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Research article

Hamster oocyte membrane potential and ion permeability vary with preantral cumulus cell attachment and developmental stage

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Abstract

Background: In vitro maturation of mammalian oocytes is an area of great interest due to its potential application in the treatment of infertility. The morphological and physiological changes that occur during oocyte development are poorly understood, and further studies are needed investigating the physiological changes associated with oocyte maturation. In this study we evaluated the membrane potential and the sodium/potassium permeability ratio of oocytes acutely isolated, and cumulus-oocyte complexes in metaphase II and preantral follicle stages.

Results: Intracellular electrical recordings revealed that cumulus-enclosed oocytes have a membrane potential significantly more negative at the preantral follicle stage than at metaphase II stage (-38.4 versus -19.7 mV, $p < 0.0005$). The membrane potential of the cumulus-free oocytes was not different between the preantral and metaphase II stages. The membrane potential of the cumulus cells forming preantral stage follicles was shown to be significantly different from that of the oocyte within the follicle (-28.6 versus -38.4 mV, $p < 0.05$). The sodium/potassium permeability measured in cumulus-enclosed oocytes at the preantral stage equaled a mean value of 0.33. The ratio was significantly lower when measured in oocytes denuded of cumulus cells or cumulus-enclosed metaphase II oocytes, 0.76, 0.79, 0.77 respectively ($p < 0.001$).

Conclusions: These data show a change in the membrane potential and Na^+/K^+ permeability ratio during ooycte development from the preantral stage oocyte to the metaphase II stage. We have also demonstrated a change in the preantral oocyte membrane potential when surrounding cumulus cells are removed; either due to membrane changes or loss of cumulus cells.

Background

The functional unit of the mammalian ovary is the developing follicle. The follicle is comprised of somatic granulosa cells of two categories, thecal cells and cumulus cells, and a single gamete cell or oocyte. If the follicle continues to develop and does not undergo atresia, it will yield an oocyte competent to undergo fertilization and eventually form a new organism. Several changes in the morphology and physiology of the oocyte and surrounding cumulus cells occur at the different stages of oocyte development. These include dynamic changes in gap junctions [1], cytokine release [2], morphology [3], and

membrane physiology [4]. Improved understanding of the basic physiological changes of follicular development is necessary for the advancement of numerous areas of clinical research, including the potential to eventually mature competent oocytes in vitro for use in reproductive therapies such as in vitro fertilization (IVF).

The function of the granulosa cells, and specifically cumulus cells, as they pertain to oocyte maturation within the follicle is currently an area of intense interest. The presence of both gap junctional communication and autocrine/paracrine effect has been established [5]. These avenues of communication may play a role in the maintenance of membrane properties, providing a means for initiating progression or imposing repression of the oocyte's meiotic stage. It is also possible for the opposite effect to be present; membrane function of the oocyte to have an effect on gap junction regulation and cytokine release.

The membrane physiology of the follicular cells includes the electrical properties of the cell, such as the membrane potential, cell coupling, ion channel activity and ion permeability. The membrane physiology of the oocyte and surrounding cumulus cells has been shown to change during germinal vesicle breakdown (GVBD), a late developmental stage [6–8]. No studies have addressed the potential changes in membrane physiology from earlier stages (i.e. preantral follicles). Additionally, changes in membrane physiology at earlier stages of development in relation to the effects of attached cumulus cells have not been described. In order to address thisarea of follicular maturation, we have measured the membrane potential (E_m) and permeability ratio of sodium (Na^+) and potassium (K^+) (P_{Na}/P_K) of oocytes denuded of surrounding cells, and in complex together. Metaphase II (MII) and preantral follicle oocyte-cumulus complexes were used for evaluation.

