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Differential Regulation of Oocyte Maturation and Cumulus Expansion in the Mouse Oocyte–of Cyclic Adenosine Monophosphate

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In the present study, we have examined how differential distribution of cyclic adenosine 5′-monophosphate (cAMP)-dependent protein kinase isozymes within the mouse oocyte–cumulus cell complex might influence the physiological response of the complex to cAMP, by determining the actions of site-selective cAMP analogs on oocyte maturation and cumulus expansion. Five different analogs of cAMP were utilized: 8-thiomethyl-cAMP and 8-bromo-cAMP, which bind to site 1 on the type II regulatory subunit (RII) of cAMP-dependent protein kinase A (PKA); 8-aminohexylamino-cAMP, which binds to site 1 on the type I regulatory subunit (RI) of PKA; N^6-monobutyryl cAMP, which binds to site 2 on either RI or RII; and 8-piperidino-cAMP, which binds to either site 1 on RII or site 2 on RI. These analogs were tested alone or in paired combinations that synergistically activate either the type I or type II PKA isozyme. When tested alone, analogs that can bind to, and presumably activate, type I PKA were the most potent inhibitors of germinal vesicle breakdown (GVB) in both cumulus cell-enclosed and denuded oocytes. Consistent with this result was the finding that paired combinations of analogs that selectively activate type I PKA were also most effective in preventing GVB. On the other hand, pulsing meiotically arrested cumulus cell-enclosed oocytes with high concentrations of analogs that bind to PKA II, or with paired combinations of analogs that selectively activate type II PKA, led to induction of GVB; stimulation with analogs or combinations thereof that presumably stimulate type I PKA was less effective. Cumulus expansion in response to PKA stimulation showed similar selectivity in that type II PKA-stimulating treatments were considerably more effective in provoking expansion than type I PKA-stimulating treatments. 8-N_3-[^{32}P]cAMP photoaffinity labeling of PKA regulatory subunits revealed that only RI was present in oocyte extracts, while extracts from oocyte–cumulus cell complexes contained both RI and RII. These results support the hypothesis that type II PKA mediates cAMP-stimulated cumulus expansion and resumption of meiotic maturation, while direct elevation of type I PKA within the oocyte is instrumental in maintaining meiotic arrest.

INTRODUCTION

Fully grown, meiotically competent mammalian oocytes will spontaneously resume meiotic maturation, manifested as germinal vesicle breakdown, if isolated from the ovary and cultured *in vitro*. Supplementing culture medium with analogs of cyclic adenosine 5′-monophosphate (cAMP) or cAMP phosphodiesterase inhibitors or treatment with agents that stimulate an increase in cAMP production will suppress this meiotic response, maintaining the oocyte in prophase I arrest (reviewed by Tsafriri *et al.*, 1982; Eppig and Downs, 1984). Thus, cAMP has been implicated as an important negative regulator of germinal vesicle breakdown. Yet this cyclic nucleotide also has a positive role in oocyte maturation, as it is an important mediator of the gonadotropin-induced reinitiation of meiotic maturation in preovulatory follicles (Lindner *et al.*, 1977). A possible explanation for these contradictory actions of cAMP on oocyte maturation has been proffered by Dekel *et al.* (1988), in which the response of the gamete is dictated by the relative level of cAMP the oocyte is exposed to. Hence, sustained elevated levels of cAMP maintain oocytes in meiotic arrest, while a transient increase may trigger germinal vesicle breakdown (Dekel *et al.*, 1988). According to this hypothe-

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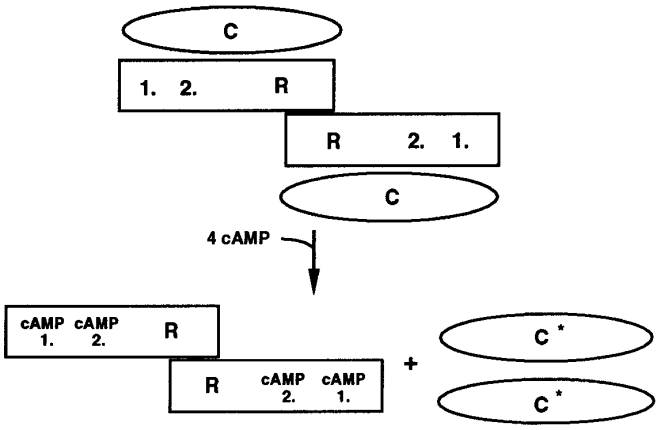

FIG. 1. Scheme for cAMP-dependent protein kinase (PKA) activation. The inactive holoenzyme is a tetramer, consisting of a dimer of regulatory subunits (R), each bound to one inactive catalytic subunit (C). Each R has two cAMP binding sites, designated site 1 and site 2. Upon binding of four cAMP molecules to the R subunits, the C subunits dissociate and become activated (C*).

sis, the magnitude of the change in cAMP may be the important stimulus for oocyte maturation, not necessarily the absolute level of cAMP reached in the cell.

When cAMP is absent, cAMP-dependent protein kinase, or protein kinase A (PKA), exists as an inactive tetramer, comprised of a dimer of regulatory subunits (R) bound to two catalytic subunits (C). cAMP acts by binding to R, which leads to the release of two active C monomers that phosphorylate proteins at serine and threonine residues (Fig. 1; recently reviewed by Spaulding, 1993; Francis and Corbin, 1994). There are two intrasubunit cAMP-binding sites on each R, sites 1 and 2 (Corbin *et al.*, 1978; Doskeland, 1978), and when both sites are occupied by cAMP, the affinity of R for C is dramatically reduced, leading to dissociation of the holoenzyme (Hemmings, 1975; see Fig. 1). Two major isozymes of PKA have been described in cells, types I and II, based on their elution positions from DEAE cellulose columns (Corbin *et al.*, 1975). Classification of the holoenzyme as type I or II is based on the presence of RI or RII subunits. In addition to their distinct ionic properties, RI and RII exhibit different affinities for cAMP and the catalytic subunits, and the holoenzyme state may be differentially activated by cAMP analogs (Beebe and Corbin, 1986).

Site-selective cAMP analogs are distinguished by their affinity for intrasubunit binding sites 1 or 2 on R. Binding of cAMP to R shows positive cooperativity, such that binding to site 1 stimulates binding at site 2 and vice versa (Rannels and Corbin, 1981; Robinson-Steiner and Corbin, 1982); this leads to positive cooperativity in PKA activation (Robinson-Steiner and Corbin, 1983). In addition, combining cAMP analogs specific for one binding site with analogs specific for the other binding site results in synergistic activation of PKA, as the kinase is activated to a level greater than the sum of the activities produced by single treatment with each of the analogs (Robinson-Steiner and Corbin, 1983; Ogreid and Doskeland, 1983; Beebe *et al.*, 1984). Further, by the proper combination of cAMP analogs, either type I or type II PKA can be selectively activated (Beebe *et al.*, 1988).

