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Mechanisms regulating intracellular pH are activated during growth of the mouse oocyte coincident with acquisition of meiotic competence

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Abstract

Oocytes grow within ovarian follicles, and only gain the ability to complete meiosis when they are nearly fully grown. We have found that both of the major types of intracellular pH regulatory mechanisms in the mammal – the Na^+/H^+ and HCO_3^-/Cl^- exchangers – were essentially inactive in mouse oocytes over most of the course of their growth. However, as oocytes approached full size, Na^+/H^+ and HCO_3^-/Cl^- exchangers became simultaneously active, and, at the same time, the intracellular pH of isolated oocytes increased sharply by about 0.25 pH unit. This activation of intracellular pH regulatory mechanisms and increase in pH occurred coincident with the acquisition of meiotic competence. The activation of pH regulatory mechanisms during oocyte growth represents a previously unknown milestone in the development of the capacity of the oocyte to function independently upon ovulation.
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Introduction

In each reproductive cycle, a cohort of primordial follicles in the ovary, each consisting of an oocyte enclosed by a single layer of granulosa cells, is recruited to grow. During growth, the mammalian oocyte increases in size from ~20 μm to a species-dependent final size of 70–120 μm, all the while remaining arrested in first meiotic prophase and exhibiting a prominent prophase nucleus, termed the germinal vesicle (GV). Simultaneously, the surrounding granulosa cells proliferate and form an antral (Graafian) follicle from which the mature egg is ovulated. When ovulation is triggered, the fully grown GV oocyte completes first meiosis (MI) and then is rearrested in second meiotic metaphase (MII) as a mature egg, a process termed meiotic maturation. A major developmental milestone during oocyte growth is the acquisition of meiotic competence—the ability to undergo germinal vesicle breakdown (GVBD) and proceed through meiotic maturation when released from the follicle. Although the molecular basis for the acquisition of meiotic competence is not well understood, it is known that growing oocytes become competent only when they reach ~80% of full size (Sorensen and Wassarman, 1976; Wassarman et al., 1979). In addition, the oocyte also undergoes numerous developmental changes during growth, including progressively losing its reliance upon metabolic and homeostatic support of the granulosa cells and becoming capable of existing as an independent cell when ovulated.

Alterations in intracellular pH (pH_i) and activation of pH_i regulatory mechanisms are associated in a number of cell types with developmental changes or metabolic activation. This includes, for example, the stimulation of pH_i regulatory mechanisms by growth factor signaling and metabolic activation (Ganz et al., 1989), and the increase in pH_i that is integral to egg activation in some marine invertebrates (Johnson et al., 1976). pH_i in mammalian cells is regulated by electroneutral transport mechanisms. These include Na^+/H^+ exchangers of NHE gene family which export H^+ (in exchange for Na^+

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moving down its normally inward concentration gradient) to correct low pH_i, and the HCO_3^-/Cl^- exchangers of the AE gene family which export HCO_3^- (in exchange for Cl^- moving down its normally inward concentration gradient) to correct increases in pH_i (Alper, 1994; Orlowski and Grinstein, 1997, 2004; Romero et al., 2004; Roos and Boron, 1981). Although little is known about pH_i regulation in the growing oocyte, there is good evidence that robust pH_i regulation is required after fertilization and throughout preimplantation mammalian embryo development. HCO_3^-/Cl^- exchange was shown to be highly active in preimplantation mammalian embryos from the pronuclear egg through the blastocyst stages, including in mouse, hamster, and human (Baltz et al., 1991; Lane et al., 1999a; Phillips et al., 2000; Phillips and Baltz, 1999; Zhao and Baltz, 1996), and exchanger activity is required for mouse embryos to maintain pH_i and for normal embryo development (Zhao et al., 1995). Similarly, Na^+/H^+ exchange is active in regulating pH_i in preimplantation embryos, including those of mouse, hamster, and human (Gibb et al., 1997; Harding et al., 2002; Lane et al., 1998; Steeves et al., 2001).

The picture is more complex around the time of fertilization. In several marine invertebrates and amphibians, it is well-established that pH_i is low and pH_i regulatory mechanisms are inactive in unfertilized eggs, while fertilization causes an increase in pH_i and activates regulatory mechanisms (Dube et al., 1985; Dube, 1988; Dube and Eckberg, 1997; Epel, 1988; Freeman and Ridgway, 1993; Johnson et al., 1976; Webb and Nuccitelli, 1981). The best-known example is the Na^+/H^+ exchanger in the sea urchin, which is quiescent in the egg but becomes highly active within minutes of fertilization, permanently increasing pH_i and triggering subsequent embryo development (Epel, 1988; Johnson et al., 1976). No such increase in pH_i, however, appears to follow fertilization in mammals (Ben Yosef et al., 1996; Dale et al., 1998; Kline and Zagray, 1995; Phillips et al., 2000; Phillips and Baltz, 1996). Nevertheless, pH_i regulatory transporters were also found to be quiescent in the unfertilized mammalian egg, and activated after fertilization: in the mouse egg, the HCO_3^-/Cl^- exchanger is inactive in the egg with activity gradually increasing following fertilization to reach maximal activity when pronuclei form (Phillips and Baltz, 1999), while it has been shown that the Na^+/H^+ exchanger is similarly activated after fertilization in the hamster egg (Lane et al., 1999b). Thus, pH_i regulatory mechanisms appear to become fully active following fertilization in a number of species, including at least some mammals.

