Development 133, 591-599 doi:10.1242/dev.02246

Granulosa cells regulate intracellular pH of the murine growing oocyte via gap junctions: development of independent homeostasis during oocyte growth

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Oocytes grow within ovarian follicles in which the oocyte is coupled to the surrounding granulosa cells by gap junctions. It was previously found that small growing oocytes isolated from juvenile mice and freed of their surrounding granulosa cells (denuded) lacked the ability to regulate their intracellular pH (pH_i), did not exhibit the pH_i-regulatory HCO_3^-/Cl^- and Na^+/H^+ exchange activities found in fully-grown oocytes, and had low pH_i. However, both exchangers became active as oocytes grew near to full size, and, simultaneously, oocyte pH_i increased by approximately 0.25 pH units. Here, we show that, in the more physiological setting of the intact follicle, oocyte pH_i is instead maintained at ~7.2 throughout oocyte development, and the growing oocyte exhibits HCO_3^-/Cl^- exchange, which it lacks when denuded. This activity in the oocyte requires functional gap junctions, as gap junction inhibitors eliminated HCO_3^-/Cl^- exchange activity from follicle-enclosed growing oocytes and substantially impeded the recovery of the oocyte from an induced alkalosis, implying that oocyte pH_i may be regulated by pH-regulatory exchangers in granulosa cells via gap junctions. This would require robust HCO_3^-/Cl^- exchange activity in the granulosa cells, which was confirmed using oocytectomized (OOX) cumulus-oocyte complexes. Moreover, in cumulus-oocyte complexes with granulosa cells coupled to fully-grown oocytes, HCO_3^-/Cl^- exchange activity was identical in both compartments and faster than in denuded oocytes. Taken together, these results indicate that growing oocyte pH_i is controlled by pH-regulatory mechanisms residing in the granulosa cells until the oocyte reaches a developmental stage where it becomes capable of carrying out its own homeostasis.

KEY WORDS: pH-regulation, Oocyte, Granulosa cells, Gap junctions

INTRODUCTION

Oocytes grow within ovarian follicles, multicellular units comprising the oocyte and its surrounding granulosa cells. Oocyte growth takes approximately 15 days in mice, during which time the oocyte increases in size from about 15 μm to a final diameter of 70-80 μm. Growth of the oocyte is accompanied by proliferation of the granulosa cells, to eventually form the antral follicle from which the mature egg is ovulated. Throughout growth, the oocyte remains arrested at prophase I of meiosis, which is characterized by a prominent nucleus (the germinal vesicle; GV). Oocyte growth is an essential component of oogenesis, since oocytes only become capable of resuming meiosis when they reach about 80% of their final size in the mouse (Sorensen and Wassarman, 1976; Wassarman et al., 1979). Meiosis normally resumes as a result of the pre-ovulatory surge in luteinising hormone, and progresses to metaphase II (a process termed meiotic maturation), whereupon the oocyte is ovulated in most mammals, including the mouse, and re-arrests pending successful fertilisation.

It has long been recognised that follicular cells are essential for oocyte growth. Oocytes grow in vitro only when surrounded by a granulosa shell (Eppig, 1977; Eppig, 1979; Eppig and Wigglesworth, 2000). Indeed, the rate of oocyte growth in vitro can be directly related to the number of attached granulosa cells (Brower and Shultz, 1982). Communication between the oocyte and its granulosa cells is bidirectional (Eppig, 2001; Matzuk et al., 2002) and occurs by at least two distinct mechanisms. Intercellular signals pass extracellularly via paracrine factors that may trigger signaling pathways. Conversely, small molecules (~<1 kDa) can pass directly between the oocyte and granulosa cells through gap junctions (Anderson and Albertini, 1976; Ducibella et al., 1975; Kidder and Mhawi, 2001), which are intercellular channels formed by the docking of multimeric hemichanels, each comprising six transmembrane proteins known as connexins (Bruzzone et al., 1996; Sosinsky and Nicholson, 2005). Molecules thought to be transferred between oocyte and granulosa cells include nutrients, nucleotides, amino acids and possibly meiotic signals (Buccione et al., 1990a; Eppig, 1991a). The unequivocal importance of gap junction communication within the follicle has been demonstrated by targeted gene deletion; oocyte growth is retarded in mice deficient for either GJA4 (Cx37) or GJA1 (Cx43), connexins principally responsible for oocyte-granulosa and granulosa-granulosa gap junctions, respectively (Ackert et al., 2001; Simon et al., 1997).

Regulation of intracellular pH (pH_i) is a key homeostatic function carried out by virtually all cells. In mammals, pH_i is controlled mainly by HCO_3^-/Cl^- exchangers of the anion exchanger (AE) gene family and Na^+/H^+ exchangers of the Na^+/H^+ exchanger (NHE) family. HCO_3^-/Cl^- exchangers export HCO_3^- in exchange for extracellular Cl^-, thereby correcting alkalosis; Na^+/H^+ exchangers extrude protons thereby correcting acidosis (Alper, 1994; Orlowski and Grinstein, 1997; Orlowski and Grinstein, 2004; Roos and Boron, 1981; Romero et al., 2004). Alterations in pH_i are known to be associated with changes in cell growth and proliferation. For example, many mitogens stimulate an activation of Na^+/H^+ exchange, which is necessary for the growth and proliferation of cultured cells (Grinstein et al., 1989). Moreover, growth and

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Accepted 13 December 2005

proliferation is impaired in some pH_i regulation-compromised cells when intracellular pH_i is disturbed (Grinstein et al., 1989; Kapus et al., 1994), and pH_i dysregulation can lead to decreased cell survival (Pouyssegur et al., 1984). The ability to correct pH_i perturbations is of particular importance in early mammalian development, as inhibition of pH_i regulatory mechanisms hinders pre-implantation development in mouse and hamster embryos (Lane et al., 1998; Zhao et al., 1995).

