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Na^+/H^+ Antiporter Activity in Hamster Embryos Is Activated during Fertilization

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This study characterized the activation of the regulatory activity of the Na^+/H^+ antiporter during fertilization of hamster embryos. Hamster oocytes appeared to lack any mechanism for the regulation of intracellular pH in the acid range. Similarly, no Na^+/H^+ antiporter activity could be detected in embryos that were collected from the reproductive tract between 1 and 5 h post-egg activation (PEA). Activity of the Na^+/H^+ antiporter was first detected in embryos collected at 5.5 h PEA and gradually increased to reach maximal activity in embryos collected at 7 h PEA. Parthenogenetically activated one-cell and two-cell embryos demonstrate Na^+/H^+ antiporter activity, indicating that antiporter activity is maternally derived and initiated by activation of the egg. The inability of cycloheximide, colchicine, or cytochalasin D to affect initiation of antiporter activity indicates that antiporter appearance is not dependent on the synthesis of new protein or recruitment of existing protein to the cell membrane. In contrast, incubation of one-cell embryos with sphingosine did inhibit the appearance of Na^+/H^+ antiporter activity, showing that inhibition of normal protein kinase C activity is detrimental to antiporter function. Furthermore, incubation of oocytes with a phorbol ester which stimulates protein kinase C activity induced Na^+/H^+ antiporter activity in oocytes in which the activity was previously absent. Incubation with an intracellular calcium chelator also reduced the appearance of antiporter activity. Taken together, these data indicate that the appearance of Na^+/H^+ antiporter activity following egg activation may be due, at least in part, to regulation by protein kinase C and intracellular calcium levels.

Key Words: **oocytes; intracellular pH; parthenogenetic activation; protein synthesis; protein kinase C.**

INTRODUCTION

Intracellular pH (pHi) regulates a multitude of cellular processes including enzyme activity, cell division, and cytoskeletal dynamics (Begg and Rebhun, 1979; Regula *et al.*, 1981). The most commonly mechanism utilized by cells for the regulation of pHi in the acid range is the Na^+/H^+ antiporter (NHE). NHE activity regulates cytoplasmic pH, Na^+ concentration, and cell volume (Shrode *et al.*, 1996), as well as being a trigger for growth and proliferation (Boron, 1983). NHE activity also has an important role during fertilization and initiation of development in marine species such as the sea urchin (Johnson *et al.*, 1976; Shen and Steinhardt, 1978; Dube *et al.*, 1985) or surf clam (Dube and Eckberg, 1997). In these species, there is a significant rise in pHi in eggs after activation by spermatozoa. This alkalization with an accompanying spike in intracellular Ca^{2+} levels is necessary for subsequent development to proceed and initiates both protein and DNA synthesis as well as other biosynthetic processes (Vacquier, 1974; Grainger *et al.*, 1979; Winkler and Steinhardt, 1981). In contrast to the sea urchin, although there is an increase in pHi in the *Xenopus* egg after activation, it is not due to activity of the NHE although it is present in the egg (Nuccitelli *et al.*, 1981; Webb and Nuccitelli, 1981). Unlike those of marine species or *Xenopus*, oocytes of mice (Kline and Zagray, 1995; Phillips and Baltz, 1996), rats (Ben-Yosef *et al.*, 1996), or human (Dale *et al.*, 1998) do not change pHi during egg activation or fertilization.

Previous studies on the regulation of pHi in mammalian embryos have reported conflicting results. Two-cell em-

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TABLE 1
Compositions (mM) of Media Used

Component	H3t	bfHH3t[a]	bsfHH3t[b]	HECM-10
NaCl	113.8	113.8	—	113.8
KCl	3.0	3.0	3.0	3.0
$MgCl_2 \cdot 6H_2O$	0.5	0.5	0.5	2.0
$CaCl_2 \cdot 2H_2O$	2.0	2.0	2.0	1.0
$NaHCO_3$	25.0	—	—	25.0
Na lactate[c]	1.0	1.0	—	4.50
Ca lactate[c]	—	—	1.0	—
Glutamine	0.5	0.5	0.5	0.2
Taurine	0.5	0.5	0.5	0.5
Choline chloride	—	—	113.8	—
Hepes	—	25.0	25.0	—
Amino acids, each[d]				0.01
Panthothenate				0.003
Polyvinyl alcohol				0.1 mg/ml

[a] Medium was adjusted to pH 7.35 with NaOH.
[b] Media were adjusted to pH 7.35 with KOH.
[c] Concentration of L-isomer (Na lactate was added as 50% D/L isomers).
[d] Amino acid solution consists of asparagine, aspartate, cysteine, glutamate, glycine, histidine, lysine, proline, serine.

bryos from some strains of mice are reported to lack active Na^+/H^+ exchange (Baltz *et al.*, 1990, 1991) although the mRNA for the antiporter is present in oocytes from the same strain (Barr *et al.*, 1998), indicating a possible role for NHE in oocyte pHi regulation. In this study we report that hamster oocytes do possess the NHE; however, its activity is not activated until 5–7 h post-egg activation.

