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Short Contribution

Age and gonadotropins control Ca^{2+}-spike acquisition in mouse oocytes isolated from early preantral follicles

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ABSTRACT The action of gonadotropins upon the oocyte is known to be crucial at later stages of follicular development in mammals. However, its influence on oocytes at early preantral stages is still a matter of debate. In the present study we evaluated the onset of mouse oocyte's capacity to exhibit calcium spikes during preantral stages of follicular development, prior to meiotic competence acquisition. In particular, through the use of confocal microscopy, we probed for the specific effects of age and gonadotropin stimulation upon the calcium dynamics of preantral follicle oocytes. We found that important developmental changes on the Ca^{2+} signalling mechanisms take place early during follicular development. Specifically we demonstrate that both age and gonadotropin stimulation increase the capacity of oocytes recovered from preantral follicles to exhibit calcium spikes. We propose that a strictly morphological staging of follicular development is insufficient to predict oocyte behaviour and must take in consideration animal age and gonadotropin environment.

KEY WORDS: *preantral follicles, ovary, incompetent oocytes, calcium signalling, PMSG*

In mammals, proper follicular and oocyte development depends on the action of gonadotropins FSH and LH, as well as that of yet poorly understood cellular interactions and local regulatory factors (Albertini and Anderson, 1974; Dong *et al.*, 1996; Simon *et al.*, 1997; for review see Buccione *et al.*, 1990; Albertini, 1992; Albertini and Carabatsos, 1998). Accordingly, the positive effect of gonadotropin stimulation upon oocyte development is known to be mediated by somatic follicular compartments, namely on late antral stage events such as GVBD and the reaching of metaphase II stage (Downs *et al.*, 1988, Plancha and Albertini, 1994; for review see Buccione *et al.*, 1990). However, gonadotropin influence over early preantral events is still a matter of debate (Richards, 1994; Roy and Greenwald, 1996). In the mouse, oocyte meiotic competence is acquired near the completion of the growth phase, by the time of follicular antrum formation (Szybek, 1972; Sorensen and Wassarman, 1976; for review see Albertini and Carabatsos, 1998). Likewise, the effects of gonadotropins upon oocyte events leading to meiotic competence acquisition remain poorly understood.

Calcium signalling is involved in important events in oocytes, such as meiotic competence acquisition (Carroll *et al.*, 1994; Lefèvre *et al.*, 1995; for review see Homa, 1995; Carroll *et al.*, 1996). In particular, it was shown that the phosphoinositide/calcium signalling pathway is involved in the oocyte capacity to exhibit spontaneous calcium oscillations after follicular release (Lefèvre *et al.*, 1997; Pesty *et al.*, 1998). Moreover, it has been demonstrated that specific inhibition of phosphoinositide metabolism, as well as intracellular calcium chelation, blocks FSH-dependent resumption of meiosis (Coticchio and Fleming, 1998). In the present study, we characterised the onset of cytoplasmic calcium spikes in oocytes recovered from a morphologically homogeneous preantral follicle population, prior to meiotic competence acquisition.

Preantral ovarian follicles with no signs of isolation damage, atresia or oocyte abnormalities were selected (Fig. 1). Follicles used had 2-3 granulosa cell layers and a mean diameter of 115.8 μm (SD: 14.0 μm). As expected, oocytes released from these preantral follicles were at the GV stage, but not yet fully-grown, exhibiting a mean diameter of 58.6 μm (SD: 6.4 μm). A positive control group was composed by fully-grown oocytes recovered from large antral follicles from PMSG-stimulated adult animals. As expected, control oocytes were significantly larger (diameter: 81.4 μm; SD: 4.5 μm, $p<0.0001$) than those from preantral follicles.

Abbreviations used in this paper: PMSG, pregnant mare serum gonadotropin; FSH, follicle stimulating hormone; LH, luteinizing hormone; AM, acetoxymethyl ester; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; GVBD, germinal vesicle breakdown; SD, standard deviation.

