计划生育生殖生物学国家重点实验室简况及论文集

State Key Laboratory of Reproductive Biology Brief Introduction and Papers

1999

中国科学院动物研究所
Institute of Zoology, Chinese Academy of Sciences
计划生育生殖生物学国家重点实验室
State Key Laboratory of Reproductive Biology

实验室简介

实验室主任:

祝　诚研究员

副主任:

段恩奎研究员、朴允尚研究员

学术委员会主任:

庄临之研究员

副主任:

刘以训研究员，科学院院士

李伟雄研究员

学术委员会委员（按姓氏笔画）:

王永潮	教授	北京师范大学生物系
王寒正	研究员	上海计划生育所
石其贤	研究员	浙江医科院计划生育所
庄临之	研究员	中国科学院动物研究所
刘以训	研究员 科学院院士	中国科学院动物研究所
孙曼霁	研究员 科学院院士	军科院药理毒理研究所
李伟雄	研究员	国家计生委科技所
肖碧莲	研究员 工程院院士	国家计生委科技所
严缘昌	研究员	中科院上海细胞所
陈大元	研究员	中国科学院动物研究所
祝　诚	研究员	中国科学院动物研究所
顾芝萍	研究员	中科院上海药物所
裘岳亭	研究员 科学院院士	中科院上海生化所
曹咏清	研究员	中国科学院动物研究所
薛社普	研究员 科学院院士	医科院基础医学研究所

学术委员会顾问:

赵白鸽	研究员	国家计生委科技司司长
朱耀华	副研究员	国家计生委科技司
程治平	教授	哈尔滨医科大学
李载平	研究员	中科院上海生化所
王　宾	副教授	美国宾州大学医学院
Puett, D. J.	教授	美国佐治亚大学生化系主任
Fathalla, M. F.	教授	美国人口委员会杰出专家
Seagal, S. J.	教授	美国洛氏基金会人口科学部顾问
Luong, C. K. P.	教授	加拿大不列颠哥伦比亚大学妇产科系
Tsang, K. B.	教授	加拿大渥太华大学妇产科系生殖生物中心主任

研究组:

本实验室共设七个研究组，在学科和技术方面各有特长，从不同角度和不同水平探讨生殖规律。

1、性腺生物学研究组　　负责人：刘以训研究员
2、受精生物学研究组　　负责人：孙青原研究员
3、胚胎生物学研究组　　负责人：段恩奎研究员
4、生殖生理学研究组　　负责人：祝　诚研究员
5、生殖免疫学研究组　　负责人：彭景榔副研究员
6、生殖内分泌学研究组　　负责人：王　红研究员
7、细胞和分子生物学研究组　负责人：朴允尚研究员

研究方向:

1995 年经学术委员会讨论，计划生育生殖生物学国家重点实验室的研究方向已集中为三大方面：(1)配子发生、成熟、排放以及黄体形成、萎缩的分子机理；(2)受精的分子机理和生殖过程；(3)胚胎着床的分子机理和抗着床作用。目的是从形态学、生理学、生物化学、细胞生物学、分子生物学等不同角度，在细胞和分子水平上探讨生殖调控的基本规律，同时为发展有效、安全、经济、实用的避孕方法奠定基础。

课题申请指南:

所在课题申请将围绕计划生育生殖生物学国家重点实验室的三大研究方向进行。

1. 生殖内分泌学研究
2. 生殖腺的细胞和分子生物学研究
3. 生殖细胞发育、成熟和排放机理的研究
4. 受精机理和生殖工程研究
5. 胚泡着床分子机理的研究
6. 妊娠早期人胎盘的细胞和分子生物学研究
7. 与生殖相关的生物活性物质（蛋白质、细胞因子、激素）的基因调控与基因工程研究
8. 有关避孕药物作用机制、开发应用与产业化的研究

1999 年资助课题一览表

（1999.05—2000.04）

序号	课 题 名 称	申请人	资助额(万元)
1998 年延续课题：			
1	异种哺乳动物间核移植的研究。	李劲松 (孙青原)	2.0
2	RU486 用于紧急避孕对于宫内膜整合素及 MUC-1 蛋白表达的影响。	郑淑蓉 (彭景榎)	2.0
3	RU486 对早孕恒河猴胎盘形成的影响。	王训立 (刘以训)	2.0
4	纤蛋白溶酶原激活因子和抑制因子对精子功能的影响。	邹如金 (刘以训)	2.0
5	小鼠植入启动过程中雌激素与表皮生长因子(EGF)的作用及相互关系。	赵兴绪 (段恩奎)	2.0
6	肾素血管紧张素系统对卵母细胞成熟的调控研究。	夏国良 (王 红)	2.0
7	大鼠卵巢颗粒细胞凋亡机制的分子生物学研究---颗粒细胞凋亡与线粒体机能、bc1---2、EGF 及其受体基因表达的相互关系。	于晓光 (祝 诚)	2.0
1999 年新申请课题：			
1	实验动物体细胞克隆的若干影响因素的研究	王敏康 (孙青原)	2.0
2	蛋白激酶在卵细胞周期调控中的作用 cAMP 和 PKC 对 MAPK 活性的影响	孙青原	2.0
3	胚胎滋养层 MHCII 类抗原表达调控机理的研究	彭景榎	2.0
4	花生四烯乙 3 醇胺在着床中的作用及其调节	肖爱珍 (段恩奎)	2.0

