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A novel H^+ permeability dominating intracellular pH in the early mouse embryo

Jay M. Baltz[1,2,*], John D. Biggers[1,3] and Claude Lechene[4]

[1]Laboratory of Human Reproduction and Reproductive Biology, [2]Department of Obstetrics, Gynecology and Reproductive Biology and [3]Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115, USA
[4]Laboratory of Cellular Physiology, Brigham and Women's Hospital, and Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

*Present address for correspondence: The Loeb Medical Research Institute, Ottawa Civic Hospital, 1053 Carling Avenue, Ottawa, Ontario, K1Y 4E9, Canada

SUMMARY

Most cell types are relatively impermeant to H^+ and are able to regulate their intracellular pH by means of plasma membrane proteins, which transport H^+ or bicarbonate across the membrane in response to perturbations of intracellular pH. Mouse preimplantation embryos at the 2-cell stage, however, do not appear to possess specific pH-regulatory mechanisms for relieving acidosis. They are, instead, highly permeable to H^+, so that the intracellular pH in the acid and neutral range is determined by the electrochemical equilibrium of H^+ across the plasma membrane. When intracellular pH is perturbed, the rate of the ensuing H^+ flux across the plasma membrane is determined by the H^+ electrochemical gradient: its dependence on external K^+ concentration indicates probable dependence on membrane potential and the rate depends on the H^+ concentration gradient across the membrane. The large permeability at the 2-cell stage is absent or greatly diminished in the trophectoderm of blastocysts, but still present in the inner cell mass. Thus, the permeability to H^+ appears to be developmentally regulated.

Key words: pH regulation, mouse, preimplantation, embryo, H^+

INTRODUCTION

In most mammalian cells, regulation of intracellular pH (pH_i) is effected by membrane transport proteins, which move acid or base equivalents into or out of the cell in response to perturbed pH_i (Boron, 1987). The most common of these transporters that function to relieve acid loads are the Na^+/H^+ antiporter, which extrudes H^+ in exchange for importation of extracellular Na^+, and the $Na^+,HCO_3^-/Cl^-$ exchanger, which imports HCO_3^- and Na^+, while exporting Cl^-. HCO_3^-/Cl^- exchangers, which export HCO_3^- in exchange for importing extracellular Cl^-, relieve intracellular alkalosis. Such mechanisms are activated by perturbations in pH_i from the normal value, and thus perform a homeostatic function, protecting cells from acidosis and alkalosis and maintaining a stable pH_i. In addition, changes in pH_i or its regulation have been implicated in the response to growth factors and other growth-stimulating processes, and may play a role in development (Moolenaar, 1986; Ganz et al., 1989; Grinstein et al., 1989).

The preimplantation mouse embryo is unlike most other cells in that it does not exhibit detectable activity of any of the specific mechanisms for relieving acid loads; we were unable to detect activity attributable to a Na^+/H^+ antiporter, a $Na^+,HCO_3^-/Cl^-$ exchanger or any other acidosis-relieving mechanism at the 2-cell stage (Baltz et al., 1990; 1991a). This stage does, however, have HCO_3^-/Cl^- exchanger activity, which rapidly relieves alkalosis (Baltz et al., 1991b).

Even though 2-cell-stage embryos apparently possess no specific means for eliminating excess intracellular acid, we found that they did, nonetheless, recover from experimentally induced acid loads (Baltz et al., 1990; 1991a). From this it could be inferred that excess H^+ is removed from the cytoplasm, either by leaving the cell or by being sequestered internally. Since the latter mechanism must have finite capacity, and is therefore saturable, it was likely that H^+ was actually leaving the cells by an unusual mechanism. The identity of this mechanism, and its mode of action, were unknown. However, there was preliminary evidence that H^+ (or the equivalent) crossed the plasma membrane in a rheogenic (i.e., charge translocating) fashion: baseline pH_i of 2-cell embryos was very sensitive to external K^+ concentration and therefore probably membrane potential (Baltz et al., 1990), implying that H^+ is in electrochemical equilibrium across the membrane; this would also indicate that the membrane is highly permeable to H^+.

We hypothesized, therefore, that 2-cell-stage mouse embryos are permeable to H^+ and that H^+ flux through this permeability pathway is the mechanism of recovery from

acid loads (Baltz et al., 1990). In this paper, we show that the changes in pH_i that are observed are the result of H^+ crossing the plasma membrane, and that the rate of H^+ flux across the membrane is dependent on the K^+ gradient. However, transmembrane H^+ flux apparently does not require the movement of K^+ down its gradient. Together, these findings are indicative of a passive H^+ permeability in 2-cell-stage embryos, which allows the movement of H^+ across the membrane under the influence of the H^+ electrochemical gradient. We also find that, unlike 2-cell-stage embryos, the external surface of the blastocyst-stage embryo is no longer permeable to H^+; however, the inner cell mass retains the permeability.

MATERIALS AND METHODS

Chemicals

2′,7′-Bis(2-carboxyethyl)-5(and 6)-carboxy fluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, OR; 1 mg/ml stock in dimethyl sulfoxide stored at −20°C) was added to solutions for a final concentration of 1.2 μM. Fresh stocks of $HgCl_2$ (200 mM), diamide (1 M), dithiothreitol (DTT, 1 M) and $ZnSO_4$ (1 M) were prepared in the experimental medium and those of p-chloromercuriphenylsulfonic acid, monosodium salt (PCMBS, 0.5 M) in dimethyl sulfoxide; these stocks were then diluted to the required concentrations in the appropriate medium immediately prior to an experiment. These reagents, and the components of the media, were obtained from Sigma (St Louis).