MATERIALS AND METHODS

Culture Media and Inhibitors

The basic medium used in this study was a Hepes-buffered modification of HECM-3 supplemented with 0.5 mM taurine (bfHH3t; Table 1; Lane *et al.*, 1998a). Medium bfHH3t was adjusted to pH 7.35 with NaOH. For some experiments in which a sodium-free medium was required, all sodium in bfHH3t was replaced with choline and the medium adjusted to pH 7.35 with KOH (sbfHH3t; Table 1). For assessment of Na^+-dependent HCO_3^-/Cl^- exchange, 25 mM Hepes was replaced with 25 mM sodium bicarbonate (H3t; Table 1).

Medium for prolonged embryo culture was hamster embryo culture medium-10 (HECM-10; Table 1; Lane *et al.*, 1998b). Medium HECM-10 was prepared from stocks the day before culture and stored at 4°C.

All chemicals were obtained from Sigma Chemical Co. unless otherwise stated. 5-(*N*-ethyl-*N*-isopropyl)amiloride, hydrochloride (EIPA; Molecular Probes, Eugene, OR); cycloheximide; cytochalasin D; colchicine; lumicolchicine; phorbol-12-myristate 13-acetate (PMA), inactive phorbol ester; phorbol-12-myristate 13-acetate, 7-*O*-methyl (MPMA); 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; *N*,*N*′-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-](acetyloxy)-methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester (BAPTA-AM); and sphingosine were dissolved as ×1000 stocks in DMSO and added to the culture medium immediately prior to use.

Medium for calibration of pH consisted of 100 mM KCl (Miyazaki and Igusa, 1981), 25 mM NaCl, 21 mM Hepes, and 75 mM sucrose (Baltz *et al.*, 1990, 1991; Lane *et al.*, 1998). The medium was adjusted to 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 ×1000 stock solutions dissolved in DMSO (Baltz *et al.*, 1990, 1991). Using this procedure it is possible to routinely obtain standard curves with regression coefficients of greater than 0.98.

Animals

Oocytes and embryos were collected from 3- to 4-month-old golden hamster females. Multiple ovulations were induced by an ip injection of pregnant mare's serum gonadotropin (Pregnyl; Diosynth, Chicago, IL) on the day of proestrus. Oocytes were collected from unmated females immediately following ovulation. For collection of embryos, females were mated to males on day 4 of the estrous cycle and embryos collected at specific intervals post-egg activation (PEA) by sperm (timings described by Bavister *et al.*, 1983). One-cell embryos collected at 3 h PEA regularly had only one pronucleus and around 35–55% had not yet extruded the second polar body. At 6 h PEA embryos have two pronuclei and two polar bodies; however, the cytoplasmic rearrangements are incomplete (Barnett *et al.*, 1996; Lane and Bavister, 1998), while by 9 h PEA these processes are completed.

Oocytes and embryos were flushed from the oviduct with medium bfHH3t at 37°C. Oocytes and early embryos were denuded of surrounding cumulus cells by incubation with 0.5 mg/ml hyaluronidase (Sigma Chemical Co.) for 1 min.

Measurement of Intracellular pH

Intracellular pH was assessed using the pH-sensitive probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF; Molecular Probes). Oocytes and embryos were loaded with 1.2 μM BCECF using the acetoxymethyl ester for 15 min at 37°C in bfHH3t. Between 8 and 12 oocytes or embryos were washed twice in medium without the probe and embryos placed in a temperature-controlled chamber (Biophysica, Baltimore, MD). Solutions were changed by flushing 20 ml of medium through the chamber using a syringe pump. Measurement of pHi was achieved using a Nikon 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 530 nm and the ratio of fluorescence intensities of excitation wavelengths of 500 (pH sensitive) to 450 nm (pH insensitive) was obtained for each embryo every 60 s for baseline readings and every 30 s for measuring changes in pHi induced by medium changes. Fluorescence ratios calibrated *in situ* using a nigericin/high K^+ method (Thomas *et al.*, 1979; Baltz *et al.*, 1990) were found to be linear over the pH range 6.7–7.8.

Induction of Intracellular Acidosis

Intracellular acidosis was induced by a NH_4Cl pulse after measurement of baseline pHi. Oocytes or embryos were incubated for

10 min with 25 mM NH_4Cl, which results in an immediate alkalization due to rapid equilibration of NH_3 across the membrane. A slower movement of NH_4^+ results in a slow acidification. Subsequent removal of the NH_4Cl causes acidification as the NH_3 leaves the cell rapidly, leaving behind the H^+ which entered the cell as NH_4^+ (Boron and DeWeer, 1976; Roos and Boron, 1981).

Determination of Na^+/H^+ Antiporter Activity in Recovery from Acidosis

Recovery from acidosis due to the activity of the NHE was identified by being Na^+-dependent and sensitive to 50 μM EIPA (Baltz *et al.*, 1991; Lane *et al.*, 1998a). A further criterion of NHE activity was the immediate acidification of the blastomeres after removal of Na^+ from the medium (Baltz *et al.*, 1991; Lane *et al.*, 1998a).

Embryo Culture

One-cell embryos were cultured in groups of 10 in medium HECM-10 at 37°C in an atmosphere of 10% CO_2, 5% O_2, and 85% N_2. Development to the morula/blastocyst stage was assessed after 72–81 h depending on the timing of collection (total development time of 82 h PEA).