Although they are quiescent in the mature MII oocyte before it is fertilized, both HCO_3^-/Cl^- exchange (Phillips et al., 2002) and Na^+/H^+ exchange (FitzHarris and Baltz, unpublished) were nonetheless found to be fully active in the mouse GV oocyte. This implies that these pH_i regulatory mechanisms are specifically inactivated during progression through meiotic metaphase, and this was directly shown for HCO_3^-/Cl^- exchange in the mouse oocyte (Phillips et al., 2002). The reason for the inactivation of pH_i regulatory mechanisms during meiotic maturation remains unknown.

Thus, our current understanding in the best-studied mammalian example, mouse, is that pH_i regulatory mechanisms are maximally active in the fully grown oocyte prior to ovulation and the resumption of meiosis. Nothing, however, is known about pH_i regulation before this period, during oocyte growth and follicle development in the ovary. Since pH_i regulation is dynamic and necessary for development in the fully grown oocyte and early embryo, and is associated with developmental and metabolic changes in other cells, we have undertaken the present studies to determine whether growing oocytes have the capacity to regulate pH_i, and whether growing oocytes exhibit developmental changes in either pH_i or the mechanisms which regulate it.

Materials and methods

Chemicals

All chemicals and drugs were obtained from Sigma (St. Louis, MO) unless otherwise noted. 4,4′-Diisocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), amiloride, and carboxyseminaphthorhodafluor-1-acetoxymethyl ester (SNARF-1-AM) were obtained from Molecular Probes (Eugene, OR). Stock solutions were prepared in water (dibutyrladenosine 3,5-cyclic monophosphate (dbcAMP)), ethanol (nigericin), or dimetyl sulfoxide (DMSO; SNARF-1-AM, valinomycin, DIDS, amiloride).

Solutions

All media were based on KSOM mouse embryo culture medium (Lawitts and Biggers, 1993) that contains (in mM) 95 NaCl, 2.5 KCl, 0.35 KH_2PO_4, 0.2 $MgSO_4$, 10 Na lactate, 0.2 glucose, 0.2 Na pyruvate, 25 $NaHCO_3$, 1.7 $CaCl_2$, 1 glutamine, 0.01 tetra sodium EDTA, 0.03 streptomycin SO_4 and 0.16 penicillin G, and 1 mg/ml bovine serum albumin (BSA) or polyvinyl alcohol (PVA). For collection of oocytes, HEPES-KSOM was used (21 mM HEPES replacing equimolar $NaHCO_3$, pH adjusted to 7.4). For all fluorophore-loading and pH_i measurements, 9 mM Na lactate was replaced with NaCl (total 104 mM NaCl and 1 mM Na lactate) and neither BSA nor PVA was included. In experiments where HCO_3^--free media were used, fluorophore loading was done in HEPES-KSOM from which HCO_3^- was also entirely omitted. Cl^--free media were produced by replacing all Cl^- salts with corresponding gluconate salts. Na^+-free media were produced by replacing Na^+ salts with corresponding choline salts. For ammonium-containing KSOM, 35 mM NaCl was replaced with equimolar NH_4Cl. HCO_3^-/CO_2-buffered media were equilibrated with 5% CO_2/air. Where specified, HCO_3^--free media were used in which 21 mM of $NaHCO_3$ was replaced by HEPES and pH adjusted to 7.4, and equilibrated with air.

Growing and mature oocyte collection

Mature GV oocytes were obtained from primed CF1 female mice (Charles River, St-Constant, PQ, Canada), approximately 44 h after eCG injection (5 IU, intraperitoneally). The ovaries were removed and minced to release cumulus-enclosed oocytes. Cumulus cells were then removed mechanically, by repeated pipetting through a narrow-bore pipet.

Growing oocytes were obtained from female mice on days 5–21 postnatal. A coordinated wave of follicular development begins shortly after birth in mice, so oocyte growth and follicular development are directly related to postnatal age. Neonatal females have only nongrowing oocytes, but during days 5–21, a large cohort of oocytes grow to full size, becoming meiotically competent starting on about day 15. This initial wave of oocyte growth recapitulates cyclical follicle development in the mature female, and thus juvenile mice have proven a valuable model for obtaining large numbers of synchronized, growing oocytes and follicles at defined stages of growth and competence. To obtain growing oocytes, ovaries were removed from mice at the appropriate age. The oocytes were isolated enzymatically by incubation in Ca^{2+}- and Mg^{2+}-free

