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Electro-rotation of mouse oocytes: single-cell measurements of zona-intact and zona-free cells and of the isolated zona pellucida

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Passive electrical properties of oocytes and of zonae pellucidae, and the mechanical coupling between them, can be elucidated by means of rotating-field-induced rotation. In low-conductivity media (25–100 $\mu S/cm$) rotation of mouse oocytes (with or without their zonae) requires fields in the 1–100 kHz frequency range. However, an isolated zona shows weak rotation in the opposite direction to that of a cell, and in response to much higher field frequencies (approx. 1 MHz). In zona-intact mouse oocytes, the rotation of cell and zona are not rigidly coupled: thus rotation of the cell can still be induced when the zona is held stationary. However, rotation of freely suspended zona-intact cells is much slower than that of zona-free cells and requires an optimum field frequency that is approximately 1.5 kHz higher. These observations show that the electrical properties of the oocyte that are measured by rotation are altered by the presence of the zona pellucida, even though no such influence has been detected using micro-electrodes. The data are consistent with the zona acting as a porous shell with a conductivity of 40 $\mu S/cm$ (preliminary estimate made at a single medium conductivity of 26 $\mu S/cm$). Measurements on cells from which the zonae had been removed gave values for the membrane capacity and resistivity of 1.2–1.3 $\mu F/cm^2$ and 400 $\Omega \cdot cm^2$, respectively. These values may reflect the presence of plasmalemma microvilli. The results strongly suggest that the technique may be useful for studies of cell maturation and for in vitro fertilization, because the cells may be further cultured after measurement.

Introduction

The state of maturation of the mammalian oocyte is thought to be extremely important for successful in vitro fertilization [1]. In order to reduce the necessity for repeated attempts at fertilization [2], it is a great advantage if a reliable forecast can be made of whether or not an egg is ready to be fertilized. However the only non-invasive tests of egg maturity are morphological ones,

Abbreviations: f_c, 'characteristic frequency' of a rotation peak (that field frequency that gives fastest rotation); F10, cell cultivation medium known as Ham's F10 (with glutamine, penicillin and streptomycin, bicarbonate buffered); HCG, human chorionic gonadotrophin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PMSG, pregnant mare serum gonadotrophin.

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such as are currently in use [1,3]. For these tests a highly experienced observer is required.

On the other hand, electrophysiological methods show that maturation and fertilization are accompanied by large changes in the membrane resistivity and/or capacity in mouse [4], rabbit [5], and frog [6] eggs.

The use of intra-cellular micro-electrodes, as in the above measurements, precludes the further cultivation of the oocytes. A non-invasive electrophysiological technique such as the method of single-cell rotation [7–11] may therefore be interesting for the study of oocytes and their fertilization. To test this possibility, we studied the electro-rotation of mouse oocytes, both with and without their zonae pellucidae (oocyte morphology is shown in Fig. 1). As the zona pellucida has properties that are also important in fertilization [12–14], we also looked for a rotational response of the zona itself, and investigated whether its presence influenced oocyte rotation.

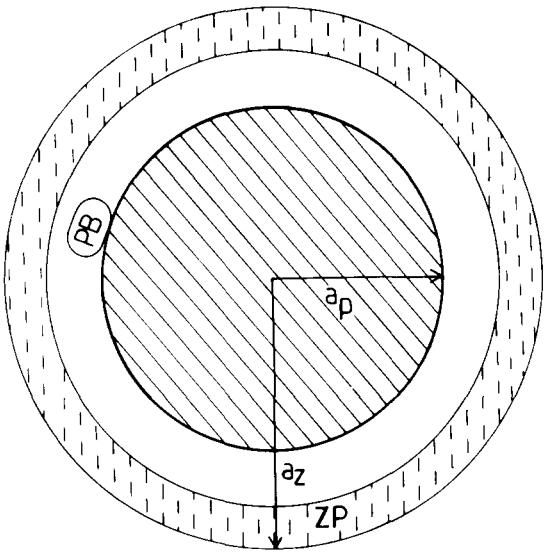

Fig. 1. Representation of the structure of an oocyte as used in this study. ZP, zona pellucida; PB, polar body. The diagram was scaled to data obtained from 10 oocytes measured in inositol medium. The measurements (±S.D.) were: a_p (plasmalemma radius) = 35.4±0.9 μm; inner radius of zona = 46.2±1.8 μm; a_z (outer radius of zona) = 55.1±1.5 μm. It is known that the morphology of the zona can change during culture [20]. The above shows that in inositol-based rotation medium the thickness of the zona could be taken as 8.9 μm, and the width of the perivitelline space as 10.8 μm.

