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Intracellular pH regulation in human preimplantation embryos

Karen P.Phillips[1,3], Marie-Claude Léveillé[2,4], Paul Claman[2,4] and Jay M.Baltz[1,2,3,4,5]

[1]Loeb Research Institute, Ottawa Hospital and Departments of [2]Obstetrics and Gynecology, (Division of Reproductive Medicine), [3]Cellular and Molecular Medicine, University of Ottawa and [4]Human IVF Program, Ottawa Hospital, Ottawa, Ontario, Canada K1Y 4E9

[5]To whom correspondence should be addressed at: Loeb Research Institute, Ottawa Hospital, Civic Site, 725 Parkdale Ave, Ottawa, Ontario K1Y 4E9, Canada

We report here that intracellular pH (pH_i) in cleavage-stage human embryos (2–8-cell) is regulated by at least two mechanisms: the HCO_3^-/Cl^- exchanger (relieves alkalosis) and the Na^+/H^+ antiporter (relieves acidosis). The mean pH_i of cleavage-stage embryos was 7.12 ± 0.008 (n = 199) with little variation between different stages. Embryos demonstrated robust recovery from alkalosis that was appropriately Cl^--dependent, indicating the presence of the HCO_3^-/Cl^- exchanger. This was further confirmed by measuring the rate of intracellular alkalinization upon Cl^- removal, which was markedly inhibited by the anion transport inhibitor, 4,4′-diisocyanatostilbene-2,2′-disulphonic acid, disodium salt. The set-point of the HCO_3^-/Cl^- exchanger was between pH_i 7.2 and 7.3. Embryos also exhibited Na^+-dependent recovery from intracellular acidosis. Na^+/H^+ antiporter activity appeared to regulate recovery up to about pH_i 6.8; this recovery was HCO_3^--independent and amiloride-sensitive, with a pH_i set-point of ~6.8–6.9. A second system that was both Na^+- and HCO_3^--dependent appeared to mediate further recovery from acidosis up to about pH_i 7.1. Thus, pH_i of early human preimplantation embryos appears to be regulated by opposing mechanisms (HCO_3^-/Cl^- exchanger, Na^+/H^+ antiporter, and possibly a third acid-alleviating transporter that was both Na^+- and HCO_3^--dependent) resulting in the maintenance of pH_i within a narrow range.

Key words: embryo/HCO_3^-/Cl^- exchanger/Na^+/H^+ antiporter/pH

Introduction

Intracellular pH (pH_i) regulation is an important component of mammalian cell homeostasis. There are three major pH_i-regulatory mechanisms: the HCO_3^-/Cl^- exchanger, which alleviates alkalosis, and the Na^+/H^+ antiporter and Na^+,HCO_3^-/Cl^- exchanger, both of which alleviate acidosis. pH_i regulation has been well-examined in some mammalian embryos, such as those of mouse (Zhao *et al*., 1995; Phillips and Baltz, 1999) and hamster (Lane *et al*., 1998, 1999a,b), but little is known about the mechanisms of pH_i regulation in the human preimplantation embryo.

HCO_3^-/Cl^- exchangers mediate the electroneutral exchange of extracellular Cl^- for intracellular HCO_3^-, thus decreasing pH_i (Alper, 1991, 1994). HCO_3^-/Cl^- exchangers are members of the *AE* (anion exchanger) gene family, which includes the erythrocyte band 3 protein (AE1) and at least two other isoforms with wide tissue distributions (AE2 and AE3). We have shown that mRNA encoding two members of the *AE* family, *AE2* and *AE3*, are expressed in preimplantation mouse embryos, with *AE2* mRNA also present in the unfertilized egg (Zhao *et al*., 1995; Phillips and Baltz, 1999). HCO_3^-/Cl^- exchanger activity is present in all stages of preimplantation embryo in the mouse and hamster and is required for maintenance of normal pH_i and recovery from intracellular alkalosis in mouse embryos (Baltz *et al*., 1991; Zhao *et al*., 1995; Lane *et al*., 1999b). Activity is very low in the unfertilized mouse and hamster egg and gradually becomes activated following fertilization, reaching maximal activity around the time of pronuclear formation (Lane *et al*., 1999b; Phillips and Baltz, 1999).

The Na^+/H^+ antiporter mediates the electroneutral exchange of extracellular Na^+ for intracellular H^+ to increase pH_i. Na^+/H^+ antiporter is encoded by members of the *NHE* gene family (*NHE1–6*; Orlowski and Grinstein, 1997). *NHE-1* mRNA expression has been demonstrated in mouse eggs and in all stages of mouse embryo development, and immunohistochemistry of mouse blastocysts has shown NHE-1 protein localization to the blastocoel membrane (Barr *et al*., 1998). NHE-3, whose mRNA has only been detected in unfertilized mouse eggs, immunolocalizes to the outer surface of the blastocyst (Barr *et al*., 1998). pH_i is regulated by Na^+/H^+ antiporter in hamster embryos (Lane *et al*., 1998), and to varying extent in mouse embryos depending on the strain of mouse (Gibb *et al*., 1997; C.L.Steeves, M.Lane, K.P.Phillips, B.Bavister and J.M.Baltz, unpublished). Na^+/H^+ antiporter activity becomes activated following fertilization in the hamster (Lane *et al*., 1998, 1999a), similar to HCO_3^-/Cl^- exchanger upregulation at fertilization in the mouse (Phillips and Baltz, 1999) and hamster (Lane *et al*., 1999b).

The Na^+, HCO_3^-/Cl^- exchanger imports HCO_3^- and Na^+ in exchange for Cl^- and is amiloride-insensitive, inhibited instead by stilbene derivatives such as 4,4′-diisocyanatostilbene-2,2′-disulphonic acid, disodium salt (DIDS) (Grinstein *et al*., 1989). To date, Na^+, HCO_3^-/Cl^- exchanger activity has not been reported in mammalian embryos.