Recently it was shown that isolated (denuded) growing oocytes from CF1 mice exhibited minimal evidence of HCO_3^-/Cl^- or Na^+/H^+ exchanger activity (Erdogan et al., 2005). However, both activities developed during the final stages of growth, coincident with the acquisition of meiotic competence. At the same time, resting pH_i in denuded oocytes increased abruptly by approximately 0.25 pH units (Erdogan et al., 2005). Thus, although fully-grown GV-stage oocytes posses robust HCO_3^-/Cl^- and Na^+/H^+ exchange activities, smaller growing oocytes are apparently a rare example of a mammalian cell unable to regulate their own pH_i. However, it was not determined whether oocyte pH_i behaves similarly in the more physiological setting of the intact follicle. Our results here indicate that growing oocyte pH_i is controlled by pH_i-regulatory mechanisms in the granulosa cells, acting through the gap junctions.

MATERIALS AND METHODS

Chemicals and solutions

Chemicals and drugs were obtained from Sigma (St Louis, MO) unless otherwise noted. 4,4′-Diisocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), carboxyseminaphthorhodafluor-1-acetoxymethyl ester (SNARF-1-AM), and SNARF-1-dextran were obtained from Molecular Probes (Eugene, OR). Stock solutions were prepared in water [dibutyrladenosine 3,5-cyclic monophosphate (dbcAMP)], ethanol (nigericin), or dimethyl sulfoxide (DMSO; SNARF-1-AM, valinomycin, DIDS, 1-octanol, 18α-glycyrhetinic acid).

All media were based on KSOM mouse embryo culture medium (Lawitts and Biggers, 1993), which 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). HEPES-KSOM was used for oocyte collection and microinjection (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 BSA was excluded. Cl^--free media were produced by replacing all Cl^- salts with corresponding gluconate 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.

Oocyte and follicle collection

Germinal vesicle-stage oocytes (GV) were obtained from primed CF1 female mice (Charles River, St-Constant, PQ, Canada), approximately 44 hours after equine chorionic gonadotropin (eCG) injection (5 IU, intraperitoneally). Ovaries were removed and minced to release cumulus-enclosed oocytes. Cumulus cells were subsequently removed by repeated pipetting through a narrow-bore pipette. Growing oocytes and pre-antral follicles were obtained from female mice on postnatal days (P) 9-21. A wave of follicular development occurs shortly after birth in mice, such that oocyte size and follicular development are related to postnatal age (Sorensen and Wassarmann, 1976; Eppig, 1991b). Thus growing oocytes and follicles of the required size can be obtained from mice of the appropriate age. Oocytes and follicles were isolated mechanically by fine mincing of the ovary with a razor blade, as previously described (Erdogan et al., 2005). The diameters of the isolated denuded oocytes were measured using an eyepiece reticle. Diameters of follicle-enclosed oocytes were determined from SNARF-dextran fluorescence images calibrated against eyepiece-reticle measurements.

Microinjection and oocytectomy

Microinjection and oocytectomy were performed using Narishige micromanipulators mounted on a Zeiss Axiovert microscope. Injections were performed in a drop of HEPES-KSOM under oil. Oocytes or follicles were immobilized using a holding pipette while the injection solution was delivered through an injection pipette using a pressure-pulse controlled by a microinjection apparatus (Harvard Apparatus-Holliston, MA). Injection volumes were an estimated 5% of oocyte volume based upon cytoplasmic displacement.

Oocytectomy is a microsurgical technique for removing the oocyte from an intact cumulus-oocyte complex such that granulosa cell function can be examined in the absence of the oocyte. Oocytectomy was performed according to the protocol first described by Buccione and co-workers (Buccione et al., 1990b). Briefly, cumulus-oocyte complexes were immobilized using a holding pipette under moderate negative pressure. An injection pipette was then passed through the COC into the holding pipette. At this point, the negative pressure causes the oocyte to be aspirated into the holding pipette, leaving an intact cumulus shell surrounding an empty zona pellucida. Only complexes in which all ooplasm had been removed (as determined using the light microscope) were used for pH_i measurements.

Fluorescence microscopy

pH_i measurements were performed using a quantitative fluorescence imaging microscopy system (Inovision, Durham, NC). pH was measured using SNARF-1 which was either microinjected in dextran-coupled form (SNARF-dex; 10 kDa, estimated final concentration 0.5-1.0 mM), or loaded as the acetoxymethyl ester derivative (SNARF-AM 5 μM, 30 minutes). SNARF was illuminated using 545 nm light, and emission monitored at 600 and 640 nm. The ratio of the two intensities (640/600) was calculated by dividing the images after background subtraction. Where shown, exemplar images are of the 640 nm emission. Calibration was performed using the nigericin/high K^+ method with valinomycin added to collapse the K^+ gradient (Baltz and Philips, 1999). Resting pH_i was determined in KSOM after a 15-minute stabilization period. pH_i was averaged for oocytes within 5 μm increments for each experiment. All measurements were performed in a temperature- and atmosphere-controlled chamber (37°C, 5% CO_2/air). dbcAMP (100 μM) was included in all experiments to prevent spontaneous oocyte maturation. Because slight differences in injection volume are inevitable when injecting growing oocytes of different sizes, we have confirmed that moderate changes in the concentration of SNARF-dex do not affect pH_i measurements. It was found that injections of twice the normal volume had no effect upon the recorded resting pH_i or rate of pH_i increase upon removal of Cl^- (see below for details), in denuded GV oocytes or one-cell embryos.

To assess oocyte-granulosa cell-coupling, follicle enclosed oocytes were microinjected with a 2.5 mg/ml solution of fluorescein. Follicles were examined 30-60 minutes after microinjection using a LM 35 inverted microscope (Carl Zeiss) fitted with fluorescence optics and a camera controlled by SPOT-Advanced software (Diagnostic Instruments, Sterling Heights, MI). For each follicle, regions of interest were placed within the oocyte and the granulosa regions to calculate fluorescence intensities (using Adobe PhotoShop). The amount of dye transfer was quantified as the relative fluorescence intensity in the granulosa/oocyte regions after background subtraction.

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

HCO_3^-/Cl^- exchanger activity was quantified using the Cl^- removal method. On exposure to Cl^--free solution, the HCO_3^-/Cl^- exchanger runs in reverse, causing intracellular alkalinization due to HCO_3^- influx. A pH_i increase 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-containing oocytes were placed in the chamber, equilibrated for 15 minutes, and then measurements were taken for 10 minutes, after which the solution was changed to Cl^--free, low-lactate KSOM. The initial rate of intracellular alkalinization upon Cl^- removal was determined using linear regression (Sigma Plot 8.0, Chicago, IL), and exchanger activity was reported as the change in pH_i per minute (pHU/minute). Inhibition by DIDS (500 μM) 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; Erdogan et al., 2005; Phillips and Baltz, 1999; Phillips et al., 2002; Zhao and Baltz, 1996).