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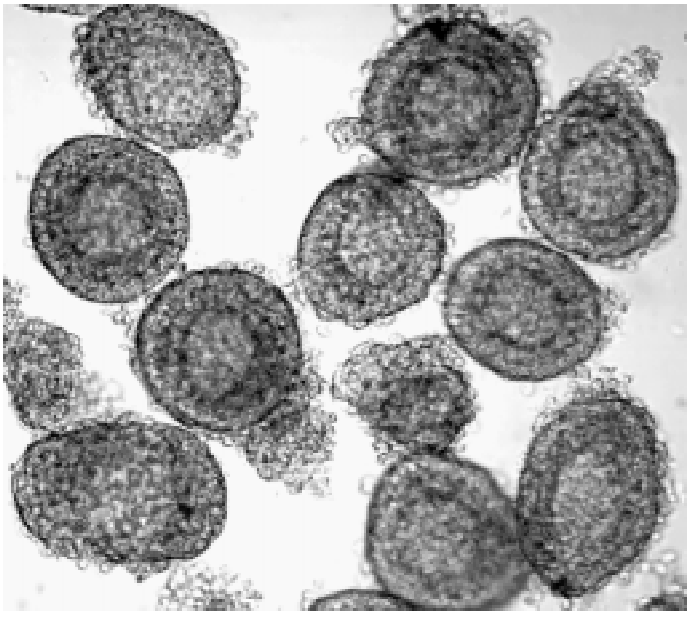

Fig. 1. Representative early preantral follicles used in this study.

Oocytes recovered from preantral follicles (Fig. 1) from juvenile (12 days old; n=37) and prepubertal (24 days old; n=26) animals did not exhibit calcium peaks (Figs. 2A,C and 4). In contrast, 46.9% of the oocytes (n=46) recovered from similar follicles in adult animals (2-3 months old) exhibited some calcium spikes during the 20 min recording period (Figs. 2E,4). Most fully grown GV oocytes from the positive control group (72.7%; n=21) exhibited Ca^{2+} spikes (Fig. 2G). Age is shown to significantly increase the percentage of oocytes exhibiting Ca^{2+} peaks in morphologically similar follicles (Table 1). The existence of such different patterns is interesting because it argues against the present belief that ovarian follicle development is equivalent throughout the female reproductive life (for review see Hirshfield, 1991; Fortune, 1994). Animal age seems thus to be a critical parameter for oocytes in morphologically similar preantral follicles to acquire calcium signalling mechanisms.

In oocytes recovered from PMSG-stimulated juvenile mice, calcium spikes occurred only in one case (n=40), while in prepubertals 13.6% of the oocytes (n=44) showed Ca^{2+} peaks (Figs. 2B,D and 4). Most of the preantral follicle oocytes (69.7%; n=33) recovered from adult PMSG-stimulated animals demonstrated Ca^{2+} spikes (Figs. 2F,3,4). Therefore, exogenous PMSG stimulation significantly increased the percentage of oocytes undergoing Ca^{2+} peaks in morphologically similar follicles (Table 1). Gonadotropin stimulation is thus shown to be another crucial parameter able to influence oocyte behaviour during early preantral follicular stage. Again, this result argues in favour of the idea of functional diversity within a population of morphologically similar preantral follicles.