Different cAMP analogs do not always exhibit comparable potency in mediating cAMP-dependent cellular responses. The spontaneous maturation of mouse and rat oocytes, monitored as the breakdown of the germinal vesicle, is effectively suppressed by dibutyryl cAMP (dbcAMP), but 8-bromo-cAMP (8-Br-cAMP) has a very limited effect (Tornell *et al.*, 1984; Eppig, 1989). In the rat, dbcAMP prevents hormone-induced germinal vesicle breakdown in follicle-enclosed oocytes, yet continuous exposure of follicles to 8-Br-cAMP stimulates maturation (Hillensjo *et al.*, 1978). In addition, 8-Br-cAMP is more effective than dbcAMP in stimulating cumulus cell progesterone production (Tornell *et al.*, 1984) or expansion of the cumulus oophorus (Eppig, 1989). While differences in cell permeability of the cAMP analogs might explain these observations, it is more likely that differences in the distribution of PKA isozyme types in the somatic and germ cells are responsible. Indeed, Schultz (1991) has reported unpublished observations that the mouse oocyte contains predominantly RI, while several studies report that RII is the predominant subunit in ovarian extracts (Jahnsen *et al.*, 1985, 1986a; Hunzicker-Dunn *et al.*, 1985). The disparate results with dbcAMP and 8-Br-cAMP in oocytes and cumulus cells suggest that the effects of these analogs may be attributable to differential or selective activation of PKA isozymes in the two cell types (cf. Eppig, 1989).

The present study was carried out to test the hypothesis that type II PKA within the cumulus cells mediates the stimulatory effect of cAMP on meiotic resumption and cumulus expansion, while type I PKA within the oocyte mediates the suppressive action of cAMP on germinal vesicle breakdown. Both oocyte maturation and cumulus expansion experiments were conducted with site-selective cAMP analogs that specifically activate either type I or type II PKA to establish the specific PKA isozymes involved in these physiological responses. The results of these experiments strongly support the proposed hypothesis; furthermore, we have shown that the mouse oocyte contains exclusively the type I regulatory subunit of PKA, which is likely present in association with C as the holoenzyme, while cumulus cells contain both RI and RII, although the majority of holoenzyme is likely type II.

MATERIALS AND METHODS

Oocyte Isolation and Culture

For all experiments, immature 20- to 22-day-old (C57BL/6 × SJL/J) F_1 mice were used. Mice were primed with 5 IU

pregnant mare serum gonadotropin (Diosynth Inc., Chicago, IL) and killed by cervical dislocation 48 hr later. Ovaries were placed in petri dishes with 2.5 ml culture medium containing 0.2 m*M* 3-isobutyl-1-methylxanthine (IBMX; Aldrich, Milwaukee, WI) to maintain meiotic arrest, and the large Graafian follicles were punctured by sterile needles to release the oocyte–cumulus cell complexes. For some of the experiments, oocytes were denuded of their investing cumulus cells by shearing action caused by drawing complexes in and out of a sterile Pasteur pipet. Oocytes were washed four times in IBMX-free medium before allocation into the appropriate test medium. For oocyte maturation experiments, oocytes were cultured in 1 ml medium in plastic Falcon test tubes for varying periods of time before being assayed for germinal vesicle breakdown. For cumulus expansion experiments, oocyte–cumulus cell complexes were cultured for 17–18 hr in 500 μl of medium in Falcon petri dishes covered by equilibrated paraffin oil. Cumulus expansion was assessed by a subjective scale from ''0'' to ''+4'' and a cumulus expansion index was calculated (range 0–4.00) as previously described (Fagbohun and Downs, 1990).

Eagle's minimum essential medium with Earle's salts was used for all cultures. Medium contained 0.23 m*M* pyruvate, antibiotics, and 3 mg/ml lyophilized crystallized bovine serum albumin (ICN ImmunoBiologicals, Lisle, IL). All cultures were gassed with a humidified mixture of 5% CO_2, 5% O_2, and 90% N_2. Culture tubes were gassed, sealed, and placed in a water bath, while petri dishes were placed in a modulator incubator (Billups-Rothenberg, Del Mar, CA) that was gassed before incubation. With the exception of 8-piperidino-cAMP (Biolog Life Science Inst., La Jolla, CA), all cAMP analogs were purchased from Sigma (St. Louis, MO).

Statistical Analysis

Each oocyte maturation experiment was performed at least three times with at least 40 oocytes per group per experiment, and the data were reported as the mean percentage germinal vesicle breakdown ± SEM. Oocytes were assessed for maturation at the end of the culture period by removing cumulus cells and scoring them for germinal vesicle breakdown, the first observable manifestation of maturation. Maturation status was scored only for viable oocytes; viability routinely exceeded 95%. All frequencies were subjected to arcsin transformation before statistical analysis. Differences between groups were compared statistically by analysis of variance followed by Duncan's multiple range test. A P value less than 0.05 was considered to be significant.

^{32}P-Azido-cAMP Photoaffinity Labeling

Equal numbers of oocyte–cumulus cell complexes and denuded oocytes were isolated from 48-hr-primed mice in MEM containing 3 mg/ml polyvinylpyrrolidone (PVP) and 0.2 m*M* IBMX and then were washed three times in IBMX-free MEM/PVP. The tissue was transferred in a small volume to a microfuge tube on ice. Tissue from three to four isolations was pooled in each tube. Tubes were then centrifuged, the medium was removed, and cold extraction buffer was added (containing 2 m*M* EDTA, 2 m*M* EGTA, and 50 m*M* benzamidine). Samples were immediately frozen in an EtOH/dry ice bath and stored at −80°C until assayed. This labeling experiment was performed three times, and the numbers of oocytes/complexes analyzed for each experiment were: 825, 1550, and 872.

To photoaffinity label R subunits, samples were subjected to several cycles of freeze/thawing and the supernatant was incubated with 2 μM 8-N_3-[^{32}P]cAMP (ICN Biomedicals, Inc.) in the presence of 5 m*M* EGTA, 10 m*M* $MgCl_2$, and 1 m*M* ATP for 30 min at 30°C in the absence or presence of 0.1 m*M* cAMP (Hunzicker-Dunn *et al.,* 1991) and then irradiated and denatured (Hunzicker-Dunn, 1981). Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 5% acrylamide stacking and 10.5% acrylamide running gels at 50 mA/gel (Hunzicker-Dunn *et al.,* 1991). Radioactively labeled bands on autoradiographs were identified as RI at 47 kDa, RII_α at 54 kDa, or RII_β at 52 kDa by their migration relative to protein standards (Hunzicker-Dunn, 1981) and by loss of label in samples incubated with competing unlabeled cAMP. Labeling intensities of bands on autoradiographs were quantified using a Zeineh laser densitometer with a Hewlett–Packard 339A integrator.

Site Selectivity of the cAMP Analogs Used in this Study

Table 1 shows the affinities of the cAMP analogs used in this study for the intrasubunit binding sites on the R subunits of PKA, obtained from Ogreid *et al.* (1989). Early studies showed that 8-Br-cAMP binds to site 1 but indicated a lack of selectivity between RI and RII (Rannels and Corbin, 1980; Corbin *et al.,* 1982); however, the study by Ogreid *et al.* (1989) indicated site selectivity for RII. We hypothesized, based on earlier studies with rodent oocyte–cumulus cell complexes (Hillensjo *et al.,* 1978; Tornell *et al.,* 1984; Eppig, 1989), that 8-Br-cAMP binds to site 1 preferentially on RII in rodent complexes. This hypothesis has been supported by experiments detailed below. It is also important to note that 8-piperidino-cAMP (8-Pip-cAMP) binds with high affinity to two different sites, site 1 on type II PKA and site 2 on type I PKA (Ogreid *et al.,* 1985, 1989). Consequently, either type I or type II PKA isozyme can be selectively activated with 8-Pip-cAMP, depending on which second cAMP analog it is combined with, making it an especially useful analog in analyzing possible differential actions of the two protein kinase isozymes.