Oocyte rotation modelled

Rotation of a particle in a rotating field may be in either direction (with the field or against it), and may change direction as the field frequency is scanned. Conclusions may be drawn about the nature of the particle from the rotation spectrum. Thus the field frequency needed to give fastest anti-field rotation has been used to deduce the capacity and resistivity of the plasmalemma or other bounding membrane [7,8,10,15]. In general, the technique can yield similar information to that given by dielectric spectroscopy [16,17].

At the time of writing, there are three well understood classes of rotation that can be expected to be seen in studies of cells such as oocytes or of zonae pellucidae. Experimentally, these rotations can be characterised according to their direction and the field frequency that elicits them. It must be remembered that medium conductivity has a strong influence on rotation: in what follows we refer to observations taken in low-ionic media of 20–200 μS/cm conductivity.

At frequencies between 1 and 100 kHz (or higher in high-conductivity media), cells exhibit an anti-field rotation. The period of the field frequency that gives fastest rotation is identical with the time-constant of charging of the cell membrane, and the amplitude and direction are also described by calculation of the net dipole produced by this membrane-charging process [9,16]. The double layer is not thought to be directly responsible for this rotation for reasons given at the end of this section. This is the rotation that was initially described for plant protoplasts and other cells [7,8], and the rotation spectrum is illustrated by the theoretical curves A to D in Fig. 2. Curve A was modelled using cell parameters (see legend to Fig. 2) derived from our measurements on zona-free eggs; curves B to D show the effect of adding a hypothetical surface conductance to the model (see Discussion).

At frequencies above 300 kHz (depending on the medium conductivity) rotation of cells reverses and increases to a second maximum. According to theoretical predictions [9], this should lie at frequencies of 10–40 MHz (depending principally on the high-frequency value of the inner conductivity), assuming that the permittivity of the medium

approximates that of water. As these frequencies are beyond the range of present equipment, this peak will not be further discussed. However, the start of this peak is visible as the high-frequency end of curves A to D.

A third class of rotation is the co-field peak exhibited by yeast cell walls at frequencies of the order of 1 MHz in 10 μS/cm media [18]. It was shown that this rotation was due to the cell wall conductivity being a factor of 3 higher than that of the medium. The indirect origin of this conductivity lies in the fixed charges present in many cell walls. The resulting concentration of counter-ions can be many times that in the bulk, especially in low-ionic media [19]. Curves E to H show how the possession by the zonae of plausible values of conductivity should cause them to also exhibit co-field rotation near to 1 MHz.

It may be noted that the dielectric relaxation of the ionic double layer (which for cell-sized objects should occur at lower frequencies than measured here [17]) might also be expected to give rise to rotation. No such primary rotational response appears to have been observed yet in the case of cells, although a double layer will result in a surface conductance and so exert a minor effect on the rotation that is due to membrane-charging (see Eqn. 2 below). Even synthetic vesicles made from uncharged lipids (presumably lacking a double layer) show this anti-field, membrane-charging rotation (Arnold, W.M. and Zimmermann, U., unpublished data). As expected, charged polystyrene particles show no anti-field rotation [17], but instead a co-field rotation at low frequencies (< 1 kHz) which may be due to the double layer. As emphasized in [17], the double layer does not necessarily rotate as part of the particle.

Rotation analysis

Rotation depends on the interaction of an induced dipole with the inducing field, therefore rotation speed is always found to be proportional to the square of the field strength. For presentation purposes, this relationship was used to normalise results obtained at different generator outputs.

A general equation for the rotation of a spherical cell (or particle) with or without surface con-

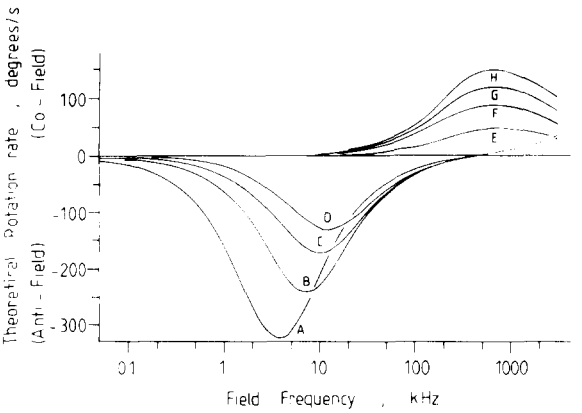

Fig. 2. Theoretical rotation rates of oocytes and isolated zonae. The rotating field strength of 58 V/cm and medium viscosity of 1.27 cP are the same as used for the measurements shown in Fig. 3. Curve A represents a zona-free oocyte modelled according to the data derived in this paper: plasmalemma radius 36 μm; membrane capacity and resistivity 1.25 μF/cm^2 and 400 $\Omega \cdot$cm^2, respectively. The internal conductivity was assumed to be 5 mS/cm, and relative permittivity values of 70 were assumed for inside and outside media. In curves B to D surface conductances of 100, 250 and 400 nS, respectively, were added to the above model. Curves E to H represent an isolated, washed zona pellucida (inside conductivity equal to the outside) of thickness 8.9 μm and outside radius 55 μm. The conductivity of the zona was modelled as 39, 52, 65 and 78 μS/cm, respectively.