Recently, it has been shown that human preimplantation embryos, at all stages from zygote to blastocyst, have the ability to recover from intracellular alkalosis, which was

induced by increased external pH (Dale *et al*., 1998). In contrast, Dale *et al*. could not detect any recovery from mild acidosis induced by decreased external pH in human embryos until the blastocyst stage. Thus, they concluded that preimplantation human embryos lack mechanisms needed for recovery from intracellular acidosis until the blastocyst stage, but possess a mechanism such as the HCO_3^-/Cl^- exchanger for alleviating alkalosis throughout preimplantation development.

Here, we report the results of our investigations into the mechanisms used by human embryos to regulate pH_i. We have used methods which were successfully employed in mouse and hamster embryos to reveal the identity and active ranges of pH_i regulatory mechanisms present in human embryos.

Materials and methods

Eggs and embryos

Immature and mature eggs and cleavage-stage embryos, excess to requirements for standard IVF or intracytoplasmic sperm injection (ICSI) protocols at the Human IVF Clinic, Ottawa Hospital were obtained for research with written patient consent. Patients were stimulated using standard ovarian stimulation protocols which included a gonadotrophin releasing hormone analogue (Lupron; Abbott Pharmaceuticals, Montreal, QC, Canada), followed by ovarian stimulation with human menopausal gonadotrophin (Humegon; Organon Canada; Scarborough, ON, Canada) or recombinant FSH (Puregon; Organon Canada) with dose adjusted according to follicular response and serum oestradiol concentrations (Rattanachaiyanont *et al*., 1999). Oocyte retrieval was performed 36 h following i.m. administration of 10 000 IU human chorionic gonadotrophin (HCG, Pregnyl®; Organon Canada). Ninety-six embryos used for this study were obtained following IVF while 126 embryos were obtained following ICSI. For IVF, each cumulus-enclosed egg was inseminated with 0.125×10^6/ml motile spermatozoa following standard sperm washing procedures. 17–19 h post-insemination, eggs were stripped of cumulus cells and assessed for the presence of pronuclei. For ICSI, eggs were stripped of cumulus cells using 80 IU/ml hyaluronidase (Type VIII Bovine Testes; H-3757, Sigma, St Louis, MO, USA) in HEPES-buffered human tubal fluid with 0.5% bovine serum albumin (HTF-BSA) (HTF; Meditech 1ST Canada Inc., Montreal, QC, USA) 2–4 h post-egg retrieval and assessed as immature if there was a germinal vesicle (GV) or no polar body (metaphase I: MI), or mature (metaphase II: MII) if there was no GV and one polar body. Only MII eggs were used for ICSI. Immature eggs and some mature eggs (MII) were released for research (within 24 h of oocyte retrieval) and were not exposed to spermatozoa. One immature egg (MI) did mature (MII) after several hours in culture after release for research. In total, four GV eggs, nine MI eggs and nine MII eggs were released for research and used for this study.

ICSI was performed on an inverted microscope at ×400 magnification using Hoffman modulation contrast. 1–2 µl washed spermatozoa were placed in HEPES-buffered HTF-BSA containing 10% polyvinylpyrrolidone (PVP K-90; Irvine Scientific, Santa Ana, CA, USA) and injected using standard techniques. Following injection, each egg was cultured individually in a 20 µl drop of HTF-BSA covered with paraffin oil at 37°C, 5% O_2/5% CO_2/90% N_2 for about 17–19 h. Embryos were then assessed for the presence of pronuclei (PN). Pronuclei detected in embryos donated for research included (assessed 17–19 h post-IVF/ICSI): 0PN, 26 embryos; 1PN, 43 embryos; 2PN, 120 embryos; 3 PN, 23 embryos; 4 PN, 1 embryo. Embryos were maintained in culture for 2–4 days post-IVF/ICSI during which time embryo cleavage was assessed. Embryos were graded from 1 to 5 (Rattanachaiyanont *et al*., 1999) according to the quality of blastomeres and the presence of fragmentation with 5 indicating the best-quality embryos. Embryos used for this study included 11 Grade 1; 53 Grade 2; 122 Grade 3; 21 Grade 4; and 6 Grade 5 embryos. Eggs were not rated.

Chemicals and solutions

All chemicals and drugs were obtained from Sigma (St Louis, MO, USA) unless otherwise noted. SNARF-1-AM (carboxyseminaphthorhodafluor-1-acetoxymethyl ester) and DIDS were obtained from Molecular Probes (Eugene, OR, USA).

Media used for pH_i measurements were based on KSOM mouse embryo culture medium (Lawitts and Biggers, 1993). This medium supports the culture of human embryos, and its use allows direct comparisons with our previous results in the mouse. The modified KSOM contained (in mol/l) 104 NaCl, 2.5 KCl, 0.35 KH_2PO_4, 0.2 $MgSO_4$, 1 Na^+ lactate, 0.2 glucose, 0.2 Na^+ pyruvate, 25 $NaHCO_3$, 1.7 $CaCl_2$, 1 glutamine, 0.01 tetrasodium EDTA, 0.03 streptomycin SO_4 and 0.16 K penicillin G. Media were equilibrated with 5% CO_2/ air except where noted. HEPES-KSOM (Lawitts and Biggers, 1993), used for egg/embryo handling, was produced by replacing 21 mmol/l (of 25 mmol/l) $NaHCO_3$ with equimolar HEPES (pH adjusted to 7.4 with NaOH or KOH, as appropriate). HCO_3^--free solutions were similarly produced by replacing 21 mmol/l $NaHCO_3$ with equimolar HEPES and the remaining 4 mmol/l with equimolar NaCl. For Cl^--free solutions, all Cl^- salts were replaced with corresponding gluconate salts. For Na^+-free (< 1 mmol/l) solutions, NaCl was replaced with equimolar choline Cl^-, and Na^+ pyruvate, Na^+ lactate and $NaHCO_3$ were replaced by K^+ pyruvate, lactic acid and choline HCO_3^-, respectively. To induce alkalosis, solutions containing 35 mmol/l NH_4Cl were used (NH_4^+-KSOM), wherein 25 mmol/l $NaHCO_3$ was reduced to 12 mmol/l; NaCl was 69 mmol/l. To inhibit recovery from alkalosis, 0 Cl^- NH_4^+-KSOM was used in which NH_4Cl was replaced with NH_4SO_4 and all other Cl^- salts replaced with corresponding gluconate salts. Acidosis was induced by a 10 min pulse of 35 mmol/l NH_4Cl (NH_4^+-KSOM or NH_4^+-HEPES-KSOM, as specified) containing 25 mmol/l $NaHCO_3$.

