

### An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes

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#### Abstract

Reinitiation of meiosis in meiotically competent, fully grown mammalian oocytes is governed by a fall in intraoocyte cAMP concentrations and the subsequent inactivation of protein kinase A (PKA). A similar reduction in intraoocyte cAMP concentrations in growing, meiotically incompetent rat oocytes not leading to resumption of meiosis, questions the involvement of PKA in the regulation of meiosis at this early stage of oocyte development. We examined the possibility of whether PKA activity maintains growing oocytes in meiotic arrest and further explored the mode of activation of PKA under conditions of relatively low cAMP concentrations. Our experiment demonstrated that inactivation of PKA stimulates growing rat oocytes to resume meiosis, and elevates the activity of their maturation-promoting factor (MPF). We also found that the expressions of type I and type II regulatory subunits (RI and RII) of PKA are higher in growing and fully grown oocytes, respectively. In addition, we revealed that the common 1:1 ratio between the regulatory (R) and catalytic (C) subunits of PKA is apparently not abrogated and, in accordance PKA activity in growing oocyte - cell extract is fully dependent on cAMP. Finally, we identified in growing oocytes, the A kinase anchoring protein (AKAP) 140, which was previously depicted in fully grown oocytes. We conclude that an active PKA prevents growing oocytes from resuming meiosis. Our findings further suggest that relatively high abundance of the PKA I isoform and/or its subcellular compartmentalization, through interaction with AKAP140, could possibly account for the high basal PKA activity at relatively low intraoocyte cAMP concentrations.

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#### Introduction

Meiosis of mammalian oocytes is initiated during embryonic life, proceeds up to the diplotene of the first prophase and is arrested at this stage around birth. Meiotically arrested oocytes are characterized by the presence of diffused chromosomes surrounded by an intact nuclear membrane termed 'germinal vesicle' (GV). This stage corresponds to the G2 phase of the cell cycle. Resumption of meiosis, also known as oocyte maturation, represents the transition from G2 to M phase and involves condensation of the chromosomes, dissolution of the nuclear membrane referred to as germinal vesicle breakdown (GVB) and formation of the first metaphase (MI) spindle. The first meiotic division is completed by the emission of the first polar body (PBI) and is immediately followed by the second metaphase (MII). Resumption of meiosis takes place in sexually mature females at each reproductive cycle, in response to the preovulatory surge of luteinizing hormone (LH). However, oocyte maturation

in mammals can also occur spontaneously upon removal of the oocytes from the ovarian follicle (reviewed by Dekel 1995).

Spontaneous maturation cannot be observed in oocytes that are isolated from female mice, hamster and rats younger than 15, 23 and 22 days postpartum, respectively (Szybik 1972, Iwamatsu & Yanagimachi 1975, Bar-Ami & Tsafriri 1986). Furthermore, the ability to resume meiosis spontaneously in a sexually mature female is not shared by all the ovarian oocytes (Balakier 1978, Harrouk & Clarke 1995), but acquired progressively during oocyte growth (Christmann *et al.* 1994, Chesnel & Eppig 1995a). Accordingly, oocytes that are incompetent or competent to resume meiosis are referred to as growing or fully grown, respectively (Iwamatsu & Yanagimachi 1975, Sorensen & Wasserman 1976).

A key regulator of the G2 to M phase transition is maturation-promoting factor (MPF), a complex of a cyclin-dependent kinase, p34cdc2, and cyclin B. The binding of cyclin B to p34cdc2 forms the pre-MPF

complex, the activation of which is achieved by dephosphorylation on Thr-14 and Tyr-15 of p34cdc2. The activity of MPF is elevated in oocytes upon reinitiation of meiosis, reaches a maximal level at MI and then decreases prior to PBI emission. MPF is reactivated upon entry into the second meiotic division and remains highly active until fertilization (Choi, *et al.* 1991, Zernicka-Goetz *et al.* 1997, Josefsberg & Dekel 2002).

Experimental manipulations, initially performed in mouse oocytes, have shown that high intraoocyte cAMP levels prevent spontaneous resumption of meiosis (Cho *et al.* 1974). This view has been reinforced by the finding that LH-induced resumption of meiosis in mouse (Schultz *et al.* 1983) and rat (Dekel *et al.* 1984) is associated with a decrease in intraoocyte cAMP concentrations. It has been later proposed that rat oocytes do not produce inhibitory levels of cAMP and that the cyclic nucleotide is provided by the somatic follicular cells, via diffusion through gap junctions (Dekel 1988a). In contrast to this approach, it was recently demonstrated that meiotic arrest in mice is maintained by the oocytes Gs protein (Mehlmann, *et al.* 2002, 2004, Mehlmann 2005) and that rat and mouse oocytes express adenylyl cyclase 3 (Horner *et al.* 2003) that apparently contributes to the control of intraoocyte cAMP levels. Regardless to its cellular origin, there is no doubt that a reduction in intraoocyte cAMP level is a prerequisite for oocyte maturation.

The linkage between cAMP and the downstream biochemical events that govern cell division is provided by the cAMP-dependent protein kinase A (PKA). The fact that an active PKA is responsible for the maintenance of fully grown oocytes in prophase arrest has been initially demonstrated in amphibians (Maller & Krebs 1977), and later confirmed for mammalian oocytes (Bornslaeger *et al.* 1986). However, no such information is presently available with regard to growing oocytes. Furthermore, the phospho-proteins which serve as a substrate for PKA remained unknown and the downstream events subjected to regulation by the cAMP/PKA signal transduction pathway remain unidentified. Nevertheless, it has been shown that MPF activation at the onset of meiosis is dependent on the reduction in intraoocyte cAMP (Josefsberg & Dekel 2002).