ductance can be obtained by combining Eqns. 3 and 8 presented by Fuhr and Kuzmin [9]: the resulting equation was used to plot the curves given in Fig. 2. The theory used refers to a single-shell model: in curves E to H this shell is the zona pellucida, whereas in curves A to D it is the relatively thin membrane, which can be electrically described by its capacity and resistivity (C_m and R_m) *. Indistinguishable curves can be obtained from the simpler equations derived in terms of membrane parameters by Schwan [16].

The theory used to derive curves A to D of Fig. 2 can only be considered to be valid when the plasmalemma forms the electrical and the hydro-

* The Fuhr equations are written in terms of bulk permittivities and conductivities (units: F/m and S/m, respectively). In order to use them with the usual membrane parameters (C_m, F/m^2) and (R_m, $\Omega \cdot$m^2), we interconverted these quantities by use of the membrane thickness d: ε_2 (the absolute permittivity of the membrane) $= d \cdot C_m$, and σ_2 (the conductivity of the membrane material) $= d/R_m$.

dynamic boundary of the cell. In the case of mammalian oocytes, the presence of a thick external layer of larger radius (the zona pellucida, see Fig. 1) means that the results should be interpreted with caution. On the one hand the zona can be expected to increase the hydrodynamic drag on the cell, so that the rotation speed of a rigid, zona-intact cell must be lower than that of a zona-free cell. On the other hand, the zona may exert an electrical 'screening" effect, which will decrease the field seen by the plasmalemma. An example of the screening effect was seen in the case of the cell wall of yeast cells [18], even though this is relatively thin (0.3 μm). A similar or greater effect can be expected in the case of the 9 μm thick zona pellucida.

Although the full equation used to plot the curves in Fig. 2 is too complicated to warrant presentation here, a simplified theory can be used to predict and analyse the field frequency (f_c) that gives maximum cell rotation in the anti-field direction. If the medium conductivity is held much lower than that of the cell interior, this frequency can be expressed as [10,15,16]:

$$f_c = \frac{1}{2 \cdot \pi \cdot a \cdot C_m}\left(2 \cdot \sigma + \frac{a}{R_m}\right) \quad (1)$$

where a is the cell radius, and σ is the medium conductivity. Notice that a plot of f_c against σ should be linear, having gradient $1/(\pi \cdot a \cdot C_m)$ and intercept $1/(2 \cdot \pi \cdot R_m \cdot C_m)$.

The effect of 'surface conductance' * (K_s) is neglected in Eqn. 1. In the case of cells (which have a high internal conductivity), a high K_s has an identical effect on rotation spectra to that of a low value of R_m. As shown by Schwan (literature quoted in Ref. 16), the magnitude of K_s that has the same effect as a given R_m depends strongly on the cell radius (a). At relatively low frequencies such as used here the equivalence of these two quantities is:

$$2 K_s \cdot R_m = a^2 \quad (2)$$

In contrast to the situation with cells, the effect of K_s on particles with internal conductivities equal to or less than that of the medium is to generate a co-field rotation [17] which is very similar to the rotation caused by conduction within cell walls [18]. For the case of modelled zonae pellucidae, this rotation is shown in Fig. 2 as curves E to H.

Results

(a) Zona rotation relative to vitellus

The rotation speed of the zona was in all cases slower than that of the oolemma-bounded cell, which rotated as a rigid sphere. Polar bodies, which were sometimes prominent in the perivitelline space (Fig. 1), were swept round at an intermediate speed. It therefore appears that no rigid mechanical coupling existed between zona, polar bodies, or plasmalemma. We assume that the zona was driven round by viscous coupling to the rotating cell. The lack of a rigid connection between zona and plasmalemma was further demonstrated by the observation that a cell continued to rotate even after the zona had become stuck to the floor of the chamber (which occurred frequently until the floor material was changed from glass to methacrylate). It must be noted that in the case of the human eggs that were available to us the perivitelline space was much smaller and also asymmetrical: in these cases the complete cell rotated as a rigid body.