Results

The mean E_m of cumulus-enclosed oocytes from preantral follicles in control Ringers Salt Solution (RSS) at 22°C was -38.4 ± 4.5 mV (n = 10), all values are listed with standard error (Table 1). Metaphase II oocytes with cumulus cells attached were significantly different, E_m = -19.7 ± 0.8 mV ($p < 0.001$, n = 10). When the E_m of denuded (without cumulus cells) oocytes at the preantral and the metaphase II stages were investigated the mean values were -19.7 ± 0.5 mV, n = 10 and -19.8 ± 0.5 mV, n = 10 respectively, these values are statistically insignificant. The E_m of the cumulus cells attached to preantral oocytes was measured to be -28.6 ± 1.2 mV (n = 3).

P_{Na}/P_K values in oocytes collected from preantral follicles varied whether cumulus cells were retained or removed, the values were 0.33 ± 0.03, 0.79 ± 0.014 respectively ($p < 0.001$, n = 4). Metaphase II oocytes did not show a change in P_{Na}/P_K whether the cumulus cells were retained or stripped away. P_{Na}/P_K was statistically different when compared across maturational stage if cumulus cells were present ($p < 0.001$ n = 4), but if removed the ratio of P_{Na}/P_K became closer to 1.0 and no longer varied between stages (Table 1).

Discussion

In this study, P_{Na}/P_K and E_m, were evaluated in early and late oocyte development. These data indicate a significant change in P_{Na}/P_K and E_m between oocytes collected at the metaphase II stage and those from preantral follicles, and the effect from removal of cumulus cells. First, cumulus intact oocytes were compared to denuded oocytes to determine the amount of regulation from cumulus cells in a stage dependant manner. Secondly, comparisons made between two chosen stages of oocyte development (preantral and metaphase II) determined the amount of change in both variables tested over a broad maturational time. These stages were chosen to look at the early stages of oocyte maturation compared to late in an effort to bridge the gap present in the current literature, in which the changes occurring at transitions from GV to MII have been studied.

Table 1: Differences in the resting membrane potential (R_m) and the permeability ratio

	CUMULUS ENCLOSED		DENUDED	
	Preantral	Metaphase II	Preantral	Metaphase II
OOCYTE R_m n = 10	-38.4 ± 4.5 mV$^{+,++}$	-19.7 ± 0.8 mV	-19.7 ± 0.5 mV	-19.8 ± 0.5 mV
CUMULUS R_m n = 3	-28.6 ± 1.2 mV*			
OOCYTE P_{Na}/P_K n = 10	0.33 ± 0.03**	0.76 ± 0.04	0.79 ± 0.014	0.77 ± 0.02

These data indicate that while E_m of denuded oocytes at the MII stage were no different than cumulus enclosed MII oocytes, denuded oocytes from preantral follicles versus cumulus enclosed of the same stage did vary. Furthermore, if cumulus was removed from preantral stage oocytes the membrane potential became more positive and no longer significantly different than metaphase II oocytes, regardless of cumulus cell attachment. While present, heterologous gap junctions between the oocyte and cumulus cells may play a key role in the maintenance of oocyte membrane potential. Previous studies in several species have shown functional gap junctions in the cumulus-oocyte complex present during many stages and required for meiotic resumption [[11] for review]. When the cumulus cells begin to undergo cumulus expansion, cytoplasmic extensions protruding through the zona pellucida contacting the oolema are retracted, thus breaking heterologous gap junction communication. The loss of gap junctions during cumulus expansion could account for the shift in oocyte membrane potential observed between early and late maturation stages. The mechanism could be either direct reduction in ion diffusion through gap junctions, or indirectly by causing the oocyte to adjust oolemma properties in response to loss of a cumulus derived signal.

The data presented here support previous studies (11) and could explain the change in membrane potential seen when cumulus cells are chemically removed, as in the case of the preantral oocyte experiments or physiologically removed as in the case of the metaphase II oocyte. Work done by Gilula, Epstein and Beers, 1978 describes a similar conclusion, ionic coupling of cumulus cells and oocytes decrease to zero from preovulatory to postovulatory specimens [12]. Other work from the pig model shows a loss of gap junctions in the same maturational time, progression from metaphase I to metaphase II [13].