pH_i measurements

pH_i was measured using the pH-sensitive fluorophore, SNARF-1, loaded into eggs/embryos by incubating them with 5.0 µmol/l SNARF-1-AM at 37°C for 30 min in HEPES-KSOM (House, 1994; Zhao *et al*., 1995; Zhao and Baltz, 1996; Phillips and Baltz, 1996; Baltz and Phillips, 1998; Phillips *et al*., 1998). After SNARF-1 loading, eggs/embryos were washed several times with HEPES-KSOM and placed in a temperature-controlled chamber (Biophysica, Baltimore, MD, USA) that was modified to allow solution changes and control of the atmosphere. Complete exchange of solutions in the chamber was obtained after ~1 min (unpublished measurements). During measurements, eggs/embryos were maintained at 37°C (± 0.5°C). For most experiments, HEPES-KSOM was immediately replaced with KSOM.

The methods used for pH_i measurements have been previously described in detail (Baltz *et al*., 1990; Zhao *et al*., 1995, 1997; Baltz and Phillips, 1998; Phillips *et al*., 1998). Briefly, simultaneous measurements were made of groups of eggs/embryos with data recorded for each individual egg/embryo. SNARF-1 fluorescence was detected using an intensified CCD camera with output to an image storage and quantification system (Inovision, Durham, NC, USA). Two fluorescence emission wavelengths were detected, 640 nm (pH_i-sensitive) and 600 nm (pH_i-insensitive), using an excitation wavelength of 535 nm. The ratio of the two emission intensities

(640/600), dependent only on pH_i, was calculated by dividing the images after background subtraction. Ratio was calibrated to pH_i using calibration solutions containing 10 μg/ml nigericin and 5 μg/ml valinomycin with 100 mmol/l K^+ (Thomas *et al*., 1979; Baltz *et al*., 1990). pH_i calibration curves were generated regularly and were indistinguishable between eggs and all embryo stages. SNARF-1 loading and exposure to excitation illumination does not adversely affect mouse eggs or embryos, as it was previously shown that mouse eggs could be fertilized by IVF and will cleave following pH_i measurements (Phillips *et al*., 1998).

Recovery from induced alkalosis

To determine whether eggs/embryos were able to recover from an increase in pH_i, intracellular alkalosis was induced using the permeant weak base NH_4Cl (Boron and DeWeer, 1976; Roos and Boron, 1981; Zhao and Baltz, 1996). Following steady-state pH_i measurements, the solution was changed to NH_4^+-KSOM (12 mmol/l HCO_3^-) for 20 min. HCO_3^- was reduced to 12 mmol/l to maximize recovery from intracellular alkalosis (external HCO_3^- acts as a competitive inhibitor; Baltz *et al*., 1991). Where appropriate, HCO_3^-/Cl^- exchanger activity was inhibited by omitting external Cl^-. The rate of recovery from alkalosis was determined by fitting the recovery to a single exponential by non-linear regression. The first derivative was used to calculate the rate of recovery as a function of pH_i. For statistical analysis, recovery rates were compared at every 0.1 pH_i increment between 7.1 and 7.7.

Cl^- removal assay for HCO_3^-/Cl^- exchanger activity

Upon exposure of cells to Cl^--free solution, any HCO_3^-/Cl^- exchanger present will run in reverse, resulting in intracellular alkalinization due to HCO_3^- influx coupled to Cl^- efflux (Nord *et al*., 1988). Intracellular alkalinization upon Cl^- removal is therefore indicative of HCO_3^-/Cl^- exchanger activity and the initial rate of alkalinization serves as a measure of activity (Nord *et al*., 1988; Zhao and Baltz, 1996). In this assay, following 10 min of steady-state pH_i measurement, the solution was changed to Cl^--free KSOM for 20 min. The initial rate of intracellular alkalinization upon Cl^- removal was determined using linear regression. For some experiments, pH_i was measured during Cl^- removal for 10 min, after which external Cl^- was replaced (KSOM) for 10 min and then a second Cl^- removal was performed for an additional 20 min.

Recovery from induced acidosis

To determine whether embryos were able to recover from a decrease in pH_i, intracellular acidosis was induced by a 10 min pulse of NH_4^+-containing solution (NH_4^+-KSOM or NH_4^+-HEPES-KSOM) followed by a 15 min period in KSOM. This protocol results in the net acidification of the cytoplasm after the NH_4^+ pulse (Boron and DeWeer, 1976; Roos and Boron, 1981; Zhao and Baltz, 1996). Generally, any possible recovery from acidosis was assessed by measuring pH_i for 15 min following the NH_4^+ pulse. In some experiments, however, a 15 min period in Na^+-free solution was followed by another 15 min in Na^+-containing solution. The rate of recovery from acidosis was determined by fitting the recovery to a single exponential by non-linear regression. The first derivative was used to calculate the rate of recovery as a function of pH_i. For statistical analysis, recovery rates were compared at every 0.1 pH_i increment between 6.5 and 7.1.

Statistics

Data are presented as the mean ± SEM. In all cases, $P < 0.05$ was considered significant. Descriptive statistics were obtained using SigmaPlot 1.0 (Jandel Scientific, San Rafael, CA, USA) or SigmaPlot 5.0 (SPSS, Chicago, IL, USA). Throughout, '*n*' indicates the total number of eggs or embryos. Statistical comparisons were done using InStat (GraphPad, San Diego, CA, USA). For statistical comparisons of three or more groups Bartlett's test for homogeneity of variances was first used to determine whether parametric ANOVA (pANOVA) or non-parametric ANOVA (nANOVA) was appropriate. Comparisons were then made using pANOVA or nANOVA followed by the Tukey–Kramer multiple comparisons test or Dunn's test, respectively. For two groups of data, an *F*-test was performed to test for equality of variances prior to selecting a *t*-test for analysis. Statistical comparisons of two groups of data were made using Student's *t*-test or Welch's alternate *t*-test for equal or unequal variances, respectively. Linear regression was used to determine the initial rate of alkalinization upon Cl^- removal and to determine the rate of pH_i change when recovery from acidosis was inhibited. Non-linear regression, using the appropriate exponential curves as specified, was used to determine the rates of recovery from alkalosis and acidosis.