(b) Effect of the zona on oocyte rotation (Fig. 3)

Cells complete with zona pellucida (points 'Δ') rotated more slowly than those from which the zona had been removed (points '□'). This was in spite of the fact that the zona-intact cells had to be measured in free suspension in order to avoid adhesion of the zona to the floor of the chamber (which occurred even to methacrylate). Both sorts of oocytes rotated in the opposite direction to the field over the 1–100 kHz range of frequencies. The agreement between the spectra of zona-free cells (points '□' in Fig. 3) and the appropriate model

* Surface conductance refers to a tangential conductance in what should strictly be an infinitessimally thin layer around the surface of a particle. The physical basis of this conductance is not usually defined by may be the increased ion concentrations within the electrical double layer and/or the conductance of a thin surface coating (in cells, this may be the glycocalix or cell wall).

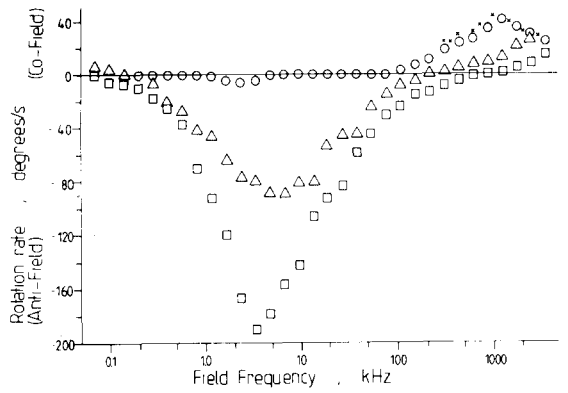

Fig. 3. Rotation spectra of: an oocyte without zona pellucida (□); an intact oocyte (△); and two examples of an empty zona pellucida (○ and ×). The medium conductivities were 49 μS/cm, 78 μS/cm and 26 μS/cm, respectively. All data refer to a rotating field strength within the external medium of 58 V/cm. This was the actual value used for the zona-free oocytes, the intact oocyte and the empty zona were examined at a higher field strength (80 V/cm); the rotation speed data was therefore normalised by division by the square of the field strength ratio. Frequency range 0.0707 kHz to 3.28 MHz.

(curve A of Fig. 2) is very good, except that the theoretical rotation rate is consistently higher (by about 60%). This was probably because the zona-free cells could be rotated on the floor of the chamber, which aided observation but can be expected to have raised the hydrodynamic resistance above the theoretical, free-suspension value. From other measurements, we found that the ratio between the rotation speeds of zona-free and zona-intact cells, both measured in free suspension, was 2.8 : 1.

Zona-intact cells also exhibited consistently higher f_c values (see Fig. 4 and Table I): this and the speed observations can be most simply explained by assuming that the zona conductivity is higher than that of the medium. We attempted to model the effect of this higher conductivity by giving a surface conductivity (K_s) to the cell specification used for Fig. 2 curve A: curves B–D show how increasing this conductivity caused both the cell rotation rate to decrease and the f_c to increase. However, the increase in f_c is larger than actually observed: this is probably because the surface-conductance model of the effect of the zona does not take account of the separation between plasmalemma and zona, or of the appreciable thickness of the zona.

(c) Rotation of isolated zonae

Empty zonae (isolated by micro-manipulation and repeated washing) rotated quite differently from zona-intact or zona-free cells. Rotation occurred in the opposite direction to the cell rotation (that is, it was co-field), and in response to much higher frequencies (≈ 1 MHz), as shown by the series of points '○' and '×' in Fig. 3. The similarity of this rotation to that given by yeast cell walls [18] suggested that it might be explained by the possession by the zona of a conductivity in excess of that of the medium.

We therefore modelled the rotation of a thick conductive shell with dimensions based on those given in the legend to Fig. 1. Progressive increases of the conductivity of the shell above that of the medium gave curves E to H of Fig. 2, and it can be seen that curve E matches the experimental curves (Fig. 3, '○' and '×') closely with regard to both frequency-dependence and magnitude. Interpolation of the experimental amplitude between the theoretical results shows that a conductivity of 41 μS/cm is required to account for the experimental results. This value applies to a homogeneous-shell model: it is possible that the zona could be better modelled as two or more layers of higher and lower conductivity than the above value.

The above does not take account of the possibility that the permittivity of the zona could be considerably different from that of water. Other modelling exercises (not depicted) demonstrated that a positive peak of the required amplitude close to 1 MHz could also be produced by assuming that the zona had the same conductivity as the medium, but a relative permittivity of only 45. However, the 'peak' produced by this model is much flatter than the experimental results demand, and it was concluded that at least the predominant cause of the observed rotation was a zona conductivity of about 40 μS/cm.