The presence of gap junctions is well documented but the regulation of these connexin pores is not well defined in any model. Thus it is interesting to note the difference in the membrane potential of the cumulus cells and oocyte at the preantral stage. This indicates that while the two cells are highly electrically coupled before ovulation [12], they maintain a different membrane potential.

When the E_m and P_{Na}/P_K of denuded and cumulus-intact oocytes were compared within their maturational stage, either preantral or MII, there was a significant difference during the preantral stage ($p < 0.0001$, 0.001 respectively) but not at MII. This substantiates the above importance of cumulus regulation. Grenfield, Hackett, and Linden investigated Xenopus oocyte K^+ currents in 1990 to reveal that an outward K^+ current in response to cAMP is abolished by inhibition of gap junctions or removal of cumulus cells [14], effecting the permeation of the cell to K^+ and substantiating these data. The loss of gap junctions in Grenfield's paper caused the outward potassium current to decrease, just as the P_{Na}/P_K value became closer to one in our studies.

Results for the membrane potential of MII oocytes is similar to values obtained by Racowsky and Saterlie [9]. This value (-19.7 ± 0.8 mV) from the cumulus-enclosed oocyte is less negative than the potential we report from oocytes removed from preantral oocytes (-38.4 ± 4.5 mV, $p < 0.001$). Racowsky and Saterlie have also investigated the importance of changes in oocyte and cumulus cell membrane potential during the resumption of meiosis and progression to metaphase II [10]. The aforementioned authors indicate that a shift in E_m to a more positive value does not seem to be requisite for this progression. Our data suggest there is a requisite change in membrane potential from the early stage to late stage oocytes. The change in oocyte membrane potential and P_{Na}/P_Kwe describe here suggest there is a requisite change in oocyte development between the preantral and metaphase II stage. This alteration in membrane potential may not be necessary for progression from germinal vesicle to MII, but it is likely that it is required to produce an oocyte competent for fertilization. This may be a more gradual change or a multi-step rise in the E_m and decrease in P_{Na}/P_K from preantral to GVBD.

Materials and methods

Media

Human Tubal Fluid (HTF) media is the standard for handling oocytes in the clinical setting and thus was chosen for manipulation of the cells prior to electrical recordings. HEPES buffered RSS (124 mM NaCl, 2.6 mM KCl, 2.7 mM CaCl, 1.7 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 10 mM HEPES) was used as the perfusion media in recording E_m and as the control solution in calculation of P_{Na}/P_K. The use of RSS allowed manipulation of the ionic constituents of the perfusion media during electrical recording. To verify that RSS was acceptable for use with oocytes, E_m recordings from oocytes in HTF media were compared to cells bathed in RSS (data not shown). Reciprocal dilution of sodium with potassium was done in RSS by replacing molar concentrations of sodium salts with the potassium equivalent.

Preantral oocyte preparation

Preantral follicles were collected from Syrian Golden Hamsters euthanized by cervical dislocation followed by removal of the ovaries into artificial human tubal fluid medium (HTF). Tissue preparation was adapted from previous work [15] and experience in this laboratory. Use of this technique has produced viable follicles for physi-