PKA is a serine/threonine kinase, composed of two catalytic subunits (C) held in an inactive state in association with a regulatory subunit (R) dimer. The binding of two cAMP molecules to each regulatory subunit allows the catalytic subunits to dissociate and results in phosphorylation of substrate proteins. Two major isoenzymes of PKA, type I and type II containing RI and RII respectively, were depicted (Corbin *et al.* 1975, Spaulding 1993). These two isoenzymes, PKAI and PKAII differ in their cAMP responsiveness and in particular, subcellular localization. The RI isoforms are predominantly diffuse in the cytoplasm and are more sensitive to cAMP, whereas the RII isoforms are localized

in cellular organelles and less responsive to cAMP signaling (Chen *et al.* 1997, Keryer *et al.* 1999, Skalhegg & Tasken 2000). We found that fully grown oocytes in rats express both regulatory subunits of PKA and further demonstrated their cellular translocation upon resumption of meiosis (Kovo *et al.* 2002).

As mentioned, reinitiation of meiosis in fully grown oocytes is associated with a fall in intraoocyte cAMP concentrations, an initial step in a cascade of events leading to oocyte maturation. However, a comparable decrease in intraoocyte cAMP concentrations taking place in rat-growing oocytes does not lead to resumption of meiosis (Goren *et al.* 1994). These findings suggest that meiosis in growing oocytes is independent of regulation by cAMP and question the role of PKA in maintaining meiotic arrest at this early stage of oocyte development. Furthermore, the possible involvement of PKA in the negative control of meiosis in growing oocytes raises some intriguing questions regarding the mode of regulation of its activity at basal cAMP concentrations. Herein, we provide experimental evidence that meiotic arrest in rat-growing oocytes is maintained by an active PKA. We suggest that the relatively high abundance of PKAI in growing oocyte and/or its subcellular compartmentalization by a PKA anchoring protein (AKAP) may account for the high basal activity under conditions of relatively low cAMP concentrations.

## Materials and Methods

### Oocytes collection and culture

Experiments with animals were performed in accordance with the United States National Academy of Science legal requirements. Ovaries from sexually immature 19-day-old Wistar female rats were the source of growing incompetent oocytes (Goren *et al.* 1994). The oocytes were isolated from ovaries containing homogenous population of preantral follicles. Twenty-four-day old Wistar female rats, treated with 10 IU of pregnant mares serum gonadotropin (PMSG, Sanofi Sante Nutrition Animale, France) for induction of follicular development and sacrificed 48 h later, were used for the recovery of fully grown oocytes that were isolated from large antral follicles. The ovaries of the above-mentioned rats were removed and placed in Leibovitz's L-15 tissue culture medium (Gibco), supplemented with 5% fetal bovine serum (Biolab, Jerusalem, Israel), penicillin (100 IU/ml) and streptomycin (100 µg/ml, Gibco). When isolated from the ovarian follicles, the oocytes were arrested at the first prophase. Growing oocytes remain meiotically arrested for at least 24 h of incubation. To maintain meiotic arrest in fully grown oocytes, the phosphodiesterase inhibitor, isobutylmethylxantine (IBMX) that prevents cAMP degradation (0.2 mM, Sigma), was included in the medium of incubation (Dekel 1988b). The follicles were punctured under a

stereoscopic microscope in order to release the cumulus-oocyte complexes. The cumulus cells were removed by repetitive pipeting after collagenase (Sigma) treatment (50 IU/ml, 30 min, 37 °C). Denuded oocytes were incubated in the above medium in a 37 °C humidified incubator. Oocytes were examined for maturation by differential interference contrast (DIC) microscopy. In the presence of GV, the oocytes were classified as meiotically arrested. Following approximately 4 h of incubation in IBMX-free medium, fully grown oocytes resumed meiosis, as indicated by GVB.

### Oocyte culture

The collected oocytes were incubated with or without the following agents: PKA inhibitors H-89 [*N*-(2-((*P*-Bromo-cinnamyl) amino)ethyl]-5-isoquinolinesulfonamide, 2HCl], H-7 [1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, 2HCl], and H-8 [*N*-(2-(Methylamino)ethyl]-5-isoquinolinesulfonamide, 2HCl] (Calbiochem, San-Diego, CA, USA).

### PKI microinjection

The oocytes were transferred into 10 µl drops of L-15 tissue culture medium (Gibco), supplemented with 5% fetal bovine serum (Biolab, Israel), under 3.5 ml of paraffin oil in a 35 mm 1006 Falcon Petri dish placed on a heated (37 °C) stage of an inverted (Nikon, Diaphot, Tokyo, Japan) microscope equipped with DIC optics. Microinjections were performed using a threeD motor coarse control micromanipulator (Narishige, McHenry, IL, USA), equipped with IM-I88 and IM-6 microinjectors connected to a holding (1.0 and 0.75 mm outer diameter (OD) and inner diameter (ID) respectively) and injecting (3–5 and 2–4 µm OD and ID respectively) glass pipettes (Humagen Fertility Diagnostics, Charlottesville, VA, USA). Following microinjection (10 pl of PKA inhibitor, PKI, 6.0 mg/ml Calbiochem, USA), the oocytes were rinsed and incubated in the above medium in a 37 °C humidified incubator. Oocytes microinjected with buffer solution (5 mM MES, pH=6.5 Calbiochem, USA), as well as noninjected oocytes served as controls. Oocytes were examined for maturation after 14 h of incubation by DIC microscopy, as described above.

### Immunofluorescent analysis

Oocytes were fixed with 3% *p*-formaldehyde in PBS, followed by their permeabilization with 1% Triton X-100, and immunostained with monoclonal anti-RI and anti-RII antibodies (1:150), followed by incubation with Cy3-conjugated AffiniPure donkey anti-mouse antibodies (1:300, Jackson Immunoresearch Lab. Inc., West Grove, PA, USA). For DNA staining, DAPI (4', 6'-diamidino-2-phenylindole) was added.