Results

Steady-state pH_i in human eggs and embryos

Steady-state pH_i was measured in KSOM (5% CO_2, 37°C) in eggs (GV, MI and MII) and cleavage-stage embryos (2–8 cell). pH_i ranged from about 6.9 to 7.55 among different eggs and embryos with no significant difference between stages ($P > 0.05$; nANOVA) or fertilization protocol (ICSI versus IVF; Welch's *t*-test; $P > 0.05$). Embryo morphology rating generally had no effect on pH_i, although pH_i of embryos rated 2 or 3 was significantly lower than pH_i of embryos rated 5, probably due to variation and small sample size in the latter group (nANOVA; $P < 0.05$). Mean pH_i (± SEM) were as follows: GV eggs: 7.04 ± 0.07 ($n = 4$); MI eggs: 7.03 ± 0.04 ($n = 9$); MII eggs: 6.98 ± 0.02 ($n = 9$); and cleavage-stage embryos: 7.12 ± 0.01 ($n = 199$).

Recovery from intracellular alkalosis in embryos

Cleavage-stage embryos were alkalinized by exposure to NH_4Cl to assess whether embryos could recover from intracellular alkalosis (Figure 1A). Recovery rates were determined by fitting a single exponential to the recovery by non-linear regression for every 0.1 pH_i increment between pH_i 7.1 and 7.7 (Figure 1C). There was no significant difference in recovery rates between stages of embryo development (compared at pH_i 7.6; $P > 0.05$; nANOVA), and thus, the recovery rates for cleavage-stage embryos were pooled ($n = 15$; Figure 1A). When embryos ($n = 8$) were alkalinized in the absence of external Cl^- (Figure 1B,C) recovery from alkalosis was significantly inhibited ($P = 0.001$, Welch's *t*-test). Thus, embryos demonstrate Cl^--dependent recovery from alkalosis, which was active above about pH_i 7.2–7.3 (Figure 1C).

Assessment of HCO_3^-/Cl^- exchanger activity in embryos by Cl^- removal assay

Cleavage-stage embryos demonstrated rapid intracellular alkalinization upon Cl^- removal (Figure 2A,B). There was no significant difference in rate between cleavage stages, morphology rating or fertilization protocol ($P > 0.05$; nANOVA). The mean rate (± SEM) of intracellular

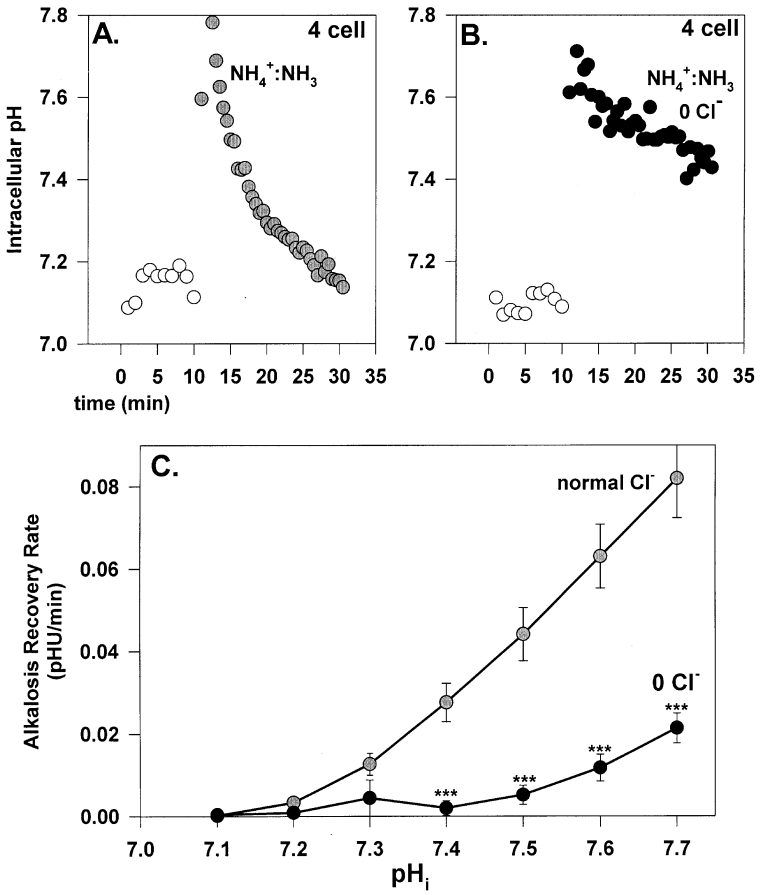

Figure 1. Recovery of cleavage-stage embryos from intracellular alkaline load. (**A**, **B**) Representative traces of pH_i demonstrating steady-state pH_i, measured in modified KSOM (○), followed by alkalinization in the presence (**A**; ⊜) or absence (**B**; ●) of external Cl^-. (**C**) Mean rate of recovery (± SEM) from NH_4^+-induced alkaline load was calculated from exponential fits to recovery data as described in the text for every 0.1 pH unit between 7.1 and 7.7 for cleavage-stage embryos (2–8 cells) in the presence ($n = 15$) or absence ($n = 8$) of external Cl^-. ***Statistical significance ($P < 0.0001$; Welch's *t*-test).

alkalinization for pooled cleavage-stage embryos was 0.053 ± 0.008 ($n = 32$). For some embryos, a second Cl^- removal was performed in which Cl^- was replaced following the first Cl^- removal (Figure 2A,B). There was no significant difference between the initial rate of intracellular alkalinization (first Cl^- removal) and the rate upon the second Cl^- removal (Figure 2A,C; paired Student's *t*-test, $P > 0.05$; $n = 6$). However, there was a significant inhibition of the rate of intracellular alkalinization when the anion transport inhibitor DIDS was present during the second Cl^- removal (Figure 2B,C; paired Student's *t*-test; $P = 0.0016$; $n = 9$).