TABLE 1

Relative Affinities of cAMP Analogs for Binding Sites on the Regulatory Subunits of cAMP-Dependent Protein Kinase[a]

	RI		RII	
cAMP Analog	Site 1	Site 2	Site 1	Site 2
8-Thiomethyl[b,c]	2.9	0.84	1.5	0.043
8-Bromo[d,e]	1.0[f]	1.3	6.8	0.11
8-Aminohexylamino[e,g]	1.6	0.11	0.29	0.021
8-Piperidino[g]	0.065	2.3	3.2	0.046
N^6-Monobutyryl[e]	0.093	3.6	0.041	0.74

[a] Relative affinities for all analogs are from Ogreid *et al.* 1989. These are based on the ability of the indicated analog to compete with cAMP for binding to sites 1 and 2 on RI and RII and are expressed as the ratio of the apparent inhibition constant for cAMP to that for the analog. An underlined affinity denotes the site selectivity for the respective analog. The superscripts after each analog reference additional papers where site selectivity has been determined.

[b] Beebe *et al.*, 1984.

[c] Beebe *et al.*, 1986

[d] Rannels and Corbin, 1980.

[e] Corbin *et al.*, 1982.

[f] Rannels and Corbin (1980) and Corbin *et al.* (1982) reported that 8-Br-cAMP binds to site 1 but shows no selectivity between RI and RII, though the data in Table 1 are contradictory. We propose that in mouse oocyte–cumulus cell complexes, this analog preferentially binds to site 1 on RII.

[g] Ogreid *et al.*, 1985.

RESULTS

Maintenance of Meiotic Arrest by Site-Selective cAMP Analogs

Cumulus cell-enclosed oocytes were cultured for 3 hr in inhibitor-free medium or in medium containing increasing concentrations of one of five cAMP analogs before being assessed for germinal vesicle breakdown. In the absence of inhibitor, 98% of the oocytes resumed maturation (Fig. 2). The two analogs that presumably bind to RII, 8-Br-cAMP and 8-thiomethyl-cAMP (8-SMe-cAMP), were the least inhibitory of the agents tested: 8-Br-cAMP had little effect and 8-SMe-cAMP was only slightly inhibitory at the highest concentration tested (1 m*M;* 71% germinal vesicle breakdown). On the other hand, the other three analogs, 8-aminohexylamino-cAMP (8-AHA-cAMP), monobutyryl-cAMP (Mb-cAMP), and 8-Pip-cAMP, all of which can bind to RI, each dose-dependently suppressed the resumption of maturation. Moreover, at the highest concentration tested, 8-AHA-cAMP and 8-Pip-cAMP completely prevented germinal vesicle breakdown.

Results from this initial experiment indicated that analogs that were more selective for binding to type I PKA were more potent inhibitors of spontaneous meiotic maturation than analogs selective for type II PKA. The next experiment was therefore conducted to determine whether stimulation of different PKA isotypes by paired combinations of site-selective analogs could differentially affect oocyte maturation. Cumulus cell-enclosed oocytes were cultured for 3 hr in medium containing all possible pairings of the five analogs, each added at 0.1 m*M*, a concentration which had only a very limited effect on oocyte maturation in the first experiment. Consistent with the above results, the most inhibitory combinations were the two that putatively activated type I PKA: 8-AHA-cAMP + 8-Pip-cAMP and 8-AHA-cAMP + Mb-cAMP; all other combinations were less effective (Fig. 3). These results are consistent with the idea that activation of type I PKA is preferentially more inhibitory to spontaneous germinal vesicle breakdown than activation of type II PKA.

To demonstrate further the synergistic action for site-selective analogs, a dose–response experiment was carried out with Mb-cAMP and 8-AHA-cAMP. Mb-cAMP binds to site 2 on RI, while 8-AHA-cAMP binds to site 1 on RI (Corbin *et al.*, 1982). Hence, as a result of positive cooperativity, these two analogs should produce synergistic stimulation of type I PKA, which would be expected to produce concomitant synergistic inhibition of germinal vesicle breakdown. Cumulus cell-enclosed oocytes were cultured for 3 hr in

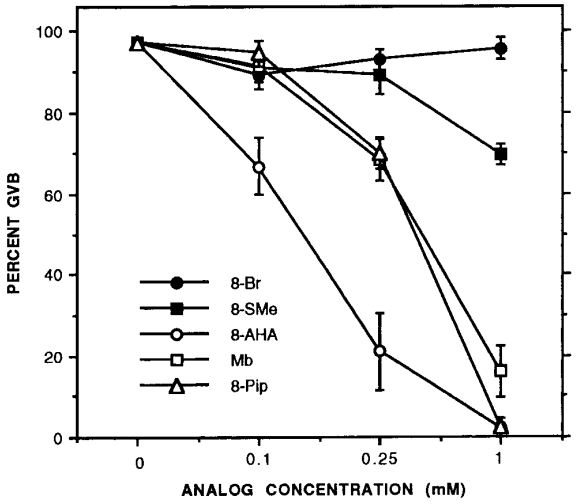

FIG. 2. Effect of individual cAMP analogs on the maturation of cumulus cell-enclosed oocytes. Cumulus cell-enclosed oocytes were cultured for 3 hr in medium containing increasing concentrations of one of five different cAMP analogs. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of at least three experiments. The percentage of maturation of oocytes cultured in the lowest concentration of 8-Br-cAMP (89%), but not in the higher two concentrations, was significantly different from the control frequency (97%). All concentrations of each of the other four analogs produced significant inhibition of germinal vesicle breakdown.

medium containing increasing concentrations (from 0.025 to 0.1 m*M*) of each of these analogs alone or in combination. As shown in Fig. 4, Mb-cAMP alone was not inhibitory at any of the concentrations and 8-AHA-cAMP alone produced inhibition at only the highest concentration tested. However, when added together, a synergistic effect was observed, with a level of meiotic arrest that exceeded the sum of the inhibitory action of the individual analogs when added alone. This can be expressed mathematically by calculation of a synergism quotient (SQ), determined by dividing the percentage of inhibition of the two analogs together by the sum of each percentage of inhibition of the individual analog treatments. Thus, any value over 1.0 denotes a synergistic response (cf. Beebe *et al.*, 1986). At all three concentrations of analog tested, synergism was evident (SQ range: 1.9–2.6; see Fig. 4).

In the next set of experiments, the actions of the cAMP analogs on maturation of denuded oocytes were examined. Denuded oocytes were cultured for 3 hr in control medium or medium containing one of the five cAMP analogs at a concentration of 0.15 or 1 m*M* and scored for germinal vesicle breakdown. Similar to the results obtained with cumulus cell-enclosed oocytes, 8-Br-cAMP and 8-SMe-cAMP produced little meiotic arrest, but 8-AHA-cAMP, Mb-cAMP, and 8-Pip-cAMP were all potent inhibitors of spontaneous maturation, particularly at the higher concentration (Fig. 5).