The oocytes were visualized by a laser confocal microscope (BioRad, Radiance 2000/AGR-3 Confocal Imaging System connected to microscope Zeiss, Axiovert 100/TV).

### Western immunoblotting

Oocytes were lysed in a boiling sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol). The samples were subjected to 10% SDS-PAGE, followed by a transfer to a polyvinylidene difluoride (PVDF) membrane. After blocking (5% dried nonfat milk in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20), the regulatory (RI and RII) and the catalytic (C) subunits were detected by incubating the membranes overnight at 4 °C with polyclonal anti-RI (1:1000), RII (1:1000) and C (1:2000) antibodies, previously characterized (Chestukhin *et al.* 1996) and kindly provided by the late Prof. Shmuel Shaltiel (The Weizmann Institute of Science, Rehovot, Israel), or with monoclonal anti-RI (1:250), RIIα (1:250) and C (1:1000, Transduction Labs, BD Biosciences, San Jose, CA, USA) antibodies. Exposure of the PVDF membrane to the above-mentioned antibodies was followed by TTBS washes and further incubated for 1 h with horse radish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse or goat anti-rabbit 1:2000, Jackson Immuno Research Labs Inc., USA). When indicated, the PVDF membrane was exposed to the first antibody that had been preincubated with the relevant recombinant peptide to confirm specificity. The immunoreactive proteins were detected by ECL (Amersham).

### MPF activity – H1 kinase assay

Histone H1 is routinely used as a substrate to assess MPF activity (Fulka *et al.* 1992). MPF activity was determined in lysates of 65 growing or 50 fully grown oocytes, frozen and then thawed in 10 l kinase buffer (15 mM MOPS, 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM MgCl<sub>2</sub>, 0.1 mM PMSF, 10 µg/ml leupeptine, 10 µg/ml aprotinin, 10 µg/ml PKI, a cAMP-dependent protein kinase inhibitor (PKI) peptide). Kinase reactions were initiated by adding 10 µl of substrate buffer, 2 mg/ml histone H1 (Sigma), 2 mM dithiothreitol (DTT), 5 µCi [<sup>32</sup>P] ATP, and incubated at 30 °C for 30 min. Kinase reaction products were subjected to SDS-PAGE and autoradiography.

### PKA activity assay

The activity of PKA was tested in a cell-free reaction system using vitronectin (Vn) as a substrate. The assay was conducted as previously described (Korc-Grodzicki *et al.* 1990, 1988). Oocytes were lysed in a buffer containing 50 mM β-glycerophosphate, 1 mM NaF

1.5 mM EGTA, 1% NONIDET P-40, 1 mM EDTA, 0.1 mM PMSF, 10 µg/ml leupeptine, 10 µg/ml aprotinin and 1 mM DTT. Phosphorylation was carried out in a final volume of 50 µl containing 50 mM HEPES (pH 7.5), 10 mM MgCl<sub>2</sub> and 0.5 µg Vn (kindly provided by the late S Shaltiel in our department). Phosphorylation was initiated by the addition of 10 µM [ $\gamma$ -<sup>32</sup>P] ATP (6 Ci/mmol). The reaction was allowed to proceed for 20 min at room temperature (RT) and arrested by the addition of sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2%  $\beta$ -mercaptoethanol) and boiled for 3 min at 95 °C. The samples were subjected to 10% SDS-PAGE and autoradiography. The cAMP dependency of this phosphorylation was established by the assay, performed in the absence and in the presence of cAMP (10 mM) and PKA specificity was determined by the addition of the specific PKA inhibitor PKI (Walsh *et al.* 1971) (30 µM). Phosphorylation of Vn by the C subunit of PKA, purified from rabbit skeletal muscle (5 mg/ml), served as a positive control.

### RII overlay procedure

The overlay procedure is a modified Western blot analysis (Carr & Scott 1992). Oocyte proteins were separated on SDS-PAGE and transferred to Immobilon. After blocking with 5% skimmed dry milk to prevent nonspecific binding, the nitrocellulose membrane was probed with radiolabeled RII $\alpha$ . Recombinant RII $\alpha$  was radiolabeled by incubation with the catalytic subunit of PKA and <sup>32</sup>P-ATP. After separation from free <sup>32</sup>P-ATP, the <sup>32</sup>P-RII (500 000 c.p.m./10 ml Blotto) was incubated with the blot for 4 h, followed by washing and autoradiography.

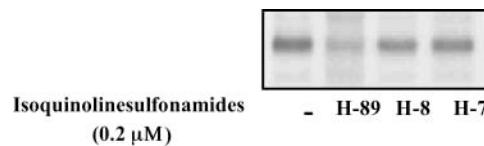
### Statistical analysis

Statistical significance (Fisher's protected least significance difference (LSD)) was determined by the ANOVA to assess differences between multiple experimental groups.

## Results

### Effect of PKA inhibitors on meiosis in growing oocytes

Meiotic arrest in growing oocytes that is independent of regulation by cAMP, could possibly be maintained by a constitutively active PKA. In order to explore this possibility, growing oocytes were incubated with H-89, an isoquinolinesulfonamide derivative that binds PKA with a relatively high affinity ( $K_i=0.05$  µM), inhibiting its action (Chijiwa *et al.* 1990). Other isoquinolinesulfonamide derivatives with lower affinities towards PKA (H-7 and H-8,  $K_i=3.0$  and 2.3 µM respectively, Hidaka *et al.* 1984) served as negative controls. Our initial experiment



**Figure 1** PKA phosphorylation assay in growing oocytes. Extracts of growing oocytes (110 oocytes per lane) were subjected to a PKA phosphorylation assay in the presence of cAMP without or with 0.2 µM of either H-89 or the other indicated isoquinolinesulfonamides. The result of one representative experiment out of three repetitions is shown.