序号	课 题 名 称	申请人	资助额（万元）
5	卵巢的留素---血管紧张素与卵泡发育和闭锁	吴尔若 (王 红)	2.0
6	恒河猴胚胎植入过程中母胎界面细胞外基质和整合素的协同表达	季维智 (朴允尚)	1.4
7	青春期 SF-1 对睾酮的调控	沙家豪 (朴允尚)	1.3
8	Nesterone 对垂体促性腺细胞作用的分子机理	冷 颖 (朴允尚)	1.3
室内课题资助:			
9	受精机理及生殖工程研究	孙青原	3.5
10	精卵发生和黄体萎缩的基因调控	刘以训	3.5
11	动情期及早期妊娠金属蛋白酶在大鼠卵巢、子宫中的表达	祝 诚	3.0
12	滋养层组织金属蛋白酶的表达调控	朴允尚	3.0
13	细胞因子和整合素对植入的调节机理	段恩奎	3.0
14	细胞因子对胚胎着床的作用机理及其应用	彭景榴	3.0
15	子宫内膜血管易变性调节机制的探讨	王 红	3.0

论文与专著目录

（一） 研究论文

性腺功能及配子发生

受精机理

胚胎着床机理

*1. Hu Zhaoyuan, Liu Yixun, Liu Kui, Byrne S, Tor NY, Feng Qiang and Ockleford CD (1999) Expression of tissue type and urokinase type plasminogen activators as well as plasminogen activator inhibitor type-1 and type-2 in human and rhesus monkey placenta. J. Anatomy 194: 183-195 ------165

*2. Wang Yanling, Mather JP, Wooddroff T, Luo Shuyi and Zhuang Lizhi (1999) Regulation of activin A in cell proliferation and hormone secretion by human normal trophoblast cells. Chinese Science Bulletin 44(7): 609-612 --------178
王雁玲，Mather JP, Wooddroff T, 罗淑宜，庄临之 (1999) 激活素 A 对人正常滋养层细胞增殖和激素分泌的调节. 科学通报 44(1): 53-56

3. 闫建设，郭椋昊，刘疆，王红 (1999) 孕酮对人早孕子宫蜕膜细胞活性肾素分泌的调节. 生理学报 51(2): 211-218 ----------186

4. 唐美仪，焦丽红，张崇理 (1999) 促肾上腺皮质激素释放激素对人早期胎盘组织绒毛膜促性腺激素释放的调节作用. 动物学报 45(3): 323-331 ----------194

5. 刘 疆，焦丽红，王 红 (1999) 血管紧张素 II 对人子宫蜕膜催乳素分泌的调节. 中国学术期刊文摘 5(7): 922-924 ----------203

6. Li Bin, Zhou Chunxi, Yang Jun, Wang Liming, Chen Youzhen and Chao Yongqing (1999) Dynamic Changes of Lymphocyte Homing Receptors at Peri-implantation Stage in Mouse. Developmental & Reproductive Biology 8(1): 9-15 ----------206

7. Zhao Yunge, Cao Xiumei, Xiao Aizhen and Zhu Cheng (1999) Expression of MMP-2,-9, TIMP-1, -2, -3 mRNA in Rat Uterus during Estrous Cycle. Developmental & Reproductive Biology 8(2): 1-10 ----------213

8. 朱艳，陈贵安，冯强，刘以训 (1999) 人正常胚胎和葡萄胎绒毛中纤蛋白溶酶原激活与抑制因子及其尿激酶受体的表达. 生殖医学杂志 8(1): 24-27 ----------223

9. 陈 云，王妮，陈幼珍，曹咏清 (1999) 人早孕胎盘绒毛组织中整合素异二聚体α链免疫印迹检测. 生殖医学杂志 8(1): 48-50 ----------227

10. 曹咏清，孙德明，陈幼珍，朱蓬弟 (1999) 人重组γ-干扰素抗生育效应及其机理研究. 生殖医学杂志 8(2): 98-102 ----------230

11. 胡冬梅，陈竹钦，曹咏清，颜建华，王妮 (1999) 外源性细胞因子对早孕蜕膜调节自然杀伤细胞活性的作用. 生殖医学杂志 8(3): 155-159 ----------235

12. 胡冬梅，陈竹钦，颜建华，曹咏清，王妮 (1999) 正常早孕蜕膜非免疫细胞免疫活性的实验研究. 中华妇产科杂志 34(9): 533-535 ----------240

13. 曹宇静，张春雨，段恩奎，曾国庆 (1999) 纤粘连蛋白对小鼠胚胎体外发育和体外着床的作用. 动物学报 45(2): 187-193 ----------243

其它内容

（二）综述及科普文章

白酶. 生物化学与生物物理进展 26(3): 223-228 ----------319
3. 蔡理全，段恩奎，祝诚 (1999) 白血病抑制因子促进胚泡植入的研究进展. 生物化学与生物物理进展 26(2): 125-127 ----------325
4. 蔡理全，段恩奎 (1999) 整合素及其在胚泡植入中的作用. 生物化学与生物物理进展 26(4): 308-309 ----------328
5. 卢 青，刘冀珑，陈大元 (1999) 胞质钙信号. 生命的化学 19(2): 78-79 ----------330
6. 王雁玲，庄临之 (1999) 人胎盘滋养层细胞的功能分化及其调节. 生殖与避孕19 (6): 335-341 ----------332
7. 李辉，沈孝宙 (1999) 转基因－从实验动物开始的一种生物工程技术. 实验动物科学与管理16(2): 32-37 ----------339
8. 铁国栋，赵兴绪，段恩奎 (1999) 胚泡植入的调控研究进展. 甘肃农业大学学报 34(4): 335-341 ----------345
*9. Liu Yixun (1999) Regulation of plasminogen activator system in the ovary. Biological Signals and Receptors 8: 160-177 ----------352