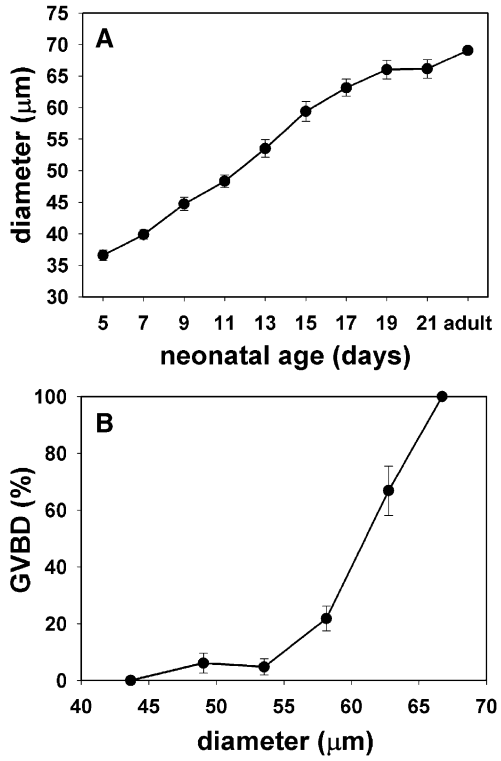

Fig. 1. Oocyte growth and acquisition of meiotic competence. (A) Oocyte diameter is shown as a function of neonatal age. Ovaries were removed from mice 5–21 days after birth at 2 day intervals, and enzymatically digested to release oocytes (see text). In addition, GV stage oocytes were collected from adult ovaries. The diameters of all oocytes collected from neonatal ovaries, and all GV oocytes collected from adult ovaries, were measured, and the mean diameter was calculated for the oocytes collected. 4–11 mice were assessed on each neonatal day, for a total of 223–779 individual oocytes measured for each day; 11 adult mice were assessed, for a total of 458 GV oocytes. Each data point represents the mean ± SEM of the diameters calculated on each day. (B) Meiotic competence is shown as a function of oocyte diameter. The diameters of oocytes isolated from neonatal ovaries (days 13–21) were measured, and their ability to undergo GVBD in culture assessed as described in the text. The percentage of oocytes that reentered meiosis (as assessed by GVBD) within each 5 μm increment of diameter is shown. Each point represents the % GVBD in oocytes from 6–17 mice (mean ± SEM), plotted vs. the mean (±SEM) diameter with each 5 μm diameter increment. Where error bars are not visible, they are smaller than the size of the symbol.

KSOM with collagenase (Type I, 2 mg/ml) and DNAse (0.01 mg/ml) for 30 min (Eppig, 1976), or, where specified, by mechanical isolation following fine mincing of the ovary with a razor blade. The diameters of the isolated denuded oocytes were then measured using an eyepiece reticle and grouped within 5 μm increments. To determine mouse age vs. mean oocyte diameter, all oocytes recovered from neonatal females of a given age were measured without first separating them by size.

Intracellular pH measurement

pH_i measurements were performed using a quantitative fluorescence imaging microscopy system (Inovision, Durham, NC) as previously described in detail (Baltz and Phillips, 1999; Zhao and Baltz, 1996). pH_i was determined in oocytes loaded intracellularly with the pH-sensitive fluorophore, SNARF-1, by a 30 min incubation with the acetoxymethyl ester derivative (SNARF-1-AM, 5 μM). Two emission wavelengths were used, 640 and 600 nm, with an excitation wavelength of 535 nm. The ratio of the two intensities (640/600), which is dependent mainly on pH_i, was calculated by dividing the images after background subtraction.

Calibration of ratio with pH_i was done by the nigericin/high K^+ method with valinomycin added to collapse the K^+ gradient. Resting pH_i was determined in KSOM solution after a 15 min stabilization period. It should be kept in mind, however, that this method of calibration is more accurate for determining the values of pH_i changes than for determining absolute pH_i values.

Each replicate consisted of simultaneous measurements made up groups of 5–25 oocytes. pH_i was averaged for oocytes within each 5 μm increment at each time point. All measurements were done in a temperature- and atmosphere-controlled chamber (37°C, 5% CO_2/air for HCO_3^-/CO_2-buffered media, or air for HCO_3^--free media). dbcAMP (100 μM) was included during oocyte isolation, to prevent spontaneous maturation of meiotically competent oocytes after removal from the ovary.

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

HCO_3^-/Cl^- exchanger activity was quantified by the Cl^- removal method (Baltz and Phillips, 1999; Phillips et al., 2002; Phillips and Baltz, 1999; Zhao and Baltz, 1996). On exposure of cells to Cl^--free solution, the HCO_3^-/Cl^- exchanger will run in reverse, resulting in intracellular alkalinization due to HCO_3^- influx coupled to Cl^- efflux (Nord et al., 1988). Increased pH_i upon Cl^- removal thus indicates HCO_3^-/Cl^- exchanger activity, and the initial rate of alkalinization provides a quantitative measure of activity (Nord et al., 1988). Here, SNARF-1-loaded oocytes were placed in the chamber, equilibrated for 15 min, and then measurements taken for 10 min, after which the solution was changed to Cl^--free, low-lactate KSOM for 20 min. The initial rate of intracellular alkalinization upon Cl^- removal was determined using linear regression (Sigma Plot 8.0, Chicago, IL), and exchanger activity is reported as the change in pH_i per minute (pHU/min). Inhibition by DIDS (500 μM) present in the Cl^--free medium was used to confirm that any observed alkalinization was mediated by HCO_3^-/Cl^- exchange. This assay for HCO_3^-/Cl^- exchanger activity has been extensively described and validated in mouse oocytes and embryos (Baltz and Phillips, 1999; Phillips et al., 2002; Phillips and Baltz, 1999; Zhao and Baltz, 1996). Although 20 μM DIDS is sufficient to maximally inhibit anion exchange in embryos (Zhao and Baltz, 1996), a higher concentration of DIDS (500 μM) was used in most experiments to produce immediate inhibition even if mixing was not immediately complete during solution changes.

NH_4Cl method for assessing recovery from alkalosis

Alkalosis was induced by introduction of 35 mM NH_4Cl as an isosmolar substitution for NaCl, as previously described (Phillips and Baltz, 1999; Zhao