NH_4Cl method for assessing recovery from alkalosis

Alkalosis was induced by the introduction of 35 mM NH_4Cl as an isosmolar substitution for NaCl, as previously described (Phillips and Baltz, 1999; Zhao 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 alkalinisation. The subsequent recovery in the continued presence of NH_4Cl provides a measure of the cell's ability to recovery from alkalosis, which may be mediated by HCO_3^-/Cl^- exchanger activity (Baltz, 2003; Baltz and Phillips, 1999).

Statistics and data analysis

For experiments in which oocytes of different sizes were compared, oocytes were grouped in 5 μm increments within each experiment, as previously described (Erdogan et al., 2005). Data points presented are the mean±s.e.m. of all replicates performed. Means of replicates were compared using *t*-tests (two groups) or ANOVA (three or more groups). Where ANOVA was used, Tukey-Kramer's and Dunnett's post-hoc tests were applied as appropriate [using Instat (GraphPad, San Diego, CA)].

RESULTS

The intact follicle maintains oocyte pH_i during growth

To address the question of whether the follicular environment influences oocyte pH_i, we set out to determine pH_i in follicle-enclosed growing oocytes. Preliminary attempts to measure oocyte pH_i within intact pre-antral follicles or cumulus-oocyte complexes from antral follicles (COCs) using the membrane-permeable form of SNARF-1 (SNARF-AM) were ineffective, as granulosa cells accumulated a significant amount of the fluorophore, obscuring the relatively small amount of SNARF-1 fluorescence due to SNARF-1-AM that was able to pass through the granulosa shell and accumulate in the oocyte (not shown). Therefore, as a strategy for directly measuring pH_i in follicle-enclosed oocytes, we instead microinjected a dextran-conjugated form of SNARF-1 (SNARF-dex; 10 kDa) into follicle-enclosed oocytes. Because gap junctions that couple the oocyte to the granulosa cells only permit the passage of small molecules (~<1 kDa), SNARF-dex was restricted to the oocyte, providing a means of recording oocyte pH_i in the absence of contaminating fluorescence from granulosa cells (Fig. 1A).

Using this method, we determined resting pH_i in follicle-enclosed growing oocytes compared with that of denuded oocytes. Oocytes ranging in size from 45-80 μm were obtained from neonatal CF1 mice aged 9-21 days. In each experiment, follicle-enclosed and denuded oocytes were microinjected with SNARF-dex and imaged simultaneously (Fig. 1A). As previously, pH_i increased with size in denuded oocytes, such that small oocytes (45-50 μm diameter) exhibited a resting pH that was about 0.3 pH units lower than their fully-grown counterparts (70-80 μm; Fig. 1B). In striking contrast, the pH_i of follicle-enclosed oocytes was approximately 7.2-7.3 regardless of oocyte size, indicating that the follicle sets the pH_i of the oocyte during growth.

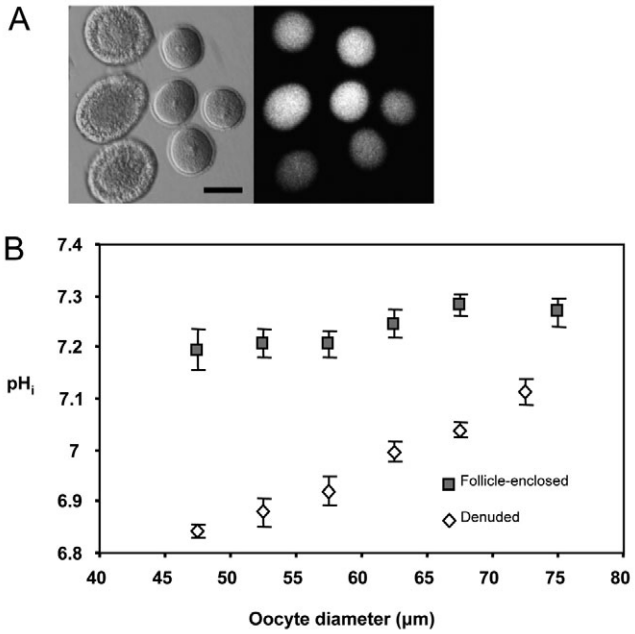

Fig. 1. The follicle sets oocyte pH_i during growth. (**A**) An example of one experiment in which follicle-enclosed and denuded oocytes were microinjected with SNARF-dex to determine pH_i. Brightfield (left) and fluorescence (right) images are shown. Note that fluorescence is restricted to the oocyte. Scale bar 50 μm. (**B**) Oocytes within each experiment were grouped in 5 μm increments. pH_i increased significantly with increasing size in denuded oocytes ($P<0.0001$), whereas there is no significant difference in pH_i between size groupings in follicle-enclosed oocytes ($P>0.25$). Each point comprises between four and 17 oocytes from two to eight replicates.

The small growing oocyte exhibits substantial HCO_3^-/Cl^- exchange activity only when granulosa cells are present

To further explore the impact of the follicle upon pH_i regulation in the enclosed oocyte, we compared the effect of removing Cl^- from the bathing media in follicle-enclosed and denuded oocytes. Cl^- removal causes the HCO_3^-/Cl^- exchanger to run in reverse, so that an increase in pH_i indicates active HCO_3^-/Cl^- exchange (see Materials and methods). As previously shown (Erdogan et al., 2005), Cl^- removal triggered only minimal pH_i change in small denuded oocytes, but considerable pH_i increases in fully-grown oocytes, consistent with an upregulation of HCO_3^-/Cl^- exchange during oocyte growth (Fig. 2A, top row). As before, this upregulation occurred predominantly in the final stages of growth. By contrast, Cl^- removal stimulated robust pH_i increases in follicle-enclosed oocytes of all sizes that were assessed (Fig. 2A, bottom row). DIDS, a HCO_3^-/Cl^- exchange inhibitor, inhibited all pH_i increases in follicle enclosed oocytes (Fig. 2A, bottom right). Analysis of HCO_3^-/Cl^- exchange activity (see Materials and methods) confirmed that HCO_3^-/Cl^- exchange increases with oocyte size in denuded oocytes, but remains constant in follicle-enclosed oocytes (Fig. 2B). Granulosa-cell accompaniment thus furnishes the growing oocyte with the HCO_3^-/Cl^- exchange activity that it lacks when denuded.