Parthenogenetic Activation of Oocytes

Oocytes were collected and denuded in bfHH3t and transferred to medium HECM-10. Oocytes were parthenogenetically activated by a technique described by Tateno and Kamiguchi (1997). Denuded oocytes were incubated in HECM-10 containing 3 μM A23187 calcium ionophore (Sigma) for 10 min, washed twice, and placed in culture for 30 min in HECM-10. Treated oocytes were then incubated in 7% ethanol in HECM-10 for 5 min, again washed, and placed into culture for 18 h. The following morning parthenogenetically activated one-cell embryos with two polar bodies and two-cell embryos were used for pH analysis.

Determination of Protein Synthesis by Embryos

Rates of protein synthesis were assessed by incorporation of [^{3}H]phenylalanine into TCA-precipitable fraction using the technique described by Thompson *et al.* (1998). Briefly, groups of 10 embryos were incubated in 10 μM L-[2,3,4,5,6-^{3}H]phenylalanine in medium HECM-10 at 37°C in 10% CO_2, 5% O_2, and 85% N_2 for 2 h. After 2 h embryos were washed three times in HECM-10 with 4% BSA (Miles Pentex, Bayer Diagnostics, Kankakee, IL) and precipitated with trichloroacetic acid (Sigma) and stored at 4°C. The following day the TCA precipitate was collected onto a glass-fiber disk (Whatman, Maidstone, UK) and radioactivity determined using a scintillation counter.

Statistical Analysis

Differences in rates of recovery from acid loading between different stages of development were determined by analysis of variance followed by the Bonferroni procedure for multiple comparisons. Embryo development in culture was assessed using linear-logistic regression in which the error distribution was assumed to be binomial. The null hypothesis of no treatment effect against a treatment effect was tested using the log-likelihood ratio statistic. Analysis of rates of recovery in the presence of inhibitors was compared to the control recoveries and rates of protein synthesis were examined using a paired *t* test.

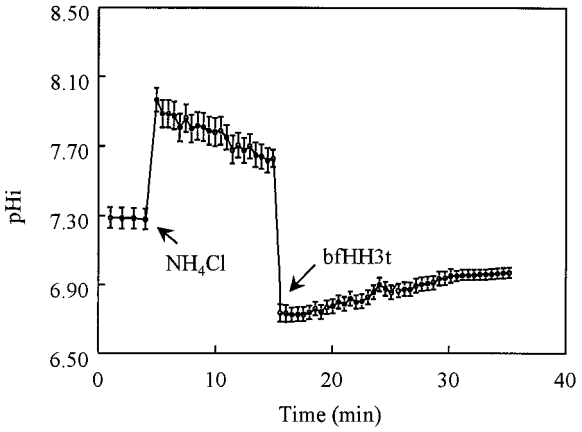

FIG. 1. Recovery from acidosis by hamster oocytes. Baseline pHi was determined in bfHH3t for 5 min, followed by a pulse of 25 mM NH_4Cl for 10 min to induce intracellular acidosis. Recovery from acidosis was assessed in bfHH3t for 20 min. Data represent means ± SEM of a replicate experiment of 10 embryos.

RESULTS

Examination of pHi Regulatory Systems in Hamster Oocytes

Na^+/H^+ antiporter. In bicarbonate-free medium (bfHH3t), recovery from acidosis by hamster oocytes was found to be relatively slow (Fig. 1). In most cases there was a very small recovery of around 0.2 pH units over 20 min. However, pHi did not recover to initial values even after 60 min (data not shown). This recovery from acidosis by hamster oocytes was not dependent on Na^+ in the medium as initial recovery rates were similar in medium with Na^+ (0.027 ± 0.009 pH units/min) or without Na^+ (0.032 ± 0.009 pH units/min, n = at least 40 embryos/treatment). Recovery from acidosis by hamster oocytes was also not sensitive to the presence of 50 μM EIPA (0.025 ± 0.007 pH units/min; n = 37 embryos. Removal of Na^+ from the medium also did not affect pHi of oocytes (Fig. 2). Therefore, oocytes do not appear to have NHE activity.

Na^+-dependent HCO_3^-/Cl^- exchanger. To determine if hamster oocytes have Na^+-dependent HCO_3^-/Cl^- exchange activity, the recovery experiments were repeated in the presence of bicarbonate (H3t). Recovery from acidosis was not affected by the presence of bicarbonate in the medium (Fig. 3). Similarly, recovery from acidosis was not affected by the presence of 100 μM DIDS (which inhibits HCO_3^-/Cl^- exchange) or 50 μM EIPA (Fig. 3). Therefore, Na^+-dependent HCO_3^-/Cl^- exchange activity was not detected.