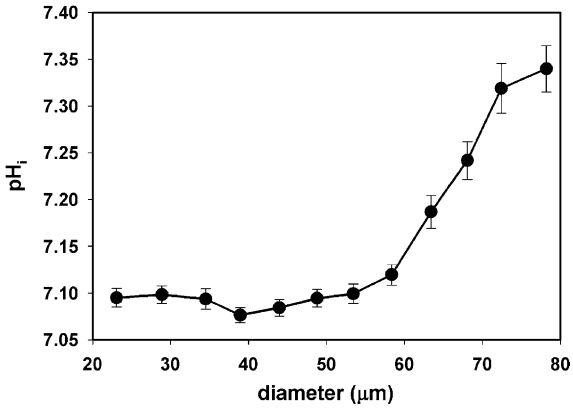

Fig. 2. pH_i as a function of oocyte diameter. pH_i was determined for individual oocytes from neonatal mice (days 5–21) whose diameters were measured, and the mean pH_i determined for oocytes within each 5 μm increment. Each point represents the mean ± SEM of the pH_i of 34–169 oocytes that fell within the specified size ranges in 16–37 separate experiments, except for the lowest and two highest diameter groups, where fewer oocytes fell within those ranges (12–23 oocytes in 5–11 experiments). These data were plotted vs. the mean (±SEM) diameter with each 5 μm diameter increment. Where error bars are not visible, they are smaller than the size of the symbol. Data were analyzed as described in the text.

and Baltz, 1996). Briefly, membrane-permeable NH_3, in equilibrium with NH_4^+ in the medium, enters the cell through the membrane, where it then quickly complexes with H^+ to establish equilibrium, resulting in a large, essentially instantaneous intracellular alkalinization. The subsequent recovery in the continued presence of NH_4Cl is a measure of HCO_3^-/Cl^- exchanger activity (Baltz and Phillips, 1999). There is, however, always a substantial component of the apparent recovery that is nonspecific, due largely to the slower influx of NH_4^+. This nonspecific recovery was determined in the presence of DIDS (500 μM), added 10 min before NH_4Cl to ensure complete inhibition, and the recovery mediated by HCO_3^-/Cl^- exchange was then taken to be the difference between the recovery in the absence and presence of the inhibitor.

Na^+ removal assay for Na^+/H^+ exchange activity

Na^+/H^+ exchanger activity was quantified by the Na^+ removal method. Analogous to the Cl^- removal assay for HCO_3^-/Cl^- exchanger activity, removal of external Na^+ causes Na^+/H^+ exchangers to operate in reverse, resulting in cytoplasmic acidification. Upon Na^+ reintroduction, recovery from the induced acidosis occurs, revealing Na^+/H^+ activity (Steeves et al., 2001). The initial rate of recovery was determined by linear regression (as above), providing a measure of Na^+/H^+ exchanger activity. Mediation of the recovery by Na^+/H^+ exchange was confirmed by inhibition with amiloride (1000 μM, added 10 min before Na^+ removal).

Statistics and data analysis

Graphs were created using SigmaPlot 8.0 (Chicago, IL), with all values plotted as mean ± SEM unless otherwise noted. The relationship between dependent and independent variables was determined by Univariate ANOVA with General Linear Models, using SPSS 12.0 (SPSS, Inc. Chicago, IL). For analyses where oocyte diameter was an independent variable, oocyte diameters were first grouped into bins of 5 μm width, as described above, with each such diameter category (diameter range) considered a nominal value of the independent variable.