Granulosa cells have an impact upon oocyte pH_i regulation in cumulus-oocyte complexes

To determine whether oocyte pH_i is also influenced by granulosa cells in fully-grown COCs retrieved from antral follicles of adult mice, resting pH_i was monitored simultaneously in cumulus-enclosed and denuded GV-stage oocytes obtained from eCG-primed adult mice (Fig. 3A). Denuded GV oocytes exhibited a resting pH_i of 7.07±0.02, and exhibited a robust pH_i increase upon Cl^- removal. Cumulus-enclosed oocytes had a significantly increased resting pH_i (7.16±0.02; $P<0.05$), and Cl^- removal caused a significantly greater

rate of pH_i increase ($P<0.01$; Fig. 3B). Thus, even in fully-grown oocytes that have already developed a substantial HCO_3^-/Cl^- exchange activity of their own, the influence of the granulosa cells upon pH_i is significant.

Gap-junction inhibitors block pH regulation in follicle-enclosed, small growing oocytes

Growing oocytes are coupled to the surrounding granulosa cells by gap junctions that permit the passage of small molecules between the two compartments (Kidder and Mhawi, 2002). We reasoned that gap junctions may potentially provide a route by which follicular cells influence oocyte pH_i. We therefore examined the effect of gap junction inhibition upon pH regulation in follicle-enclosed oocytes. Because gap junction inhibitors may have some non-specific actions, we employed two structurally unrelated compounds: 1-octanol and 18α-glycyrhettinic acid (AGA) to inhibit all gap junctions in the follicle. Both inhibitors significantly inhibited Cl^- removal-induced pH_i increases in small (45-60 μM diameter) follicle-enclosed oocytes, when compared with controls (Fig. 4A,B; $P<0.01$). Importantly, similar drug treatments did not prevent Cl^- removal-induced pH_i increases in fully-grown denuded GV oocytes, confirming that the drugs are not compromising our ability to monitor pH_i changes and are not directly affecting HCO_3^-/Cl^- exchange (Fig. 4A, insets). The ability of AGA and octanol to inhibit gap junction communication in the follicle under these conditions was verified by microinjecting fluorescein into follicle-