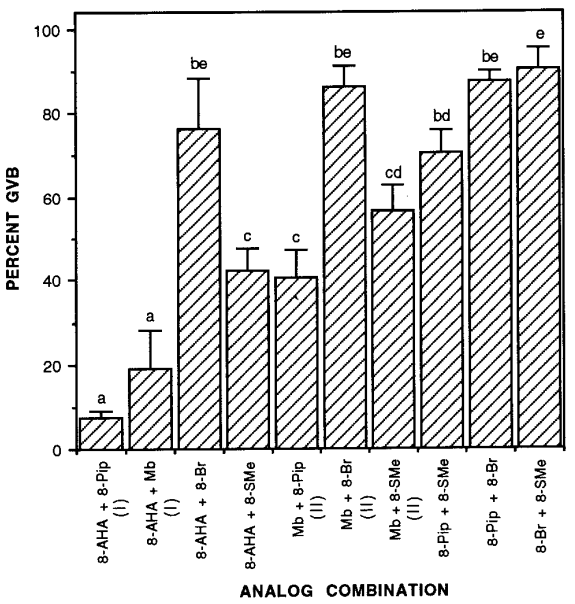

FIG. 3. Effect of site-selective cAMP analog pairs on the maturation of cumulus cell-enclosed oocytes. Cumulus cell-enclosed oocytes were cultured for 3 hr in medium containing paired combinations of cAMP analogs, each at a concentration of 100 μM. The isozyme type specifically activated is designated in parentheses. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of at least three experiments. Groups with at least one identical letter at the top of the bar are not significantly different.

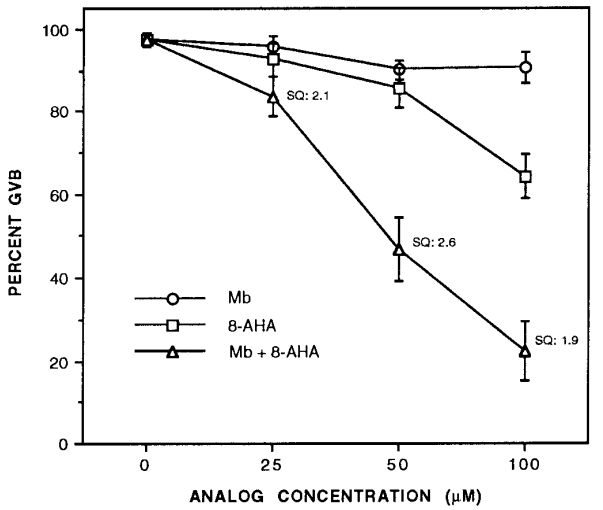

FIG. 4. Synergistic interaction of Mb-cAMP and 8-AHA-cAMP on oocyte maturation. Cumulus cell-enclosed oocytes were cultured for 3 hr in medium containing either Mb-cAMP alone, 8-AHA-cAMP alone, or the two analogs together, in one of three concentrations from 25 to 100 μM. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of three experiments. A synergism quotient (SQ) was calculated for each of the concentrations as follows. The extent of inhibition by each of the analogs alone or by combination of the two analogs was determined by subtracting the percentage of germinal vesicle breakdown in each group from the percentage of germinal vesicle breakdown in the control group. The percentage of inhibition in the combination group was then divided by the sum of each of the two inhibition values for the individual treatments. Synergism is indicated by a value greater than 1. As can be seen, a value of about 2 was achieved for each of the combinations.

To ascertain if combinations of site-selective cAMP analogs that specifically activate type I PKA would elicit the most potent suppression of maturation, denuded oocytes were cultured for 3 hr in the 10 paired combinations of analogs before being scored for germinal vesicle breakdown. As observed previously with cumulus cell-enclosed oocytes, the two analog pairs that are known to preferentially activate type I PKA were the most potent inhibitors of oocyte maturation (Fig. 6).

Opposing Actions of 8-Br-cAMP in Cumulus Cell-Enclosed and Denuded Oocytes

Because 8-Br-cAMP was the least inhibitory of any of the analogs tested in the above experiments, an experiment was carried out to determine if this analog could alter the inhibitory action of 8-AHA-cAMP on oocyte maturation. Cumulus cell-enclosed and denuded oocytes were maintained in

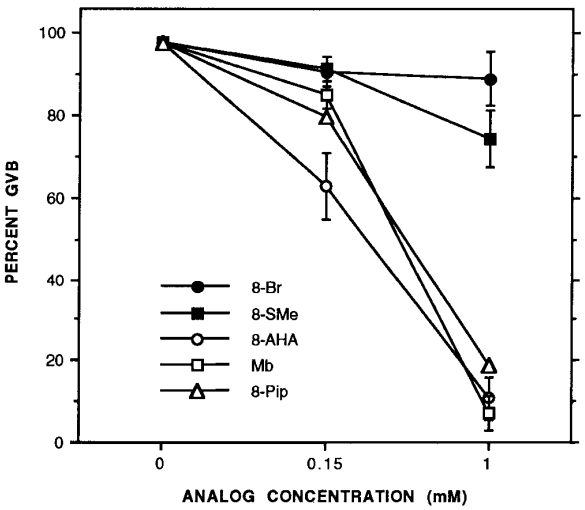

FIG. 5. Effect of individual cAMP analogs on the maturation of denuded oocytes. Denuded oocytes were cultured for 3 hr in medium containing one of five cAMP at either 0.15 or 1 m*M*. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of at least three experiments. Neither of the two concentrations of 8-Br-cAMP and only the highest concentration of 8-SMe-cAMP were inhibitory, while both concentrations of 8-AHA-cAMP, Mb-cAMP, and 8-Pip-cAMP were inhibitory.

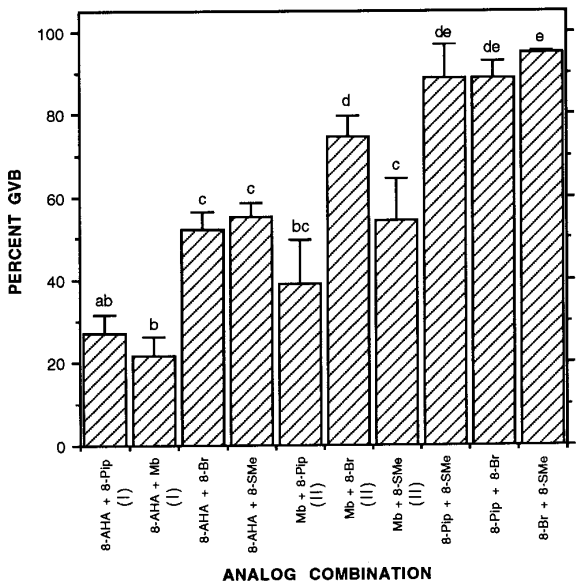

FIG. 6. Effect of site-selective cAMP analog pairs on the maturation of denuded oocytes. Denuded oocytes were cultured for 3 hr in medium containing paired combinations of cAMP analogs, each at a concentration of 150 μM. The PKA isozyme specifically activated is designated in parentheses. The data are presented as the mean percentage of germinal vesicle breakdown ± SEM of at least three experiments. Groups with at least one identical letter at the top of the bar are not significantly different.