(d) Rotation after membrane puncture

The rotation spectrum was usually scanned from high frequencies down as far as 1 kHz, because puncture of the plasmalemma was found to occur at lower frequencies. If the frequency was taken lower than 1 kHz, one or sometimes two breaks in the plasmalemma often occurred. These were visi-

ble because the contents of the vitellus flowed into the peri-plasmic space (but appeared to be retained by the zona pellucida). At this stage the filled zona still showed a rotation spectrum resembling, but much weaker than, that of the zona-intact cell. However, the amplitude decreased with time, perhaps due to loss of ions through the zona. (In the case of human cells, a polar body was prominantly visible, and could be induced to rotate within the zona. Thread-like components of the cytoplasm also rotated and appeared to be 'wound up' by this motion.)

(e) Measurement of C_m and R_m

The frequency of the anti-field rotation maximum (f_c) of single cells was found to increase linearly with the medium conductivity as shown in Fig. 4. Although this is as expected from Eqn. 1 (Rotation analysis) for a simple membrane-bounded cell, it was also found to be accurately true for zona-complete cells. The near unity values of the correlation coefficients (r) of least-squares fits to the data (Table I) show how accurately linear the data is. Accordingly we used Eqn. 1 (Rotation analysis) to deduce values for the membrane capacity and resistivity of all the cells. In the case of zona-less cells, these values may be taken literally. However, as shown above the presence of the zona appears to strongly affect the rotation speed and we also found that the f_c values of zona-intact cells were always higher than those of zona-free cells. Therefore the values of C_m and R_m for cells with a zona must be taken as 'apparent'.

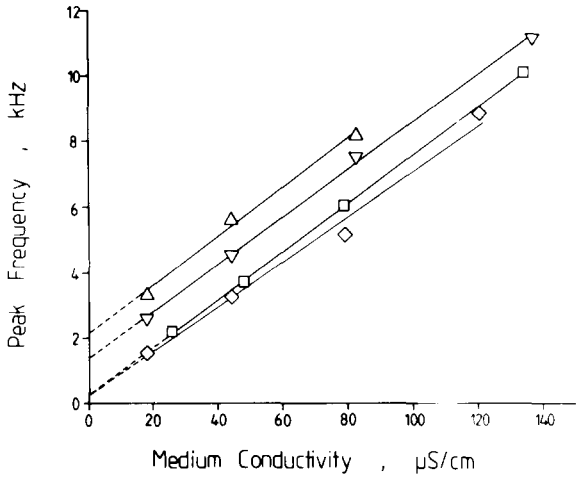

Fig. 4. The increase in the frequency giving maximum rotation speed of oocytes as a function of medium conductivity. Each line is the least-squares fit to the data obtained from a single cell (see Table I). The cells were: △, an oocyte with $a_p = 35$ μm, $a_z = 51$ μm; ▽, an oocyte with $a_p = 36$ μm, $a_z = 52$ μm; □, a zona-free oocyte with $a_p = 37$ μm; ◇, a zona-free oocyte with $a_p = 36$ μm.

TABLE I

DERIVATION OF APPARENT MEMBRANE CAPACITIES (C_m) AND RESISTIVITIES (R_m) OF SINGLE CELLS FROM PLOTS OF ROTATIONAL f_c AGAINST MEDIUM CONDUCTIVITY

For each of the four cells the gradient of the regression line (Fig. 4) was used to calculate the membrane capacity (Eqn. 1), after which use of the intercept (the value of f_c extrapolated to $\sigma = 0$) allowed calculation of the membrane resistivity. LSR, abbreviation for least-squares regression.

Symbol in Fig. 4 and oocyte type	△ with zona	▽ with zona	□ no zona	◇ no zona
Plasmalemma radius (μm)	35	36	37	36
LSR regression coefficient (r)	0.9976	0.9997	0.9997	0.9920
Gradient of LSR (Hz·cm/μS)	73.3	72.3	72.2	67.6
Apparent C_m (μF/cm^2)	1.24	1.22	1.19	1.31
Intercept of LSR (kHz)	2.1	1.4	0.3	0.3
Apparent R_m (Ω·cm^2)	61	93	400	400

At the start of this work, the measurement of the f_c values was not found to be reproduceable, especially in the case of zona-intact cells. Inconsistencies were invariably of the type that the initially measured f_c was too high, presumably because the zona hindered the complete replacement of culture medium by low-conductivity inositol solution. The problem was eliminated by careful adoption of the 8-fold wash procedure described in Methods.

(f) Effect of field exposure

Cells which had been measured by rotation in several conductivities were found to still have a functioning membrane, as judged by appearance of fluorescence inside the cell after fluorescein diacetate was added to the medium.

A preliminary experiment was performed to further test the viability of the cells after exposure to the field. The f_c values of 14 cells were measured, for which it was necessary to use a field strength of 58 V/cm for 5 s each. Directly after the f_c measurements the cells were returned to F10 in individual microwells in the CO_2 incubator. After 24 h incubation, none of the cells showed plasmalemma lysis, although the cytoplasm of five cells had a granular appearance. Experiments to test the longer-term effects of the rotating-field exposure could not be made because the rotation work was not performed under sterile conditions.