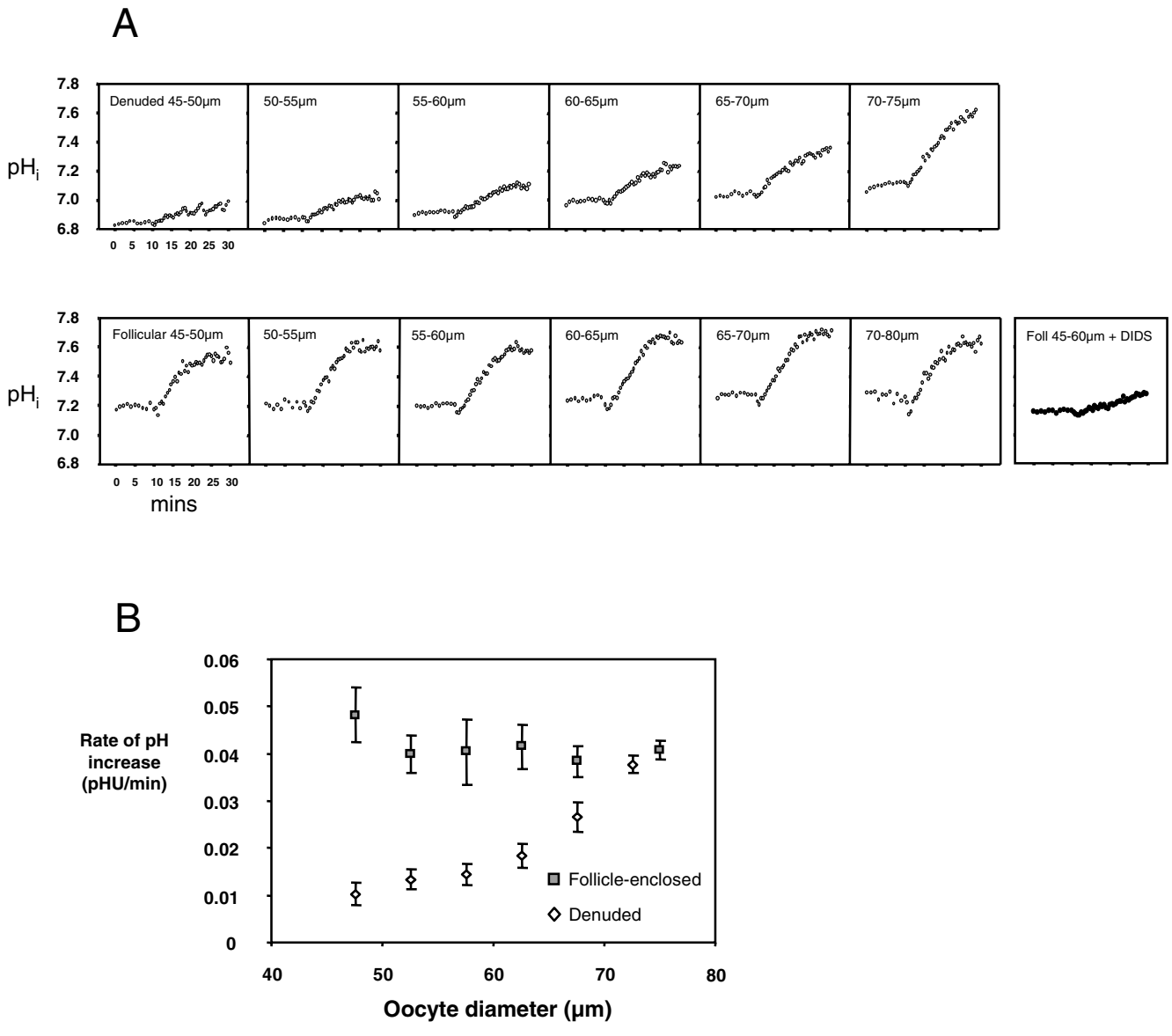

Fig. 2. The follicle affords the growing oocyte HCO_3^-/Cl^- activity. (**A**) pH_i was monitored in denuded (top row) and follicle-enclosed (bottom row) oocytes simultaneously. Cl^- was removed from the bathing medium at 10 minutes in each experiment. Traces shown are the mean of all experiments performed. Note that Cl^- removal causes sharp increases in pH_i in all follicle-enclosed oocytes, but only in larger denuded oocytes. (**B**) Plot of the rate of pH_i increase, which provides an indication of HCO_3^-/Cl^- activity. HCO_3^-/Cl^- exchange rate increases significantly with increasing oocyte size in denuded oocytes ($P<0.0001$), whereas there is no difference between sizes in follicle-enclosed oocytes ($P>0.8$). DIDS inhibited Cl^--induced pH_i increases in follicle-enclosed oocytes (A, bottom row, far right). Each point comprises between four and 17 oocytes from two to eight replicates.

enclosed oocytes; both AGA and octanol inhibited the passage of fluorescein from the oocyte to the granulosa cells (Fig. 4C). Analysis of relative fluorescence intensities in the oocyte and follicular compartments confirmed significant inhibition of dye transfer in the AGA- and octanol-treatment groups when compared with controls ($P<0.01$; see Materials and methods for further details). Thus, blocking gap junction permeability using gap junction inhibitors eliminates HCO_3^-/Cl^- exchange activity in the granulosa-enclosed oocyte.

The normal physiological function of the HCO_3^-/Cl^- exchanger is to correct alkaline loads. Therefore, a second strategy for examining HCO_3^-/Cl^- exchange is to monitor recovery from an induced alkalosis. It was previously shown that, whereas fully-grown denuded oocytes recover from an ammonium-induced alkalosis within 20 minutes, recovery is incomplete in smaller oocytes (Erdogan et al., 2005). Here, we used a similar approach to determine whether gap junction inhibition affects the recovery of follicle-enclosed oocytes from alkalosis. The effect of NH_4Cl (35 mM) was therefore compared in small follicle-enclosed oocytes (45-60 μm) in the presence of octanol, AGA and DMSO (Fig. 5). NH_4Cl addition resulted in a similar degree of alkalosis in all three treatment groups (the difference in pH_i increase between groups being highly insignificant; $P=0.98$), pH_i increasing from a resting value of pH 7.1-7.2 to approximately pH 8.3-8.4. Rapid recovery from alkalosis occurred in control oocytes, pH_i recovering by 1.02±0.01 pH units, to pH 7.40±0.01, within the first 10 minutes, and resting baseline pH_i was almost fully restored within 20 minutes. By sharp contrast, 10 minutes after NH_4Cl addition, pH_i remained significantly more elevated in AGA- ($P<0.01$) and octanol- ($P<0.01$) treated oocytes than in controls, indicating that gap junctions participate in the recovery from an induced alkalosis in follicle-enclosed oocytes. Inhibition by AGA was apparently only transient, as resting pH_i was subsequently restored 30 minutes after NH_4Cl addition. Because the effect of AGA was permanent in the Cl^- removal experiment (see Fig. 4), this may indicate that AGA is inactivated by ammonium. Nevertheless, oocyte pH_i remained substantially elevated 40 minutes after NH_4Cl addition in octanol-treated follicles (0.32±0.10 pH units above resting pH_i). Therefore, the presence of the intact follicle apparently enables enclosed growing oocytes to recover quickly from alkalosis, providing gap junction communication is maintained.

Granulosa cells posses robust HCO_3^-/Cl^- exchange activity

The experiments above indicate that gap junctions permit the follicle to regulate oocyte pH_i. A likely explanation for these results is that the pH regulatory exchangers of the granulosa cells regulate the pH of the ooplasm during growth, presumably by controlling oocyte pH_i via gap junctions. Such a model implies that the granulosa cells should themselves possess significant pH_i regulatory ability. To test this prediction, we assessed HCO_3^-/Cl^- exchange activity in cumulus-oocyte complexes from which the oocyte was first removed by oocytectomy. This technique allowed us to examine intact shells of granulosa cells in the absence of the enclosed oocyte (Fig. 6A). Oocytectomized (OOX) complexes were loaded with SNARF-AM and HCO_3^-/Cl^- activity determined. Cl^- removal resulted in robust and rapid pH_i increases in all OOX complexes (0.91±0.02 pH units/minute; Fig. 6B). This rate of pH_i increase is more rapid than we have previously recorded in denuded oocytes or embryos of this mouse strain (CF1; see above) (see also Baltz, 2003). Finally, if this model is correct, induced pH_i changes should occur in granulosa cells at least as quickly as they do in the enclosed oocyte. To test this, COCs were partially denuded by careful pipetting with a fine-bore pipette, so that both oocyte and cumulus could be simultaneously loaded with SNARF-1 using SNARF-AM. Using this method, we were able to simultaneously monitor pH_i within the oocyte and granulosa cells (Fig. 7). Cl^- removal resulted in rapid pH_i increases within the oocyte and granulosa cells, which were simultaneous and indistinguishable. The rate of increase was 0.089±0.003 pH units/minute within the oocyte, which is substantially steeper than that of denuded GV oocytes (0.067±0.01 pH units/minute; see Fig. 3), indicating that even a partial covering of cumulus cells can influence oocyte pH_i regulation. These results are all consistent with the notion that the robust pH_i-regulatory activity of granulosa cells is conferred to the adjoining oocyte.