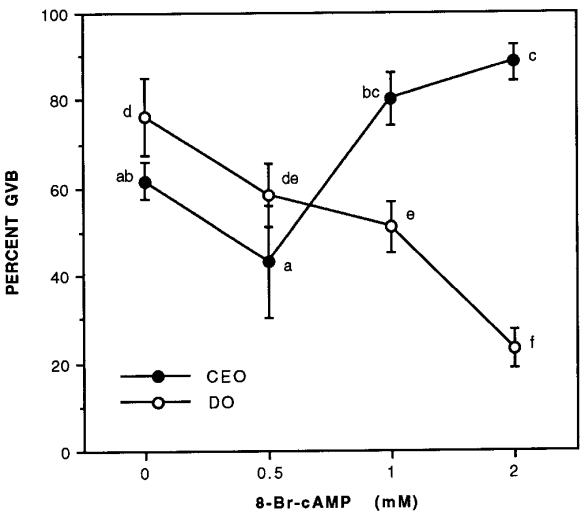

FIG. 7. Dose–response effect of 8-Br-cAMP on oocyte maturation in 8-AHA-cAMP-treated cumulus cell-enclosed and denuded oocytes. Cumulus cell-enclosed oocytes (CEO) or denuded oocytes (DO) were cultured for 17–18 hr in medium containing 0.1 or 0.15 m*M* 8-AHA-cAMP, respectively. 8-Br-cAMP was added in increasing concentrations from 0.5 to 2 m*M*. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of three experiments. The cumulus cell-enclosed and denuded oocyte treatments were statistically analyzed separately, and groups with at least one identical letter are not significantly different.

meiotic arrest for 17–18 hr with 8-AHA-cAMP (0.1 and 0.15 m*M*, respectively) and 8-Br-cAMP was added in increasing concentrations from 0.5 to 2 m*M*. When 8-Br-cAMP was added to meiotically arrested cumulus cell-enclosed oocytes, a dose-dependent reversal of meiotic arrest was, indeed, observed (an increase from 60% at 0 m*M* 8-Br-cAMP to 87% germinal vesicle breakdown at 2 m*M;* Fig. 7). Interestingly, just the opposite result was observed in denuded oocytes, as 8-Br-cAMP dose-dependently suppressed oocyte maturation (a decrease in germinal vesicle breakdown from 77 to 22%). Therefore, differential effects of 8-Br-cAMP on oocyte maturation were determined by the presence or absence of the cumulus oophorus and are consistent with the idea that elevation of cAMP within the cumulus cells has a stimulatory effect on oocyte maturation while direct elevation of cAMP within the oocyte is inhibitory.

Induction of Meiotic Maturation by cAMP Pulsing of Cumulus Cell-Enclosed Oocytes

It has previously been shown that a brief, 3-hr exposure of cumulus cell-enclosed oocytes to high levels of cAMP can relieve the meiotic arrest maintained by hypoxanthine and dbcAMP (Downs *et al.*, 1988; Eppig, 1989). We hypothesized that activation of type II PKA within the cumulus

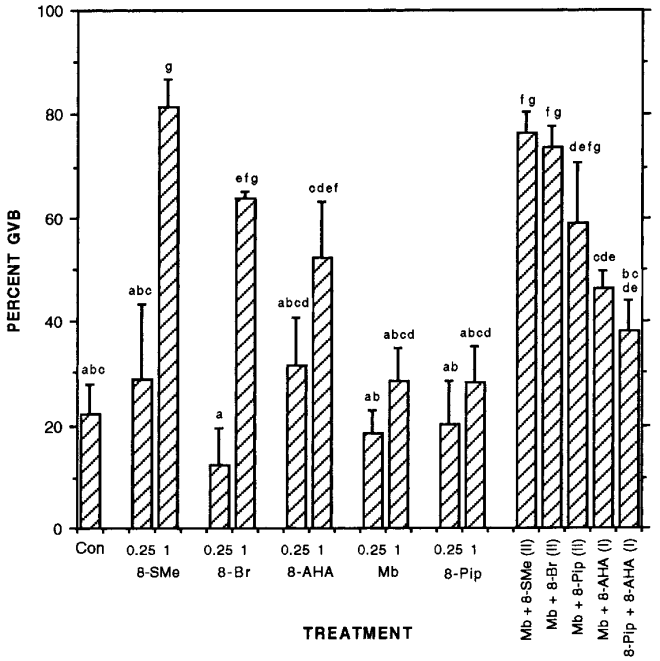

FIG. 8. The effect of pulsing cumulus cell-enclosed oocytes with cAMP analogs on meiotic maturation. Cumulus cell-enclosed oocytes were cultured for 20–21 hr in medium containing 300 μM dbcAMP. Controls (Con) received no other treatment, but all other groups were pulsed during the first 3 hr of culture with one of five cAMP analogs alone at a concentration of 0.25 or 1 m*M* or paired combinations of site-selective cAMP analogs, each at a concentration of 0.25 m*M*. Oocytes were then washed free of the analog(s) and cultures were continued for 17–18 hr in 300 μM dbcAMP. The PKA isozyme activated by the site-selective pairs is given in parentheses. Data are presented as the mean percentage of germinal vesicle breakdown (GVB) ± SEM of three experiments. Groups with at least one identical letter at the top of the bar are not significantly different.

cells is responsible for the stimulation of meiotic maturation achieved by this approach. Hence, the effects of the different cAMP analogs were tested using the following experimental protocol. Cumulus cell-enclosed oocytes were cultured for 3 hr in medium containing 300 μM dbcAMP plus or minus one of two doses of each of the five analogs alone (0.25 or 1 m*M*), or combinations of two analogs (each at 0.25 m*M*) that presumably activate either type I or type II PKA. Oocytes were then washed in inhibitor-free medium and returned to dbcAMP-supplemented medium, where cultures were continued for 17–18 hr before scoring for germinal vesicle breakdown. These results are shown in Fig. 8. In control cultures that were exposed to only dbcAMP, about 20% of the oocytes underwent maturation. The frequency of maturation was increased to 81 and 62% after a 3-hr pulsing with the highest concentrations of 8-SMe-cAMP and 8-Br-cAMP, respectively. Furthermore, the paired combinations that were most stimulatory were Mb-cAMP + 8-SMe-cAMP (78% maturation), Mb-cAMP + 8-Br-cAMP (75% maturation), and Mb-cAMP + 8-Pip-cAMP (59% maturation), all of which should preferentially activate type II PKA. 8-AHA-cAMP, Mb-cAMP, and 8-Pip-cAMP alone, or combinations thereof, were generally less stimulatory, although the highest concentration of 8-AHA-cAMP increased the maturation frequency to 52%. Thus, it appears that activation of type I PKA is inhibitory to oocyte maturation but that activation of the type II kinase is more effective in eliciting a positive maturation response in cumulus cell-enclosed oocytes.

Earlier work has shown that ligand-induced resumption of meiotic maturation in meiotically arrested oocytes in culture is mediated by the follicle (cumulus) cells, consistent with the hypothesis that a positive stimulatory factor(s) is generated in these cells and transferred to the oocyte where it triggers germinal vesicle breakdown (Downs *et al.*, 1988; Fagbohun and Downs, 1991). If this is the mechanism employed to bring about maturation, then a 3-hr pulse of elevated cAMP to denuded oocytes should not be stimulatory to maturation. This was tested following the same procedure as in the preceding experiment but using only individual cAMP analogs at 1 m*M* for the initial 3-hr culture. As shown in Fig. 9, there was no positive effect in any of the cultures, indicating that the cumulus cells are required for the meiosis-inducing action of cAMP.

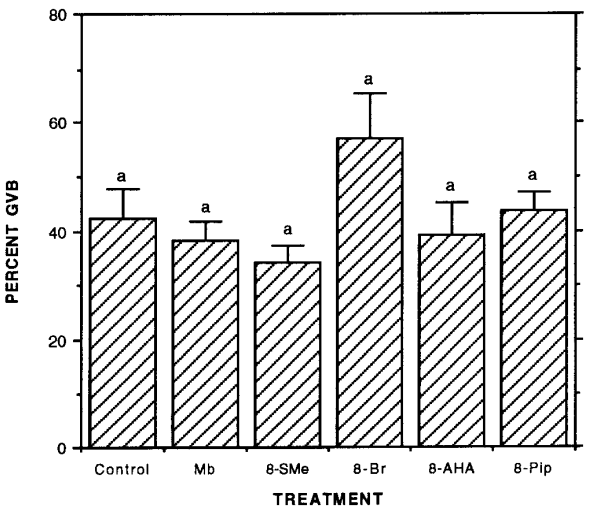

FIG. 9. The effect of pulsing denuded oocytes with cAMP analogs on meiotic maturation. Denuded oocytes were cultured for 20–21 hr in medium containing 300 μM dbcAMP. Controls received no other treatment, but all other groups were pulsed during the first 3 hr with one of five cAMP analogs at a concentration of 1 m*M*. The oocytes were then washed free of the analog and cultures were continued for 17–18 hr in 300 μM dbcAMP. Data are presented as the mean percentage of germinal vesicle breakdown ± SEM of three experiments. No significant differences were observed between any of the six groups.