Discussion

The co-field rotation of the zonae can be explained by a conductive-shell model in which the shell has a thickness of 8.9 μm and a conductance of approx. 40 μS/cm. The surface conductance model found quantitatively suitable for yeast walls and cells [18] cannot explain the results in this system. This is presumably because the zona is unlike a cell wall in that its thickness cannot be neglected relative to the cell radius, nor does it lie directly on the surface of the plasmalemma.

A zona pellucida conductivity that is higher than that of the medium is quite plausible in relationship to the known structure of this organelle: a porous matrix composed of acidic glycoproteins. The predominant (47% of the total) component is somewhat heterogeneous and has a molecular mass near to 120 kDa [20]. Biosynthetic studies [21] have shown that the mannose-containing core of ZP2 is homogeneous, and has an isoelectric point of 6.5. Complex glycosylation reduces the pI to an average of 5.5. The majority of the fixed charge demanded by the high-conductivity model is therefore presumably associated with the complex part of the polysaccharide side chains. The positive ions required for electrical neutrality will be free to move through the porous glycoprotein matrix. In bacterial cell walls impedance spectroscopy has measured a large conductivity due to a similar effect [22], but zonae have a conductivity that is relatively small compared to that of bacterial walls. A similar conductivity (33 μS/cm at 10 μS/cm medium conductivity) to that seen in zonae seems to occur in the cell wall of yeast [18], where it may be due to the presence of phosphomannan.

We are not able to exclude the possibility that the rotation of the zonae could also have been influenced by their permittivity, although the shape of the rotation peak indicates that the conductivity effect is dominant. In the case of yeast walls [18] it was possible to exclude a permittivity effect; the difference is due to the very much greater thickness of the zonae, which (according to model calculations) accentuates the permittivity effect. However, it is difficult to imagine how the permittivity of the zonae could be very different from that of the medium, because they are very porous with a dry matter content of approx. 2% *. Results obtained at several conductivities may enable the more accurate estimation of any permittivity effects.

Removal of the zona was found to decrease the f_c of oocytes, as expected if the zona were more conductive than the medium. It may be thought that there is a second interpretation of this decrease in f_c, namely that the pronase or trypsin used for the digestion led to an artifactual increase in membrane resistivity. The data of Fig. 4 and Table I would demand a resistivity increase of the order of 6 : 1 to be explained in this way. However, micro-electrode measurements [4,23] could detect no change in the membrane properties after removal of the zona pellucida. As micro-electrode techniques can measure membrane resistivity rather accurately, it seems unlikely that an enzymic degradation of the membrane must be considered.

On the other hand, the observation that the rotation of oocytes is affected by the presence of the zona pellucida may seem to be at variance with the observation of Powers and Tupper [23] that no influence of the zona on the cell properties could be seen by micro-electrode measurements. The reason is the totally different current flow pattern and frequencies that are used in the two

* According to Ref. 20, the mouse zona contains 4.8 ng protein, which is 80% of the dry weight. The dimensions of the zonae used here (see legend to Fig. 1) imply a zona weight (wet) of 290 ng, so that protein constitutes 1.6% of the zona. The remaining 0.4% of the wet weight is presumably carbohydrate.

methods. Micro-electrode measurements cause the current to flow radially, so that the relatively small radial impedance of the zona is insignificant compared to the membrane impedance at low frequencies. In addition, the zona can only appear considerably more conductive in relation to almost non-ionic media; such media are rarely if ever used in micro-electrode set-ups.

The measurements of membrane capacity and resistivity presented here are the first true single-cell measurements that have been reported using the rotation technique. This is because separation of these two membrane parameters requires rotation data at several conductivities [10]. In work that has obtained such data, it has usually proved fastest to wash 6–20 cells by centrifugation, measure and discard them and then proceed to the next conductivity. However, the relative scarcity and value of oocytes made it necessary to re-use each cell several times. The true single-cell technique has a considerable further advantage in that differences between cells cannot decrease the closeness of the correlation between f_c and medium conductivity.

The value of 1.2–1.3 $\mu F/cm^2$ deduced here for the membrane capacity is a little less than the values of 1.5 ± 0.2 $\mu F/cm^2$ [23] and 1.6 $\mu F/cm^2$ [24] measured on mouse oocytes by means of micro-electrodes. On the other hand, a membrane resistivity of 400 $\Omega \cdot cm^2$ is considerably lower than that seen elsewhere. For example, Powers and Tupper [23] found a value of 2610 ± 38 $\Omega \cdot cm^2$, and Okamoto et al. [24] found a still higher value. Although rotation measurements can give apparently low values of R_m due to surface conductance (K_s) effects, this is very unlikely with larger cells such as eggs. This is because the value of K_s required to account for a given value of R_m increases with the square of the cell radius (Eqn. 2, in Rotation analysis).