Solutions

The media used have been described previously (Baltz et al., 1991b) and were based on the mouse embryo culture medium, M2 (Fulton and Whittingham, 1978; Hogan et al., 1986). The medium designated '0bicHM' contained 122 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20.8 mM Hepes, 5.6 mM glucose, 1.7 mM $CaCl_2$, 0.06 mg/ml Penicillin G (K salt), and 0.05 mg/ml Streptomycin sulfate. The medium designated '100K0bicHM' was identical except that the total K^+ concentration was increased to 100 mM by substituting KCl for NaCl (KCl increased to 98.6 mM and NaCl decreased to 28.3 mM). The medium designated '0K0bicHM' was nominally K^+-free, with NaCl replacing KCl, NaH_2PO_4 replacing KH_2PO_4 and the Na^+ salt of Penicillin G replacing the K^+ salt. Solution pH was adjusted to the desired values using NaOH, monitored with a Radiometer (Copenhagen) PHM 26 pH meter with a Corning (Medfield, MA) calomel combination electrode. Penicillin-free 0bicHM (Penicillin omitted) was used in experiments where Hg^{2+} was present and phosphate-free 0bicHM (KH_2PO_4 replaced by equimolar KCl) was used in experiments where Zn^{2+} was present, since Hg^{2+} and Zn^{2+} precipitate with Penicillin and PO_4^{2-}, respectively.

Animals and embryos

The embryos were obtained from superovulated (5 IU pregnant mare serum gonadotropin, PMSG, followed 48 hours later by 5 IU human chorionic gonadotropin, hCG, both intraperitoneal) BDF or CF1 female mice mated to BDF males (virus-antigen free, Charles River, Canada). 2-cell-stage embryos were flushed from the oviducts 43-47 hours post-hCG. Blastocysts were flushed from the uterine horns 91-95 hours post-hCG.

Blastocysts were used for experiments either intact or collapsed. Collapsed blastocysts were prepared by aspirating them several times through a narrow-bore pipet causing the blastocoel to be breached. Blastocysts thus collapsed had a visible breach in the trophectoderm, which did not reseal over the course of these experiments, and thus the basolateral (inner) surface of the trophectoderm and the inner cell mass were accessible to the external medium.

Isolated inner cell masses were prepared from blastocysts by immunosurgery (Solter and Knowles, 1975). Zonae were first removed by brief exposure to acid Tyrode's solution. The blastocysts were then incubated in 10× diluted rabbit anti-mouse antiserum (30 minutes, 37°C), washed three times (5 minutes each) and incubated in 10× diluted guinea pig complement (Gibco BRL, Gaithersburg, MD) (30 minutes, 37°C). Remnants of trophectoderm were completely removed by gentle pipetting.

pH_i measurements

The methods used for pH_i measurements have been detailed previously (Baltz et al., 1990). Briefly, pH_i was measured using the pH-sensitive intracellular fluorophore, BCECF, loaded into the cells by incubation with 1.2 μM of the acetoxymethyl ester derivative for 5-15 minutes. pH_i was determined from the ratio of the emission intensity (measured at 530 nm) excited at 500 nm to that excited at 450 nm; this ratio is linearly proportional to pH_i, and is dependent only on pH_i and not on dye concentration, cell thickness, etc. 2-cell embryos were oriented with the blastomeres side-by-side; intact blastocysts were oriented with the inner cell mass lying to one side; collapsed blastocysts and isolated inner cell masses were randomly oriented. Fluorescence intensity measurements were obtained as the integrated intensities within 24×22 μm areas of each image chosen to overlie areas of the embryos. Such fluorescence intensities from individual blastomeres of ten 2-cell embryos, or from chosen locations of blastocysts, collapsed blastocysts or isolated inner cell masses were simultaneously recorded using a video image analyzer (Image 1, IVS, Concord, MA), operating on the image provided by a double-intensified silicon target video camera (ISIT-66, Dage-MTI, Michigan City, IN) on a Zeiss IM-405 inverted microscope with a 10×, 0.30 n.a. objective. Acquiring each pair of images took approximately 10 seconds, and they are taken at 1 or 2 minute intervals. The intensity ratios for each measurement area were converted to pH after calibration by the nigericin/high K^+ method (Thomas et al., 1979; Baltz et al., 1990).

Temperature control

Temperature was regulated in a temperature-controlled chamber with an approximate 2 ml volume and a heating element in the chamber walls regulated by feedback from a thermistor (Biophysica, Baltimore, MD). The temperature immediately adjacent to the embryos was monitored independently using a copper/constantan thermocouple (PT-6, Sensortek, Clifton, NJ) mounted in the chamber and displayed on a BAT-12 digital thermometer (Sensortek). Temperature was constant to within ±0.5°C at steady-state, and constant to within ±1°C across the chamber. The transition from room temperature to 37°C took approximately 5 minutes and exhibited no detectable overshoot.

To control for the possibility of any detrimental effects arising from unknown properties of the temperature regulation, we performed several experiments in which the chamber was warmed by flushing it with warm medium rather than by activating the thermoregulator. This method of warming gave identical results in experiments like those described below where the temperature was increased by the chamber regulator, thus confirming that the effects that we observed were due to the temperature change itself, rather than any other property of the temperature-regulation mechanism.

Data analysis

The intensity ratios (linearly proportional to pH) were displayed as a function of time. Initial rates of change of ratio were calculated by fitting a line to the initial, linear portion of the data using linear least squares regression. The rate of change of ratio, and hence pH_i, was determined for each individual blastomere in 2-cell embryos

or a small portion of each blastocyst. For pH_i measurements, data are reported as the means±s.e.m. of these rates of change of pH_i, combining the data from several identical experiments; an N value is also given, which corresponds to the number of readings, with each reading in a 2-cell embryo resulting from one blastomere and each reading in a blastocyst resulting from an area defined over a portion of the embryo as stated in the text. Thus, there were approximately one-half as many embryos as readings (i.e., the number of embryos is about *n*/2). Our instrumentation has been set up so that a change of one ratio unit equals a change of one pH unit over the pH_i range used (to within ±5%). Thus, we use rates of change of ratio or pH_i interchangeably. In this paper, a negative rate of ratio or pH_i change with time denotes a fall of pH_i (i.e., an increase in intracellular H^+ concentration), while a positive rate corresponds to an intracellular alkalinization.