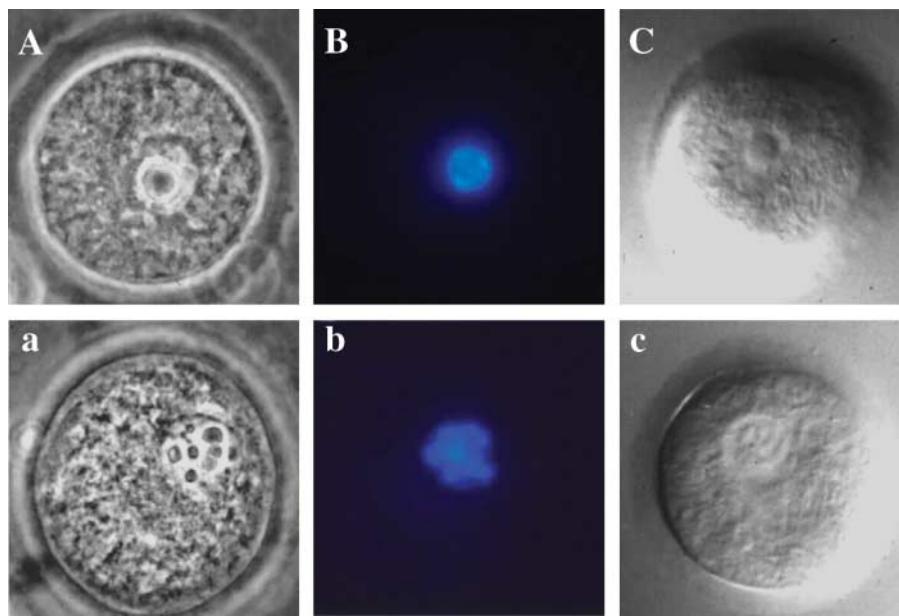
confirmed the inhibitory effect of H-89 on PKA activity in cell extracts of growing oocytes, whereas a relatively lower inhibition was exhibited by the other isoquinolinesulfonamides employed in this study (Fig. 1).

If indeed meiotic arrest in growing oocytes is maintained by an active PKA, inhibition of its activity would induce reinitiation of meiosis. In order to test this assumption, oocytes morphology was examined microscopically after an overnight incubation with or without the different isoquinolinesulfonamide derivatives. As expected, we found that the growing oocytes incubated in an inhibitor-free medium displayed an intact GV (Fig. 2A, C), whereas H-89 induced dissolution of the nucleolus and condensation of the chromatin (Fig. 2A, C). Fluorescent DNA staining confirmed these findings (Fig. 2B). The effect of H-89 on oocytes morphology was dose-dependent, with an ED<sub>50</sub> at 4 µM (Fig. 3A). The maximal effective dose (10 µM) induced chromatin condensation in 58% of the oocytes. No such effect could be demonstrated by similar or even higher concentrations of the isoquinolinesulfonamide derivatives that exhibit lower affinity towards PKA (Fig. 3B).

Complementary support for our hypothesis was provided by the use of the PKI that binds to the dissociated catalytic subunit of PKA inhibiting its activity (Walsh *et al.* 1971). PKI (10 pl of a 6 mg/ml solution) was microinjected into growing oocytes that were microscopically examined 14 h later to assess their meiotic status. This experiment revealed that inhibition of PKA induces GVB in 63.3±3.2 of the growing oocytes. Oocytes that were either injected or not injected with a buffer solution exhibited 36.15±0.65 and 20.5±15.6% GVB, respectively (Table 1).

### Effect of PKA inhibitors on MPF activity in fully grown and growing oocytes

As mentioned in the Introduction, the key regulator of reinitiation of meiosis controlling the G2 to M-phase transition is MPF, the activity of which is monitored by the histone H1 kinase activity assay (Fulka *et al.* 1992). In fully grown oocytes MPF activation, associated with reinitiation of meiosis, can be experimentally prevented by elevating intraoocyte cAMP concentrations (Josefsberg *et al.* 2003). In order to evaluate the mediatory role



**Figure 2** The effect of H-89 on growing oocytes. Growing oocytes were incubated in the absence (A, B, C) or the presence (a, b, c) of H-89 (10  $\mu$ M) for 24 h. At the end of incubation, the oocytes were either immediately monitored by DIC (C, c) or have their DNA stained for examination by fluorescent microscopy (B, b). The pictures demonstrate an intact GV and a nucleolus containing diffuse chromatin in the control group (A, B, C). Treatment with H-89 induced dissolution of the nucleolus and condensation of the chromatin (a, b, c).

of PKA in the inhibitory effect of cAMP on MPF activity, we incubated fully grown oocytes for 24 h with the phosphodiesterase-inhibitor isobutylmethylxantine (IBMX 0.2 mM) that prevents cAMP degradation along with H-89. We found that the inhibitory effect of IBMX on histone H1 kinase activity was clearly reversed by H-89 (Fig. 4A).

Taking these findings into account, we assumed that if growing oocytes indeed possess a persistently active PKA, no activation of MPF would occur in spite of the relatively low cAMP concentrations (Goren *et al.* 1994). We further anticipated that these conditions could be reversed by H-89. In order to evaluate this possibility, we performed the histone H1 kinase activity assay on growing oocytes incubated with or without H-89 at different time points after their removal from the ovarian follicle. We found that the constant, relatively low activity of MPF in growing oocytes throughout the first 8 h of incubation was substantially elevated by H-89 (Fig. 4B, C). This extent of elevation was comparable to that previously described for mice (Choi *et al.* 1991) and rat (Josefsberg & Dekel 2002), just prior to GVB.