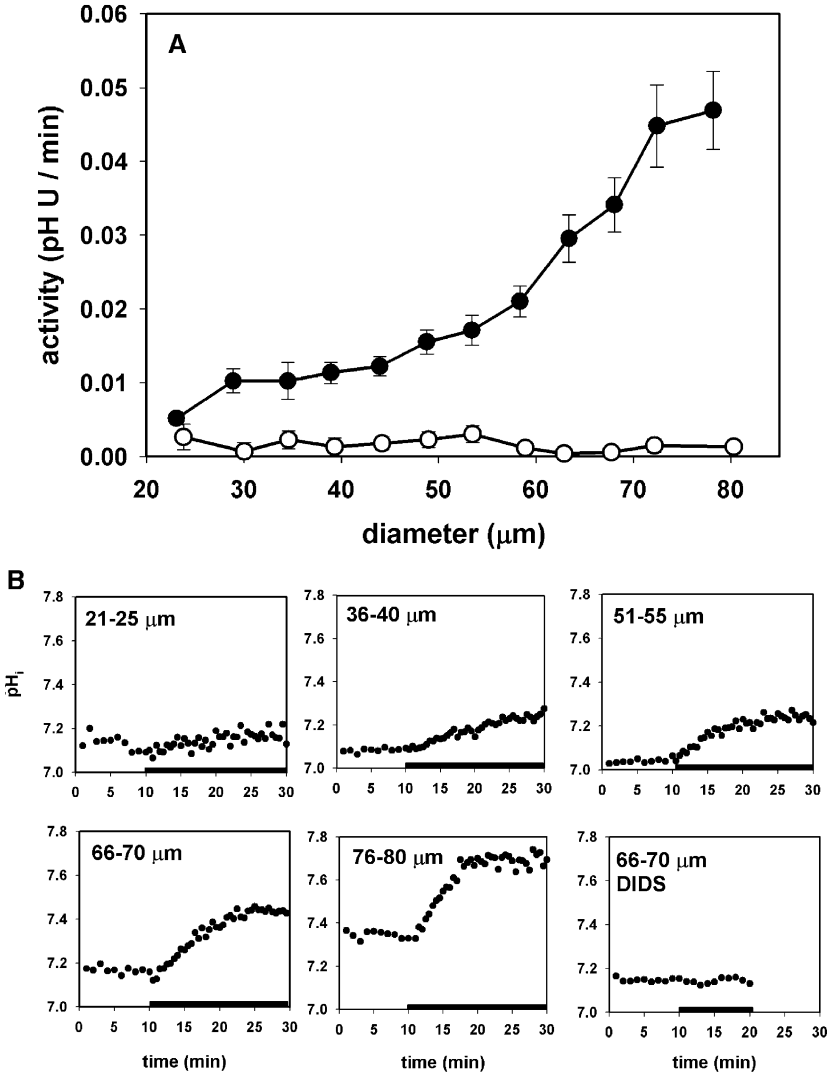

Fig. 3. HCO_3^-/Cl^- exchanger activity determined by Cl^- removal assay as a function of oocyte diameter. (A) The initial rate of alkalinization upon Cl^- removal, as a measure of HCO_3^-/Cl^- exchanger activity in oocytes, is shown as a function of the mean (±SEM) diameter with each 5 μm diameter increment (filled circles). Nonspecific activity was determined in the presence of the inhibitor, DIDS (500 μm, open circles). Each point represents the mean ± SEM of the initial rate of alkalinization vs. the mean (±SEM) diameter with each 5 μm diameter increment, and are data from the same population of oocytes shown in Fig. 2 (closed circles), or the data from 12–169 oocytes that fell within the specified size ranges in 5–23 separate experiments (DIDS data, open circles; except for smallest and largest groups where only 2 oocytes were obtained for each). Data were analyzed as described in the text. (B) Examples of results of Cl^- removal assay for oocytes of several diameters (indicated in panels). pH_i was measured as a function of time in Cl^--containing medium and then after replacement with Cl^--free medium (indicated by black bar starting at 10 min), where an increase in pH_i indicates HCO_3^-/Cl^- exchanger activity. The final trace shows the complete inhibition by DIDS (500 μM). Each trace is the mean of 2–4 oocytes in a single experiment.

Results

Oocyte growth and acquisition of meiotic competence

Oocytes were obtained from neonatal female mice on days 5–21 after birth in 2-day increments. The diameters of oocytes from these CF1 females ranged from a mean of about 36 μm on day 5 to about 66 μm on day 21, while unselected GV oocytes from superovulated CF1 females approximately 7 weeks of age had a mean diameter of about 69 μm (Fig. 1A). For subsequent experiments, oocytes were grouped by diameter in 5 μm increments, with the smallest group (from day 5 neonatal mice) being 20–25 μm, and the largest group (from the oldest neonatal ages and adult mice) being 75–80 μm. Thus, oocytes could be obtained which represented nearly the entire period of oocyte growth.

In order to determine at which size CF1 oocytes acquired meiotic competence, we collected oocytes from females on neonatal days 13–21, and placed them into culture overnight. When competent, mouse oocytes will spontaneously undergo GVBD in vitro, and thus we scored for loss of the germinal vesicle as a function of oocyte diameter. Virtually no oocytes below 55 μm diameter exhibited GVBD, while the proportion that was meiotically competent increased from less than 20% for oocytes in the 55–60 μm range to 100% above 65 μm (Fig. 1B).

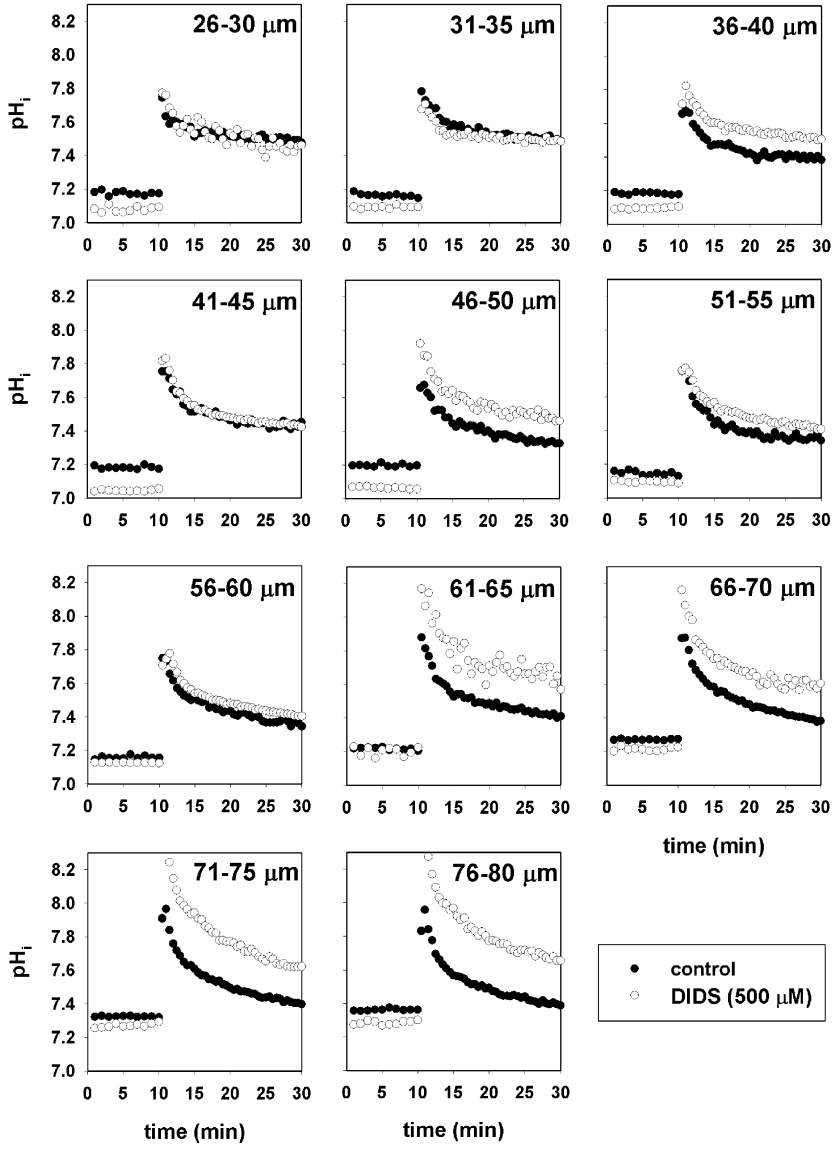

Fig. 4. Recovery from intracellular alkalosis induced by exposure to NH_4Cl by growing oocytes. pH_i of oocytes, grouped by diameter as indicated in each panel, was measured for 10 min in normal medium and then following exposure to 35 mM NH_4Cl, inducing a marked alkalosis. The recovery from alkalosis was followed for 20 min without any addition (closed symbols) or in the presence of 500 μM DIDS (open symbols) to inhibit HCO_3^-/Cl^- exchanger activity. Each trace represents the mean pH_i vs. time of five or more oocytes that fell within the specified ranges of diameter in 3–7 separate experiments (except for the largest group in DIDS, where oocytes fell in this range in only 2 experiments).