Verification of BCECF measurement technique

This technique has been used extensively for measuring pH_i in embryos (Baltz et al., 1990; 1991a,b), with appropriate controls. We have performed a further series of controls to ensure that the dye was accurately measuring pH_i at both 37°C and 23°C.

A cytoplasmic localization of the dye was confirmed in several ways. First, mechanical disruption of the plasma membrane of dye-loaded embryos (2-cell stage) by means of a glass microneedle (tip <1 μm diameter, pulled on a pipet puller; model 720, Kopf, Tujunga, CA), showed that all dye was lost after the plasma membrane was breached, even though all of the visible cytoplasmic components remained within the zona. The mean half-time for loss was 12 seconds at 36°C and 13 seconds at 23°C (s.e.m.=2, data from 5 embryos, for each temperature). All detectable dye was lost from the embryos after disruption at either temperature.

Digitonin treatment (which supposedly disrupts plasma membranes preferentially) also caused complete loss of dye from the embryos. At 100 μg/ml, digitonin caused immediate loss of all detectable dye at 23°C or 37°C; at 50 or 10 μg/ml, embryos remained unaffected for varying times, but when dye loss began, all the dye was lost immediately. Partial retention of dye was never observed.

It was also possible that the dye itself changed fluorescence as a function of temperature, independent of pH, which would give the illusion of a pH change. To assess this possibility, we made a microcuvette containing 20 μg/ml BCECF (free acid) in pH 7.3 Hepes-buffered medium (0bicHM) using a section of a 5 μl Wiretrol pipet (Drummond, Broomall, PA) sealed at both ends. This was submerged in water in the temperature-controlled chamber and the fluorescence ratio measured for the dye as a function of temperature. We found that the ratio fell by an amount corresponding to an apparent pH shift of 0.12 (±0.02) pH U when the temperature was raised from 23°C to 37°C, but the ratio remained constant at constant temperature; when the pH of 0bicHM medium was determined as a function of temperature using the pH meter, we found that the pH fell by 0.08 (±0.02) pH U over this temperature range due to shifts in the buffer's pK_a. There is no significant difference between the pH_i changes measured by the dye or pH meter, so we conclude that the dye does not exhibit a significant temperature-dependent change in fluorescence over this range; changes in the fluorescence ratio are entirely due to actual changes in pH.

Experimental design

In order to measure the rate of flux of H^+ across the plasma membrane, we used embryos depleted of H^+ and measured the rate of increase of intracellular H^+ concentration (i.e., pH_i decrease) as H^+ entered the cell from the external medium. This design was used because it is possible to control the external H^+ concentration easily, while it is difficult to set pH_i reproducibly to a range of different values. H^+-depleted embryos (i.e., with high pH_i) were obtained by flushing the embryos from the female tract with HCO_3^-/CO_2-free medium at room temperature. We also found that these embryos remained at high pH_i for an extended time if retained at room temperature. However, when the temperature was raised, pH_i was no longer stably alkaline, an effect that we show below is apparently due to increased H^+ permeability of the membrane. For isolated inner cell masses, which necessarily were exposed to prolonged incubation at 37°C during immunosurgery, the cells were alkalinized by a 30 minute incubation in pH 8.3 medium (0bicHM) prior to loading with BCECF.

We used these alkaline embryo preparations to measure H^+ flux: the embryos were loaded with BCECF at room temperature and the temperature then raised to 37°C. The initial rate of change of pH_i was determined by examining the rate immediately after the temperature reached 37°C. This manipulation was performed with different external K^+ and/or H^+ concentrations. Therefore, each experiment in which the effect of external K^+ or H^+ concentration is examined consists of BCECF-loading H^+-depleted embryos with pH_i kept stably alkaline at room temperature, raising the temperature to 37°C and then measuring the initial rate of H^+ flux by monitoring pH_i as a function of time.

One series of experiments was also done on acidified embryos. For these, 2-cell embryos were incubated in pH 6.5 medium (0bicHM) for 1 hour at 37°C before being loaded with BCECF.

Possible identities of the H^+ permeability, which is the subject of this study, were examined using several inhibitors: Zn^{2+} inhibits voltage-gated H^+ channels (Thomas and Meech, 1982; Barish and Baud, 1984; DeCoursey, 1991); the sulfhydryl-active mercurials, Hg^{2+} and PCMBS, inhibit water channels (Harris et al., 1990; Harvey et al., 1991) and these mercurials and several other sulfhydryl-active compounds (e.g., diamide and DTT) activate or inhibit several transporters of other ions (see Discussion).

RESULTS

Behavior of H^+-depleted 2-cell embryo pH_i at 23°C and 37°C

We observed that the pH_i of H^+-depleted 2-cell embryos (flushed at room temperature) had a very strong response to an increase in temperature. Fig. 1 shows the mean pH_i of one group of ten 2-cell embryos in 0bicHM medium, pH 7.3, initially at 23°C. Embryos remained alkaline as long as they were maintained at 23°C; there was only a small constant drop in pH_i at 23°C averaging −0.005±0.001 pH U/minute (*n*=60) in three identical experiments.