注：标*为 SCI 收录论文

（三） 专 著

专著

刘以训 (1999)
促性腺激素及其分泌的调节 I－下丘脑-垂体-卵巢系统 14: 239-275
促性腺激素及其分泌的调节 II－下丘脑-垂体-睾丸系统 15 : 276-301
《现代神经内分泌学》谢启文主编
上海高等教育出版社 （ISBN 7-5627-0502-X/R 474）

专著中的章节

1. Chen Yijun, Feng Qiang and Liu Yixun (1999) Expression and regulation of steroidogenic acute regulatory protein in rat corpora lutea. 《Recent Progress in Molecular Comparative Endocrinology 》Hyuk BK, Jean MPJ, Susumu I 主编 Hormone Reserch Center, Chonnam National University, Public of Korea 出版 63-70 ----------370
2. Liu Yixun, Feng Qiang and Liu Kui (1999) Comparative studies on the role of plasminogen activators, inhibitor type 1 in luteal regression in rhesus monkey, rat and mouse. 《Recent Progress in Molecular Comparative Endocrinology》Hyuk BK, Jean MPJ, Susumu I 主编 Hormone Reserch Center, Chonnam National University, Public of Korea 出版 57-62 ----------378

3. Zhang Chongli, Cheng Liren, Li Xiaojie, Jiao Lihong and Huang Weiquan (1999) The presence and function of GnRH in human early placenta. 《Recent Progress in Molecular and Comparative Endocrinology》Hyuk BK, Jean MPJ, Susumu I 主编 Hormone Reserch Center, Chonnam National University, Public of Korea 出版 297-301 ----------384
4. Zhang Fuchun, Wang Ni, Liu Dongmei, Jian Youli, Chen Youzhen, Shen Xiaozhou, Cao Yongqing and Wang Bin (1999) Development of Female Contraceptive Vaccine Through DNA Inoculation of Human Chorionic Gonadotropin Beta Subunit (hCG β). Methods in Molecular Medicine. 《DNA Vaccines (methods and protocols)》 Douglas BL, Robert GW 主编 Humana Press 出版 439-449 ----------389
5. 陈大元 (1999) 动物显微受精与克隆研究. 《中国动物科学研究》中国动物学会主编 中国林业出版社 59-64 ----------400

Reprod. Fertil. Dev., 1999, 11, 81–86

cAMP inhibits mitogen-activated protein (MAP) kinase activation and resumption of meiosis, but exerts no effects after spontaneous germinal vesicle breakdown (GVBD) in mouse oocytes

Q. Y. Sun[ABCD], Q. Lu[AD], H. Breitbart[B] and D. Y. Chen[A]

[A]*State Key Laboratory of Reproductive Biology, Institute of Zoology, The Chinese Academy of Sciences, Beijing 100080, China.*
[B]*Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel.*
[C]*To whom correspondence should be addressed, email. sunqy@panda.ioz.ac.cn*
[D]*Q. Y. Sun and Q. Lu contributed equally to this work.*

Abstract. Various signaling molecules have been implicated in the oocyte G2/MII transition, including protein kinase C (PKC), cAMP and mitogen-activated protein (MAP) kinases. However, the cross-talk among these signaling pathways has not been elucidated. The present study demonstrates that both germinal vesicle break down (GVBD) and MAP kinase phosphorylation (activation) are inhibited when intraoocyte cAMP is increased by treating the GV-intact oocytes with dibutyryl cyclic AMP (dbcAMP), forskolin, or isobutylmethylxanthine (IBMX). Okadaic acid, a specific inhibitor of protein phosphatase-1 and -2A, completely overcame this effect. Calphostin C, a specific inhibitor of PKC, accelerated both GVBD and MAP kinase phosphorylation, and this effect was attenuated by increased intraoocyte cAMP, whereas PKC activation inhibited these events. Once GVBD occurred, the progression of oocyte maturation and MAP kinase phosphorylation were independent of cAMP. These results indicate that an increase in intraoocyte cAMP, in synergy with PKC activation, initiates a cascade of events resulting in inhibition of MAP kinase phosphorylation and GVBD in the mouse oocyte.

Extra keywords: meiotic cell cycle, oocyte maturation, protein kinase A (PKA), protein kinase C (PKC), protein phosphatase.

Introduction

Mammalian oocytes, arrested at the G2 phase of the cell cycle, can resume meiosis spontaneously *in vitro* upon their release from antral follicles. This spontaneous resumption of meiosis can be blocked by the addition of dibutyryl cyclic AMP (dbcAMP), a membrane-permeable cAMP analogue, by isobutyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor, or by forskolin, an adenylate cyclase activator (Cho *et al.* 1974; Schultz *et al.* 1983*a*). A drop in intracellular cAMP levels followed by inactivation of the cAMP-dependent protein kinase A (PKA) is associated with resumption of meiosis (Schultz *et al.* 1983*b*; Bornslaeger *et al.* 1986*a*). Therefore, the cAMP/PKA pathway is assumed to play a critical role in maintaining meiotic arrest. However, little is known about the biochemical events occurring downstream to PKA inactivation.