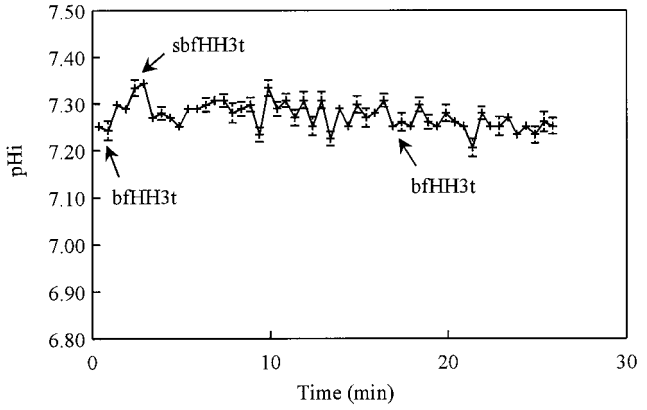

FIG. 2. Effect of Na^+ removal from the medium on pHi of hamster oocytes. Baseline pHi was determined in bfHH3t for 5 min, followed by incubation for 10 min in sbfHH3t. After 15 min total incubation time, bfHH3t medium was returned and pHi examined for a further 10 min. Data represent means ± SEM of a replicate experiment of 10 embryos.

Buffering Capacity of Oocytes and One-Cell Embryos

The intrinsic buffering capacity of oocytes and 10-h PEA one-cell embryos was calculated from the immediate increase in pHi after incubation with NH_4^+ as described by Baltz *et al.* (1991). To avoid interexperimental error both oocytes and 10-h PEA one-cell embryos were examined at the same time. Six replicates were performed with four to five oocytes and four to five embryos per replicate. The buffering capacity of oocytes was 51.4 ± 2.4 mM/pH. This was significantly higher than the buffering capacity calculated for 10-h PEA one-cell embryos of 17.9 ± 0.5 mM/pH ($P < 0.05$).

Determination of Na^+/H^+ Antiporter Activity in Embryos during Fertilization

Rates of recovery from acidosis of embryos collected at 1, 2, 3, 4, or 5 h PEA were slow, similar to those observed for oocytes. Recovery from acidosis by 1- to 5-h PEA one-cell embryos was Na^+-independent and was not sensitive to EIPA, indicating an absence of NHE activity (Fig. 4).

Recovery from acidosis by one-cell embryos collected between 6 and 10 h PEA was significantly reduced by the absence of Na^+ in the medium or the presence of EIPA (Fig. 4). The absence of Na^+ or presence of EIPA resulted in recovery rates not different from those of embryos collected between 1 and 5 h PEA. Furthermore, removing Na^+ from the medium did not affect pHi of embryos collected between 1 and 5 h PEA. However, removing Na^+ from the medium resulted in a decrease in pH for embryos collected at 5.5–10 h PEA (data not shown). Therefore, all embryos collected at 5.5 h PEA or later demonstrated NHE-dependent recovery from acidosis (Fig. 4). The rate of recovery from acidosis in Na^+-containing medium increased between 5.5 and 7 h PEA, when it reached a plateau at around 0.2 pH units/min (Fig. 4).

Na^+/H^+ Antiporter Activity in Parthenogenetically Activated Embryos

Recovery from acidosis by parthenogenetically activated one-cell or two-cell embryos was found to be both Na^+-dependent and EIPA sensitive. The rate of recovery of parthenogenetically activated one-cell embryos was 0.113 ± 0.018 pH units/min, which significantly decreased to 0.037 ± 0.009 pH units/min ($P < 0.05$) in the absence of Na^+ and 0.028 ± 0.005 pH units/min ($P < 0.05$) in the presence of EIPA (n = at least 30 embryos per experiment; minimum of three replicates). The rate of recovery in control medium bfHH3t of parthenogenetically activated 10-h PEA one-cell embryos was reduced compared to that of similar-aged 10-h PEA fertilized embryos (0.178 ± 0.015; $P < 0.05$). Activity of the NHE in parthenogenetically activated two-cell embryos (0.124 ± 0.021; n = at least 40 embryos; four replicates) was also reduced compared to fertilized 10-h PEA one-cell embryos ($P < 0.05$). Recovery from acidosis by parthenogenetically activated two-cell embryos was reduced by the absence of Na^+ (0.037 ± 0.010 pH units/min; $P < 0.05$; n = 42 embryos; four replicates) or presence of EIPA (0.029 ± 0.008 pH units/min; $P < 0.05$; n = 44 embryos; four replicates).

Development of One-Cell Embryos Collected at Various Stages after Egg Activation

Development of embryos collected at 1 and 2 h PEA was poor with only 9.5 and 11.7% of embryos developing to the blastocyst stage, respectively (Table 2). While

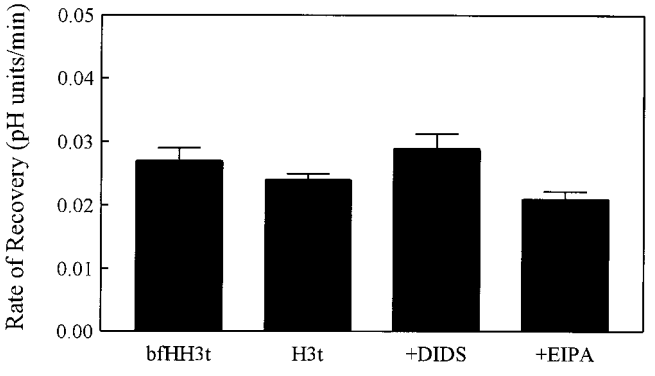

FIG. 3. Rates of recovery from acidosis by hamster oocytes in a medium containing bicarbonate. Recovery from acidosis by oocytes was assessed in medium H3t, H3t with 100 μM DIDS, or H3t with 50 μM EIPA and compared to that in medium bfHH3t. n = at least 3 replicates (8–12 embryos per replicate).