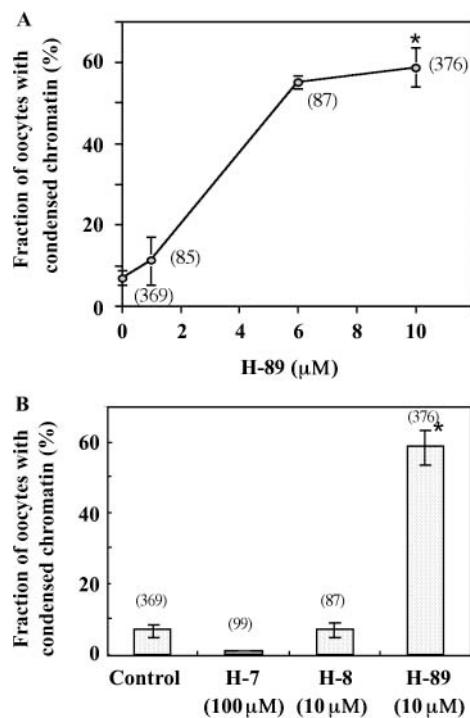
#### Expression of PKA subunits in growing oocytes

Fully grown oocytes that exhibit a cAMP-regulated PKA activity express the common 1:1 C to R ratio. Alternatively, high basal activity of PKA in growing oocytes may reflect abrogation of this ratio with a relatively lower level of R expression that leads to the presence of a free C subunit. Immunostaining demonstrated that growing oocytes express clusters of C, as well as both RI and RII subunits of PKA, homogenously distributed in the ooplasm (Fig. 5). Densitometric quantification of the

results obtained by a western blot analysis (Fig. 6A), followed by normalization of total R (RI and RII) against C (Spaulding 1993) in each oocyte sample, could not demonstrate a difference in the R to C ratio in growing, as compared to fully grown oocytes (Fig. 6B). Interestingly, the level of expression of each of the two major isoforms of the R subunit, RI and RII in growing and fully grown oocytes is clearly different. Specifically, the expression of RI is clearly higher in growing, as compared to fully grown oocytes. On the other hand, oocytes that reached their final size, express higher levels of RII, than that observed in growing oocytes. High abundance of PKAI may account for a constitutive PKA activity under conditions of relatively low cAMP concentrations.

#### Analysis of PKA activity in growing oocytes

Under conditions with similar abundance of C and R, constitutive PKA activity in growing oocytes could be explained by compartmentalization of the enzyme at distinct cellular loci. Nonetheless, if this is indeed the case, destruction of the cellular compartments should exhibit PKA activity that is fully dependent on cAMP. The dependency of PKA activity on cAMP in oocyte cell extracts was determined using a PKA phosphorylation assay. PKI, the specific PKA inhibitor (Walsh *et al.* 1971), was employed in this experiment to confirm specificity of the phosphorylation reaction. Figure 7 demonstrates that the activity of PKA in cell-free system preparations of growing and fully grown oocytes is, indeed, fully dependent on cAMP. These results support our previous findings that the abundance of the C subunit in growing oocytes is not higher than that of the R subunit of PKA.



**Figure 3** (A) The effect of H-89 on growing oocytes: a dose response curve. Growing oocytes incubated with the indicated concentrations of H-89 for 24 h were monitored by DIC microscopy. The fraction of oocytes showing disappearance of the nucleolus and chromatin condensation was calculated for each experimental point. The total number of oocytes examined for each experimental point is indicated in parenthesis. The data represent the mean  $\pm$  S.E.M. of the results obtained in at least three individual experiments. Significantly different values ( $P < 0.05$ ) are marked by an asterisk. (B) The effect of different isoquinolinsulfonamides on growing oocytes. Growing oocytes incubated with H-7, H-8 and H-89 at the indicated concentrations for 24 h were monitored microscopically for disappearance of the nucleolus and chromatin condensation. The total number of oocytes in each experimental group is indicated. The data represent mean  $\pm$  S.E.M. of the results obtained in at least three individual experiments. Significantly different values ( $P < 0.05$ ) are marked by an asterisk.

### Growing oocytes express AKAP140

Compartmentalization of PKA at distinct cellular loci could possibly account for the high basal PKA activity in growing oocytes at relatively low intraoocyte cAMP concentrations. Expression of AKAP140 has been previously demonstrated in fully grown oocytes of rat

**Table 1** Growing oocytes were microinjected with PKI (10 pl of a 6 mg/ml solution) or buffer solution. Noninjected oocytes served as an additional control. Oocytes were examined for maturation after 14 h of incubation by differential interference contrast (DIC) microscopy. The percentage of oocytes undergoing GVB is presented. Mean  $\pm$  S.E.M. of the results obtained are presented. The value obtained by PKI injections is significantly different ( $P < 0.05$ ) from the control groups.

Material injected	PKI	Buffer solution	None
Total no. of oocytes	106	83	109
GVB (%)	$63.3 \pm 3.2$	$36.15 \pm 0.65$	$20.5 \pm 19.6$

(Kovo *et al.* 2002) and mouse (Brown *et al.* 2002). We further employed the RII overlay procedure (Carr *et al.* 1991, Carr & Scott 1992) to examine rat-growing oocytes for the presence of AKAPs. As illustrated in Fig. 8, similar to fully grown oocytes, growing oocytes express an AKAP 140. Since an equal number of oocytes was loaded, the higher intensity of the signal obtained from fully grown oocytes may represent either an enhanced level of AKAP 140 expression or a higher affinity of this AKAP to RII. On the other hand, the higher protein content in fully grown as compared to the growing oocytes reported in our previous study (Goren *et al.* 1994), could possibly contribute to the different signal intensities obtained in samples of equal oocyte numbers.