However it must be stressed that the value of R_m that is measured by rotation is a high-frequency value. If there were a dielectric dispersion in the properties of the membrane at frequencies below 1–2 kHz, the R_m apparent in electro-rotation can be expected to be considerably lower than that measurable with micro-electrodes. In this case it is also to be expected [25] that the high frequency capacity will be lower than that seen at low frequencies, as indeed consistent with the values discussed above. More precisely expressed, there is a proportionality between the conductivity increment ($\Delta\sigma$) and the absolute permittivity decrement ($\Delta\varepsilon$) encountered during the traversal of the frequency domain of a dielectric dispersion. If the dispersion is due to processes with a single time constant, it has a single 'characteristic frequency' f_o and then:

$$\Delta\sigma = 2\pi \cdot f_o \cdot \Delta\varepsilon \tag{3}$$

In order to apply this equation to rotation work, the quantities σ and ε must be calculated for a single cell. This is usually done by considering the isotropic sphere that is dielectrically equivalent to a cell of the same radius a. In this case very good approximation for these 'effective particle' σ and ε can be expressed as $\sigma = a \cdot G_m$ and $\varepsilon = a \cdot C_m$ (at frequencies lower than the membrane-charging dispersion, see Ref. 16 and literature quoted there). G_m is the membrane conductivity, which is more convenient to use than R_m ($G_m = 1/R_m$). Substitution into Eqn. 3 yields:

$$f_o = \Delta G_m / (2\pi \cdot \Delta C_m) \tag{4}$$

This allows the calculation of the characteristic frequency of a dispersion occurring below the so-called β-dispersion of a single cell (or of a homogeneous cell population) from the dispersion magnitudes of membrane conductivity and capacity. If we assume that the discrepancies between the rotation and the micro-electrode values for G_m and C_m are purely the result of a dispersion process, then the dispersion magnitudes are given by $\Delta G_m = (2.5 - 0.4) = 2.1$ mS/cm^2, and $\Delta C_m = (1.55 - 1.25) = 0.3$ $\mu F/cm^2$. From these values we obtain from Eqn. 4 a characteristic frequency of 1.1 kHz for the dispersion (assumed to have a single time constant). Although this is only a rough estimate, it does support the hypothesis that the discrepancy between the membrane parameters measured by electro-rotation and those measured with internal microelectrodes is due to a dispersion in the low-frequency range. (1 kHz is too low to have had an effect on the rotation measurements, but too high to have affected the earlier microelectrode work.)

There exist other examples of dispersions in

membrane properties that are apparent in measurements with external electrodes. Thus a low-frequency dielectric dispersion associated with erythrocyte ghost membranes has been observed which gives a sufficiently large decrement in membrane resistivity to give a high frequency value of less than 200 $\Omega \cdot cm^2$, even if the membrane is assumed to be perfectly insulating at low frequencies [26,27].

A possible cause for a low-frequency dielectric dispersion in ova may be membrane microvilli. The presence of these on the oolemma is well known [12]. Irimajiri et al. [28] have shown that microvilli can indeed give rise to a dielectric dispersion, at least in rat basophil leukaemia cells. It is also accepted that the microvilli of frog [29] and starfish [30] eggs have a large effect on the membrane capacitance, as measured with micro-electrodes.

It may be significant that we have preliminary rotation results from hamster and human ova that indicate values for C_m that are 2–3-times greater than those for mouse oocytes, with R_m values being proportionally lower. This may reflect a greater contribution from microvilli in these species.

Oocytes are considerably larger than other cells that have been investigated with electro-rotation, therefore consideration must be given to the induced membrane voltages. This is because integration of the Laplace equation shows that the membrane voltage increases linearly with cell size. In a spherical cell of radius a cm and charging time constant τ s, a linear field of frequency f and peak strength E V/cm induces a membrane voltage given by [31,32]:

$$V_m = \frac{1.5 \cdot E \cdot a}{\sqrt{(1+(2\pi f\tau)^2)}} \quad (5)$$

Therefore at high frequencies the membrane voltage is low; at the rotation peak (where $2\pi f\tau = 1$), $V_m = 1.06 \cdot E \cdot a$; and at frequencies so low that $2 \cdot \pi \cdot f \ll \tau$, then $V_m = 1.5 \cdot E \cdot a$. At a field strength of 60 V/cm (the maximum used here), the membrane voltages at $f = f_c$ and $f \ll f_c$ were therefore 230 mV and 320 mV, respectively. Cells 'screened' by the zona pellucida will have seen a field strength that can be estimated (from the zona-free to zona-intact speed ratio) to have been a factor $\sqrt{2.8}$ less than the above. Despite this, the possible detrimental effects of high induced membrane voltages must be considered.