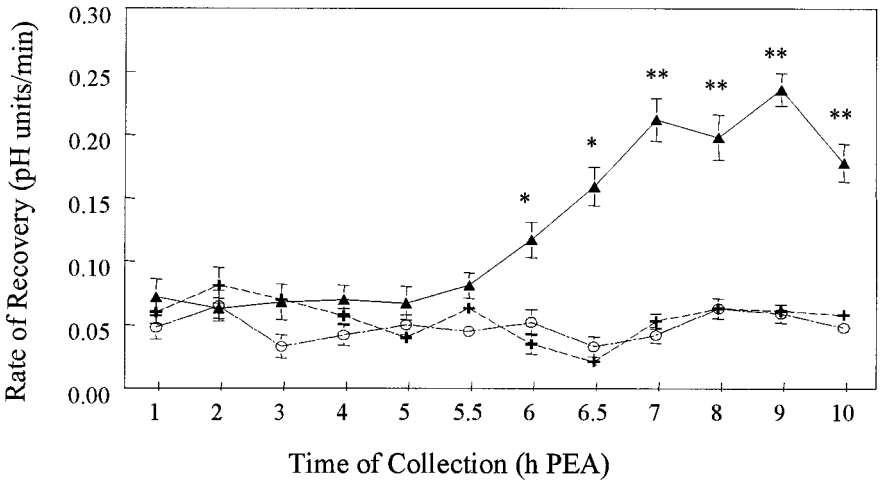

FIG. 4. Effect of Na^+ and EIPA on the recovery from acidosis by hamster one-cell embryos at various times after egg activation by the spermatozoa. Rates of recovery in control medium bfHH3t (closed triangles), rates of recovery in medium sbfHH3t (cross), and rates of recovery in medium bfHH3t with 50 μM EIPA (open circles). Rates of recovery from acidosis are significantly greater in control medium bfHH3t for embryos collected at 6–10 h PEA compared to medium without Na^+ or with EIPA ($P < 0.05$). Data represent means±SEM. n = between 24 and 36 embryos per time point (three replicates).

development to the morula/blastocyst stage was slightly higher for one-cell embryos collected between 3 and 4 h PEA, development to the blastocyst stage was still low. Significantly more embryos collected at 5 and 6 h PEA developed to the blastocyst stage, compared to embryos collected at earlier stages. However, highest rates of development were achieved when embryos were collected at 7, 8, or 9 h PEA.

TABLE 2

Development to the Morula/Blastocyst Stage of Hamster One-cell Embryos Collected at Various Times after Egg Activation

Time of collection (h PEA)	Morula/blastocyst (%)	Blastocyst (%)
1	21.7[a]	9.5[a]
2	23.5[a]	11.7[a]
3	42.8[b]	19.5[b]
4	55.5[bc]	14.8[ab]
5	50.0[bc]	38.1[c]
6	66.0[c]	45.0[c]
7	86.5[d]	68.1[d]
8	76.7[d]	65.3[d]
9	84.3[d]	67.3[d]

Note. n = between 50 and 90 embryos cultured per time point.
[ab] Values with different letters are significantly different within a column ($P < 0.05$).
[cd] Values with different letters are significantly different within a column ($P < 0.01$).

The Mechanism of Na^+/H^+ Antiporter Activation in One-Cell Embryos

In each experiment, one-cell embryos were collected from individual females at 2 h PEA and evenly distributed to culture in either HECM-10 or HECM-10 with each drug for 8 h. After 8 h the rates of recovery of pH from NH_4^+-induced acidosis were assessed. To avoid any variation between experiments, the control and drug-treated embryos were examined at the same time. Each experiment was replicated at least three times.

Effects of inhibitors of protein synthesis. There was no effect of culture for 8 h with 10 μg/ml cycloheximide on the rate of recovery from acidosis by one-cell embryos (Fig. 5a). The recovery from acidosis by embryos in both treatments was found to be Na^+-dependent. Interestingly, 10-h PEA one-cells that were collected at 2 h PEA and cultured for 8 h had a reduced rate of recovery from acidosis (0.147 ± 0.014) compared to freshly collected 10-h PEA one-cell embryos from the previous experiments (0.221 ± 0.017). To ensure that the cycloheximide had been effective at this concentration, we compared the incorporation of isotopically labeled amino acids by control and treatment groups. The incorporation of labeled amino acids by 10 groups of 10 one-cell embryos cultured for 8 h in HECM-10 or HECM-10 with cycloheximide was determined. The incorporation of labeled amino acids by embryos cultured with cycloheximide for 8 h was significantly reduced (1004 ± 34 dpm/min per 10 embryos) compared to those cultured without cycloheximide (7815 ± 75 dpm/min per 10 embryos; $P < 0.05$).