The profiles of Ca^{2+} spikes in preantral follicle oocytes were consistently composed by a sharp increase followed by a rapid decrease of fluorescence intensity (Fig. 2D-F). The duration of these spikes was 15 to 25 sec, and biphasic profiles were never observed. This is in contrast with the profiles observed in fully grown GV oocytes from the positive control group. In this case, each calcium spike started with a less sharp increase in fluorescence intensity, often presenting a biphasic profile (Fig. 2G). Moreover, the duration of these spikes was approximately two to three fold longer (35-50 sec). We suggest that this different behaviour may be due to incomplete development of the Ca^{2+} signalling machinery. According to this hypothesis, it was shown that immature oocytes possess incomplete functionality of IP3 sensitive Ca^{2+} stores (Lefèvre *et al.*, 1997). In addition, similar differences were found in the Ca^{2+}-transient profiles when these were induced, through sperm extract injection, respectively in immature and mature oocytes (Carroll *et al.*, 1994). Moreover, early preantral follicle oocytes always showed a lower frequency of calcium spikes than that exhibited by fully-grown control oocytes. Again, this may be due to an incomplete development of the Ca^{2+} signalling machinery in early preantral follicle oocytes, since rapid and regular Ca^{2+} oscillations were found to relate with meiotic competence (Lefèvre *et al.*, 1997). It is tempting to speculate that a pattern of low frequency monophasic Ca^{2+} spikes represents an intermediate step towards meiotic competence acquisition, which would be modulated by gonadotropin stimulation (Sorensen and Wassarman, 1976; Eppig and Schroeder, 1989; Eppig, 1991; Eppig *et al.*, 1992).

In short, this work shows that oocytes, even though being recovered from preantral follicles of the same stage, as assessed by morphological criteria, express different Ca^{2+} activity patterns specifically influenced by animal age and gonadotropin stimulation. Two studies have already reported age-dependent differential actions of two growth factors over preantral follicle development *in vitro* (Yokota *et al.*, 1997; Liu *et al.*, 1999). Specifically, both activin A and transforming growth factor-beta were shown to cause opposing effects if identical preantral follicles were recovered from

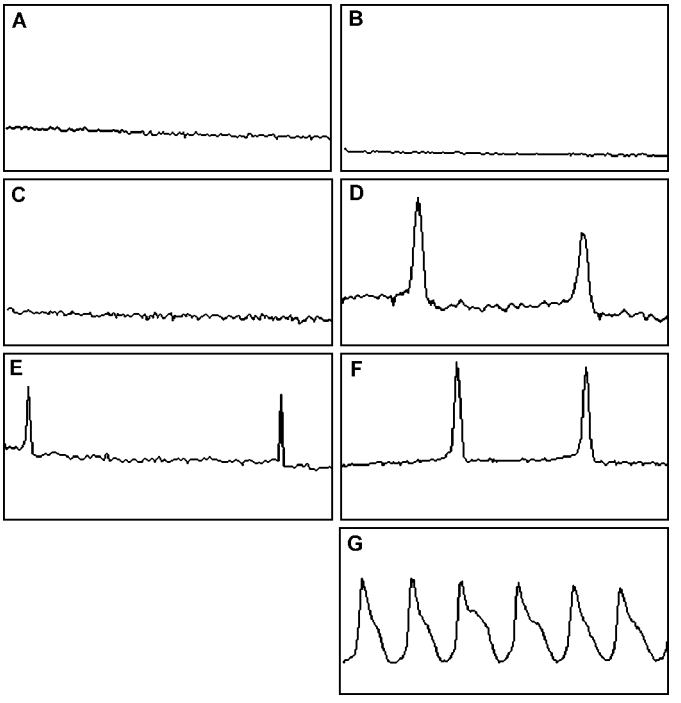

Fig. 2. Graphs representing the Ca^{2+} dynamics recordings of individual representative oocytes in a 5 min period from each group analysed. *Values in X axis are expressed in arbitrary units.* **(A,C,E)** *Recordings from the unstimulated juvenile, prepubertal and adult animal groups respectively.* **(B,D,F)** *Recordings from the PMSG-stimulated juvenile, prepubertal and adult animal groups respectively.* **(G)** *Recording of one oocyte from the positive control group (fully grown GV oocytes recovered from antral follicles in PMSG-stimulated adults).*