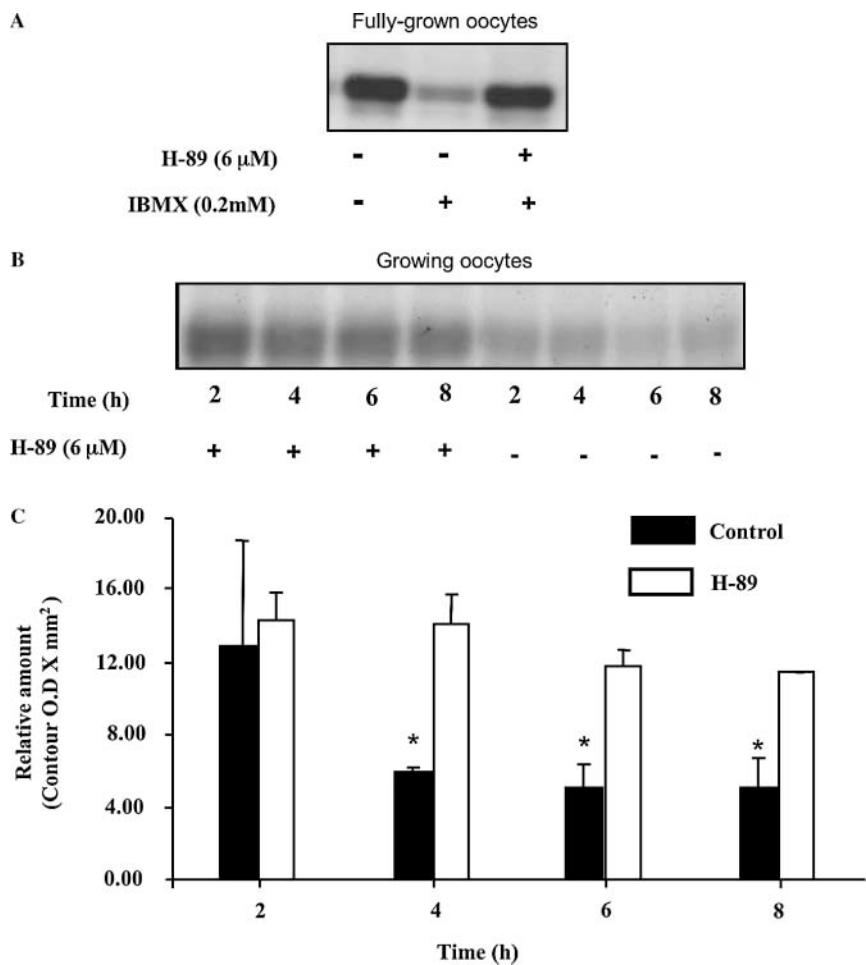
### Discussion

Our study provides experimental evidence that an active PKA prevents the resumption of meiosis in rat growing oocytes. We also demonstrate that PKA action maintains the MPF in its inactive state. The persistent state of PKA activation that apparently takes place at basal cAMP concentrations, cannot be attributed to a decreased R expression, and the subsequent release of the free C subunits. However, it may represent the relatively high abundance of PKA in growing as compared to fully grown oocytes. Tethering of PKA to a close proximity of its cellular substrate by AKAP140, could also possibly account for its high level of basal activity.

Inhibiting PKA activity in growing oocytes upon incubation with H-89, stimulated both chromatin condensation and nuclear membrane dissolution. Such an effect could not be obtained by the use of other isoquinolinsulphonamide derivatives with lower affinities to PKA. Specificity of H-89 inhibition towards PKA was further investigated by the use of PKI, the heat stable PKA inhibitor. Unlike a previous report in the mouse (Bornslaeger *et al.* 1988), rat-growing oocytes microinjected with PKI underwent GVB. Taken together, our results suggest that rat growing oocytes may possess a constitutively active PKA that controls their meiotic arrest.

MPF activation at the onset of meiosis in fully grown oocytes is conditioned to the reduction in intraoocyte cAMP (Choi *et al.* 1991, Gavin *et al.* 1991, Zernicka-Goetz *et al.* 1997, Josefsberg & Dekel 2002). Confirming previous reports in the mouse, we show herein that in rat growing oocytes, MPF activation did not occur for at least 24 h after their isolation from the ovarian follicles (Chesnel & Eppig 1995b, de Vantier Arrighi *et al.* 2000). However, our study further demonstrates that inhibition of PKA activity in these oocytes led to a substantial increase in MPF activity.

Four different regulatory subunits (RI $\alpha$ , RI $\beta$ , RII $\alpha$ , and RII $\beta$ ) and three catalytic subunits (C $\alpha$ , C $\beta$ , and C $\gamma$ ) have been identified as separate gene products (Skalhegg & Tasken 2000). Since PKA subunits are not coded by the same gene locus, there is a possibility that the level of