TABLE 2
Dose–Response Effects of cAMP Analogs on Cumulus Expansion

Treatment	*n*	Degree of cumulus expansion					CEI[a]
		0	+1	+2	+3	+4	
Control	186	170	6	10	0	0	0.14
8-Br-cAMP							
250 μM	102	92	6	0	0	0	0.15
500 μM	107	33	34	23	26	2	1.55
1000 μM	103	0	0	24	55	24	3.00
8-SMe-cAMP							
250 μM	108	94	11	2	1	0	0.17
500 μM	151	94	21	19	14	3	0.75
1000 μM	105	0	8	37	45	15	2.64
8-AHA-cAMP							
250 μM	108	105	1	1	1	0	0.06
500 μM	117	113	2	2	0	0	0.05
1000 μM	138	91	22	16	9	0	0.59
Mb-cAMP							
250 μM	149	135	6	2	4	1	0.18
500 μM	104	95	4	2	3	0	0.05
1000 μM	102	96	8	6	2	0	0.25
8-Pip-cAMP							
250 μM	131	124	3	3	1	0	0.09
500 μM	139	124	7	4	4	0	0.19
1000 μM	119	97	12	3	5	2	0.34

[a] CEI, cumulus expansion index. This represents the mean expansion value for the complexes, with 0 equal to no expansion and 4 equal to maximal expansion.

Stimulation of Cumulus Expansion by Site-Selective Analogs

A second physiological response stimulated in oocyte–cumulus cell complexes by cAMP is cumulus expansion. Treatment either with high concentrations of cAMP analogs or with ligands that stimulate increased cAMP production promotes cumulus expansion under appropriate culture conditions (Dekel and Kraicer, 1978; Eppig, 1979). We proposed that, since activation of type II PKA appears to mediate stimulatory actions within cumulus cells, this same kinase is responsible for mediating cumulus expansion as well. It would therefore follow that cAMP analogs or combinations thereof that specifically activate type II PKA should be effective mediators of cumulus expansion, whereas cAMP analogs or combinations thereof that are specific for type I PKA should be less effective.

In the first of two experiments, complexes were cultured for 17–18 hr in medium supplemented with 5% fetal bovine serum, in the absence or presence of increasing concentrations of each of the five cAMP analogs, from 0.25 to 1 m*M*. Consistent with our hypothesis, 8-Br-cAMP and 8-SMe-cAMP dose-dependently induced cumulus expansion, with cumulus expansion indices (CEIs) of 3.00 and 2.64, respectively, at the highest concentration tested (Table 2). The other analogs—8-AHA-cAMP, Mb-cAMP, and 8-Pip-cAMP—were much less stimulatory, with CEIs all less than 0.6.

The second experiment was carried out to determine whether pairs of analogs that synergistically activate type II PKA would also selectively provoke cumulus expansion. Thus, complexes were cultured with the 10 paired combinations of analogs, each at a concentration of 0.25 m*M*. As shown in Table 3, the presumed combinations specific for activation of type II PKA were most effective in bringing about cumulus expansion, with maximal CEIs ranging from 2.47 to 2.92. Note that the degree of expansion is greater than that observed when the concentration of either of the analogs is doubled (compare with Table 2). Combinations specific for activation of type I PKA were considerably less effective (CEIs from 0.47 to 0.92). Other analog combinations that served as controls produced expansion indices less than 1.00, with the exception of 8-Br-cAMP + 8-SMe-cAMP (CEI of 2.32). It is not known why this latter combination produced synergistic activation, as both analogs bind to site 1 on the regulatory subunit and should not cooperatively interact, but it is likely that the response is due to stimulation of type II PKA.

8-N_3-[^{32}P]cAMP Photoaffinity Labeling of PKA Regulatory Subunits in Extracts of Oocytes and Oocyte–Cumulus Cell Complexes

The data obtained by treatment of oocytes and complexes with cAMP analogs suggest that activation of type I PKA

TABLE 3
Effects of Combinations of Site-Selective cAMP Analogs on Cumulus Expansion

Treatment[a]	PKA isozyme activated[b]	*n*	Degree of cumulus expansion					CEI[c]
			0	+1	+2	+3	+4	
Mb + 8-AHA	I	143	59	44	33	7	0	0.92
8-Pip + 8-AHA	I	95	68	13	10	4	0	0.47
Mb + 8-Br	II	114	0	6	37	57	14	2.69
Mb + 8-SMe	II	105	0	2	24	59	20	2.92
Mb + Pip	II	150	2	18	45	69	14	2.47
8-Pip + 8-Br	—	96	53	12	14	13	4	0.99
8-Pip + 8-SMe	—	95	58	29	4	4	0	0.52
8-Br + 8-SMe	—	180	12	28	52	67	21	2.32
8-Br + 8-AHA	—	153	72	44	24	13	0	0.86
8-AHA + 8-SMe	—	214	112	57	18	23	4	0.83

[a] Complexes were cultured for 17–18 hr in MEM/FBS containing different combinations of two site-selective cAMP analogs, each at 250 μM.
[b] The presumptive PKA isozyme activated via synergistic interaction is indicated. The last five analog pairs are combinations that should not synergistically activate a particular isozyme.
[c] CEI, cumulus expansion index.

within the oocyte mediates an inhibitory action on oocyte maturation, while activation of type II PKA within the cumulus cells mediates a stimulatory action on both the resumption of meiotic maturation and cumulus expansion. Consequently, if this hypothesis is correct, then the oocyte should contain predominantly type I kinase, while the complex should exhibit both types of kinase. This expectation was borne out in the results of 8-N_3-[^{32}P]cAMP labeling of R subunits. An identical number of freshly isolated denuded oocytes and oocyte–cumulus cell complexes were used for comparison. Similar results were obtained each of the three times this experiment was performed, and the results of a representative experiment are shown in Fig. 10. Denuded oocyte extracts exhibited a prominent band at 47 kDa that corresponded to RI but had undetectable RII. On the other hand, extracts of oocyte–cumulus cell complexes contained both RI at 47 kDa and RII_α and RII_β isoforms at 54 and 52 kDa, respectively. Specificity for RI and RII binding by 8-N_3-[^{32}P]cAMP was demonstrated by adding an excess of unlabeled cAMP during the binding assay, which eliminated specific radioactive labeling of R bands. Note the presence of a nonspecific band at 45 kDa in all four lanes. When the labeling of RI bands from denuded oocytes and oocyte–cumulus cell complexes was compared by densitometry, the oocyte RI band contained 21% of the radioactivity present in the corresponding band from oocyte–cumulus cell complexes. Because equal numbers of oocytes and complexes were loaded onto each lane, this indicates that approximately 21% of the RI present in oocyte–cumulus cell complexes resides in the oocyte.