Effects of cytoskeletal disrupters. There was no effect of culture of one-cell embryos for 8 h with 100 μM cytocha-

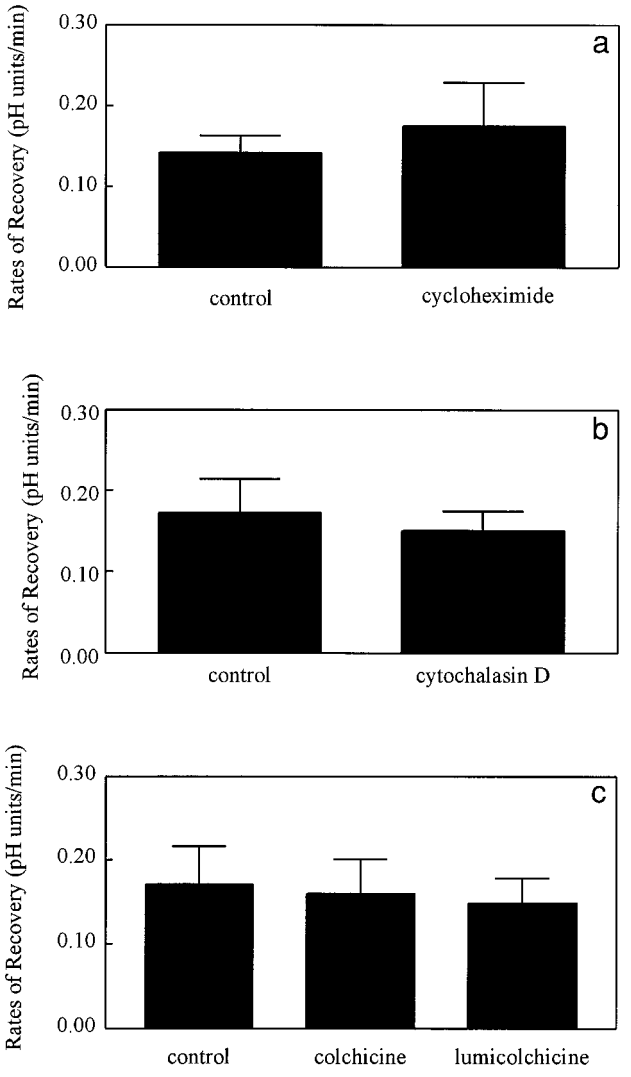

FIG. 5. Effect on the rate of recovery from acidosis of one-cell embryos cultured with (a) protein synthesis inhibitor cycloheximide (10 μg/ml), (b) cytochalasin D (100 μM), or (c) colchicine (10 μM) or lumicolchicine (10 μM). Control embryos were collected at 2 h PEA and cultured for 8 h in medium HECM-10 before measurement. Treated embryos were also collected at 2 h PEA and cultured for 8 h in HECM-10 supplemented with the indicated inhibitor.

lasin D, which disrupts actin filament polymerization, on the recovery rates from acidosis by one-cell embryos (Fig. 5b). Recovery from acidosis by all embryos was Na^+-dependent. Visualization of actin filaments by phalloidin staining using confocal microscopy (Squirrell *et al.*, 1998) confirmed that the actin in these embryos was disrupted (data not shown).

One-cell embryos cultured for 8 h with 10 μM colchicine, which disrupts the integrity of microtubules, had a similar rate of recovery from acidosis compared to embryos cultured in HECM-10 (Fig. 5c). The inactive form of colchicine, lumicolchicine (10 μM), also did not affect the rates of recovery compared to control embryos. The recovery from acidosis by all embryos was Na^+-dependent. Antibody staining and confocal microscopy (Squirrell *et al.*, 1998) confirmed that the microtubules were disrupted after culture with colchicine but not after culture with lumicolchicine or in the control embryos (data not shown).

Effects of protein kinase C inhibitors and stimulators. Culture of one-cell embryos with 500 nM sphingosine, a specific inhibitor of protein kinase C (PKC) activity (Hannun and Bell, 1989), for 8 h significantly reduced the rates of recovery from acidosis (Fig. 6), compared to the embryos cultured in medium HECM-10. The low rate of recovery from acidosis by embryos cultured with sphingosine was Na^+-independent and not affected by the presence of EIPA, indicating that recovery was not due to the NHE. To further confirm that the appearance of NHE is a PKC-regulated process, oocytes which lack NHE activity were cultured in medium HECM-10 or HECM-10 supplemented with 100 nM PMA, an activator of PKC activity. Oocytes incubated with PMA had an increased rate of recovery from acidosis (Fig. 7) and this recovery was Na^+-dependent. However, the rate of recovery observed after incubation with PMA was reduced compared to the recovery rates observed for one-cell embryos cultured in HECM-10 in the earlier experiments (mean recovery of 0.142 $\pm$ 0.007 pH units/min; $P < 0.05$). Incubation with the inactive form of the phorbol ester, MPMA (400 nM), did not affect recovery rates from acidosis (Fig. 7).