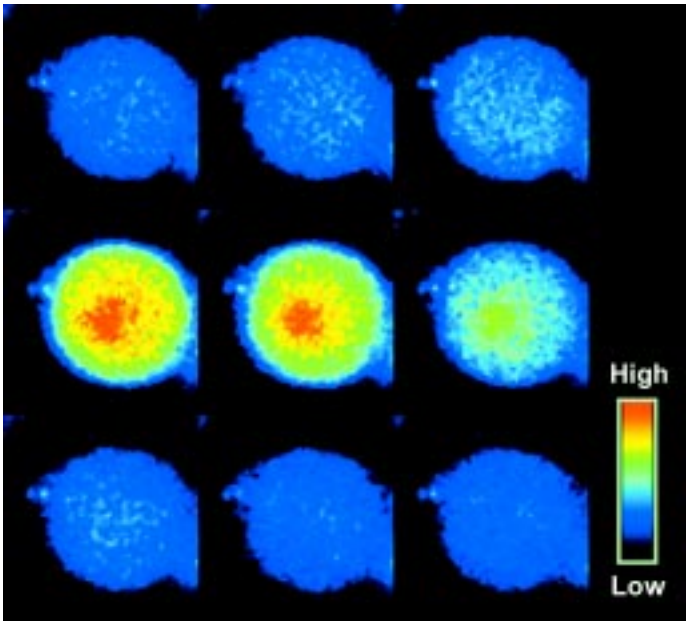

Fig. 3. Confocal sequence of images of an oocyte recovered from a preantral follicle exhibiting a Ca^{2+} spike. *Oocytes were loaded with fluo-3 AM for image acquisition.*

immature or from adult mice. These results are of relevance since the preantral follicular stage and animal ages used were comparable to the ones used in this study, and FSH is shown to be involved in this age-dependent phenomenon.

One of two mechanisms could be considered to explain the age-dependent acquisition of gonadotropin responsiveness in early preantral follicles that we have found. One possibility is a direct action of PMSG on the preantral follicle granulosa cell compartment, which would then act on the oocyte. A second possibility is a more indirect mechanism by which the oocyte responds to local regulatory factors produced by further advanced follicles under gonadotropin stimulation. This scenario is only possible in prepubertals and adults, and fits better our results.

This work is the first to establish a connection between age, gonadotropin stimulation and early preantral follicle oocyte Ca^{2+} dynamics. Specifically, we were able to identify a particular stage of follicular development (2-3 granulosa cell layers) where different combinations of gonadotropins and age elicit different oocyte behaviour. We propose that a strictly morphological staging of follicular development is insufficient to predict oocyte behaviour and must take in consideration animal age and gonadotropin environment. Adult animals seem thus more adequate for the study of oogenesis and folliculogenesis, particularly if high quality oocytes are to be obtained. When adult models cannot be used, the most adequate gonadotropin stimulation should be chosen.

TABLE 1

VALUES FROM THE LOGISTIC REGRESSION TEST OF THE PERCENTAGES OF OOCYTES EXHIBITING CA^{2+} SPIKES

Effect	Level	Odds ratio	p
Age	Juvenile (12 days old)	–	
	Prepubertal (24 days old)	6.50	<0.0001
	Adult (2-3 months old)	135.62	
PMSG	with	–	0.007
	without	3.56	

Both animal age and PMSG stimulation significantly increased the proportion of oocytes taken from preantral follicles exhibiting Ca^{2+} spikes. Total number of oocytes analysed was 226.

Experimental Procedures

Animals

Experiments were done with F1 (C57Bl6JxCBA) juvenile (12 days old), prepubertal (24 days old) and adult (2-3 months old) female mice, with a minimum of 3 animals per experimental group. Animals were sacrificed by cervical dislocation. In gonadotropin stimulated experimental groups, animals received an intraperitoneal injection of 5 IU PMSG (Chronogest, Intervet International, Boxmeer, Holland) 44-48 h prior to sacrifice.

Media and reagents

The medium used for oocyte processing was M2 (Fulton and Whittingham, 1978) supplemented with 4 mg/ml bovine serum albumin (BSA, fraction V; Sigma, St Quentin Fallavier, France). The fluorescent Ca^{2+} indicator was fluo-3/acetoxymethyl-ester (fluo-3/AM; Molecular Probes Inc., Eugene, OR, USA), which was stored at -20°C diluted (1 mM) in a 20% Pluronic F-127 solution in DMSO (Molecular Probes Inc.).