Recovery from intracellular acidosis in embryos

Recovery from induced acidosis was measured for 15 min following an NH_4^+ pulse. For some experiments, embryos were maintained in Na^+-free solution following an NH_4^+ pulse and then Na^+ was replaced for an additional 15 min (Figure 3A). Recovery rates from induced acidosis were determined by non-linear regression for every 0.1 pH_i increment spanning the pH_i range from the initial acidification pH_i to the final pH_i value measured at the end of 15 min. This pH_i range was specific for each embryo as there was considerable variation between embryos in the initial acidification pH_i and the final pH_i value. Cleavage-stage, morphology rating and fertilization protocol had no significant effect on the initial rate of recovery from acidosis ($P > 0.05$; nANOVA); thus the data were pooled. Comparing the rates of recovery from acidosis within the same pH_i range, there was no significant difference between recoveries following acidification directly into Na^+-containing solutions or the recoveries acidified first in the absence of external Na^+, followed by Na^+ replacement ($P > 0.05$; linear regression). Thus, the rates obtained from the two protocols were pooled for the analysis of rate as a function of pH_i. Rates obtained by linear regression (i.e., when recovery was inhibited and there was thus very little change in pH_i during 15 min period) are reported at the initial acidification pH_i to the nearest 0.1 pHU (e.g. the amiloride point in Figure 3B and all 0 Na^+ points).

Embryos recovered from induced acidosis in the absence of external HCO_3^- (Figure 3A,B). The absence of external Na^+ completely abolished this recovery (Figure 3A,B; $P < 0.0001$; nANOVA; Welch's *t*-test). Amiloride also significantly inhibited recovery (Figure 3A,B; $P < 0.0001$; nANOVA). This Na^+-dependent, bicarbonate-independent recovery dropped to non-specific levels at pH_i 6.8, indicating activity only below this pH_i (Figure 3B; $P > 0.05$; Welch's *t*-test).

Embryos also recovered from acidosis in the presence of external HCO_3^- (Figure 3A,C). Recovery from acidosis in the

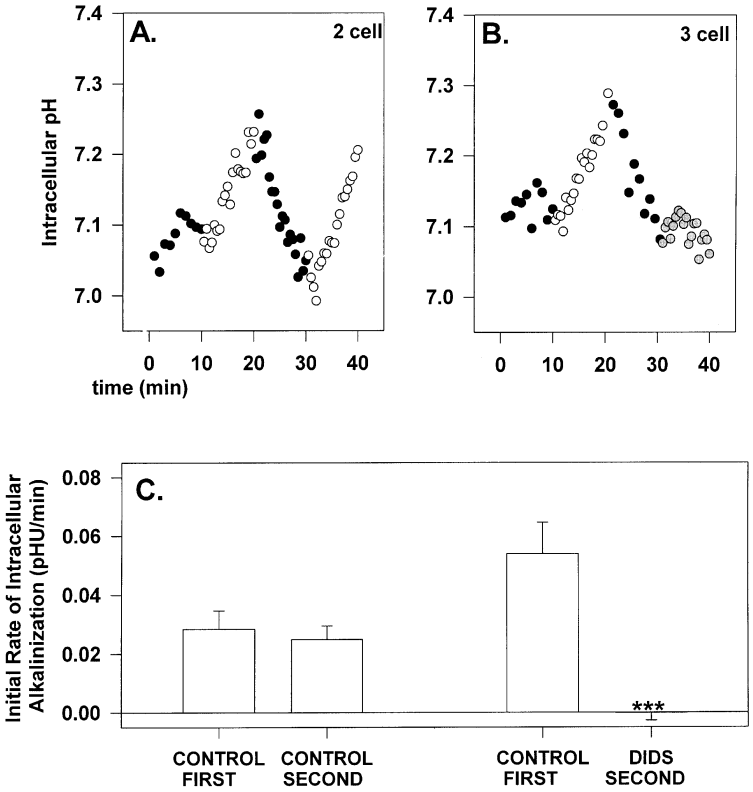

Figure 2. HCO_3^-/Cl^- exchanger activity measured by intracellular alkalinization in human embryos upon Cl^- removal. (**A**) Representative trace during two consecutive Cl^- removals. Embryo pH_i was measured for 10 min in KSOM containing Cl^- (●) and then for 10 min in the absence of external Cl^- (○). Cl^- was then reintroduced for 10 min (●) followed by a second Cl^- removal (○). (**B**) Representative trace of pH_i during two consecutive Cl^- removals (as in **A**) but with the final Cl^- removal performed in the presence of 4,4′-diisocyanatostilbene-2,2′-disulphonic acid, disodium salt (DIDS;). (**C**) Mean rates of intracellular alkalinization (mean ± SEM) upon initial (first) Cl^- removal and subsequent (second) Cl^- removal in the absence ($n = 6$) or presence ($n = 9$) of 500 µmol/l DIDS. ***Statistical significance ($P < 0.001$; paired Student's *t*-test).

pH_i range 6.5–6.9 was significantly inhibited by the absence of external Na^+ (Figure 3A, C; $P < 0.0001$; nANOVA). However, neither inhibition of Na^+/H^+ antiporter by amiloride (1 mmol/l) nor the presence of anion transport inhibitor DIDS (500 µmol/l) significantly reduced the recovery rate (Figure 3A,C; $P > 0.05$; nANOVA).

pH_i regulation in egs

HCO_3^-/Cl^- exchanger activity and recovery from acidosis were only assessed in the few eggs available to us, and thus no statistical tests were performed. HCO_3^-/Cl^- exchanger activity in eggs was assessed by the Cl^- removal assay (Figure 4A). The mean rate of intracellular alkalinization during Cl^- removal (± SEM) was 0.075 ± 0.025 pHU/min in GV eggs ($n = 2$), 0.028 ± 0.010 pHU/min for MI eggs ($n = 3$), and 0.017 ± 0.002 pHU/min in MII eggs ($n = 5$) which appear similar to the rates observed in cleavage-stage embryos. For MI eggs, a second Cl^- removal was performed in which Cl^- was replaced following the first Cl^- removal (Figure 4C). Results suggest that the initial rate of intracellular alkalinization (0.028 pHU/min) was reduced when the anion transport inhibitor DIDS was present during the second Cl^- removal (0.003 ± 0.001 pHU/min).