Intracellular pH in growing oocytes

The resting pH_i of oocytes at different stages of growth was determined after a 15 min equilibration in KSOM at 5% CO_2 and 37°C (no BSA, 1 mM lactate, external pH 7.3). Measurements were grouped according to oocyte diameter, and mean resting pH_i expressed as a function of oocyte size (Fig. 2). Growing oocytes up to about 60 μm in diameter had a mean pH_i of about 7.10, which did not vary with growth. However, pH_i increased markedly and monotonically in oocytes as mean diameter increased above 60 μm, reaching about 7.35 in fully grown oocytes above 70 μm diameter. The dependence of pH_i upon oocyte diameter was highly significant ($P < 10^{-6}$ by Univariate ANOVA). A similar dependence of pH_i upon oocyte diameter was seen in HCO_3^--free medium, where resting pH_i again increased by approximately 0.25 pH units as oocytes grew from below 60 to above 70 μm (not shown). However, in the absence of HCO_3^- and CO_2 buffering, the apparent absolute level of pH_i was only about 7.1 in the largest oocytes.

HCO_3^-/Cl^- exchanger activity in growing oocytes

Fully grown GV stage mouse oocytes have previously been shown to exhibit very robust HCO_3^-/Cl^- exchanger activity that regulates pH_i against increases (Phillips et al., 2002). Here, we determined HCO_3^-/Cl^- exchanger activity in growing mouse oocytes by recording the rate of alkalinization upon Cl^- removal. We found that activity was low in the smallest growing oocytes, but increased over the course of growth, with a rapid increase in activity occurring as oocyte diameter increased from about 60–70 μm (Fig. 3). The dependence of HCO_3^-/Cl^- exchanger activity upon oocyte diameter was significant ($P = 0.007$ by Univariate ANOVA, with diameter range and pH_i taken to be the independent variables). The rate was also highly dependent upon pH_i ($P < 10^{-6}$), reflecting the change in pH_i that also occurs with development (above). HCO_3^-/Cl^- exchanger activity was blocked completely by DIDS (500 μM; Fig. 3), revealing that a small level of exchanger activity was detectable at all stages of growth except possibly in the very smallest oocytes assessed (20–25 μm range), while DIDS-inhibitable activity increased markedly as oocytes grew past about 60 μm.

Recovery from alkalosis in growing oocytes

In order to assess the ability of the HCO_3^-/Cl^- exchanger in oocytes to mediate recovery from increased pH_i at different stages of growth, we used the NH_4Cl exposure method of inducing intracellular alkalosis, and assessed their ability to recover. Early growing oocytes possessed a limited ability to recover from induced alkalosis, with no difference in the recoveries seen in the presence vs. absence of DIDS (Fig. 4). Larger oocytes were more able to recover from high pH_i, and this was inhibitable by DIDS. In addition, the extent of alkalinization was greater in the presence of DIDS in oocytes that were nearly fully grown. A clear effect of DIDS on the extent of alkalinization and subsequent recovery was evident in oocytes that were larger than approximately 60 μm in diameter (Fig. 4). These qualitative observations were further assessed by plotting the difference between the pH_i immediately before alkalinization (at 10 min; Fig. 4) and that after 20 min of recovery (i.e., at 30 min). This revealed that the extent of recovery in the presence of DIDS did not change appreciably over the course of oocyte growth, with pH_i remaining at about 0.3–0.4 pH units above its level before alkalinization (Fig. 5). In contrast, in the absence of DIDS, the final pH_i decreased progressively with oocyte diameter, indicating that the largest oocytes exhibited the most complete recovery from alkalosis, and that recovery was mediated by the appearance of HCO_3^-/Cl^- exchanger activity.

Na^+/H^+ exchanger activity in growing oocytes

Although the appearance of HCO_3^-/Cl^- exchanger activity during oocyte growth correlated with the measured change in resting pH_i, the direction of the pH_i change was opposite to that expected if it was mediated by increased HCO_3^-/Cl^- exchanger activity. Thus, it was possible that other pH_i regulatory mechanisms normally mediating pH_i increases, such as the Na^+/H^+ exchanger, were also activated at the same point in oocyte growth. We first attempted to detect Na^+/H^+ exchanger activity in fully grown oocytes. Surprisingly, however, we found that no change in pH_i occurred upon Na^+ removal or reintroduction, in contrast to the robust activity that we detected in GV stage oocytes isolated from adult mice (not shown). One difference here was that GV oocytes had been isolated mechanically, while the fully grown oocytes from neonatal ovaries had been isolated enzymatically, using collagenase as described above. Thus, we attempted to also isolate growing oocytes mechanically, by finely mincing ovaries, to test the possibility that it was the conditions during