Isolation of preantral follicles and oocytes

Ovaries were removed and dissection of preantral follicles was performed as described elsewhere (Gomes *et al.*, 1999). Preantral ovarian follicles with 2-3 granulosa cell layers, no signs of isolation damage, atresia or oocyte abnormalities were selected. Oocytes were then mechanically released from the follicles with fine 25 gauge sterile needles. The remaining granulosa cells were carefully removed by repeated aspirations through a fine glass pipette. All procedures were performed in M2 medium, in plastic Petri dishes, under a Leica MZ12 stereo microscope, at 37°C. Oocytes were then further processed for calcium imaging within less than 30 min. All oocytes analysed were at the GV stage. Follicle and oocyte diameters (excluding theca layer or zona pellucida, respectively) were measured through the use of the length profile utility of the Bio-Rad Comos software, and expressed as the mean of two perpendicular measurements.

Fluorescent Ca^{2+} indicator loading of the oocytes

The cell-permeant Ca^{2+} indicator fluo-3/AM was dissolved to a final concentration of 5 μM in M2 medium. Oocytes were placed into a drop of the fluo-3/AM solution, under mineral oil (Sigma) and were incubated in the

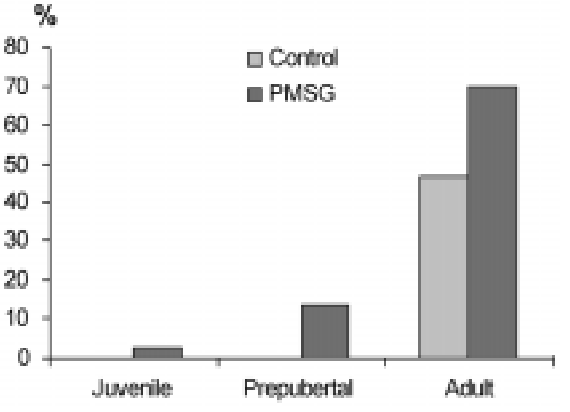

Fig. 4. Graph depicting the percentages of oocytes exhibiting Ca^{2+} spikes per experimental group. *The augmentation in these percentages observed with increasing age and with PMSG stimulation is statistically significant.*

dark for 15 min at 37°C. They were then rinsed and placed in a fresh M2 medium drop, under mineral oil, in a cell-culture chamber (Perfusion Open Closed chamber, Helmut Saur, Reutlingen, Germany) for subsequent confocal microscope observation.

Confocal microscopy of living oocytes

All measurements of fluorescence emission were performed using a Bio-Rad MRC600 confocal laser scanning imaging system interfaced with a Nikon Diaphot inverted microscope. To optimise cell viability, the 25 mW argon-ion laser was set at half power. The beam was further attenuated by means of a 1% transmittance neutral density filter and filtered through a blue high sensitive filter (BHS, Bio-Rad). To perform time-lapse calcium imaging studies, dye-loaded oocytes were viewed through a 20X objective (Fluor 20/0.75) at a single optical plane through the germinal vesicle. Calcium kinetics images of the oocytes were monitored continuously for up to 20 min with a 2-3 sec interval between two consecutive acquisitions. Full screen sequential digitised images were recorded for analysis, and a time-course curve of calcium indicator fluorescence emission was constructed using the Time Course Software for ratiometric Measurement (TCSM, Bio-Rad). Fluorescence intensity was measured in rectangles of similar areas (about 200 pixels) positioned in the oocyte cytoplasm. On these kinetic curves, data are expressed in arbitrary units uncalibrated in terms of calcium concentration. For further imaging processing Confocal Assistant 3.0 (Bio-Rad) and Adobe PhotoShop 5.0 were used.

Data collection and statistics procedure

Oocyte diameters were compared using the ANOVA Test. The proportion of oocytes generating Ca^{2+} spikes was calculated for each group, and results were compared by the Logistic Regression Test using the Intercooled Stata software. Differences were considered significant at $p<0.01$.

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