Tyrosine dephosphorylation of p34cdc2 is a prerequisite for the G2/M transition of mammalian oocytes, and protein tyrosine phosphatase-regulated dephosphorylation of p34cdc2 occurs downstream to the maturation-associated drop in the intraoocyte concentration of cAMP (Goren and Dekel 1994). Mitogen-activated protein (MAP) kinase, which is activated later than maturation-promoting factor (MPF), or immediately after germinal vesicle breakdown (GVBD), is also thought to play an important role in oocyte maturation (Maro *et al.* 1994; Verlhac *et al.* 1996; Sun *et al.* 1999*a*, 1999*b*). It has been reported by us and others that activation of PKC by phorbol ester initiates a cascade that ultimately prevents spontaneous GVBD and MAP kinase activation in mouse oocytes (Lefevre *et al.* 1992; Sun *et al.* 1999*a*).

The present study clarifies the cross-talk between cAMP and MAP kinase during mouse oocyte maturation.

Materials and methods

Oocyte collection

In order to collect preovulatory oocytes, sexually mature, unstimulated Balb/c mice (28–30 days old) were killed by cervical dislocation. Ovaries were removed and placed in Hepes-buffered CZB medium (Chatot *et al.* 1989) containing 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cumulus-free and GV-intact follicular oocytes were released from the large antral follicles into the medium by puncturing with a needle.

Oocyte maturation in vitro

The basic maturation culture medium used in the present study was Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and

1031-3613/99/020081

0.5% BSA (fraction V). All cultures were performed in Petri dishes containing microdrops of medium under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air. All chemicals and inhibitors used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) except for those specially mentioned.

Oocyte treatments

To test the effect of cAMP on the maintenance of meiotic arrest and activation of MAP kinases, denuded GV oocytes were randomly allocated to 100-μL drops of control medium or medium containing various additives, as shown in Fig. 1, and cultured for the times indicated in the Figure.

In the second experiment, in order to determine the effect of cAMP on the phosphorylation of MAP kinase and meiotic maturation after GVBD, oocytes that had recently gone through GVBD, 2.5 h after initiation of culture, were treated as shown in Fig. 2.

In the third experiment, the role of PKC inhibition in MAP kinase phosphorylation and resumption of meiosis, and the possible interaction between PKC and cAMP in regulating these events were investigated by cumulus-free GV-intact oocytes in drug-containing media for 5 h. The treatments are shown in Fig. 3.

In all experiments, the developmental stages of oocytes (GV, GVBD, MI) after culture were evaluated under an inverted microscope. MI was determined by Hoechst 33342 staining and followed by observation under an inverted fluorescent microscope. All data were evaluated by Chi-squared analysis. Differences of $P<0.05$ were considered significant.

Immunoblotting

Proteins from a total of 30 cumulus-free oocytes per treatment were extracted with double-strength electrophoresis sample buffer, and the lysates were kept frozen at −20°C. Before electrophoresis, samples were heated to

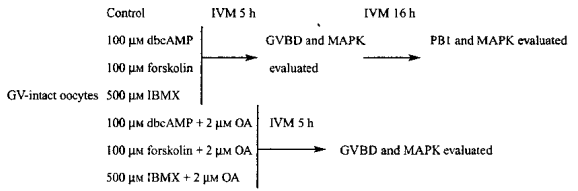

Fig. 1. Germinal vesicle-intact oocytes were subjected to various treatments to examine the effect of cAMP on the maintenance of meiotic arrest and activation of MAP kinases. GV, germinal vesicle; IVM, *in vitro* maturation; GVBD, germinal vesicle breakdown; PB1, first polar body; IBMX, isobutyl-methyl-xanthine; MAPK, mitogen-activated protein kinase; OA, okadaic acid.

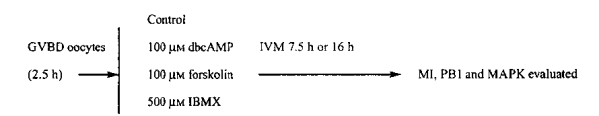

Fig. 2. Oocytes immediately following GVBD were subjected to four different treatments to examine the effect of cAMP on MAP kinase phosphorylation and meiotic maturation. MI, metaphase I. (See Fig. 1. for abbreviations.)

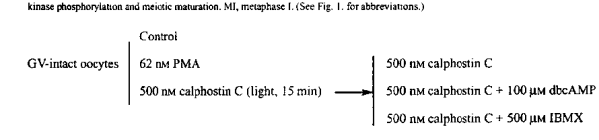

Fig. 3. GV-intact oocytes were subjected to different treatments, designed to examine the interaction between cAMP and PKC in regulating MAP kinase activity. PMA, phorbol 12-myristate 13-acetate. (See Fig. 1. for other abbreviations.)

100°C for 3 min, and then centrifuged for 5 min at 14 000g after cooling on ice for 5 min. The supernants were loaded onto gels.

Samples were separated on 10% SDS-polyacrylamid gel by using a 7 × 8 cm^2 gel apparatus (Bio-Rad, Richmond, CA, USA), and then transferred onto nitrocellulose membrane by using a Millibiot Trans-blot apparatus (Bio-Rad) for 2 h at 200 mA, 4°C, in transfer buffer. After blocking, the membrane was incubated overnight at 4°C in anti-Active™ MAPK antibody (Promega Corporation, Medacin, WI, USA) diluted 1 : 1000 with Tris-buffered saline with Tween-20 (TBST, pH 7.6) containing 1% skimmed milk, washed three times, for 10 min each time, with TBST, followed by incubation for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA, USA) diluted 1 : 2000 in TBST containing 1% skimmed milk. After washing three times, for 10 min each time, with TBST, the bands were visualized by an ECL detection system (Amersham Company). To detect the total quantity of MAP kinase in each treated group, the same blots were treated with stripping buffer (62.5 mM Tris, pH 6.7, 2% SDS, 100 mM β-mecaptoethnol) for 30 min in a 50°C water bath with occasional agitation in order to remove the bound antibody, and then reprobed with anti-ERK2 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), which reacts to both phosphorylated and dephosphorylated forms of ERK2, and to a lesser extent to ERK1, the two main forms of MAP kinase. The antibody was diluted 1 : 300 and the stripped membrane was reprobed by the same procedure described earlier. Complete stripping of the blot was verified by exposing the membrane to the ECL detection system before immunoblotting with anti-ERK2 antibody. Image processing was conducted using Photoshop 4.0 software. All experiments were repeated three times.