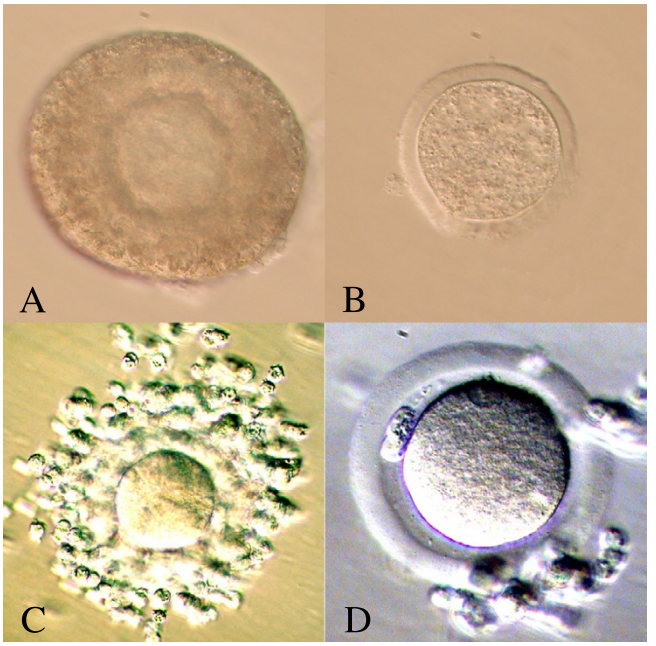

Figure 1
Representation of isolated cells. (A) Cumulus enclosed oocyte at the preantral stage. (B) Cumulus free oocyte at the preantral stage. (C) Metaphase II cumulus enclosed oocyte. (D) Metaphase II cumulus free oocyte.

ological studies previously. Tissues were minced with a scalpel and forceps in a sterile watch glass containing HTF with 3 mg/ml collagenase, Sigma Chemical Co., St. Lewis, MO and 0.1 mg/ml DNAse, Sigma Chemical Co., St. Lewis, MO. The minced tissue was then transferred to a 60 × 15 mm petri dish, Falcon, Franklin Lakes, NJ. Tissue was allowed to dissociate in the collagenase containing media for 10 minutes. The separation of follicles within the tissue was facilitated by repeatedly aspirating the sample through a glass pasture pipette during the collagenase treatment. Follicles were visualized using a low power dissection microscope and transferred into fresh HTF media with 10% BSA, w/v Sigma Chemical Co., St. Lewis, MO, using a glass pipette and washed three times. Small preantral follicles with oocytes having a diameter less than or equal to 60 μm were ultimately chosen for evaluation, transferred to HTF and maintained at 37°C, 5%CO_2 until transfer to perfusion dish. When oocytes free of cumulus cells were needed, the same protocol was followed with adjustment of the enzyme treatment timeto 15 minutes and a small-bore pipette (I.D. 100 μm) was used to strip the surrounding cells from the oocyte.

Metaphase II oocyte collection

Metaphase II oocyte-cumulus masses were collected as previously following a standard stimulation protocol.

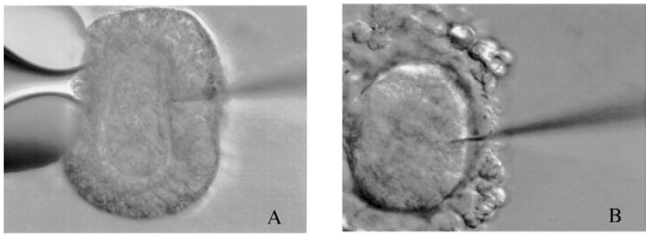

Figure 2
Impalement of cumulus enclosed oocytes. Photomicrograph depicting the method of impalement required for electrical potential measurements in cumulus-enclosed oocytes, preantral (A) and metaphase II (B).

Briefly, hamsters were injected with 100 IU pregnant mares serum gonadotropin (PMSG), DioSynth, Holland, during metestrus followed by 100 IU hCG, Sigma Chemical Co., St. Lewis, MO, injection 48–52 hours later. Fallopian tubes were removed from euthanized animals into HTF 14–16 hours post hCG. The cumulus-oocyte mass was then removed by rupturing the tubules adjacent to a visible swelling and teased out with a 28 gauge needle. When denuded oocytes were needed, hyaluronidase and a small-bore pipette (100–120 μm) were used to remove attached cumulus cells from the oocyte. All oocytes and cumulus-oocyte complexes were maintained at 37°C until use. Metaphase II oocytes were classified as such by the presence of a single polar body having been extruded following (GVBD). All tissues used are represented in figure 1.