When the temperature is increased to 37°C, the response is biphasic, with a sharp drop during the approximately 5-7 minute period over which the temperature is increasing, and then a steady, linear decrease thereafter. This initial sharp drop observed during warming is due mainly to a shift in the pK_a of the endogenous intracellular buffers with temperature, as can be inferred from several observations: the initial decrease is of the expected magnitude and direction (a decrease of about 0.24 pH U is expected when the temperature is increased from 23°C to 37°C; Somero, 1985); after correcting for the constant rate of pH_i decrease at 37°C, the initial sharp drop is a constant 0.22 pH U, regardless of the rate of fall of pH_i after reaching 37°C, even under conditions in which this rate varied widely (e.g., by varying the external pH and K^+ concentrations, see below); the initial pH_i decrease was instantaneous if temperature was changed by rapid introduction of 37°C medium into the chamber (rather

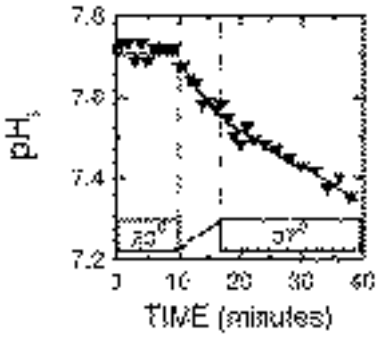

Fig. 1. Intracellular pH of 2-cell-stage mouse embryos at 23°C and 37°C. 2-cell-stage embryos were placed in medium 0bicHM, pH 7.3. pH_i was monitored at 23°C, after which the temperature was raised to 37°C. After reaching 37°C, a constant rate of acidification was observed. Each point represents the mean pH_i of a group of ten embryos at the indicated time.

than the slower temperature change effected by the regulator) and was reversed instantaneously by reintroducing 23°C medium.

After a steady 37°C was reached in pH 7.3 medium, pH_i was found to decrease at a constant rate with a mean of −0.015±0.001 pH units/minute (*n*=68). At 34°C, the decrease proceeded at an intermediate rate (about 80% of the rate at 37°C); reducing the temperature back to 23°C halted the decrease.

Dependence of rate of pH_i decrease on external pH (pH_o) and K^+

The rate of change of pH_i in 2-cell embryos after the temperature was raised to 37°C was strongly dependent both on the external pH and on the external K^+ concentration. A higher external H^+ concentration (lower pH_o) resulted in a much faster rate of appearance of H^+ in the cytoplasm, while raising the external K^+ concentration from 6 mM (in 0bicHM) to 100 mM (100K0bicHM) decreased the rate of fall at all pH_o. Thus, over the range (6.5-8.5) of pH_o tested, the fastest rate of fall was observed in 2-cell-stage embryos when pH_o was 6.5 and K^+ was 6 mM (−0.030±0.002 pH U/minute; *n*=78); the slowest rate of fall was found when pH_o was 8.5 and the external K^+ was 100 mM. Indeed, in the latter case, pH_i was steady or rose slightly (+0.0003±0.0012 pH U/minute; *n*=40). Recoveries under these two extreme conditions are shown in Fig. 2; Fig. 3 shows the rates of change of pH_i measured at 6 mM (Fig. 3A) or 100 mM (Fig. 3B) external K^+ over the entire range of pH_o employed. It is seen that the rate of pH_i decrease is faster with increasing external H^+ concentration (lower pH_o) and that raising the external K^+ concentration slows the rate over the entire range of pH_o. It can also be seen in Fig. 3 that the rate of acidification at 23°C is also dependent on pH_o, but is much slower than at 37°C.

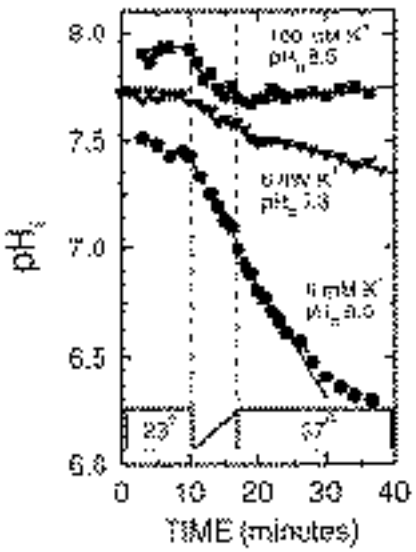

Fig. 2. Effect of varying external pH and K^+. 2-cell-stage embryos were treated as described for Fig. 1. However, the concentration of H^+ and K^+ in the medium was varied. The results in pH_o 7.3, 6 mM K^+ medium shown in Fig. 1 are replotted here for comparison. In pH_o 8.5, 100 mM K^+ medium, the acidification is eliminated or reversed. In pH_o 6.5, 6 mM K^+ medium, it occurs at a much elevated rate.

Is countertransport of K^+ necessary for H^+ flux?

The experiments described above indicated that the presence of an outwardly directed K^+ gradient increased the influx of H^+ from the external medium into 2-cell embryos. We therefore performed an experiment to determine whether transport of H^+ depended on transport of K^+ in the opposite direction. Since it is difficult to remove K^+ from within the

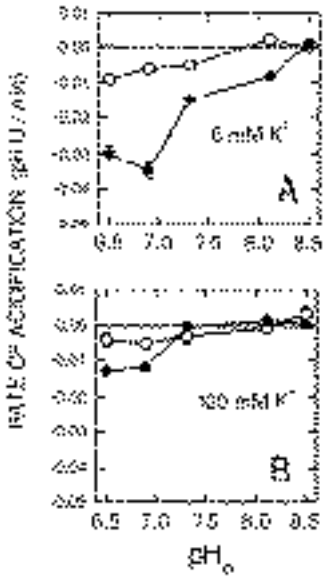

Fig. 3. Summary of effect of varying external pH and K^+ at the 2-cell stage. Experiments were carried out as described in the text and as shown in Figs 1 and 2. The initial rate of acidification, in pH U/minute, is shown as a function of pH_o at 23°C (open circles) and 37°C (closed circles) for both 6 mM (A) and 100 mM (B) K^+. It can be seen that the external pH (pH_o) has a dramatic effect on the rate of change of pH_i, especially at 37°C, and also, to a lesser degree, at 23°C. It is also evident that the rates are decreased overall when the external K^+ concentration is raised. Each point corresponds to measurements on 20 to 40 embryos in 2-5 separate experiments. The means±s.e.m. are shown.