DISCUSSION

Our experiments indicate that granulosa cells control oocyte pH_i during the early phases of oocyte growth, and that this intercellular homeostasis requires functional gap junctions. This conclusion is supported by three main lines of evidence. First, although the pH_i of denuded oocytes increases with oocyte size, granulosa cell coupling maintains oocyte pH_i within the relatively narrow range of 7.2-7.3

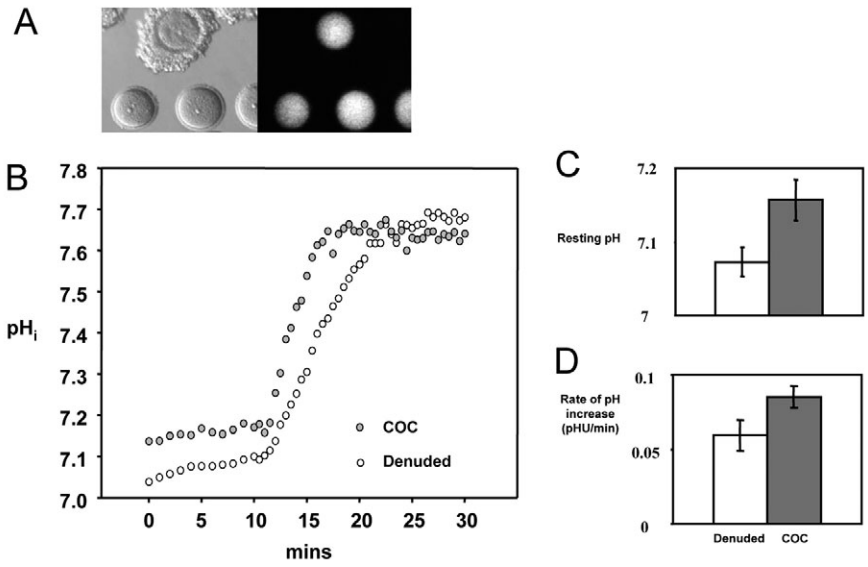

Fig. 3. Granulosa cells influence oocyte pH_i in cumulus-oocyte complexes. (**A**) Example of an experiment in which cumulus-enclosed and denuded oocytes were compared with SNARF-dextran. Brightfield (left) and fluorescence (right) images are shown. (**B**) Cl^- was removed from the bath at 10 minutes. (**C**,**D**) Cumulus-enclosed oocytes exhibited a significantly higher resting pH_i (C; $P<0.05$) and rate of pH_i increase (D; $P<0.01$) than did denuded oocytes. Data are from four replicates. Total oocytes: denuded, $n=25$; cumulus-enclosed, $n=23$.