Effects of calcium chelators. Culture of one-cell embryos with the calcium chelator BAPTA-AM (5 μM) significantly reduced the rate of recovery from acidosis compared to one-cell embryos cultured in control medium HECM-10 alone (Fig. 8). However, the rate of recovery was significantly greater than that of one-cell embryos that were

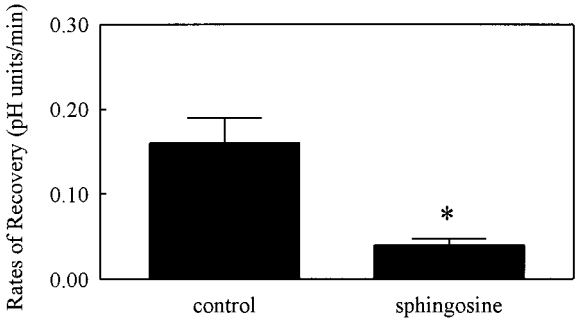

FIG. 6. Effect of culture with the protein kinase C inhibitor sphingosine (500 nM) on the rate of recovery from acidosis by hamster one-cell embryos. *Significantly different from recovery in bfHH3t ($P < 0.05$). Control embryos were collected at 2 h PEA and cultured for 8 h in medium HECM-10 before measurement. Treated embryos were also collected at 2 h PEA and cultured for 8 h in HECM-10 supplemented with sphingosine.

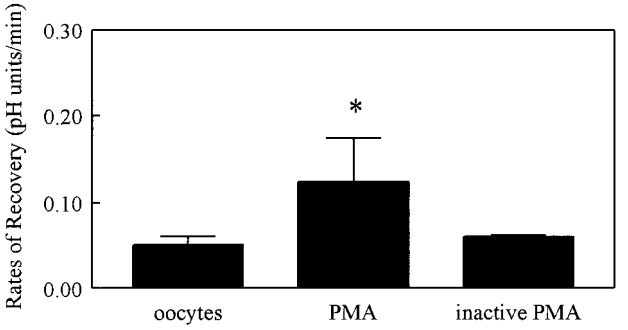

FIG. 7. Effect of culture with a phorbol ester on the rate of recovery from acidosis by hamster oocytes. *Significantly different from recovery in bfHH3t ($P < 0.05$). Control embryos were collected at 2 h PEA and cultured for 8 h in medium HECM-10 before measurement. Treated embryos were also collected at 2 h PEA and cultured for 8 h in HECM-10 supplemented with either PMA (100 nM) or MPMA (400 nM). The increase in recovery from acidosis by oocytes cultured in PMA was Na^+-dependent.

cultured with sphingosine. Removal of Na^+ from the medium resulted in a decrease of 0.09 ± 0.02 pH units from resting pHi in one-cell embryos that were cultured with BAPTA, indicating that there was still a Na^+-dependent component to regulation of pHi.

DISCUSSION

Hamster oocytes appear to lack any mechanism for the alleviation of intracellular acidosis. Neither NHE activity or Na^+-dependent HCO_3^-/Cl^- transporter activity could be demonstrated. Most cells utilize the NHE to alleviate intracellular acidosis. However, there are a few exceptions, such as erythrocytes (Boron, 1983; Frelin *et al.*, 1985), rabbit pulmonary macrophages (Bidani *et al.*, 1989), and undifferentiated chick somites (Gillespie and Greenwell, 1988). Reports by Baltz *et al.* (1990, 1991) showed that the embryos from specific mouse strains also lack NHE activity. Furthermore, similar to the hamster oocytes in this study, active Na^+-dependent HCO_3^-/Cl^- transporter activity could not be detected in these mouse embryos. Instead recovery from acidosis by both hamster oocytes and the mouse embryos in the studies by Baltz *et al.* (1990, 1991) was slow.

Hamster oocyte cytoplasm had an increased intrinsic buffering capacity (i.e., ability to buffer changes in pH) compared to that of one-cell embryos collected at 10 h PEA. This increased buffering capacity may be an evolutionary mechanism to compensate for the reduced capacity to restore pHi from acid levels due to a lack of functional NHE. The value for buffering capacity of hamster oocytes of 51 mM/pH is higher than that reported for mouse (Baltz *et al.*, 1990) or hamster (Lane *et al.*, 1998a) cleavage-stage embryos, which ranges from around 17 to 35 mM/pH.

NHE activity was not detected in hamster one-cell embryos until after 5.5 h PEA. Prior to this time recovery from acidosis was found to be both Na^+-independent and EIPA-insensitive and resembled that observed for hamster oocytes. Activity of the NHE appeared gradually over a 2-h period and by 7 h after egg activation, maximal activity of the antiporter could be detected. The rates of recovery of pHi observed in this study by 7- and 10-h PEA embryos are similar to the rates previously reported for the 10-h PEA one-cell and 32-h PEA two-cell hamster embryo (Lane *et al.*, 1998a).

Analysis of parthenogenetically activated one-cell and two-cell embryos revealed an active NHE, although the rates of recovery observed were reduced compared to normally fertilized one-cell and two-cell embryos (Lane *et al.*, 1998a). The presence of NHE activity in parthenogenetically activated embryos indicates that the appearance of the antiporter was not a result of fertilization but due to activation of the oocyte. Therefore, either the mRNA for the protein or the protein itself already exists in the oocyte, despite the lack of functional Na^+/H^+ exchange in oocytes. In support of this a recent study on the mouse demonstrated the presence of mRNA for the NHE in both oocytes and two-cell embryos (Barr *et al.*, 1998), despite the apparent absence of functional NHE activity in two-cell mouse embryos (Baltz *et al.*, 1990, 1991).