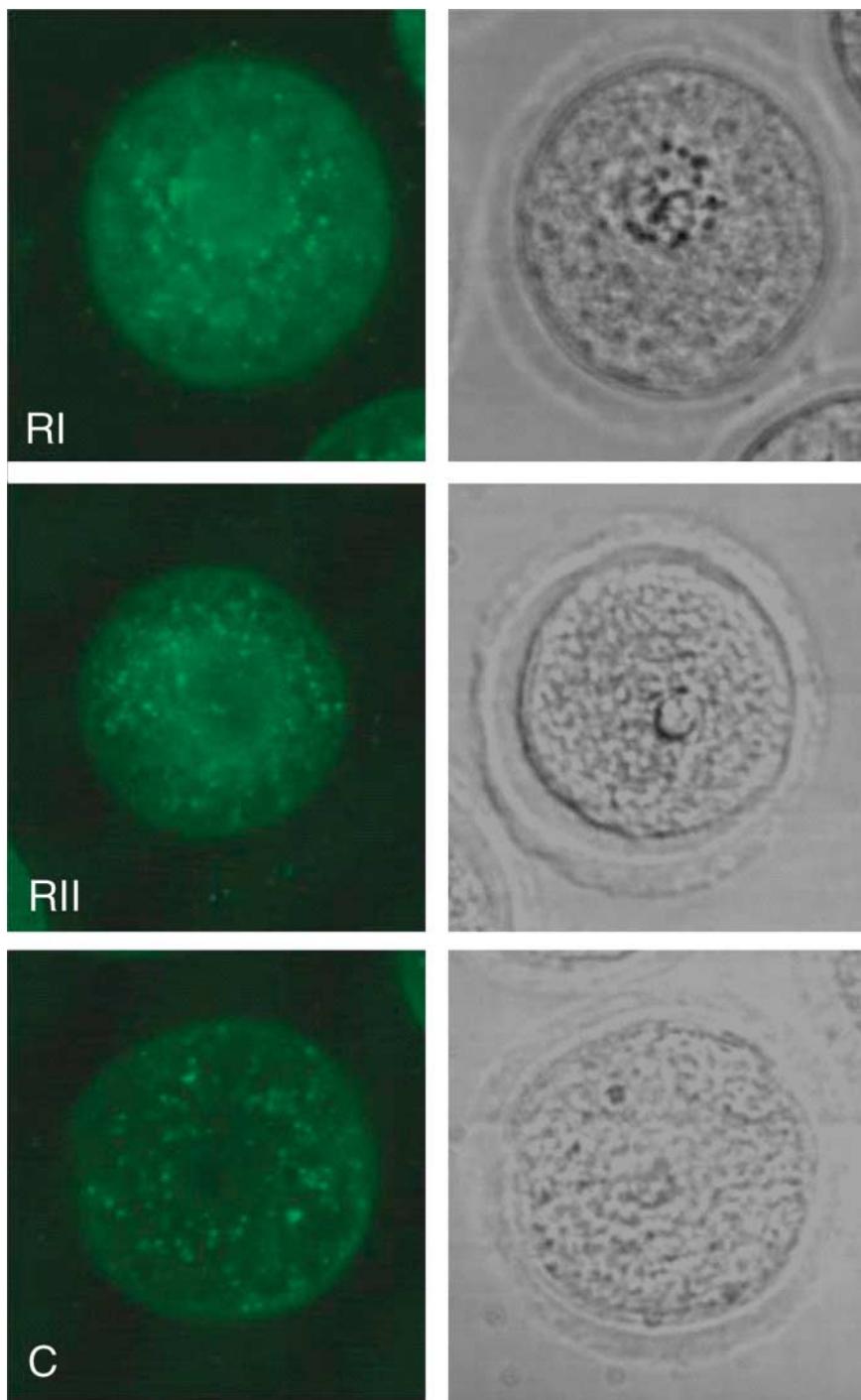
**Figure 4** MPF activity in growing and fully grown oocytes incubated with or without H-89. A. Fully grown oocytes incubated with or without IBMX (0.2 mM) and H-89 for 24 h were extracted and assayed for H1 kinase activity. B. Rat-growing oocytes were extracted at the indicated times of incubation in the presence or absence of H-89 (6  $\mu$ M) and assayed for H1 kinase activity. The result of one representative experiment out of three repetitions is shown C. Densitometric quantification of the results revealed that there was a significant difference in H1 kinase activity after 4 h of incubation with H-89.

regulatory and catalytic subunits, under some conditions, could be independently regulated (Corbin 1983). A selective expression of PKA subunits could be associated with major differences in the properties of PKA signal transduction and may generate a constitutively active PKA. Along this line, low levels of RI $\alpha$  in pancreatic  $\alpha$  cells that led to the presence of free catalytic subunit and therefore, high basal activity of PKA have been reported (Arava *et al.* 1998). Alternatively, this study further demonstrates that high levels of RI $\alpha$  expressed in a pancreatic  $\beta$  cell line produced low basal, but highly inducible catalytic activity of PKA. Another example is presented by the *Aplasia* sensory neurons. In these cells, PKA becomes persistently activated at basal cAMP concentration, due to a decreased R to C subunit ratio (Chain *et al.* 1995).

Taking this information into account, we explored the possibility that an abrogation in the common 1:1 ratio between the C and R subunits in growing oocytes provides the mechanism for the high basal activity of PKA. However, examination of the relative abundance of C and R subunits could not reveal, in growing oocytes, any deviation from the ratio present in fully grown oocytes. The absence of the free C subunit was further

confirmed by our failure to detect basal PKA activity in extracts of growing, as well as fully grown oocytes further demonstrating that enzyme activation is fully dependent on cAMP. Interestingly, we found that the levels of expression of the two major isoforms of the R subunit, RI and RII, in growing, were different than that in fully grown oocytes. Specifically, our experiments revealed that in fully grown oocytes, RII isoform exhibited a relatively higher level of expression than that in growing oocytes, whereas the RI subunit was more abundant in growing oocytes. Such distinct developmental changes in the expression of PKA subunits have been described previously in rat testes (Oyen *et al.* 1990). Specifically, whereas the RI $\alpha$ , RI $\beta$ , and C $\alpha$  subunits in male germ cells were expressed at premeiotic and meiotic stages, the expression of RII subunits could be detected only during the later stages of spermatid elongation (Oyen *et al.* 1990).