Results

Effects of cAMP and okadaic acid on MAP kinase phosphorylation and resumption of meiosis

GVBD is associated with a reduction in intraoocyte cAMP concentration followed by inactivation of PKA (Bornslaeger 1986*a*; Schultz 1983*a*, 1983*b*). This process is immediately followed by MAP kinase activation (Verlhac *et al.* 1993). We therefore wished to examine whether MAP kinase activation is induced by the drop in intracellular cAMP. MAP kinase was present in a dephosphorylated form in GV-intact oocytes (Fig. 4, lane 1), whereas it was activated when the denuded oocytes were cultured for 5 h (Fig. 4, lane 2). However, the occurrence of GVBD and the phosphorylation of MAP kinases were blocked when intracellular cAMP was increased by dbcAMP, forskolin or IBMX (Fig. 4, lanes 3, 5, 7; Table 1). The inhibition of GVBD and MAP kinase activation by cAMP, forskolin or IBMX could be completely overcome by okadaic acid, a specific inhibitor of protein phosphatase-1 and -2A (Fig. 4, lanes 4, 6, 8; Table 1). Both GVBD and MAP kinase phosphorylation were inhibited by increased cAMP even after 16 h culture with forskolin or IBMX, whereas MAP kinase was fully phosphorylated in the control oocytes that were in MII stage (Fig. 4, lanes 9–11).

Lack of effect of cAMP on MAP kinase phosphorylation and meiotic maturation after GVBD

In order to determine whether the effects of cAMP persist following GVBD, we induced elevated cAMP levels in oocytes immediately after GVBD. MAP kinase was present in a dephosphorylated form in oocytes soon after GVBD, similar to GV oocytes (Fig. 5, lanes 1, 2). However, an increase in intracellular cAMP after GVBD affected neither MAP kinase phosphorylation nor the progression of oocyte maturation. The extent of oocyte maturation and the degree of MAP kinase phosphorylation in oocytes treated by dbcAMP, forskolin or IBMX (Fig. 5, lanes 3–5) were not different from the control 16 h after culture (Fig. 5, lane 6; Table 2).

Fig. 4. Effects of cAMP modulators and okadaic acid on MAP kinase phosphorylation (activation) (top) and expression (bottom) in mouse oocytes. Lane 1: GV oocytes; lane 2: oocytes matured *in vitro* for 5 h; lane 3: GV oocytes cultured with dbcAMP for 5 h; lane 4: oocytes cultured with both dbcAMP and OA for 5 h; lane 5: GV oocytes cultured with forskolin for 5 h; lane 6: oocytes cultured with both forskolin and OA for 5 h; lane 7: GV oocytes cultured with IBMX for 5 h; lane 8: oocytes cultured *in vitro* with both IBMX and OA for 5 h; lane 9: GV oocytes cultured with dbcAMP for 16 h; lane 10: GV oocytes cultured with forskolin for 16 h; lane 11: GV oocytes cultured with IBMX for 16 h. This blot is representative of three similar experiments. (See Fig. 1. for abbreviations.)

Table 1. Effects of cAMP and okadaic acid (OA) on resumption of meiosis in mouse oocytes

Germinal vesicle-intact, cumulus-free mouse oocytes were collected and treated with cAMP modulators and the protein phosphatase inhibitor, okadaic acid. The presence of germinal vesicles was evaluated 5 and 16 h after the various treatments. Numbers in parentheses denote percent of cells.

Treatments	Germinal vesicles (%) 5 h	16 h
500 μM IBMX	55/55 (100)[a]	49/55 (89.1)[a]
100 μM dbcAMP	53/55 (96.4)[a]	52/55 (94.5)[a]
100 μM forskolin	45/50 (90.0)[a]	45/50 (90)[a]
500 μM IBMX + 2 μM OA	7/50 (14.0)[b]	0/50 (0)[b]
100 μM dbcAMP + 2 μM OA	10/36 (27.8)[b]	0/36 (0)[b]
100 μM forskolin + 2 μM OA	4/50 (8.0)[b]	0/50 (0)[b]

a v. b, $P<0.01$.

Possible interaction between cAMP and PKC in regulating MAP kinase and the meiotic cell cycle

We have previously shown that phorbol 12-myriatate 13-acetate (PMA) inhibits both MAP kinase activation and GVBD (Sun *et al.* 1999*a*). We therefore wished to determine

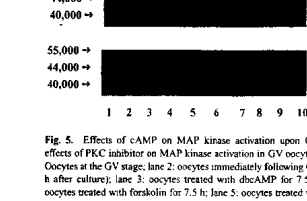

Fig. 5. Effects of cAMP on MAP kinase activation upon GVBD and effects of PKC inhibitor on MAP kinase activation in GV oocytes. Lane 1: Oocytes at the GV stage; lane 2: oocytes immediately following GVBD (2.5 h after culture); lane 3: oocytes treated with dbcAMP for 7.5 h; lane 4: oocytes treated with forskolin for 7.5 h; lane 5: oocytes treated with IBMX for 7.5 h; lane 6: oocytes cultured in medium without additions for 7.5 h (control); lane 7: oocytes cultured for 3 h; lane 8: oocytes cultured with PKC inhibitor, calphostin C, for 3 h; lane 9: oocytes that were initially treated with calphostin C for 15 min, and then treated with IBMX for up to 3 h; lane 10: oocytes that were first treated with calphostin C for 15 min, and then treated with dbcAMP for up to 3 h. This blot is representative of three similar experiments. (See Fig. 1. for abbreviations.)