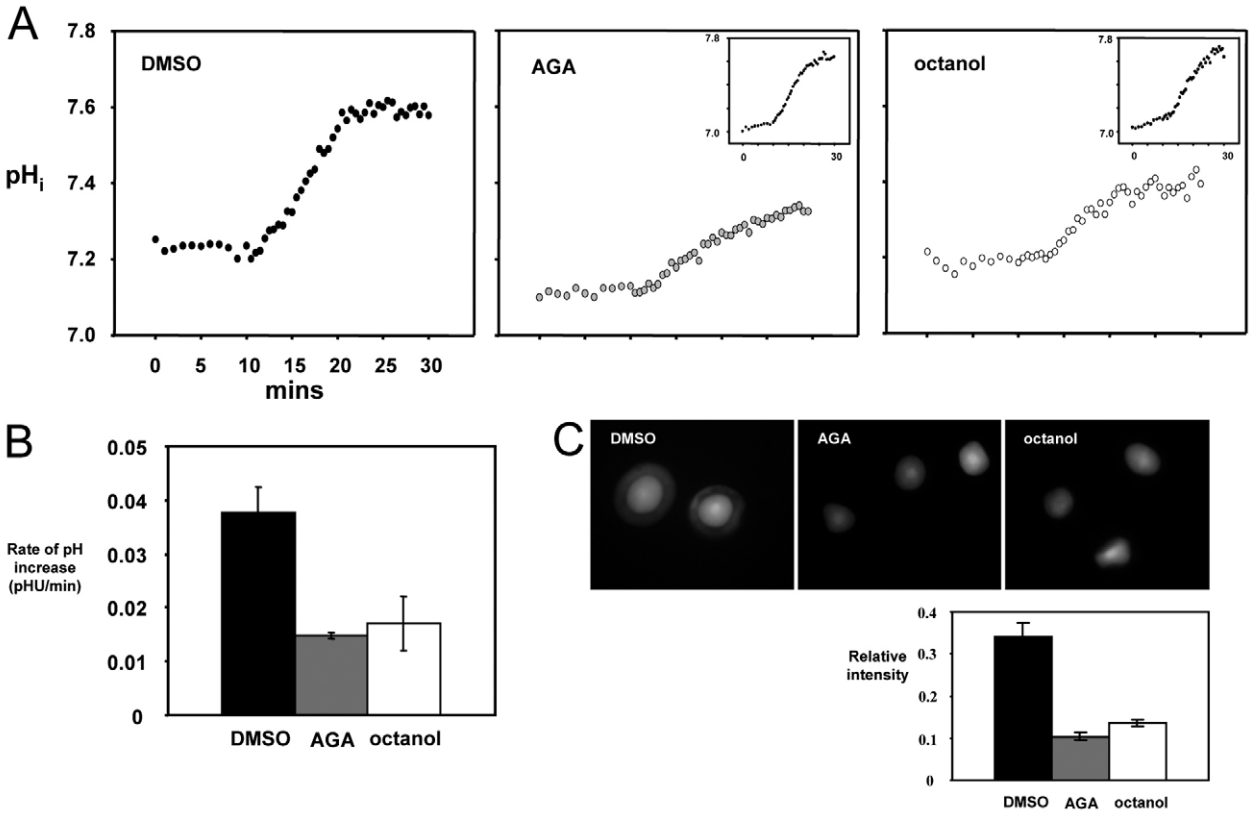

Fig. 4. Gap-junction inhibitors prevent Cl^- removal-induced pH_i increases in follicle-enclosed oocytes. (**A**) Follicle-enclosed oocytes were recovered from mice aged 10-12 days and microinjected with SNARF-dex. Cl^- removal was performed after 20 minutes in the presence of DMSO (vehicle; 0.3%), AGA (150 μM, 0.3% DMSO) or 1-octanol (1 mM, 0.1% DMSO). Only oocytes sized 45-60 μm were selected for analysis. (**B**) The rate of pH_i increase upon Cl^- removal in follicle-enclosed oocytes was quantified. Note that AGA and octanol both significantly inhibit Cl^- removal-induced pH_i increases in follicle-enclosed oocytes compared with control (P<0.01). Three replicates were performed for each treatment, a total of 17, 12 and 25 oocytes for DMSO, AGA and octanol, respectively. Insets in A show the effect of similar treatments upon fully-grown denuded oocytes (insets: AGA, n=28; octanol, n=24; both of three replicates). (**C**) Fluorescein was injected into follicle-enclosed oocytes following similar pre-incubation periods in DMSO, AGA or octanol. Images were obtained 30-60 minutes after microinjection. Note that the granulosa cells surrounding the oocyte can be clearly visualized in DMSO-treated (control) follicles, indicating that fluorescein can pass easily between the oocyte and its granulosa cells. Similar results were obtained for untreated controls in the absence of DMSO (not shown). Note also that AGA and octanol both inhibited fluorescein from entering the granulosa cells. Images presented are unprocessed, and similar microscope settings were used in each acquisition. Controls were carried out on the same days as drug treatments. Bar chart shows the analysis of relative intensity of fluorescence in the oocyte and granulosa regions of follicles from each group. The relative fluorescence intensity in granulosa cells compared with the oocyte is significantly reduced by AGA or octanol (P<0.01). A relative fluorescence value of approximately 0.1 in octanol- and AGA-treated oocytes may reflect a small amount of residual dye transfer from the oocyte to the granulosa cells, or light scattering from the oocyte. n=22, 9, 16 and 14 for untreated, DMSO, AGA and octanol treatments, respectively.

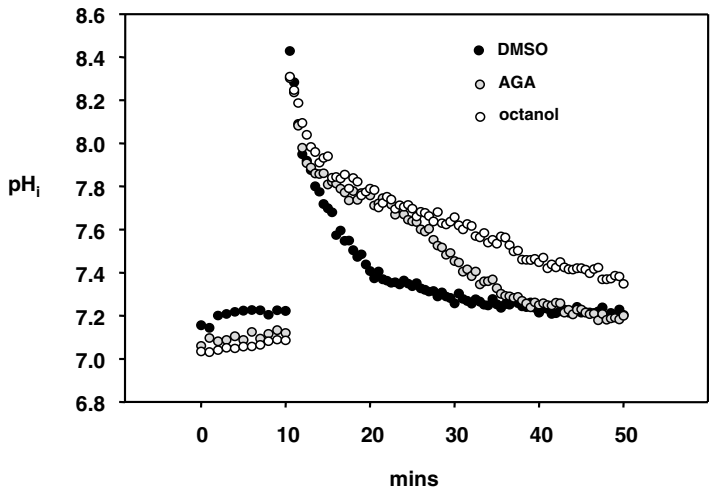

Fig. 5. Gap junction inhibitors significantly inhibit the recovery from alkalosis in follicle-enclosed oocytes. Follicle-enclosed oocytes (45-60 μm diameter) from mice aged 10-12 days were microinjected with SNARF. NH_4Cl (35 mM) was added to the bathing media 10 minutes after initiation of pH_i measurements at t=0. Drug treatments are the same as in Fig. 4. Three replicates were performed for each treatment, with a total of 18, 18, and 21 follicles for DMSO, AGA and octanol, respectively. The records shown are the average of all three replicates for each treatment.

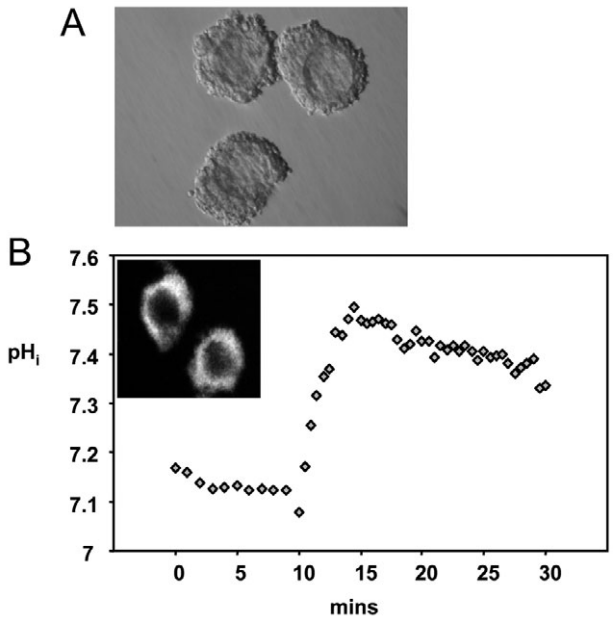

Fig. 6. Cl^- removal triggers rapid pH_i increases in oocytectomised (OOX) cumulus-oocyte complexes. (**A**) Examples of OOX complexes produced by aspirating the oocyte from within cumulus-oocyte complexes (see Materials and methods). (**B**) pH_i was recorded during removal of Cl^- (at 10 minutes) in SNARF-AM loaded complexes. Cl^- removal caused a very rapid increase in pH_i (0.91±0.02 pH units/minute). Data shown is the average of three replicates, from a total of 11 complexes. Inset shows an example of fluorescence images of OOX complexes.

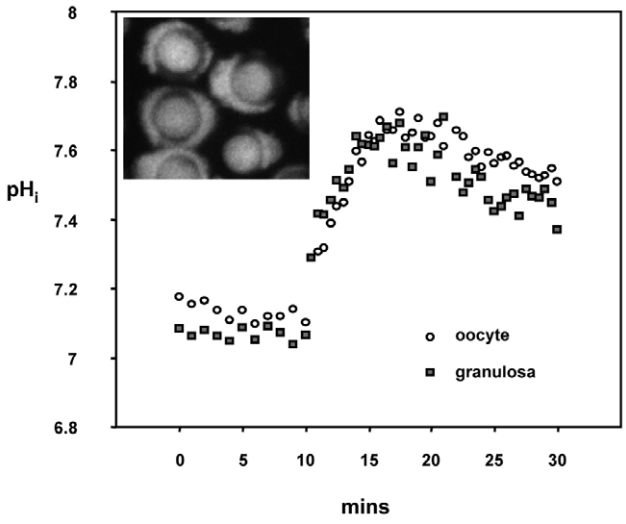

Fig. 7. Cl^- removal-induced pH_i changes in partly denuded cumulus-oocyte complexes. Cumulus-oocyte complexes were partly denuded by careful pipetting with a narrow-bore pipette, and subsequently loaded with SNARF-AM. This allowed simultaneous visualization of the oocyte and attached granulosa cells (see inset). Cl^- was removed from the bathing media at 10 minutes. Fluorescence was measured separately in regions within the oocyte and within granulosa cell areas peripheral to the oocyte in order to monitor pH_i in the two compartments. Notice that Cl^- removal causes pH_i increases within oocyte and granulosa cells that are indistinguishable. Trace shown is the average of eight complexes in one of two similar experiments.