cells to determine if H^+ influx is inhibited in experiments like those above, we performed the converse experiment, measuring H^+ efflux from acidified cells: embryos were acidified by incubation in pH 6.5 medium and the increase in pH_i measured as H^+ exited the cells upon exposure to alkaline (pH 8.3) medium at 37°C. If the movement of H^+ required countertransport of K^+, then H^+ efflux would not occur in K^+-free medium. Fig. 4 shows such experiments, where acidified embryos maintained at pH 6.5 are exposed to pH 8.3 medium, either with 6 mM K^+ (0bicHM) or nominally K^+-free (0K0bicHM). Even in K^+-free medium, a rapid rise in pH_i was observed, corresponding to an efflux of H^+ (30 embryos in three experiments). The efflux is quite rapid, although somewhat slower than that observed in 6 mM K^+; this difference in rates is discussed in the Discussion section.

Behavior of H^+-depleted blastocyst pH_i at 23°C and 37°C

Since the pH_i decrease is greatest at pH_o 6.5 (Figs 2, 3), we assessed the response of blastocysts at this pH_o (0bicHM medium) to maximize sensitivity. The blastocyst presents a more complex geometry for measurement than the 2-cell-stage embryo, since it consists of two cell types (trophectoderm epithelium and inner cell mass), and since the trophectoderm encloses a fluid-filled cavity (the blastocoel). We assessed the rate of fall of pH_i in the trophectoderm of intact blastocysts by monitoring fluorescence away from the inner cell mass. We also assessed the rate of fall of pH_i in isolated inner cell masses and in mechanically collapsed blastocysts. In the latter, the measured pH_i is some average of that of inner cell mass cells and trophectodermal cells, since the two cell types are not distinguishable and are closely apposed after the blastocoel is collapsed. Thus, we obtained pH_i measurements of trophectoderm in intact blastocysts where only the apical (outer) surface is accessible to the medium, of both inner cell mass cells and trophectoderm together in collapsed blastocysts where both surfaces are accessible, and of isolated inner cell masses free of trophectoderm.

Fig. 5 depicts the results of three similar experiments showing the pH_i of intact trophectoderm, collapsed blastocysts and isolated inner cell masses in pH 6.5 0bicHM medium at 23°C and then 37°C. It can be seen that the three preparations exhibit very different behavior: Trophectoderm of intact blastocysts is quite unresponsive to the acid external medium; the rate of fall of pH_i occurred at only at a mean rate of −0.008±0.001 (n=50) at 37°C, not much different from the rate at 23°C (−0.004±0.001, n=50). Isolated inner cell masses, however, show a large change in pH_i at 37°C (mean rate −0.058±0.004 pH U/minute, n=35). Collapsed blastocysts show an intermediate response at 37°C (mean rate −0.039±0.002 pH U/minute, n=48). It is also evident that pH_i of collapsed blastocysts does not fall as far as that of inner cell masses or of 2-cell embryos under the same conditions (compare in Fig. 5 and Fig. 2).

Fig. 5B shows an experiment identical to that in Fig. 5A, except that the pH_i of the trophectoderm of intact blastocysts and that of collapsed blastocysts were measured simul-

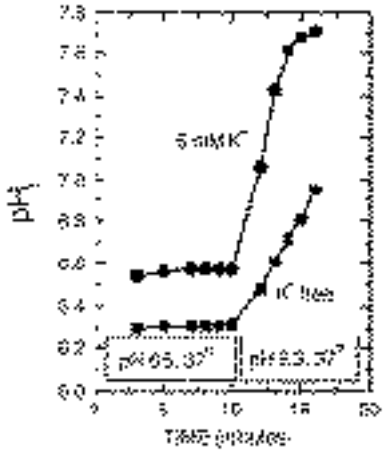

Fig. 4. Efflux of H^+ from acidified 2-cell embryos at 37°C. 2-cell-stage embryos were acidified and maintained acidotic in pH 6.5 medium (0bic HM) at 37°C. They were then exposed to alkaline (pH 8.3) medium, making this the converse of the experiments shown as the lowest curve in Fig. 2. pH_i increased upon exposure to alkaline media either with 6 mM K^+ present (0bic HM), or in nominally K^+-free medium (0K0bicHM).

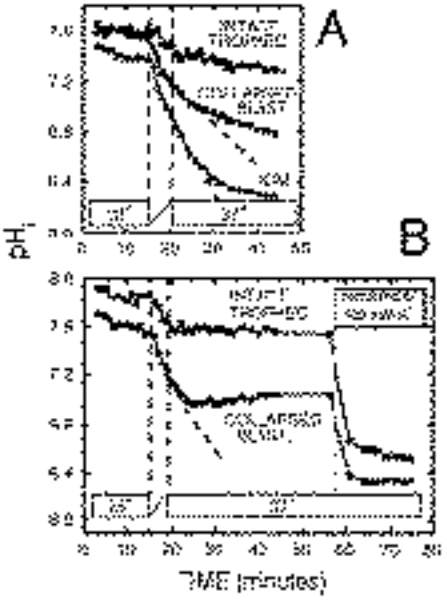

Fig. 5. Intracellular pH of intact blastocyst trophectoderm, isolated inner cell masses and collapsed blastocysts at 23°C and 37°C. Intact blastocysts, isolated inner cell masses, or blastocysts collapsed by aspiration through a narrow-bore pipet were placed in pH 6.5 medium. As for 2-cell embryos, pH_i was monitored at 23°C, after which the temperature was raised to 37°C. Isolated inner cell masses (ICM) exhibited a marked rate of acidification, while intact trophectoderm was refractory to the effect of the external acid. Collapsed blastocysts showed an intermediate response. (A) Three experiments, one with each type of preparation, with 10-20 embryos each. (B) One experiment in which both intact and collapsed blastocysts (10 of each) were assayed simultaneously (see text). At the end of this experiment, the external medium was replaced with one containing 1 μg/ml nigericin, 0.5 μg/ml valinomycin and 100 mM K^+, to equilibrate pH_i with pH_o (see text for further details).