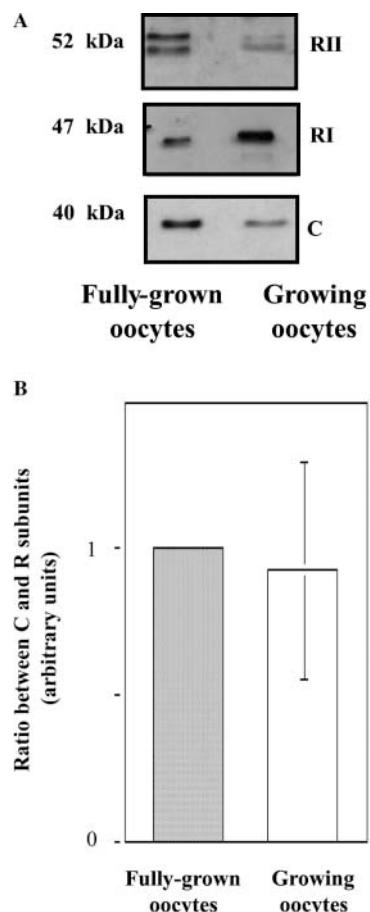
The two major isoenzyme PKAI and PKAII (containing RI and RII respectively) differ in their cAMP responsiveness. The RI isoforms that are predominantly diffuse in the cytoplasm are more sensitive to cAMP, whereas the RII isoforms are more localized in cells and less responsive to cAMP signaling (Downs & Hunzicker



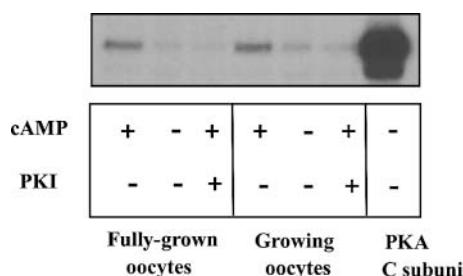
**Figure 5** Immunostaining of growing oocytes for the PKA subunits RI, RII, and C. Growing oocytes were collected and fixed as described in Materials and Methods. RI, RII and C subunits of PKA were detected using the relevant primary antibodies and a donkey anti-mouse Cy-3 secondary antibody. Phase contrast images (a, b, c) along with immunofluorescence (A, B, C) are presented.

1995, Skalhegg & Tasken 2000). Our results imply that growing oocytes express higher levels of the RI. Therefore, lower concentration of cAMP is apparently required to activate the PKA $\text{I}$  isoenzyme, which is more abundant in these oocytes. Taking these findings into account, we suggest that, in growing oocytes, low undersized amount of intraoocyte cAMP concentration left in special microenvironment would continue to

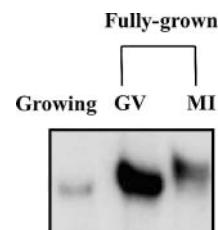
activate PKA $\text{I}$  after their removal from the ovarian follicle. As the oocytes grow, the relative expression of the two R isoforms is reversed, and in oocytes that reach their final size, RII becomes the dominant regulatory subunit. Since the affinity of this R isoform to cAMP is lower, higher intraoocytes cAMP concentrations are required to maintain the threshold level of PKA activity that would keep the oocytes in meiotic arrest. Separation



**Figure 6** Expression of the PKA subunits in growing and fully grown oocytes. (A) Extracts of growing and fully grown oocytes were separated by 12% SDS-PAGE followed by their transfer to a nitrocellulose membrane and incubation with anti RI, RII and C PKA subunits antibodies. Each lane contains 500 oocytes. The result of one representative out of three individual experiments is shown. (B) Densitometric analysis quantifying the absolute amounts of the R and C PKA subunits each, in growing and fully grown oocytes, was performed. The values obtained for total R (RI and RII) were normalized against those obtained for C. The C to R ratio in fully grown oocytes was defined as 1. No abrogation from this value is observed in growing oocytes ( $P=0.8$ ).



**Figure 7** PKA activity in extracts of growing and fully grown oocytes. Growing and fully grown oocytes (100 oocytes per lane) were subjected to a PKA phosphorylation assay in the presence or absence of cAMP or PKI. The result of one representative experiment out of four repetitions is shown.



**Figure 8** AKAP expression in rat-growing oocytes. Growing and fully grown oocytes (250 per sample) were collected. The fully grown oocytes were collected in IBMX-medium, maintaining them meiotically arrested. Their protein extracts were separated on SDS-PAGE, transferred to Immobilon and probed with  $^{32}\text{P}$ -RII $\alpha$  for RII overlay assay. The result of one representative experiment out of three repetitions is presented.

of these oocytes from the ovarian follicle, turns on the biochemical events that govern resumption of meiosis.

An alternative mechanism that may account for high basal PKA activity in growing oocytes could be provided by subcellular compartmentalization of the enzyme. This function is attributable to AKAPs that have been demonstrated to translocate the PKA holoenzyme by binding to its R subunit (Lohmann *et al.* 1984, Carr *et al.* 1991). In accordance with this idea, cellular localization of the R subunits demonstrated in growing oocytes is definitely different from that previously shown in fully grown rat and mouse oocytes (Brown *et al.* 2002, Kovo *et al.* 2002). Therefore, AKAP140 identified, herein, in growing oocytes could possibly take part in this developmentally regulated PKA translocation.

Collectively, we demonstrated that in growing, incompetent rat oocytes, an active PKA inhibits MPF activation and keeps the oocytes in a state of meiotic arrest. This persistent activity at basal concentrations of cAMP does not result from a decrease in the R to C subunit ratio. Our findings suggest that a high basal activity of PKA in growing oocytes may be maintained due to the relatively high abundance of PKA $\text{I}$ , which is more sensitive to low concentration of cAMP and/or to sequestration of the enzyme in close proximity to both its upstream and downstream effectors, possibly via AKAP140.

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