throughout growth. Second, the granulosa cells provide the small growing oocyte with access to HCO_3^-/Cl^- exchanger activity, which it otherwise lacks. Third, the follicle fails to provide the oocyte with HCO_3^-/Cl^- exchanger activity in the presence of gap junction inhibitors. The small increase in pH_i that did occur upon Cl^- removal in octanol- or AGA-treated follicles is most likely attributable to the exchangers of the oocyte itself, and is similar to the response of denuded oocytes of the same size (compare Figs 2 and 4). A requirement for gap junctions was also confirmed by our NH_4Cl experiment, in which octanol and AGA substantially retarded recovery from the induced alkalosis. Although we cannot rule out the possibility that gap junctions allow the granulosa cells to present the oocyte with a factor(s) that activates the otherwise quiescent pH-regulatory mechanisms in the oocyte, the findings that granulosa cells themselves have very robust HCO_3^-/Cl^- exchange, and that pH_i changes occur simultaneously within the two compartments in partly-denuded COCs, are more consistent with exchangers within the granulosa cells having a direct effect upon oocyte pH_i. Indeed, the observation that fully grown GV-stage oocytes with either a full or partial covering of cumulus cells adopt a similar high level of exchanger as do OOX complexes (compare Figs 3 and 6) may imply that even fully grown oocytes, which have relatively robust pH_i-regulatory mechanisms of their own, can adopt the greater exchanger activity of the granulosa cells. We conclude the most likely explanation for these results is that the oocyte is able to access the abundant pH_i-regulation activity of the granulosa cells. The unusually low pH_i of small denuded oocytes thus most likely reflects electrochemical equilibrium in the absence of sufficient Na^+/H^+ and HCO_3^-/Cl^- exchange.

Granulosa cells as regulators of oocyte pH

Although it has long been established that gap junctional communication between oocyte and granulosa cells is imperative for oocyte growth, there remain relatively few examples of basic physiological functions that are carried out by the granulosa cells on behalf of the oocyte. Metabolic cooperativity between oocyte and granulosa cells underpins oocyte energy production. Although oocytes utilize glucose poorly, granulosa cells readily metabolize glucose and supply the oocyte with substrates that it can use (Biggers et al., 1967). The granulosa cells also facilitate uptake of some amino acids and nucleotides that are ineffectively taken up by denuded oocytes, an effect dependent upon gap junctions (Colonna and Mangia, 1983; Cross and Brinster, 1974; Haghighat and van Winkle, 1990; Heller et al., 1981; Heller and Schultz, 1980). In *Xenopus*, gap junctions allow the oocyte access to Na^+/Ca^{2+} exchange activity within granulosa cells, which may influence oocyte intracellular calcium conentrations ($[Ca^{2+}]_i$) (Supplisson et al., 1991). Our results here reveal that the follicular cells, acting through gap junctions, determine the pH_i of the growing oocyte and provide it with access to the HCO_3^-/Cl^- activity that it requires to recover quickly from an induced alkalosis. Granulosa cells thus regulate the pH of the ooplasm, thereby carrying out a basic homeostatic function on behalf of the oocyte. Intriguingly, it was recently shown that oocytes stimulate granulosa cell uptake of certain amino acids, which are transferred from the granulosa cells to the oocyte; the oocyte thus promotes a granulosa cell function upon which it depends (Eppig et al., 2005). It remains to be seen whether the oocyte is similarly capable of directing its own pH_i regulation by influencing pH_i regulation in the granulosa cells.

Little is known about pH_i regulation in granulosa cells. Studies of avian granulosa cells revealed evidence of active Na^+/H^+ antiporters, as well as an unidentified Na^+-independent means of correcting

acidosis (Asem and Tsang, 1988; Li et al., 1992). Here, we have demonstrated the presence of strongly active HCO_3^-/Cl^- exchangers in granulosa shells (OOX complexes) from murine antral follicles. Moreover, our gap-junction inhibitor experiments firmly imply the presence of HCO_3^-/Cl^- exchangers in granulosa cells in pre-antral follicles. Thus it would appear that granulosa cells have been suitably equipped to be able to regulate both their own pH_i and also that of the oocyte. We speculate that granulosa cell control of oocyte pH_i is likely to be essential for normal oogenesis, although this is yet to be tested directly.

Gap junctions permit intercellular pH cooperativity

Although it is known that pH_i can modulate gap junction permeability (Morley et al., 1996; Peracchia et al., 1996; Saez et al., 2005; Sosinsky and Nicholson, 2005), there is little available information about their role in synchronising pH_i in connected cells. One recent study showed that microinjection of acid into cardiomyocytes causes detectable pH_i decreases in adjacent cells (Zaniboni et al., 2003). The effect was abrogated by AGA, confirming the expected result that protons or proton equivalents can pass intercellularly via gap junctions. However, whether such gap junctional proton permeability holds physiological relevance was unknown. Our experiments here indicate that gap junction-mediated pH cooperativity allows the granulosa cells to control oocyte pH_i, presumably by permitting the intercellular diffusion of protons or proton equivalents (Zaniboni et al., 2003). This appears to be biologically important, as growing oocytes are incapable of regulating their own pH_i, and assume a lower pH_i when denuded. Indeed, gap-junction inhibition compromises the ability of follicle-enclosed oocytes to recover from an experimentally induced alkalosis. Thus, regulation of oocyte pH_i by the granulosa cells is, to our knowledge, the first demonstration of gap junctions, allowing one cell to control the pH_i of its neighbor.

We conclude that although in CF1 mice the growing oocyte is incapable of regulating its own pH_i, granulosa cells assume this function to regulate and maintain the pH of the ooplasm on its behalf. The current study therefore adds intracellular pH regulation to an expanding list of functions for which the oocyte demands the assistance of the granulosa cell.

This work was funded by a Canadian Institutes of Health Research Operating Grant (MOP74515) to J.M.B.

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