DISCUSSION

The results of this study have shown that physiological responses of the oocyte–cumulus cell complex to cAMP can be differentially manipulated by the use of site-selective cAMP analogs. The disparate effects of the cAMP analogs are likely due to the differential distribution of PKA isozymes in the different ovarian cell types. As with the unpublished findings of Schultz and Richards (Schultz, 1991), our results of 8-N_3-[^{32}P]cAMP photoaffinity labeling of oocyte–cumulus cell complexes and denuded oocytes revealed that the only R subunit detectable within the oocyte is type I, while both RI and RII subunits are present within the cumulus cells. Accordingly, we propose that type I PKA within the oocyte mediates meiotic arrest while type II PKA within the cumulus cells mediates meiotic resumption and cumulus expansion.

The cAMP Paradox

When the actions of cAMP on oocytes are tested, paradoxical maturation responses can be elicited. cAMP analogs, cAMP-elevating agents, and phosphodiesterase inhibitors have well-established suppressive effects on the spontaneous maturation of oocytes *in vitro* (Eppig and Downs, 1984; Schultz, 1991), presumably attributable to the activity of a phosphoprotein whose phosphorylation state depends on the sustained activation of PKA (Schultz, 1991). Nevertheless, cAMP also participates in the transduction of a positive meiosis-inducing signal within the preovulatory ovarian follicle generated in response to gonadotropin stimulation (Lindner *et al.*, 1977). Thus, depending on the pattern of exposure to cAMP, either inhibition or stimulation of mammalian oocyte maturation can result. It has been hypothesized that a constant exposure of the oocyte to elevated levels of cAMP maintains the gamete in meiotic arrest, but a transient rise in cAMP may bring about a positive meiotic

response (Dekel *et al.*, 1988). Alternatively, if cAMP has a higher affinity for RI than for RII (Hoffman *et al.*, 1975; Hunzicker-Dunn *et al.*, 1985), then the absolute levels of cAMP reached within the follicle may also play an important role in regulating the meiotic response. Lower levels of the cyclic nucleotide would preferentially activate type I PKA and suppress oocyte maturation, whereas higher levels, achieved in response to the preovulatory gonadotropin surge, would activate the type II holoenzyme and lead to germinal vesicle breakdown and cumulus expansion.

Type I PKA Activation and the Maintenance of Meiotic Arrest

Persistent activation of type I PKA leads to suppression of spontaneous oocyte maturation, since germinal vesicle breakdown was blocked most effectively in both denuded and cumulus cell-enclosed oocytes under conditions that stimulate the type I PKA isozyme. Such results are consistent with the finding that RI was the only PKA subunit detected within the oocyte. It is interesting to note that RI protein and mRNA also predominate in early male germ cells (Oyen *et al.*, 1987; Pariset *et al.*, 1989; Landmark *et al.*, 1993). At high concentrations, cAMP analogs that bind to site 1 on RI (8-AHA-cAMP and 8-Pip-cAMP) were the most inhibitory to germinal vesicle breakdown. Mb-cAMP, which binds to site 2 on either RI or RII, was also a very potent inhibitor. The two least inhibitory compounds were 8-SMe-cAMP, specific for site 2 on RII, and 8-Br-cAMP, an analog we have proposed to be specific for RII in mouse oocyte–cumulus cell complexes (see below). Additional evidence was provided by the experiments in which paired combinations of the site-selective analogs were used. The two most inhibitory combinations were those that preferentially activate the type I kinase. Further, the dose–response experiment with 8-AHA-cAMP and Mb-cAMP demonstrated synergistic interaction between these two analogs at each concentration tested in suppressing spontaneous oocyte maturation.

Type II PKA Activation and the Resumption of Meiotic Maturation

Isolated cumulus cell-enclosed oocytes that are maintained in meiotic arrest can be stimulated to resume maturation by treatment with ligands such as follicle-stimulating hormone and luteinizing hormone (Dekel and Beers, 1978; Downs *et al.*, 1988). This response can be mimicked by pulsing meiotically arrested cumulus cell-enclosed mouse oocytes with a high concentration of cAMP analog (Downs *et al.*, 1988; Eppig, 1989). This latter approach was used in the present study to determine which PKA isozyme mediated the positive effect of cAMP on oocyte maturation. We hypothesized that type II PKA would be the primary isozyme involved because cumulus cells are required for the action of stimulatory ligands on oocyte maturation

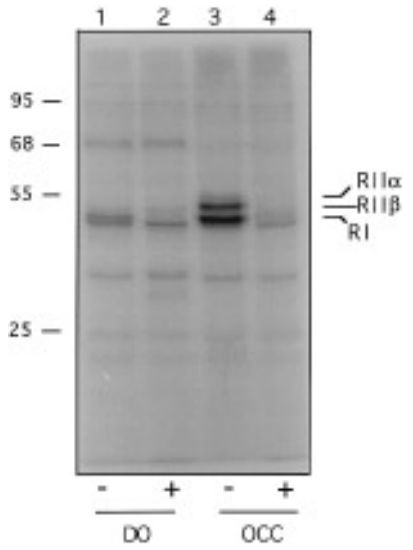

FIG. 10. Identification of R subunits in denuded oocytes and oocyte–cumulus cell complexes. Extracts were incubated with 2 μM 8-N_3-[^{32}P]cAMP from equal numbers of denuded oocytes (DO) and oocyte–cumulus cell complexes (OCC) in the absence (−) and presence (+) of 0.1 m*M* cAMP, as indicated. Molecular weights in kDa of protein standards are shown at the left. Quantification of the specific labeling of RI at 47 kDa and RII_β and RII_α at 52 and 54 kDa, respectively, by laser densitometry yielded the following (relative units): RI in DO, 2080; RI in OCC, 10021; RII in DO, 0; RII in OCC, 7974.

(Downs *et al.*, 1988), and previous studies have shown that RII predominates in the soluble extracts from rat and porcine ovaries (Jahnsen *et al.*, 1985, 1986a; Hunzicker-Dunn *et al.*, 1985). Moreover, any type II holoenzyme present in the mouse oocyte–cumulus cell complex resides in the cumulus cells.

The results of our study strongly support the proposition that type II PKA mediates the cAMP-stimulated resumption of meiotic maturation. Single analogs and paired combinations of analogs that are selective for type II PKA were most potent in stimulating germinal vesicle breakdown in dbcAMP-arrested cumulus cell-enclosed oocytes. The lack of effect of any analog on denuded oocytes confirmed that type II PKA within the cumulus cells was the site of cAMP action.

In rat ovaries, only 5% of the total R subunit protein is represented by RI, whereas two isoforms of RII constitute 95% (Jahnsen *et al.*, 1986b). In addition, RII is hormonally regulated during follicle maturation whereas RI is not, such that an increase in RII occurs during the development of preovulatory rat follicles (Jahnsen *et al.*, 1985; Hedin *et al.*, 1987) and in cultured granulosa cells stimulated by hormone or cAMP (Ratoosh and Richards, 1985; Beebe *et al.*, 1989). Furthermore, Hunzicker-Dunn *et al.* (1980) have shown that human chorionic gonadotropin (hCG) stimula-

tion of rabbit preovulatory follicles results in short-term stimulation of the type II kinase but not of type I. Such acute stimulation of type II PKA is consistent with an involvement in triggering the resumption of meiotic maturation, since germinal vesicle breakdown is initiated within only a few hours after hCG treatment. In the present study, nearly equivalent levels of RI and RII subunits were detected in mouse cumulus cells. We do not know if the relatively greater amount of RI in these cells is species-specific or is a unique feature of cumulus cells compared to mural granulosa cells. Nor is it known how much, if any, type I holoenzyme exists in mouse cumulus cells. The type II PKA holoenzyme constitutes greater than 95% of the PKA holoenzyme activity present in rat follicular granulosa cells, and the RI present in these cells is largely free of C subunits (Hunzicker-Dunn *et al.*, 1985; Hunzicker-Dunn, unpublished). Similar conditions may exist in mouse cumulus cells in that the majority of holoenzyme is type II. Nevertheless, it is important to emphasize that RII, and thus the type II holoenzyme, is absent in mouse oocytes.