Table 2. Effects of cAMP on cell cycle progression following germinal vesicle breakdown (GVBD) in mouse oocytes

Oocytes immediately following germinal vesicle breakdown were treated with different cAMP modulators. Metaphase I (MI) was evaluated by Hoechst 33342 staining and metaphase II (MII) was identified by the presence of the first polar body. Numbers in parentheses denote percent of cells.

Treatments	MI (%), 7.5 h	MII (%), 16 h
Control	30/30 (100)	61/137 (83.6)
500 μM IBMX	38/38 (100)	27/33 (81.8)
100 μM dbcAMP	34/34 (100)	79/98 (80.6)
100 μM forskolin	32/32 (100)	20/36 (83.3)

whether inhibition of PKC affects these processes. To this end, we tested the effects of PKC inhibitor on GVBD and MAP kinase activation. As can be seen in Fig. 5, lanes 7 and 8, and in Table 3, treatment of oocytes with calphostin C under light accelerated both GVBD and MAP kinase activation compared with the control groups in which calphostin C was not added or which were without light illumination of calphostin. IBMX and dbcAMP attenuated this effect (Fig. 5, lanes 9, 10; Table 3). This suggests that cAMP and PKC may interact with each other in regulating MAP kinase activity during oocyte maturation.

Discussion

MAP kinases are activated in response to extracellular signals, including growth factors, hormones and neurotransmitters. The modulation of MAP kinase activity by cAMP has been proposed in a variety of cell types; however, depending on the system, cAMP has been shown to have

Table 3. Effects of protein kinase C (PKC) modulators on resumption of meiosis in mouse oocytes

Cumulus-free, germinal vesicle-intact mouse oocytes were collected and treated with PKC modulators, or treated first with the PKC inhibitor calphostin C, for 15 min under light, and then with cAMP modulators. The presence of germinal vesicles was determined 1.5, 3 and 5 h after culture.

Treatments	Germinal vesicles (%) 1.5 h	3 h	5 h
Control	38/48 (79.2)[a]	106/386 (27.5)[c]	36/320 (11.1)[j]
162 nM PMA	60/60 (100)[b]	56/59 (94.9)[de]	56/59 (94.9)[k]
500 nM cal. (15 min, light)	13/32 (40.6)[c]	7/32 (21.9)[f]	3/32 (9.4)[l]
500 nM cal. (15 min, light) 500 μM IBMX	41/48 (85.4)[a]	28/48 (58.3)[df]	27/48 (56.3)[k]
500 nM cal. (15 min, light) 100 μM dbcAMP	40/48 (83.3)[a]	24/48 (50.0)[df]	24/48 (50.0)[k]

Cal, calphostin a vs b, a vs c, b vs c, c vs d, e vs f, a vs h, c vs h, h vs j and j vs k, $P<0.01$.

opposing effects on MAP kinase activity. In neonatal rat cardiomyocytes, PKA activators, such as forskolin, IBMX, dbcAMP or isoproterenol, significantly activate Raf-1 and MAP kinases (Yamazaki *et al.* 1997). Elevation of intracellular cAMP was also shown to activate MAP kinase in other cell types, such as PC12 cells (Frodin *et al.* 1994; Vossler *et al.* 1997; Yao *et al.* 1998). On the other hand, elevation of intracellular cAMP has been demonstrated to attenuate MAP kinase activation induced by growth factors and insulin in other cell types, such as rat hepatoma H4EII cells (Nagasaka *et al.* 1994), Schwann cells (Kim *et al.* 1997), rat smooth muscle cells (Plevin *et al.* 1997), human osteoblastic and bone marrow stromal cells (Chaudhary and Avioli, 1998), and others. The possible cross-talk between pathways utilizing cAMP and the MAP kinase pathway in the mammalian meiotic cell cycle, however, has not been analysed.

Many signaling molecules are involved in mammalian oocyte maturation, of which cAMP was the first to be identified. Resumption of meiosis is associated with a reduction in the intraoocyte concentration of cAMP, followed by inactivation of PKA. Increased intraoocyte cAMP induced by treatment of oocytes with dbcAMP, forskolin, purines or IBMX inhibits denuded mouse oocyte meiotic maturation, suggesting that cAMP may play a critical role in the maintenance of meiotic arrest in a PKA-mediated manner (Downs and Eppig 1986; Dekel 1996; Tsafrivi *et al.* 1996; Downs 1997). In the present study, we show that the increased intraoocyte cAMP levels induced by the treatment of GV oocytes with dbcAMP, forskolin or IBMX inhibit both GVBD and MAP kinase activation 5 and even 16 h after culture. This result suggests that cAMP-dependent PKA acts as a negative regulator for the G2/M transition and MAP kinase activation. Our result is consistent with that obtained in *Xenopus* oocytes, whereby injection of the catalytic subunit of PKA (PKAc) prevents progesterone-induced MAP kinase activation (Matten *et al.* 1994).