taneously. Here, ten intact and ten collapsed blastocysts taken from the same mice were placed side-by-side in the chamber and thus were exposed to identical conditions throughout the experiment. It can be seen that the different response of intact and collapsed blastocysts is evident within a single experiment. In addition, the effect of an introduction of 1 μg/ml nigericin, 0.5 μg/ml valinomycin and 100 mM K^+, which results in a large H^+ permeability in cells and sets pH_i equal to pH_o, was assessed. This resulted in an essentially instantaneous decrease of pH_i in both cases, indicating that introducing an exogenous H^+ permeability has a similar effect regardless of whether the blastocyst is intact or collapsed.

Effect of Zn^{2+} and sulfhydryl reagents

We tested the effects of Zn^{2+} and several sulfhydryl reagents on the rate of fall of pH_i in H^+-depleted 2-cell-stage embryos. These experiments were done in medium 0bicHM, again at pH_o 6.5 to maximize the rate of pH_i decrease and thus make detection of any effects easier.

Zn^{2+}, present as 1 mM $ZnSO_4$ throughout the experiment, had no effect on the rate of fall at 37°C (−0.027±0.001, *n*=20, plot not shown). Another manipulation was also carried out to assess the effect of Zn^{2+}. When embryos are flushed in warm medium and maintained at 37°C, pH_i is approximately 6.9. When such embryos are exposed to 100 mM K^+, pH_i rises dramatically (plot not shown), due to depolarization of the plasma membrane and consequent efflux of H^+ via the permeability pathway, as we have previously reported (Baltz et al., 1990). Zn^{2+} (1 mM) also did not inhibit this rise in pH_i.

We also tested a number of sulfhydryl reagents. Neither diamide (100 μM), dithiothreitol (1 mM) nor PCMBS (1 mM) prevented pH_i from falling at 37°C (plots not shown).

However, two of the sulfhydryl reagents, Hg^{2+} and diamide, did have a dramatic effect on 2-cell embryos at 23°C: 10 μM Hg^{2+}, added as $HgCl_2$, caused a fall in pH_i at 23°C comparable to that induced by raising the temperature to 37°C: pH_i immediately began to decrease at a mean rate of −0.038±0.002 (*n*=40; Fig. 6). At 100 μM Hg^{2+}, the fall was very rapid (−0.144±0.004, *n*=20); while at 1 μM, there was no effect (−0.009±0.001 before addition of Hg^+, and −0.011±0.001 after addition, *n*=40). Diamide (1 mM), when added at 23°C, caused a fall in pH_i similar to that seen with Hg^+, but with a delay of about 10 minutes (after the delay, the mean fall was −0.027±0.001, *n*=40); diamide at 100 μM had no effect over 20 minutes of observation. PCMBS (1 mM), an impermeant analog of Hg^+, had no effect at 23°C even if present for more than 20 minutes (plots not shown).

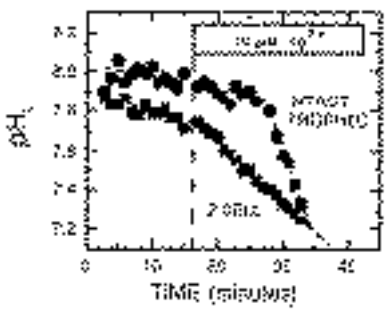

Fig. 6. Effect of Hg^{2+} on embryo pH_i. Embryos in pH 6.5 medium were exposed to 10 μM Hg^{2+} at the times indicated. This immediately caused a large acidification in 2-cell-stage embryos which continued at a steady rate. Intact trophectoderm, however, reacted to the same manipulation differently, being unaffected for 10 minutes after exposure to Hg^{2+} and then exhibiting a large acidification.

We also examined the response of intact blastocyst trophectoderm, which is refractory to the temperature-induced increase in acidification rate, to exposure to 10 μM Hg^{2+} at 23°C. We found that trophectoderm had a very different response to Hg^{2+} than 2-cell-stage embryos. In contrast to the immediate acidification produced by introduction of Hg^{2+} to 2-cell embryos, no effect was seen upon introduction of Hg^{2+} or for 10 minutes after. After 10 minutes, however, a marked acidification began (Fig. 6).

DISCUSSION

H^+-depletion of embryos

We found that embryos flushed and then maintained at room temperature in HCO_3^--free medium had a remarkably high pH_i which remained fairly stable. The origin of this alkalosis in room temperature-flushed embryos has not been determined, but it presumably is due to the loss of CO_2 upon flushing from the female tract with HCO_3^--free medium. The lack of HCO_3^- also prevents the HCO_3^-/Cl^- exchanger that is present in the preimplantation embryo (Baltz et al., 1991b) from mediating a recovery from the alkalosis. Embryos can also be H^+-depleted by incubation at 37°C in an alkaline medium. This latter method gave similar results and was used with the isolated inner cell masses.

2-cell-stage mouse embryos are permeable to H^+ at 37°C

The observations reported here support the conclusion that H^+ or the equivalent crosses the plasma membrane. We have shown that the source of the H^+ which appears in the cytoplasm after warming to 37°C is the external medium, since the rate of increase in cytoplasmic H^+ concentration is directly related to the external H^+ concentration (Figs 2, 3); reducing the concentration of external H^+ reduces the rate of appearance of H^+ in the cytoplasm and finally eliminates or reverses the flux. If the pH_i decrease was instead the result of internally generated H^+ (e.g., released from acidic compartments or produced by the cells' metabolism), then external pH should be without much influence.