Type II PKA Activation and Cumulus Expansion

The stimulatory action of cAMP on cumulus expansion in isolated oocyte–cumulus cell complexes is well documented (Dekel and Kraicer, 1978; Eppig, 1979). It was therefore of interest to establish the PKA isozyme specificity for this response. The dose–response experiment using single analogs revealed that only those analogs specific for type II PKA produced significant expansion. In addition, the three type II-directed analog combinations produced the greatest stimulation of expansion, whereas the two type I-directed combinations had only a nominal effect. The five nonspecific combinations were relatively ineffective, with the exception of 8-Br-cAMP + 8-SMe-cAMP. Although no synergism was expected in this case, it is apparent that some interaction has taken place that most likely involves activation of type II PKA.

The Use of 8-Pip-cAMP to Determine PKA Isozyme Function

8-Pip-cAMP represents a powerful tool to decipher the role of the two PKA isozyme types in various cellular functions, because either type I or type II isozyme can be selectively activated with this analog depending on what other site-selective analog it is paired with (Ogreid *et al.*, 1985, 1989). In the present study, when 8-Pip-cAMP was paired with 8-AHA-cAMP, and type I PKA was activated, germinal vesicle breakdown was inhibited but negligible induction of meiotic maturation or cumulus expansion was observed. On the other hand, combining 8-Pip-cAMP with Mb-cAMP, thereby activating type II PKA, proved to be less inhibitory to germinal vesicle breakdown during short-term culture, but more effective in inducing meiotic maturation and cumulus expansion. These results serve as compelling evidence in support of the proposed roles for the two isozymes in oocyte maturation and cumulus expansion.

Evidence that 8-Br-cAMP Preferentially Activates Type II PKA

8-Br-cAMP reportedly binds with comparable affinity to site 1 on RI and RII and is presumably not a selective activator of either PKA isozyme (Rannels and Corbin, 1980; Corbin *et al.*, 1982), though a more recent study suggests that this analog is selective for RII (Ogreid *et al.*, 1989). Earlier studies with rodent oocytes indicated that this analog was unable to block germinal vesicle breakdown but was a potent stimulator of cumulus cell-dependent events, such as progesterone synthesis, cumulus expansion, and induction of meiotic maturation (Hillensjo *et al.*, 1978; Tornell *et al.*, 1984; Eppig, 1989). This analog has been used extensively in stimulating numerous cAMP-dependent responses in cultured granulosa cells, including hormone secretion (Veldhuis *et al.*, 1982; Hsu and Hammond, 1987; Morbeck *et al.*, 1993), enzyme activation (Zor *et al.*, 1983; Daniel and Armstrong, 1984; Gomberg-Malool *et al.*, 1993; Knecht, 1987), receptor formation (Knecht and Catt, 1982; Sanders and Midgely, 1983; Golos *et al.*, 1986; Feng *et al.*, 1987), expression of RII (Ratoosh and Richards, 1985), generation of intracellular calcium signals (Asem *et al.*, 1987; Flores *et al.*, 1990), and cell proliferation (Morbeck *et al.*, 1993). Since RII and, hence, the type II holoenzyme predominates in the granulosa cells (Jahnsen *et al.*, 1985, 1986a; Hunzicker-Dunn *et al.*, 1985), and Schultz (1991) had provided unpublished information that the mouse oocyte contains primarily the RI subunit, the available data suggested that in the mammalian follicle 8-Br-cAMP selectively activates the type II isozyme. We therefore performed the present experiments with the assumption that 8-Br-cAMP was a selective analog for site 1 on RII in mouse oocyte–cumulus cell complexes.

Our data support the above assumption for the following reasons. (1) 8-Br-cAMP had little inhibitory activity on oocyte maturation when added either alone or in combination with Mb-cAMP or 8-Pip-cAMP. If 8-Br-cAMP is not discriminating between site 1 on RI and RII, one would expect some inhibition of oocyte maturation, since combination with these analogs should produce a synergistic activation of type I PKA, and other site-selective analog pairings have implicated a role for this isozyme in meiotic arresting mechanisms. (2) 8-Br-cAMP was a potent stimulator of germinal vesicle breakdown and cumulus expansion in oocyte–cumulus cell complexes, both alone and when paired with the site 2-selective analog, Mb-cAMP, a combination that should activate type II PKA. In addition, this action mimicked that of 8-SMe-cAMP, an analog with established selectivity for site 1 on RII (Beebe *et al.*, 1986). (3) Results of the dose–response experiment with 8-AHA-cAMP-arrested oocytes (Fig. 7) are of particular interest because 8-Br-cAMP actually reversed the meiotic arrest maintained in cumulus

cell-enclosed oocytes by an RI-directed analog in dose-dependent fashion. Such reversal indicates a positive, type II PKA-mediated stimulus in the cumulus cells that overrides type I PKA-mediated meiotic arrest in the oocyte. That the cumulus cells were responsible for this positive response was demonstrated by the augmentation of meiotic arrest by 8-Br-cAMP when the oocyte was denuded of its cumulus investment. This result can most likely be explained by the fact that at high concentrations the site selectivity of a particular cAMP analog becomes compromised (Francis and Corbin, 1994). We propose that within the denuded oocyte there is no RII for 8-Br-cAMP to bind and, hence, at the higher concentrations it begins to bind RI, leading to activation of type I PKA and an increase in the meiotic arrest brought about by the suboptimal concentration of 8-AHA-cAMP.

Concluding Thoughts

This study has demonstrated the usefulness of site-selective cAMP analogs in studying the role of different PKA isozymes in cAMP-mediated physiological responses within the oocyte–cumulus cell complex. Opposite effects on oocyte maturation and cumulus expansion were observed when type I or type II PKA was activated that were consistent with the distribution of R subunit isotype in the oocyte and cumulus cells. Differential distribution of isozyme types within the oocyte and cumulus cells may dictate the nature of the response to cAMP stimulation and indicates an important regulatory function in the various cAMP-mediated events in follicular physiology. A further consideration is the possible compartmentalization of isozyme within the respective cells. Proximity or accessibility of each isozyme to a cAMP-generating system, perhaps influenced by anchoring to specific binding proteins at strategic cell locations (Harper *et al.,* 1985; Moger, 1991; Carr *et al.,* 1993; Spaulding, 1993; Scott and McCartney, 1994; Inagaki *et al.,* 1994), could be a critical factor in the behavioral outcome of a particular cell or tissue. Finally, variations in relative isozyme distribution may explain species differences in oocyte sensitivity to the arresting action of cAMP. For example, the use of site-selective analog pairing may prove to be a useful strategy in optimizing meiotic arrest in species such as the bovine, which exhibit limited sensitivity to phosphodiesterase inhibitors or conventional cyclic nucleotide analogs such as dbcAMP (Homa, 1988; Sirard and First, 1988; Sirard, 1990).

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