attributed to simple Michaelis-Menten kinetics. This characteristic of pH dependence of the Na^+/H^+ antiporter has been reported for many cell types [8, 30]. The set point of the Na^+/H^+ antiporter in hamster embryos was found to be at a pH of 7.14. The set point of activation of the antiporter is very close to the physiological pH_i, indicating that it plays an important role in pH_i homeostasis. The pH of oviduct fluid is reported to be alkaline [31, 32] and therefore above the set point of the Na^+/H^+ antiporter. However, the Na^+/H^+ antiporter would still have a substantial role in vivo in the removal of acidic by-products such as the protons that result from ATP hydrolysis [33].

In the mouse, 2-cell embryos derived from a hybrid strain (BDF) do not appear to have any mechanism for the regulation of pH_i in the acid range [14, 15]. Rather, recovery from acid loading by these 2-cell embryos appeared passive [14]. In contrast, a recent study reported that 2-cell mouse embryos derived from the Quackenbush strain do utilize the Na^+/H^+ antiporter to recover from acidosis [16]. In addition, in Quackenbush 2-cell embryos, an H^+-monocarboxylate (e.g., lactate) cotransport system contributes to the alleviation of intracellular acidosis at a very acid pH_i [16].

Five different isoforms of the Na^+/H^+ antiporter have been identified by molecular cloning, and their relative abundance in a number of tissues has been determined [34]. These isoforms differ in their tissue specificity, kinetics, and pharmacological properties. The sensitivity to EIPA of 2-cell embryos in this study indicates a sensitivity to amiloride similar to the IC_{50} of 1–10 μM reported for the NHE1 (sodium hydrogen exchanger) isoform [35, 36]. The NHE1 isoform is present in most cell types and is generally found on the basolateral surfaces of polarized cells [37] as well as the plasma membrane of nonpolarized cells [34]. Furthermore, a recent study has demonstrated that mRNA for the NHE1 isoform is present in the oocyte and early mouse embryo throughout development to the blastocyst stage [38]. NHE1 is the form of the antiporter that can be activated by growth factors through phosphorylation by protein kinase C either directly or indirectly [39]. It is yet to be confirmed that the NHE1 isoform is the one present in hamster preimplantation embryos.

Inhibition of Na^+/H^+ antiporter activity did not affect development of 2-cell embryos in culture, and the majority of embryos developed to the blastocyst stage. Therefore, it appears that when the external environment is maintained at a constant pH of 7.25, 2-cell hamster embryos could maintain pH_i at levels that support development. As the antiporter set point of 7.14 is close to this pH, the activity of the antiporter would be expected to be limited and therefore the presence of EIPA would not be expected to cause a substantial decrease in development. Challenging with a small acidosis (around 0.3 pH units), by culture with either the weak acid DMO or increased CO_2, did not affect development of 2-cells to the blastocyst stage, and embryos were able to recover pH_i and develop normally. However, when the Na^+/H^+ antiporter was inhibited, the same small acid challenge sharply reduced developmental potential and only a very small number of embryos (less than 5%) were able to develop to the blastocyst stage. Therefore, Na^+/H^+ antiporter activity appears essential for the regulation of pH_i in the acid range and for protection from acidosis. The loss of functional Na^+/H^+ antiporter activity prevents the embryo from being able to regulate pH_i in the acid-to-neutral range, and this is not consistent with developmental competence. A pH_i of below 6.9 was not permissive of hamster

embryo development in this study. It has been reported that the failure of DDK mouse embryos to develop after fertilization by alien sperm is also a result of low pH_i [40]. In contrast to that of the cleavage-stage hamster embryo, development of the later stage hamster embryo is increased in culture when the CO_2 concentration is increased from 5% to 10% [41]. Therefore, it would appear that there may be a change in the set point of Na^+/H^+ exchange in the later-stage embryos, perhaps indicative of a different isoform. The sensitivity of embryo development to pH is not restricted to the acid range. The activity of the HCO_3^-/Cl^- exchanger was essential for normal development in culture when mouse embryos were challenged with an alkaline loading [25]. Whether the same would be true for the hamster embryo is currently under investigation.

Therefore, in conclusion, the hamster cleavage-stage embryo uses the Na^+/H^+ antiporter for the regulation of pH_i. Na^+/H^+ antiporter activity enables embryos to recover from an acidic challenge and also contributes to the maintenance of baseline pH_i levels. This antiporter activity was essential for maintenance of developmental competence when embryos were exposed to an acidosis.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Ralph Albrecht for generously providing equipment for analysis of pH_i levels. We would also like to thank Drs. Prather, Tasca, and Watson for their critique of the manuscript.

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