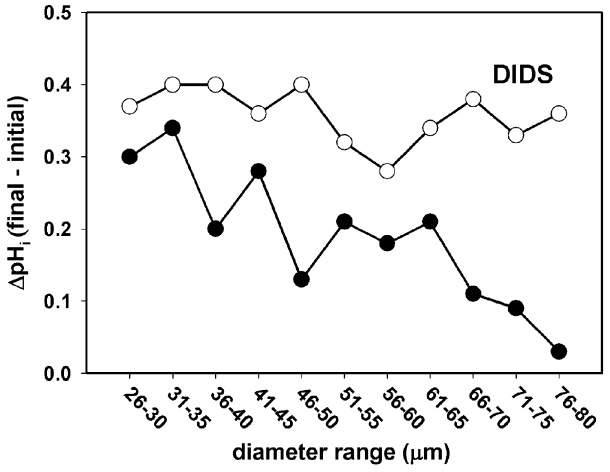

Fig. 5. Extent of recovery from alkalosis as a function of oocyte diameter. The data in Fig. 4 were analyzed by determining the difference between pH_i immediately before NH_4Cl exposure and after 20 min of recovery for each 5 μm range of oocyte diameter. For small oocytes, pH_i remained about 0.3 pH units above the initial level, while recovery in the largest oocytes was nearly complete after 20 min (closed symbols). In the presence of 500 μM DIDS (open symbols), oocyte pH_i remained approximately 0.3–0.4 pH units above the initial level for oocytes of all diameters assessed.

enzymatic treatment (i.e., collagenase and DNAse in Ca^{2+}/Mg^{2+}-free media) that caused the loss of Na^+/H^+ exchanger activity in oocytes. We were successful in obtaining oocytes from just below 50 μm through fully grown, although smaller oocytes could not be isolated mechanically. Nevertheless, since this size range spans, that where HCO_3^-/Cl^- activity had been found to appear during growth, this method was adequate for experiments designed to test whether there was a similar appearance of Na^+/H^+ exchanger activity during oocyte growth. Using the Na^+ removal assay, we found that mechanically isolated, fully grown oocytes (~70 μm) exhibited robust Na^+/H^+ exchanger activity (below). Thus, the enzymatic treatment used to isolate growing oocytes apparently causes the loss of Na^+/H^+ exchanger activity in oocytes, but it was retained in mechanically isolated oocytes.

Before proceeding, we performed a short series of experiments to test whether mechanically isolated oocytes exhibited the same appearance of HCO_3^-/Cl^- exchanger activity during growth that was demonstrated using enzymatically isolated oocytes (above). Consistent with the results obtained with enzymatically isolated oocytes, mechanically isolated oocytes with diameters <55 μm showed a lack of HCO_3^-/Cl^- exchanger activity as measured by the Cl^- removal method, while larger oocytes (~70 μm) showed robust activity (not shown). Thus, it appeared that enzymatic isolation causes the loss of Na^+/H^+ exchanger activity but does not affect HCO_3^-/Cl^- exchanger activity in oocytes. Using the Na^+-removal assay with mechanically isolated oocytes, we then found that Na^+/H^+ exchanger activity was low in the smallest oocytes obtained, with little activity evident in oocytes with diameters below