In parallel to this study, a study by Phillips and Baltz (1999) has demonstrated that activity of the HCO_3^-/Cl^- transporter which regulates pHi in the alkaline range could not be detected in the mouse oocyte. However, activity could be demonstrated beginning around 4 h postinsemination. Similar to the NHE in the hamster, the HCO_3^-/Cl^- transporter appeared gradually and reached a maximum after around 8 h postinsemination (Phillips and Baltz, 1999). Similarly, parthenogenetically activated mouse embryos also had HCO_3^-/Cl^- transporter activity, indicating that

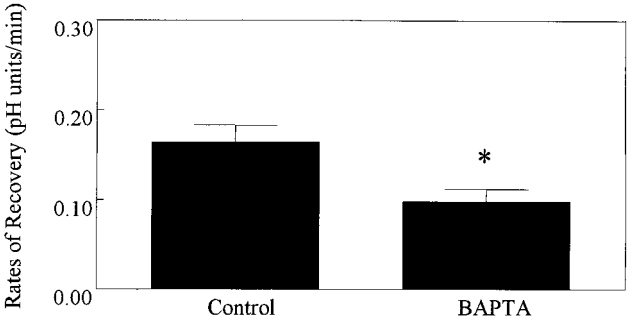

FIG. 8. Effect of culture with calcium chelator BAPTA on the rate of recovery from acidosis by hamster embryos. *Significantly different from recovery in bfHH3t ($P < 0.05$). Control embryos were collected at 2 h PEA and cultured for 8 h in medium HECM-10 before measurement. Treated embryos were also collected at 2 h PEA and cultured for 8 h in HECM-10 supplemented with BAPTA (5 μM).

like the hamster, the mRNA or protein is also found in the mouse oocyte and is initiated by activation of the oocyte (Phillips and Baltz, 1999).

NHE activity is activated in many cells by growth factors and hormones. The three major mechanisms of activation identified for this antiporter are the synthesis of new protein, transport of existing protein to the membrane, or activation of existing transporters, usually by PKC, Ca^{2+}-calmodulin kinase, or protein kinase A (reviewed by Grinstein and Rothstein, 1986; Fliegel and Frohlich, 1993). In this study, incubation of newly fertilized embryos with cycloheximide did not affect NHE activity, indicating that the appearance of antiporter activity during fertilization is not due to synthesis of new protein. Furthermore, the cytoskeleton was not involved in the trafficking of protein to the cell membrane, as disruption of either actin filaments or microtubules did not affect antiporter appearance or activity. A common mechanism for the activation of the NHE1 isoform of the NHE, the isoform thought to be present in the hamster embryo (Lane *et al.*, 1998a), is through either direct or indirect phosphorylation of the C-terminal cytoplasmic domain by PKC (Sardet *et al.*, 1989). The increase in NHE activity which occurs during sea urchin fertilization is mediated by PKC (Shen and Buck, 1990). Sphingosine specifically interferes with the activation of PKC by displacing diacylglycerol from the PKC complex (Hannun and Bell, 1989) and has been used in a variety of cell types to inhibit the biological actions of PKC (Hannun *et al.*, 1987; Hannun and Bell, 1989). Incubation of hamster embryos with sphingosine prevented the activation of NHE activity. Furthermore, incubation of oocytes which lack functional Na^+/H^+ exchange with the phorbol ester PMA, which stimulates PKC activity, induced some NHE activity. Therefore the initiation of NHE activity during fertilization in the hamster appears due to phosphorylation of existing inactive protein either directly or indirectly by PKC. Calcium oscillations initiated by sperm activation of the egg continue for around 5–6 h, which is around the time that the antiporter is activated (Miyazaki and Igusa, 1981; Bos-Mikich *et al.*, 1997). Prevention of these calcium waves by incubation with the calcium chelator BAPTA-AM in this study prevented the normal onset of NHE activity in one-cell embryos following fertilization. Therefore, it appears that these calcium waves are at least in part involved in the mechanism for the activation of the PKC regulatory pathway that initiates NHE activity in one-cell embryos around 6 h after fertilization.

An interesting finding in this study is that the onset of NHE activity occurs at the same time PEA as the observed increase in the ability of one-cell embryos to develop in culture. Embryos collected and cultured prior to the appearance of NHE activity developed very poorly in culture, indicating that these embryos may not be able to regulate pHi in culture. Inhibition of NHE in cultured hamster two-cell embryos prevented development when embryos were exposed to a small acidosis (Lane *et al.*, 1998a), indicating that the presence of functional NHE appears to have an important physiological role at least in culture, when the embryo is lacking the supportive environment of the reproductive tract.

As the mouse embryo also activates its major pHi regulatory system, the HCO_3^-/Cl^- transporter, around 4–8 h after egg activation (Phillips and Baltz, 1999), this time of embryo development may be crucial for the initiation and activation of several proteins that are essential for subsequent developmental competence. It therefore appears that the early mammalian embryo immediately following fertilization has no system for regulating pHi. Additionally, these early hamster embryos are also unable to regulate intracellular calcium homeostasis due to an inability to regulate voltage-gated calcium channels (Lane and Bavister, 1998). Interestingly, these calcium channels are also regulated by pHi, so a lack of a functional NHE may also contribute to this loss in calcium homeostasis, which resulted in a loss in developmental competence (Lane and Bavister, 1998). The acquirement of NHE activity during fertilization therefore appears important for normal embryo development.

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