We also observed that the inhibitory effect of cAMP on MAP kinase activation and GVBD could be completely overcome by okadaic acid, a protein phosphatase inhibitor. Previous studies conducted by us and others suggested that protein phosphatases control MAP kinase activation in mouse and rat oocytes (Zernicka-Goetz *et al.* 1997; Sun *et al.* 1998, 1999*a*, 1999*c*). It has been proven that protein phosphatase can inactivate the MAP kinase cascade via dephosphorylation of ERK1/2 on thr183 (Hunter 1995). Therefore, we suggest that both cAMP and protein phosphatases control the resumption of meiosis and MAP kinase phosphorylation/dephosphorylation, and that protein phosphatases act at a stage downstream to cAMP.

MAP kinase is present in a dephosphorylated (inactive) form at the time of GVBD and it is fully activated 3.5 h after incubation *in vitro*. However, in mouse oocytes that fail to go through GVBD 4 h after incubation *in vitro*, MAP kinase is not activated, suggesting that the ability of ooplasm to phosphorylate MAP kinase is a prerequisite for GVBD (Sun *et al.* 1999*a*). We showed that cAMP inhibits neither meiotic cell cycle progression nor the MAP kinase activation once GVBD occurs, suggesting that some unidentified maturation-inducing factor(s) released from the GV into the ooplasm might overcome the inhibition of MAP kinase activation by cAMP. Such maturation-inducing factor(s) would activate MAP kinase in a manner that is independent of the cAMP/PKA pathway. It is possible that cAMP may inhibit MAP kinase activation by blocking the release of such unidentified maturation-inducing factor(s) in the GV. Another possibility is that cAMP may block GVBD through inhibition of the MPF rise that occurs around the time of GVBD, and thus inhibits a series of steps, one of which is MAPK activation.

The activation of another protein kinase, PKC, induces resumption of meiosis in oocytes of the rat (Aberdam and Dekel 1985), pig (Coskum and Lin 1995) and *Xenopus laevis* (Chung *et al.* 1992), but inhibits GVBD in mouse oocytes (Bornslaeger *et al.* 1986*b*; Lefevre *et al.* 1992; Niemierko and Komar 1992). We previously reported that activation of PKC by phorbol ester entrains a cascade of events that ultimately inhibits MAP kinase activation and induces interphase arrest in mouse oocytes (Sun *et al.* 1999*a*). Whether cAMP/PKA and PKC interact with each other in the cascade that regulates MAP kinase activity is still unclear. In the present study we found that the stimulation of MAP kinase activation and resumption of meiosis by the PKC inhibitor, calphostin C, was attenuated by increased intraoocyte levels of cAMP. We used calphostin C as a PKC inhibitor, because it has been proven to be specific and potent compared with other inhibitors such as staurosporine (Sun *et al.* 1999*a*). The present results, together with the existing data, suggest that PKC and cAMP/PKA activation probably synergistically inhibit MAP kinase phosphorylation and inhibit the G2/M transition.

In summary, our data suggest that an increase in intracellular cAMP, in synergy with PKC activation, inhibits a cascade of events resulting in inhibition of the resumption of meiosis and MAP kinase activation in mouse oocytes. However, these pathways have no effect on MAP kinase activation or on the meiotic cell cycle progression following GBVD.

Acknowledgments

This study was supported by the grants from Pandeng Program, Life Science Special Fund of CAS supported by the Ministry of Finance and the SKLRB Fund to Q. Y. Sun. It was also in part supported by the Rockefeller Foundation.

References

Aberdam E. and Dekel N. (1985). Activators of protein kinase C stimulate meiotic maturation of rat oocytes. *Biochem. Biophys. Res. Commun.* **132**, 570–4.

Bornslaeger E. A., Mattei P. M. and Schultz R. M. (1986*a*). Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev. Biol.* **114**, 453–62.

Bornslaeger E. A., Poueymirou W., Mattei P., and Schultz R. M. (1986*b*). Effects of protein kinase C activators on germinal vesicle breakdown and polar body emission of mouse oocytes. *Exp. Cell Res.* **165**, 507–17.

Chaudhary L. R. and Avioli L. V. (1998). Identification and activation of mitogen-activated protein (MAP) kinase in normal human osteoblastic and bone marrow stromal cells: attenuation of MAP kinase activation by cAMP, parathyroid hormone and forskolin *Mol. Cell Biochem.* **178**, 59–68.

Chatot C. L., Ziomek C. A., Bavister B. D., Lewis J. L. and Torres I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J. Reprod. Fertil.* **86**, 679–88.

Cho W. K., Stern S. and Biggers J. D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. *J. Exp. Zool.* **187**, 383–6.

Chung D. L., Brandt-Rauf P. W., Weinstein I. B., Nishimura S., Yamaizumi Z., Murphy R. B., and Pincus M. R. (1992). Evidence that the ras oncogene-encoded p21 protein induces oocyte maturation via activation of protein kinase C. *Proc. Natl Acad. Sci. USA* **89**, 1993–6

Coskum S. and Lin Y. C. (1995). Mechanism of action of epidermal growth factor-induced porcine oocyte maturation. *Mol. Reprod. Dev.* **42**, 311–17.

Dekel N. (1996). Protein phosphorylation/dephosphorylation in the meiotic cell cycle of mammalian oocytes. *Rev. Reprod.* **1**, 82–8

Downs S. M. and Eppig J. J. (1986). The role of purines in the maintenance of meiotic arrest in mouse oocytes. *Tokai J. Exp. Clin. Med.* **11**, 463–9.