It is known, for example, that ionic channels in oocytes may be activated by hyperpolarization [33], although how these channels may respond to alternating voltages is unclear. In addition, alternating voltages of 100 mV or more can cause membrane breakdown. In the case of artificial lipid membranes, longer pulse lengths [34] or lower frequencies of alternating field [35] were found to be particularly damaging. This presumably explains why destruction of the plasmalemma was only seen when rotation spectra scans were allowed to go too low in frequency.

Cells which had been subjected only to field frequencies near to the f_c could be further cultivated and retained their ability to be stained by fluorescein diacetate. It seems that the short exposure to the medium-frequency field necessary for a rotation measurement is not necessarily harmful. We therefore hope to be able to use the technique to differentiate between ripe and unripe cells before attempting in-vitro-fertilization, and to characterise the electrical properties of fertilized and unfertilized cells.

Materials and Methods

(a) Chemicals and media

Unless otherwise stated, hormones and enzymes were obtained from Sigma. Water was double-distilled and sterile. Rotation media used inositol as osmoticum, or else a mixture of inositol and Nycodenz (a non-ionic density-modification material from Nyegaard, Oslo, Norway). Following cryoscopic measurements, the osmotic strength was adjusted to 290 ± 5 mosM. Conductivity, initially 5 μS/cm or less, was increased to 25 μS/cm by the addition of small volumes of 200 mM Hepes buffer (adjusted to pH 7.2 with NaOH). Further adjustment of conductivity up to 50–120 μS/cm was made with isotonic saline.

(b) Oocytes and zonae

Superovulation was induced in 24–48 day old sexually immature CB6F1 mice. 5 IU PMSG in saline was given intraperitoneally, followed 48 h

later by 5 IU HCG. Injections were performed at 8:00 pm, so that eggs could be removed at 9:00 am, 13 h after the HCG injection.

After killing the mice by cervical dislocation, the oviducts were removed and placed in a drop of F10. Under observation through a dissecting microscope, the swollen part of the transparent oviduct was punctured to enable the mass of cumular-surrounded eggs to be pressed out. 20–30 oocytes could be harvested per mouse.

Oocytes were freed from cumulus cells by incubation for 3 min in F10 containing 0.5% hyaluronidase. After washing the eggs three times, they were incubated in F10 supplemented with 5 mg/ml bovine serum albumin and incubated (5% CO_2, 37 °C) until use.

Removal of zonae pellucidae was carried out by treatment with 0.5% pronase, followed by washing three times in F10. Preparation of empty zonae pellucidae was performed using a micromanipulator (Leitz). Cells were clamped by suction applied through a holding pipette (50–80 μm bore). This allowed an incision to be made with a capillary that had been drawn to a needle, following which the vitellus was pressed out of the zona. Before rotation, the zonae were washed eight times as described for the cells.

The human eggs used were non-viable (polyspermic), obtained from an in vitro fertilization program.

(c) Rotation

Directly before rotation, 8 drops (each approx. 200 μl) of a 290 mosM inositol or inositol/Nycodenz solution of the required conductivity were arranged on a plastic Petri dish. A cell was taken from culture and washed through this series of drops. In order to avoid carry-over of salts, the entire micro-pipette was washed out in each solution before the egg was carried into the next. In the meantime the rotation chamber was washed out repeatedly with the same solution. The cell was pipetted as nearly as possible into the centre of the chamber for measurement of the f_c or for the taking of the rotation spectra. Some cells were measured at several different conductivities: in this case the 8-fold wash was repeated at each new conductivity.

The rotating field was produced by superposition of two linear, phase-quadrature, alternating fields at right angles. A four-electrode chamber and a generator of four, 90°-phase shifted voltages are required. We used a rotation chamber of the square box type [7] with platinum electrodes at a spacing of 3.0 mm. The base was interchangeable and made of glass (a microscope slide) or methacrylate to enable observation through an inverted microscope (40 × long-working-distance objective). Two types of four-phase generators were used. One generator was capable of automatically scanning the frequency range from 3.28 MHz to 0.0707 kHz with 2 or 4 steps per octave. In conjunction with a video camera and recorder with single-frame playback, rotation spectra could be obtained by measuring the angular displacement of the cell over 20 video frames at each field frequency. The second rotation apparatus was of the type described in Ref. 11 that can produce two contra-rotating fields having different frequencies which are related by a constant ratio. Under direct observation and using a single rotating field, the approximate rotation maximum is found by eye. At this point the second field is switched in and enables the accurate measurement of rotation peak frequency by a zero-crossing method.

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