Intracellular acidosis was induced in MI and MII eggs by a pulse of NH_4^+-KSOM, followed by a 15 min period in Na^+-free KSOM, with any recovery from acidosis measured during subsequent Na^+ replacement (Figure 4B,D). The mean rates (± SEM) of recovery from acidosis were as follows: MI eggs: 0.006 ± 0.002 pHU/min, 0 Na^+: 0.004 ± 0.003 pHU/min ($n = 4$); and MII eggs: 0.028 ± 0.013 pHU/min, 0 Na^+: 0.003 ± 0.002 pHU/min ($n = 4$). These recovery rates from acidosis appear to suggest only non-specific activity in MI eggs, and possibly a low Na^+-dependent activity in MII eggs.

Discussion

We have found that human preimplantation embryos, like mouse (Zhao and Baltz, 1996, Phillips and Baltz, 1999) and hamster (Lane *et al.*, 1999b) embryos, appear to alleviate intracellular alkalosis by HCO_3^-/Cl^- exchanger activity. Recovery of human cleavage-stage embryos from NH_4Cl-induced alkalosis depended on the presence of external Cl^-, as expected for mediation by HCO_3^-/Cl^- exchange. HCO_3^-/Cl^- exchanger activity was also evidenced upon external Cl^- removal, whereupon an intracellular alkalinization occurred which was inhibited by the HCO_3^-/Cl^- exchanger inhibitor DIDS.

We also found that human cleavage-stage embryos rapidly recovered from acidosis. Recovery from acidosis, induced by an NH_4Cl pulse, in the absence of external HCO_3^- and CO_2, was Na^+-dependent and completely inhibited by the presence of the Na^+/H^+ antiporter inhibitor amiloride. This suggests that Na^+/H^+ antiporter activity is present in the human

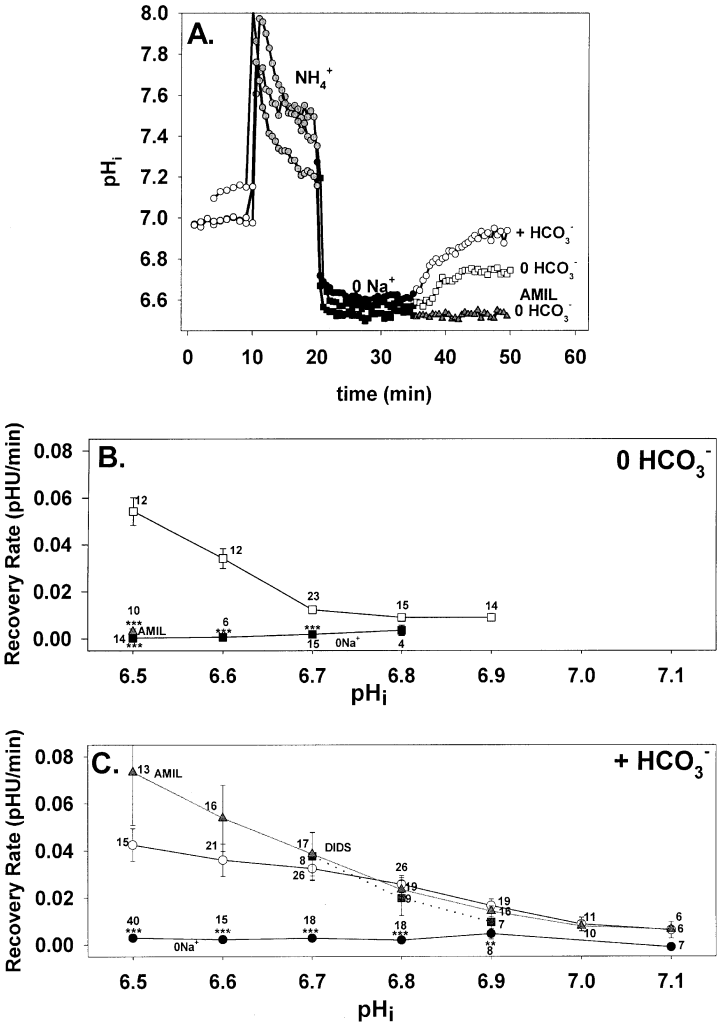

Figure 3. Rate of recovery from intracellular acid load in human embryos. (**A**) Representative trace of pH_i during recovery from intracellular acid load in the presence (○; 9 cell embryo) or absence (□; 7 cell) of external HCO_3^-. pH_i was also measured in the presence of 1 mmol/l amiloride (AMIL) with no external HCO_3^- (△; 5 cell). All recoveries were measured following a period with no external Na^+ in the presence (●) or absence (■) of external HCO_3^-/CO_2, where no recovery occurred. (**B**) Mean rate of recovery (± SEM) from acidosis in the absence of HCO_3^-/CO_2 was calculated as described in the text for every 0.1 pH unit in the range shown (▨). Recovery was measured in the presence (▨) or absence of external Na^+ (■), or in the presence of 1 mmol/l amiloride with external Na^+ ( ). ***Statistical significance ($P < 0.0001$; nANOVA; Welch's *t*-test). (**C**) Mean rate of recovery (± SEM) from acid load was calculated as in (**B**), but in the presence of external HCO_3^-/CO_2. Recovery was measured in the presence (○) or absence of external Na^+ (●), or in the presence of 1 mmol/l amiloride (△), or 500 µmol/l DIDS (▩), each with Na^+ present. Asterisks indicate statistical significance (***$P < 0.0001$ or **$P < 0.001$). Numbers above points represent number of embryos. AMIL = amiloride.

cleavage-stage embryo which is consistent with the presence of Na^+/H^+ antiporter activity in hamster (Lane *et al.*, 1998) and mouse embryos of various strains (Gibb *et al.*, 1997; C.L.Steeves, M.Lane, K.P.Phillips, B.D.Bavister and J.M.Baltz, unpublished data).