Downs S. M. (1997). Hypoxanthine regulation of oocyte maturation in the mouse: insights using hypoxanthine phosphoribosyltransferase-deficient animals. *Biol. Reprod.* **57**, 54–62.

Frodin M., Peraldi P. and Van Obbergben E. (1994). Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. *J. Biol. Chem.* **269**, 6207–16.

Goren S. and Dekel N. (1994). Maintenance of meiotic arrest by a phosphorylated $p34^{cdc2}$ is independent of cyclic adenosine 3′,5′-monophosphate. *Biol. Reprod.* **51**, 956–62.

Hunter T. (1995). Protein kinases and protein phosphatases: the Yin and Yang of protein phosphorylation and signaling. *Cell* **80**, 225–36.

Kim H. A., DeClue J. E. and Ratner N. (1997). cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. *J. Neurosci. Res.* **15**, 236–47.

Lefevre B., Pesty A., Koziak K. and Testart J. (1992) Protein kinase C modulators influence meiosis kinetics but not fertilizability of mouse oocytes. *J. Exp. Zool.* **264**, 206–13.

Maro B., Kubiak J. Z., Verlhac M. H. and Winston N. J. (1994). Interplay between the cell cycle control machinery and the microtubule network in mouse oocytes. *Semin Dev Biol* **5**, 191–8.

Matten W., Daar I. and Vande Woude G. F. (1994). Protein kinase acts at multiple points to inhibit *Xenopus* oocyte maturation. *Mol. Cell Biol.* **14**, 4419–26.

Nagasaka Y., Kaku K., Nakamura K. and Kaneko T. (1994) cAMP inhibits the insulin-stimulated mitogen-activated protein kinase pathway in rat hepatoma H4EII cells. *Biochem Biophys. Res Commun.* **29**, 1104–12.

Niemierko A. and Komar A. (1992). Effect of phorbol ester PMA and puromycin on the maturation of mouse oocytes. *Cytobios* **70**, 7–17.

Pievin R., Malarkey K., Aidulis D., McLees A. and Gould G. W. (1997). Cyclic AMP inhibits PDGF-stimulated mitogen-activated protein kinase activity in rat aortic smooth muscle cells via inactivation of c-Raf-1 kinase and induction of MAP kinase phosphatase-1. *Cell Signal* **9**, 323–8.

Schultz R. M., Montgomery R. R., Ward-Bailey P. S. and Eppig J. J. (1983*a*). Regulation of oocyte maturation in the mouse: possible roles of intercellular communication, cAMP and testosterone. *Dev. Biol.* **95**, 294–304.

Schultz R. M. Montgomery R. R. and Belanoff J. R. (1983*b*). Regulation of mouse oocyte maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev. Biol.* **97**, 264–73.

Sun Q. Y., Luria A., Rubiastein S. and Breitbart H. (1998). Protein kinase inhibitors induce the interphase transition by inactivating mitogen-activated protein kinase in mouse eggs. *Zygote* **6**, 277–84.

Sun Q. Y., Rubinstein S. and Breitbart H. (1999*a*). MAP kinase activity is down-regulated by phorbol ester during mouse oocyte maturation and egg activation in vitro. *Mol. Reprod. Dev.* **52**, 310–18.

Sun Q. Y., Blumenfeld Z., Rubinstein S., Goldman S., Gonen Y. and Breitbart H. (1999*b*) Mitogen-activated protein kinase in human eggs. *Zygote* **7**, 181–5.

Sun Q. Y., Lax Y., Rubinstein S., Chen D. Y. and Breitbart H. (1999*c*). Mitogen-activated protein kinase and cell cycle progression during mouse egg activation induced by various stimuli. *Z. Naturforsch* **54c**, 285–94.

Tsafriri A., Chun S. Y., Zhang R., Hsueh A. J. and Conti M. (1996). Oocyte maturation involves compartmentalization and opposing changes of cAMP level in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. *Dev. Biol* **178**, 393–402.

Urner F., Herrmann W. L., Baulieu E. E. and Schorderet-Slatkine S. (1983) Inhibition of denuded mouse oocyte meiotic maturation by forskolin, an activator of adenylate cyclase. *Endocrinology* **113**, 1170–2.

Verlhac M. H., de Pennart H., Maro B., Cobb M. H. and Clarke H. J. (1993). MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* **158**, 330–40.

Verlhac M. H., Kubiak J. Z., Weber M., Geraud G., Colledge W. H. Evans M. J. and Maro B. (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development* **122**, 815–22.

Vosseir M. R., Yao H., York R. D., Pan M. G., Rim C. S. and Stork J. S. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89**, 73–82.

Yamazaki T., Komuro I., Zou Y., Kudoh S., Mizuno T., Hiroi Y., Shiojima I., Takano H., Kinugawa K., Kohmoto O., Takahashi T. and Yazaki Y. (1997). Protein kinase A and protein kinase C synergistically activate the Raf-1 kinase/mitogen-activated protein kinase cascade in neonatal rat cardiomyocytes. *J. Mol. Cell Cardiol* **29**, 2491–2501.

Yao H., York R. D., Misra-Press A., Carr D. W. and Stork P. J. S. (1998) The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated protein kinases and gene expression by nerve growth factor. *J. Biol. Chem.* **273**, 8240–7.

Zernicka-Goetz M., Verlhac M. H., Geraud G. and Kubiak J. Z. (1997). Protein phosphatases control MAP kinase activation and microtubule organization during rat oocyte maturation. *Eur. J. Cell Biol.* **72**, 30–8.

Manuscript received 8 June 1999; revised and accepted 27 September 1999.