Dependence of the rate of H^+ flux on the K^+ gradient

We showed that the rate of H^+ flux depends on the K^+ gradient across the plasma membrane, and that H^+ flux into embryos is inhibited by abolishing the K^+ gradient across the membrane. This could arise either because K^+ movement down its gradient is necessary for H^+ movement in the opposite direction or, since the K^+ gradient largely determines the membrane potential of cells, because H^+ flux is sensitive to membrane potential. A positive charge-carrying

influx of H^+ through the plasma membrane would be faster into a cell with a normal, inside-negative, membrane potential than in a cell that is depolarized, since influx of positive charge is favored by a negatively charged cell interior. Thus, either a countertransport mechanism, or a direct flux of H^+ across the membrane through a permeability pathway, would be expected to exhibit inhibition of H^+ influx when external K^+ concentration is increased, as observed in 2-cell embryos.

The evidence indicates that the second mechanism—direct flux of H^+ across the plasma membrane with concomitant transfer of charge—is operating in embryos. We have shown here that the countertransport of K^+ is not required for H^+ transport, since H^+ efflux out of acidified embryos occurs even in the absence of the external K^+ needed for a countertransport mechanism (Fig. 4). Rather, the flux out of acidified embryos behaves as if it depends on membrane potential; it is somewhat slowed by the hyperpolarization produced by K^+-free medium (Fig. 4) rather than completely inhibited. In addition, we have previously shown that similar H^+ efflux from 2-cell embryos subjected to a transient acid-load induced by an ammonium pulse is not dependent on the presence of external K^+ (Baltz et al., 1990).

Developmental changes in H^+ permeability

While the data indicate that the cells of 2-cell embryos are highly permeable to H^+, blastocysts exhibit a more complex behavior. Isolated inner cell masses behave similarly to 2-cell embryos; they appear highly permeable to H^+ at 37°C. Blastocysts that have been mechanically collapsed to afford access of the external medium to not only the outward-facing apical trophectoderm surface, but also the inward-facing basolateral trophectoderm surface and the blastocoelar surface of the inner cell mass, also show a pH_i drop at 37°C, but at a slower rate than isolated inner cell masses. Intact blastocysts behave very differently: pH_i of the trophectoderm drops very slowly even at 37°C. Thus, it can be inferred that the apical surface of the trophectoderm (which is the only surface exposed to pH 6.5 medium in intact blastocysts) has little permeability to H^+.

The stability of pH_i in intact blastocyst trophectoderm is not due to the presence of the blastocoel fluid, but must be a function of the impermeability of the apical surface. This can be seen in two ways, the first being the experimental result that artificially inducing a H^+ permeability in the apical membrane with nigericin (Fig. 5B) causes an immediate acidification of pH_i, indicating that the blastocoel cannot somehow protect the trophoblast cells from acidification if the outer surface is made H^+-permeable. The second line of evidence is theoretical: we could postulate that the blastocoel acts as an 'alkaline reservoir,' neutralizing the acid influx. For a thin compartment like the trophectoderm, the H^+ concentration in the cells rapidly equilibrates to:

$$\frac{[H^+]_{external} \cdot P_{apical} + [H^+]_{blastocoel} \cdot P_{basolateral}}{P_{apical} + P_{basolateral}}$$

where the *P*s denote the permeability of the surface indicated by the subscript. Thus, it can be readily shown that if, for example, the blastocoel pH is 8.0 and the external medium is pH 6.5, then the pH_i of the trophectoderm cells would be about 6.8, not the unchanged level (above 7.5) that we observe for intact trophectoderm (Fig. 5). Only if the external permeability is negligible compared to the internal permeability is the observed result expected.

This phenomenon may be the origin of the differences seen between the responses of intact trophectoderm and collapsed blastocysts after the introduction of nigericin (Fig. 5B). While the collapsed blastocyst pH_i falls instantaneously to around 6.4 and then remains constant following exposure to nigericin, the pH_i of intact trophectoderm falls instantaneously to about 6.8 and then continues falling more slowly thereafter. This result is consistent with the H^+ concentration in the trophectoderm falling to a value approximately midway between the external and blastocoel concentrations (i.e., to around pH_i 6.8), and then falling further as the blastocoel fluid is acidified by H^+ crossing the nigericin-permeablized trophectoderm.

While the foregoing analysis appears to rule out a significant intrinsic apical permeability, it should be noted that it does not rule out a basolateral (inward facing) trophectoderm permeability. The observation that Hg^{2+} can induce a permeability in trophectoderm after a delay indicates, perhaps, that the permeability pathway is present in the trophectoderm, but is either inactive or not in the apical surface. However, from the results presented in Fig. 5, it appears that there may be two populations of cells in the collapsed blastocyst—one permeable and the other impermeable to H^+. This is indicated by the observation that the pH_i of collapsed blastocysts does not fall as far as expected, as can be seen by comparing the terminal pH_i for isolated inner cell masses (Fig. 5B), or 2-cell embryos at pH_o 6.5 (Fig. 2) to that of collapsed blastocysts at pH_o 6.5 (Fig. 5B). Note that inner cell mass and 2-cell embryo pH_i fall to a low value, while that of collapsed blastocysts stays well above this range. This result for collapsed blastocysts is consistent with our having detected the signal from a population of cells with high pH_i, and one with low pH_i, which become averaged due to the geometry of the preparation. The addition of nigericin (Fig. 5B) shows that pH_i will fall to the expected value when all cell types are made H^+ permeable. Perhaps trophectoderm is indeed impermeable to H^+ on both surfaces, while inner cell mass alone is permeable.