To determine whether any HCO_3^--dependent mechanisms mediating recovery from acidosis were also present in cleavage-stage embryos, recoveries from acidosis were measured in the presence of external HCO_3^- with CO_2 present. In the presence of HCO_3^-/CO_2, human embryos exhibited a rapid, Na^+-dependent recovery from acidosis to a higher pH_i (~7.0–7.2) than in the absence of external HCO_3^-/CO_2 where pH_i recovered to only ~6.8. This suggests that a second, Na^+-dependent mechanism, which requires HCO_3^-, contributes to the alleviation of acidosis in human cleavage-stage embryos. The presence of a second system is also suggested by the inability of amiloride to inhibit recovery from acidosis in the presence of external HCO_3^-/CO_2, in contrast to the complete inhibition seen in HCO_3^-/CO_2-free medium. Such Na^+- and HCO_3^--dependent, but amiloride-insensitive, recovery would seem to implicate Na^+, HCO_3^-/Cl^- exchanger activity. We could not, however, detect a component of the recovery in the presence of HCO_3^-/CO_2 which was DIDS-sensitive, although DIDS would be expected to partially inhibit recovery by eliminating the contribution of Na^+, HCO_3^-/Cl^- exchange, leaving only Na^+/H^+ antiporter activity. It is possible, however, that the variability in recovery rates seen with human embryos precluded such detection of partial inhibition.

We have shown that pH_i regulation in the human embryo results from the concerted efforts of at least three exchangers: HCO_3^-/Cl^- exchanger, Na^+/H^+ antiporter and an Na^+, HCO_3^--dependent, amiloride-insensitive transporter which may be the

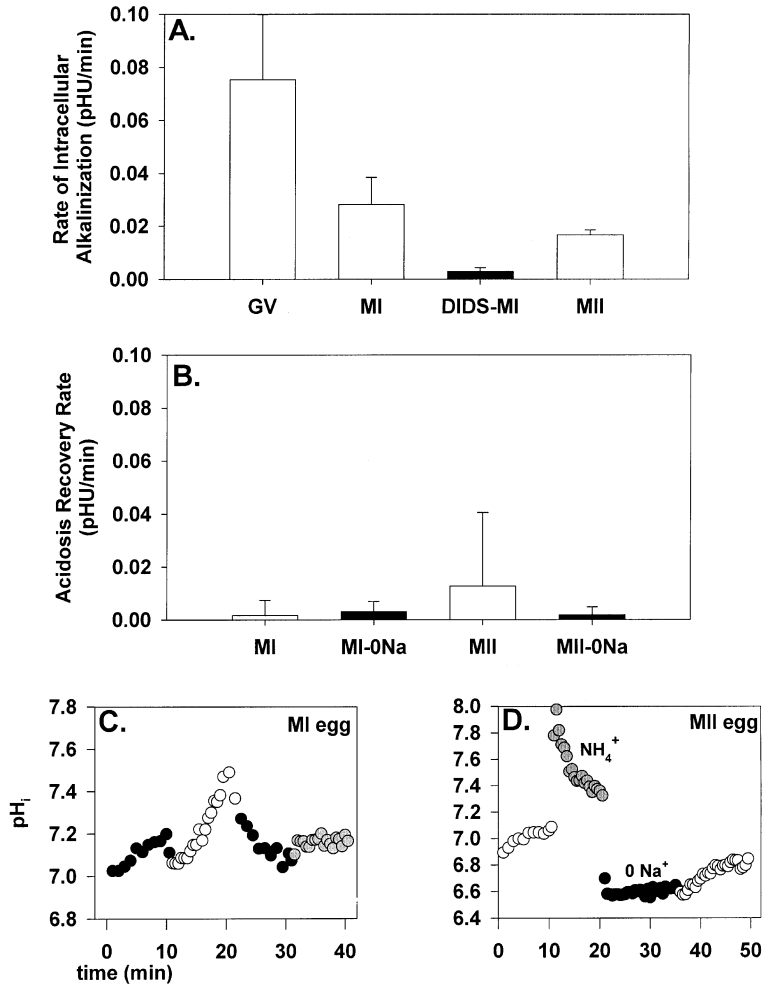

Figure 4. pH_i regulation in human eggs. (**A**) Rates of intracellular alkalinization (mean ± SEM) upon single Cl^- removal [germinal vesicle (GV); $n = 2$; and metaphase II (MII) eggs; $n = 5$] or upon Cl^- removal followed by a second Cl^- removal in the presence of 500 µmol/l 4,4′-diisocyanatostilbene-2,2′-disulphonic acid, disodium salt (DIDS) [metaphase I (MI) eggs; $n = 3$]. (**B**) MI and MII eggs were first acidified in the absence of external Na^+ followed by replacement of Na^+ (MI: $n = 4$; MII: $n = 4$). (**C**) Egg pH_i was measured for 10 min in KSOM containing 110 mmol/l Cl^- (●) followed by 10 min in the absence of external chloride (○). Representative trace of pH_i during two consecutive Cl^- removals in the absence (○) and then presence (◖) of DIDS. (**D**) Representative trace of pH_i during recovery from intracellular acid load in the presence of external HCO_3^-/CO_2.

Na^+, HCO_3^-/Cl^- exchanger, similar to reports in other cell types (Vaughan-Jones, 1988). The presence of two acid-alleviating systems (Na^+/H^+ antiporter and an Na^+, HCO_3^--dependent system) in the human cleavage stage embryo is similar to the presence of redundant pathways to alleviate pH_i in other cell types (Vaughan-Jones, 1988; Alper, 1991, 1994). Na^+/H^+ antiporter activity is important for the formation of the blastocoel cavity in the blastocyst (Barr *et al.*, 1998) and may also mediate cell volume regulation or ion homeostasis in addition to pH_i regulation in the developing embryo.

Our data indicate that human cleavage-stage embryos have the ability to effectively maintain pH_i within a range of ~7.0–7.3. We have found that the embryo's HCO_3^-/Cl^- exchanger is activated when pH_i rises above ~7.2–7.3, which therefore prevents pH_i from increasing beyond this level. We also found that, in the presence of HCO_3^-/CO_2, there appear to be two mechanisms which will prevent pH_i from becoming too low. Na^+/H^+ antiporter activity becomes activated below a threshold of ~6.8, while a second HCO_3^--dependent mechanism which may be an Na^+, HCO_3^-/Cl^- exchanger, is activated below 7.0 (Figure 3C). Together, these would effectively maintain pH_i above 7.0. The hypothesis that pH_i is maintained within a range of 7.0–7.3 by the concerted activities of these mechanisms is consistent with the baseline pH_i of ~7.1 we measured for cleavage-stage human embryos under our conditions.