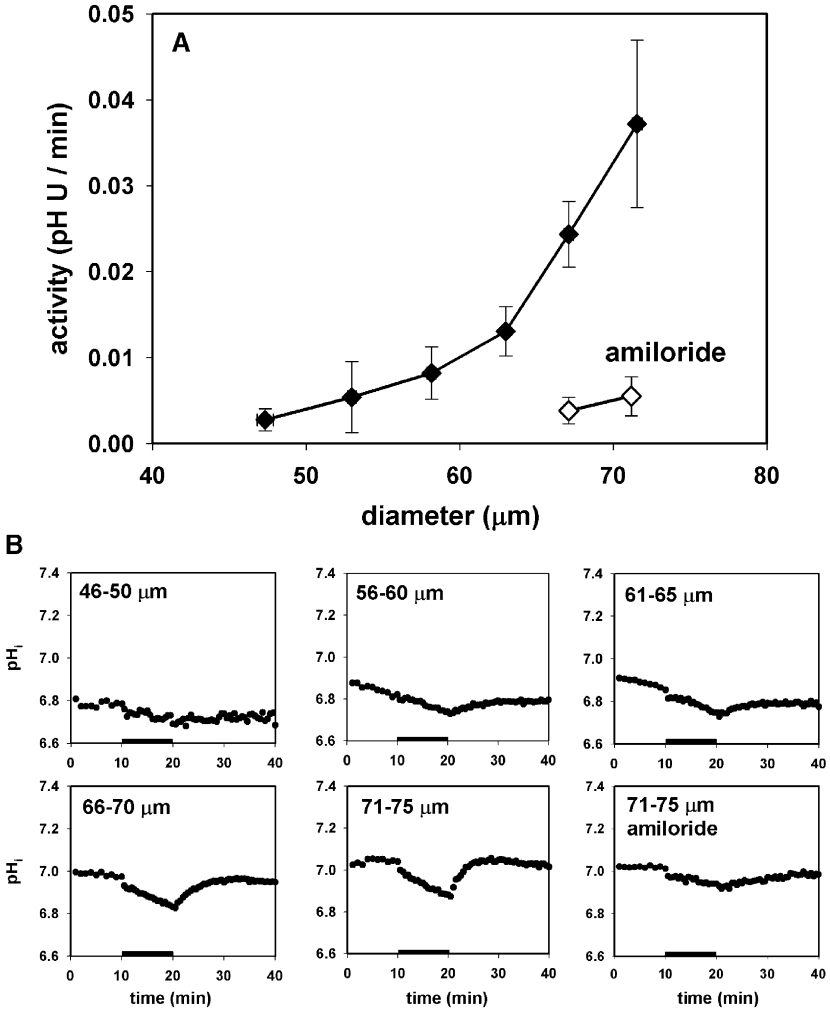

Fig. 6. Na^+/H^+ exchanger activity determined by Na^+ removal and replacement assay as a function of oocyte diameter. (A) Na^+/H^+ exchanger activity in oocytes was detected as an acidification upon Na^+ removal and a recovery upon Na^+ replacement. A quantitative measure of activity, calculated as the rate of change of pH_i upon reintroduction of Na^+, is shown as a function of mean (±SEM) oocyte diameter (filled symbols). Nonspecific activity was determined in the presence of the amiloride (1000 μM, open symbols) for the largest groups of oocytes where activity was highest. Each point represents the mean ± SEM of the initial rate of increase in pH_i after Na^+ was replaced vs. the mean (±SEM) diameter for oocytes falling within each 5 μm diameter increment. Each point represents data from 11–89 oocytes that fell within the specified ranges of diameter in 3–11 separate experiments. (B) Examples of results of Na^+ removal and replacement assay for oocytes of several diameters (indicated in panels). pH_i was measured as a function of time for 40 min, with the period in Na^+-free medium indicated by a black bar (from 10–20 min). A decrease in pH_i upon Na^+ removal and a subsequent increase upon its reintroduction indicate Na^+/H^+ exchanger activity. The initial rate of the latter was used as the measure of Na^+/H^+ exchanger activity. The final trace shows inhibition by amiloride (1000 μM). Each trace is the average of all experiments on oocytes within the specified size ranges.

about 60 μm. Activity then increased sharply as oocyte diameter increased from about 60 to 70 μm, such that oocytes 70–75 μm in diameter exhibited robust Na^+/H^+ exchange (Fig. 6). The dependence of Na^+/H^+ exchanger activity upon oocyte diameter was significant ($P = 10^{-4}$ by Univariate ANOVA, with diameter range taken to be the independent variable). Activity was completely blocked by amiloride, as expected for Na^+/H^+ exchange.

Discussion

We have found here that the mouse oocyte develops the capacity to regulate its pH_i only near the end of its growth in the ovary. Both HCO_3^-/Cl^- exchange, which acts to decrease pH_i, and Na^+/H^+ exchange, which acts to increase pH_i, became active at essentially the same stage of oocyte development, as the oocyte reached approximately 80% of its final diameter. Activation of these pH_i regulatory mechanisms occurred simultaneously with the development of the oocyte's capacity to reenter the meiotic cell cycle, so that meiotic competence, HCO_3^-/Cl^- exchange, and Na^+/H^+ exchange developed in concert as the oocyte grew from about 60–70 μm (compare Figs. 1B, 3, and 6). Although we did not directly test whether any other means of pH_i regulation was present, there was no indication in our data of any active pH_i regulation in smaller oocytes.

The development of Na^+/H^+ and HCO_3^-/Cl^- exchanger activities in growing oocytes may be analogous to the activation of pH_i-regulatory mechanisms in a number of cell types that is associated with metabolic activation or changes in proliferative state, including transition from quiescence into cell cycle progression (Epel, 1988; Ganz et al., 1989). Generally, such activation is a consequence of the action of specific signal transduction mechanisms, e.g., downstream of growth factor stimulation (Orlowski and Grinstein, 1997). Whether activation of pH_i regulatory mechanisms at this specific point of oocyte growth is also due to an as-yet-unidentified signaling event in the growing follicle remains to be determined. Alternatively, the onset of pH_i regulatory capacity could be the result of de novo synthesis and oocyte plasma membrane localization of pH_i regulatory NHE and AE family proteins as the oocyte grows.

In addition to activation of both types of pH_i regulatory mechanisms, the resting pH_i of isolated oocytes also increased markedly as they neared the end of their growth. Since Na^+/H^+ exchange mediates pH_i increases (in contrast to the opposite action of HCO_3^-/Cl^- exchange), its activation is the likely mechanism for an increase in baseline pH_i that occurs coincident with the activation of both pH_i regulatory mechanisms. In addition, a pH_i increase of similar magnitude was evident in HCO_3^--free medium equilibrated with air, where HCO_3^-/Cl^- exchange was inactive. Thus, a likely explanation for our observations is that the pH_i increase at the end of the growth period in oocytes is due to the activation of Na^+/H^+ exchange, while pH_i is thereafter maintained within a narrow preferred range by the opposing actions of both Na^+/H^+ and HCO_3^-/Cl^- exchange.

Although a relatively large pH_i increase was clearly seen during growth in isolated oocytes, it is not known if this also occurs within intact follicles, where oocytes reside in vivo. It is possible that oocytes within the follicle behave similarly, having relatively low pH_i before they reach meiotic competence, and then exhibiting a pH_i increase. However, it is also possible that pH_i in early growing oocytes is instead maintained at a higher level by the granulosa cells that surround the oocyte and are coupled to it via extensive gap junctions, but this remains to be investigated.

The data reported here clearly indicate that a previously unknown developmental event involving the simultaneous activation of pH_i regulatory mechanisms occurs at the time of acquisition of meiotic competence during growth of mouse oocytes. We speculate that this at least in part must reflect the development of the capacity of the oocyte to function independent of the follicle, as it must upon ovulation when it becomes uncoupled from the granulosa cells upon which it was dependent during its growth. Whether the activation of pH_i regulatory mechanisms or increased pH_i may also be directly involved in the acquisition of meiotic competence remains to be determined.

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