It appears, therefore, that H^+ permeability is developmentally regulated in the preimplantation mouse embryo. 2-cell-stage embryos are permeable to H^+. Intact blastocysts, however, exhibit little H^+ external (apical) permeability and are therefore not affected by pH_o in the same manner as a 2-cell-stage embryo. Readings taken over the inner cell masses of intact embryos in pH 6.5 medium (during experiments assessing the permeability of the intact trophectoderm) showed no acidification at 37°C, indicating that the inner cell mass is protected by the trophectoderm from any external acid load (data not shown). However, the inner cell mass is still H^+ permeable at the blastocyst stage, so that the blastocoel fluid, whose composition is under embryonic control, can determine pH_i of these cells.

Identity of the H^+ permeability pathway

Several mechanisms by which H^+ crosses the membrane in

those rare cell types that exhibit a permeability to H^+ have been identified. A voltage-gated H^+ channel has been identified in several cell types—the snail neuron (Thomas and Meech, 1982), the Ambystoma egg (Barish and Baud, 1984) and the rat alveolar epithelium (DeCoursey, 1991). This mechanism is characterized by the inhibitory capacity of Zn^{2+}. Since neither the fall of pH_i at 37°C nor the increase of pH_i caused by raising the external K^+ concentration to 100 mM are inhibited by Zn^{2+}, it appears unlikely that a H^+ channel similar to those found in these other cells is present in the embryo.

Another pathway by which H^+ can cross the plasma membrane is the water channel (Harris et al., 1990; Harvey et al., 1991) found in urinary bladder and frog skin. This mechanism is strongly inhibited by sulfhydryl active compounds such as PCMBS and Hg^{2+}. We found the opposite effect of Hg^{2+}: addition of as little as 10 μM Hg^{2+} to the medium caused an immediate increase in H^+ permeability and there was no effect of PCMBS, thus making a water channel an unlikely candidate for the H^+ permeability in the mouse embryo.

The ability of Hg^{2+} to induce H^+ permeability in the 2-cell mouse embryo is similar to its effect on Na^+ and K^+ permeabilities in kidney cells (Rothstein and Mack, 1991). Here, low concentrations of Hg^{2+} or PCMBS induce high permeabilities to these ions. In addition, a furosemide-sensitive NaCl cotransporter is also apparently activated by these reagents. In red blood cells diamide has been found to activate the KCl cotransporter (Lauf, 1988). It is possible that there is a transport mechanism in the mouse embryo membrane that is similarly sulfhydryl reagent activatable and which constitutes the pathway for H^+ flux. However, we can not rule out that Hg^{2+} is affecting a pathway unrelated to the one active at 37°C.

Another effect of sulfhydryl active reagents that has been reported is the induction of non-specific 'leaks' in the red blood cell membrane by SH oxidation due to diamide exposure (Deuticke et al., 1983). Here, exposure to high levels (5 mM) of diamide caused the cells to become non-specifically permeable to small molecules. However, much lower levels of Hg^{2+} (10 μM) are required to induce a H^+ permeability in 2-cell mouse embryos, indicating a specific effect.

The high permeability of embryos appears to be specific to H^+. We have data (not shown) demonstrating that the 2-cell mouse embryo exhibits low rates of Na^+ and K^+ leakage at 37°C; using electron probe X-ray microanalysis on 2-cell embryos (as described in Baltz et al., 1991b), we find that 2-cell embryos, whose Na^+,K^+ ATPase has been inhibited by ouabain and the absence of extracellular K^+, gain Na^+ and lose K^+ with a half-time of 3 hours—a slow rate reflecting a low-normal permeability and the small surface-to-volume ratio of these cells. Thus, it appears that the permeability is not due to indiscriminate leakiness to cations.

Conclusion

The data that we have presented are consistent with a model in which the 2-cell-stage mouse embryo, unlike most other cells, is highly permeable to H^+ at body temperature. This permeability pathway allows H^+ to cross the membrane, probably in a rheogenic fashion. Its identity is not yet known; specifically, the voltage-gated H^+ channels found in several other cell types and H^+ permeation through water channels have been ruled out.

This permeability has important implications for the ability of embryonic cells to regulate their pH_i. Since the major pathway for H^+ across the membrane is passive and since we have previously demonstrated the absence of specific mechanisms active in regulating pH_i in the acid and neutral range, H^+ will normally exist in electrochemical equilibrium across the membrane in this pH_i range. This is in distinct contrast to other cells, where pH_i is maintained well above equilibrium by the Na^+/H^+ antiporter and possibly by the Na^+,HCO_3^-/Cl^- exchanger. In addition, acid loads in 2-cell embryos are relieved not by activation of one of these systems, as in other cells, but by passive efflux of H^+ until equilibrium is reestablished (for a discussion of such a mechanism see Baltz et al., 1990). In contrast, pH_i is specifically regulated in the alkaline range (Baltz et al., 1991b) by a HCO_3^-/Cl^- exchanger. This is possible even in the presence of the H^+ permeability, since the H^+ gradient provides little driving force in an alkalotic cell, the H^+ concentration is small on both sides of the membrane and the exchanger operates on a much more abundant ion, HCO_3^-. Thus, the 2-cell-stage mouse embryo is unique in its mechanism of pH_i regulation, having a specific mechanism in the alkaline range, and a passive permeability in the acid and neutral range. This may be related to the high pH and HCO_3^- levels in the oviduct, where the 2-cell-stage embryo resides in vivo (see Baltz et al., 1990). By the blastocyst stage, the embryo has lost its external H^+ permeability, indicating that this phenomenon is under developmental regulation during the preimplantation period of embryo development. The inner cell mass apparently retains the H^+ permeability at this stage. Thus, the embryo is no longer sensitive to the pH of the fluid of the maternal environment, but only to the pH of the blastocoel fluid, which is under embryonic control.

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