In the absence of HCO_3^-/CO_2, our data indicate that human embryos would be less able to regulate pH_i. Since defence against alkalosis by HCO_3^-/Cl^- exchanger requires intracellular HCO_3^-, alkalosis could not be opposed in media without HCO_3^-/CO_2 where there would be little intracellular HCO_3^-. Acidosis would still be opposed, but only below ~6.8 where the Na^+/H^+ antiporter is active; the second HCO_3^--dependent mechanism which maintains pH_i above 7.0 would be inactive since it would require external HCO_3^-. This has implications for the handling of human embryos *in vitro*, which are routinely manipulated in HEPES-buffered media with low HCO_3^- concentrations at atmospheric CO_2. This would be predicted to impair the ability of embryos to maintain pH_i since the low CO_2 would result in very low intracellular HCO_3^- concentrations which would inhibit HCO_3^-/Cl^- exchange, and low

external HCO_3^- would slow any HCO_3^--dependent mechanism for alleviating acidosis. Thus, media containing sufficient HCO_3^- with appropriate CO_2 tension (i.e. 25 mmol/l HCO_3^-/5% CO_2) would be preferable.

Dale *et al*. (1998) previously found that the baseline pH_i of human eggs and zygotes was ~7.4 in HCO_3^-/CO_2-buffered medium. This value for eggs is different from the 7.0–7.1 which we found; the reason for this discrepancy could be due to differences in the media used by Dale *et al*. (1998) and those used here, but we can only speculate as to why the value for oocyte pH_i obtained by Dale *et al*. was higher than that which we have measured here. Dale *et al*. probed the ability of cleavage-stage embryos to recover from an alkalosis produced by exposing them to pH 8.0 medium (Medium 199) buffered with HEPES at atmospheric CO_2. Although the low CO_2, and hence very low intracellular HCO_3^-, might be expected to reduce HCO_3^-/Cl^- exchanger activity, the amount of intracellular HCO_3^- remaining within the embryos under their conditions (apparently used soon after removal from CO_2-buffered medium) was sufficient to permit HCO_3^-/Cl^- exchanger activity. Using this method, recovery from alkalosis was detected at every stage of embryo development, which is consistent with our findings here. In contrast, Dale *et al*. (1998) did not detect an ability in human cleavage-stage embryos to recover from acidosis until the blastocyst stage, using a protocol in which pH_i was reduced to ~7.0 by decreasing external pH to the same level, again in HEPES-buffered medium at atmospheric CO_2. This is also consistent with our findings, which indicated that Na^+-dependent recovery from acidosis in human embryos is fairly low until pH_i falls below 6.9 and thus would not have been detected with their protocol. It is unclear if the media used by Dale *et al*. (1998) for pH_i measurements contained HCO_3^-. Low or absent external HCO_3^- may also have contributed to difficulty detecting recovery from acidosis. As we have shown here, recovery from acidosis was dependent on the presence of external HCO_3^-/CO_2 between pH_i 6.8 and 7.1. We did not perform measurements on later-stage human embryos (morulae and blastocysts), and thus can only speculate that a change in transport kinetics occurs after blastocyst formation, allowing HCO_3^- independent recovery from mild acidosis in blastocysts.

We also examined pH_i regulation in a few available human eggs. pH_i of GV, MI and MII eggs was not significantly different from cleavage-stage embryos, with pH_i ranging from 7.0 to 7.1. All egg stages examined exhibited intracellular alkalinization upon Cl^- removal, similar to cleavage-stage embryos. The rate of alkalinization in MI eggs appeared to be reduced by DIDS, which suggests that HCO_3^-/Cl^- exchanger activity is also present in eggs. MII eggs exhibited a robust recovery from alkaline load, which contrasts with the recent finding that HCO_3^-/Cl^- exchanger activity in the mouse and hamster is activated upon egg activation or fertilization, with eggs (MII) having very low or barely detectable HCO_3^-/Cl^- exchanger activity (Lane *et al*., 1999b; Phillips and Baltz, 1999). However, the data are consistent with the finding by Dale *et al*. (1998) that fresh MII eggs recovered from alkalosis and that recovery was inhibited by DIDS. In contrast, recovery from induced acidosis, measured in the presence of external HCO_3^-, was barely detectable in MI eggs and did not appear to be Na^+-dependent. MII eggs demonstrated low rates of recovery from acidosis that appeared Na^+-dependent, although the recovery appeared to be much slower compared to the rates measured in cleavage-stage embryos. This is consistent with the recent finding that Na^+/H^+ antiporter activity is low in unfertilized hamster eggs (MII) and is subsequently activated upon fertilization (Lane *et al*., 1999a). Thus, it appears that although human eggs may regulate pH_i against alkalosis, they do not demonstrate robust pH_i regulation in the acid range.

Robust pH_i regulation may be particularly important for the developing cleavage-stage embryo, as the Fallopian tube has been reported to be quite alkaline in several mammalian species (rhesus: pH 7.7, Maas *et al*., 1977; rabbit: pH 7.9, Maas *et al*., 1987; rat: pH 8.0–8.2, Ben-Yosef *et al*.; 1996; mouse: pH 7.7, Y.Zhao, P.J-P.Chauvet and J.M.Baltz, unpublished data). Acid-alleviating systems may also be required by the growing embryo during this period of development to correct perturbations in pH_i due to increased metabolism and the production of intracellular protons resulting from processes such as ATP hydrolysis. We have demonstrated that human cleavage-stage embryos correct deviations in steady-state pH_i by HCO_3^-/Cl^- exchanger activity, Na^+/H^+ antiporter activity and an Na^+, HCO_3^--dependent system which together maintain embryo pH_i between 7.0 and 7.3. Thus, cleavage-stage human embryos possess the ability to maintain pH_i within a narrow physiological range, but this ability requires the presence of HCO_3^- and CO_2.

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