Electrical recording and setup for oocyte studies

Cells were bathed with a HEPES buffered Ringers Salt Solution in a perfusion chamber while held in place with a conventional glass micro-tool manufactured for holding oocytes during intra cytoplasmic sperm injection (ICSI) and a controlled pressure device. The oocyte was immobilized for impalement by pressing the cell against the bottom of the dish while being held to the holding pipette with slight negative pressure. Solutions at room temperature (22°C) were changed as needed by a gravity feed perfusion system attached to a coverslip bottom perfusion well. The effects of tissue degradation due to incubation at 22°C and successful impalement were controlled for by monitoring the oocyte morphology and successfully returning to the beginning E_m when the experiment was finished or continued measurement of membrane potential without any significant deviation for at least one minute

Electrical measurements were recorded as the potential difference between a 3 M KCl Ag/AgCl microelectrode

$$(A) \qquad e^{\frac{\Delta V}{25}} = \frac{PM}{[K^+]_i} + \frac{1\text{-}P}{[K^+]_i} \cdot [K^+]_o$$

$$(B) \qquad e^{\frac{\Delta V}{25}} = \frac{1\text{-}P}{bM} \cdot [K^+]_o$$

$$(C) \qquad P_{Na}/P_K = \frac{a}{bM + a}$$

Figure 3
Equation for calculation of P_{Na}/P_K. The GHK can rearranged to give the above linear form, with the assumptions given in the text (A). Where $P = P_{Na}/P_K$, M is the total cation concentration outside the membrane (Na^+ and K^+). When the left term from equation A is plotted against the test (K^+) cation concentration, equation A is simplified to equation B. Where *b* is the slope. B can then be rewritten as C where *a* is the intercept.

(tip impedance of 20 MΩ) inserted into the oocyte and an external 3 M KCl glass-frit electrode located downstream of the oocyte. Electrodes were coupled through Ag/AgCl half cells to a WPI model KS-700 dual channel high input impedance amplifier (WPI, New Haven, CT, USA). Recordings were displayed on a digital storage oscilloscope and stored to the hard drive of a PC using Axotape 2.0 software (Axon Instruments; Foster City, CA, USA) at a rate of 100 Hz. The oocyte membrane was pierced by passing the glass electrode through the cumulus mass and the zona pelucida to dimple the cell; a negative capacitative ring or slight tap of the micromanipulator was used to pierce the membrane (fig. 2). Cells were allowed to recover after impalement and were used for recording if the membrane potential was maintained for no less than one minute prior to and following the experiment. Possible cell damage due to room temperature experiments and damage to during impalement was monitored by assessing changes in morphology and drastic changes in membrane potential with out change in the experimental setup. Control experiments were also used to verify that there was not a change in E_m following a sham experiment where ionic concentrations were not changed in the bath.

Electrical recording and setup for cumulus studies

Cumulus cells were recorded from in-place on the preantral cumulus-oocyte complex using the same holding system as described in the above text. Cumulus cells on the surface of the follicle within two to three layers of the oocyte were recorded from by the same method as used for the oocyte. The E_m was not considered unless the value was steady for at least one minute after impalement.

Calculation of P_{Na}/P_K

Membrane potential changes were recorded during exposure to a seriesof reciprocal serial dilutions of Na^+, the control ion, replaced with K^+, the test ion. Solutions fed to the perfusion chamber were switched through a multiline gravity feed perfusion system and cells were then equilibrated in the subsequent solution ranging from 126.6 mM Na and 0 mM K to 0 mM Na and 126.6 mM K, returning to a control Ringers solution before proceeding to the next test solution. P_{Na}/P_K was calculated using a modified Goldman-Hodgkin-Katz (GHK) equation [16,17]. The exp ΔV/25, where ΔV is the difference in E_m of the test cation (K^+) and the control cation (Na^+), was then plotted as a function of the test cation concentration. The permeability ratio of these two cations was then calculated by fitting the generated data points to a least squares regression line. The slope, intercept and total alkai-metal cation concentration were substituted in the following GHK rearrangement (figure 3) from Saunders and Brown, 1977 [16]. This linear rearrangement is based on four basic assumptions pertaining to the oocyte and general ionic behavior: 1) ion activities are directly proportional to ion concentrations, 2) intracellular ion concentrations remain unchanged following changes in the ion concentration of the superfusate or perfusate, 3) the chloride ion concentration does not significantly contribute toward the oocyte membrane potential and 4) the total concentration of cations does not change.

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