# Unique protein kinase C profile in mouse oocytes: lack of calcium-dependent conventional isoforms suggested by rtPCR and Western blotting

Rathi Gangeswaran, Keith T. Jones*

*Medical Research Council Experimental Embryology and Teratology Unit, St. George's Hospital Medical School, London, SW17 0RE, UK*

Received 4 June 1997

**Abstract rtPCR and Western blotting were used to determine which members of the PKC family are present in both immature and mature mouse oocytes. Using isoform-specific PCR primers and antibodies PKC-δ and -λ were detected while such techniques failed to observe the conventional isoforms of PKC-α, -β, -γ. This isoform profile was confirmed using an alternative PCR strategy, which allowed discrimination of PCR products derived from conventional and novel PKC isoforms. In addition PKC-ε, -η, -θ and -ζ were not detected by rtPCR. These results suggest that the predominant isoforms in oocytes are PKC-δ and -λ.**



*Key words:* Protein kinase C; Oocyte; Calcium; Fertilization; Mouse; PCR

## 1. Introduction

Protein kinase C (PKC) exists as a family of serine/threonine kinases which are broadly classified into three groups depending on their cofactor requirements. Conventional isoforms (cPKC-α, -β and -γ) require $Ca^{2+}$ and diacylglycerol (DAG) for maximal activity, novel isoforms (nPKC-δ, -ε, -η and -θ) are $Ca^{2+}$ independent but require DAG as do the related kinases protein kinase D and PKUμ; and a typical isoforms (aPKC-ζ, -ι and -λ) which require neither $Ca^{2+}$ or DAG for activity. The family of kinases is complex with differing cofactor requirements probably reflecting separate and even opposing roles in cellular processes [1].

Sperm induces fertilization by a series of $Ca^{2+}$ oscillations. One mechanism whereby sperm is believed to trigger these oscillations is by binding to an oolemma receptor. This receptor is coupled to a phosphatidylinositol-specific phospholipase C (PI-PLC) through either a tyrosine kinase or a G-protein [2]. Activation of PI-PLC produces the bifurcating second messengers DAG and inositol trisphosphate ($IP_3$), and thereby $Ca^{2+}$. By this mechanism PKC is also likely to be activated at fertilization due to the production of DAG and $Ca^{2+}$. Indeed phorbol esters, most notably phorbol 12-myristate 13-acetate, have been used in some studies to successfully mimic events of oocyte activation, such as meiotic resumption in both mouse [3] and hamster oocytes [4], as well as inducing cortical granule exocytosis [5] which prevents polyspermy. However, pharmacological studies using PKC activators/inhibitors on mammalian oocytes have been inconsistent. One study has suggested that phorbol ester-induced cortical granule release is PKC independent [3], while another has shown that both hamster and mouse oocytes do not undergo meiotic resumption in the presence of phorbol esters [6].

Due to the differences that have arisen from the pharmacological assessment of PKC at fertilization this study set out to examine which common members of the PKC family are present in oocytes by a strategy based on rtPCR and Western blotting. This methodology may give some insight to their role at fertilization. The presence of cPKC-α, -β, -γ, nPKC-δ, -ε, -η, -θ, and aPKC-λ, -ζ was examined and it is reported that only nPKC-δ and aPKC-λ could be detected.

## 2. Materials and methods

### 2.1. Preparation of gametes and tissues

Twenty-one- to 24-day-old B6CB (C57BL/6JLac×CBA/CaLac) F1 hybrid mice were primed with peritoneal injection of 7.5 IU of pregnant mares' serum gonadotrophin (PMSG). Forty-eight hours later, fully grown germinal vesicle (GV) stage oocytes were released into phosphate-buffered saline with 1 mg/ml polyvinyl alcohol (PBS+PVA) by puncturing the antral follicles of ovaries with a sterile needle. The GV stage oocytes were denuded of cumulus cells by pipetting through a fine-bore pipette. For mature metaphase II (M2) oocytes, mice were superovulated 44–52 h after PMSG by peritoneal injection of 5 IU human chorionic gonadotropin (hCG). Oocytes were released from the ampullary region of the oviducts at 14–15 h post-hCG into PBS+PVA and the cumulus cells were removed with hyaluronidase [7].

For mRNA isolation the zonae pellucidae were removed by a brief incubation in acid Tyrode's solution followed by several washes in PBS+PVA. This was necessary to avoid any contamination with attached cumulus cells. The oocytes were frozen immediately in liquid $N_2$ with a minimal quantity of medium and stored at −70°C.

Whole-mouse brains and ovaries were excised from the animals used for oocyte collection. The tissues were washed in ice-cold PBS+PVA and snap frozen in liquid $N_2$. Frozen tissues were crushed in a mortar and pestle before extraction of mRNA.

### 2.2. Isolation of mRNA, cDNA synthesis and PCR

mRNA was extracted from oocytes, ovary and brain using a Micro-FastTrack Kit (Invitrogen) version 2.2. At least 400 oocytes were used for mRNA extraction. Before first-round cDNA synthesis it was necessary to DNase treat mRNA using 6 IU DNase, 6 IU RNase inhibitor and 9 μM $MgCl_2$, with incubation at 37°C for 1 h. DNase was inactivated by incubation at 95°C for 10 min. cDNA was synthesised using a cDNA Cycle Kit(Invitrogen) version 3.2. First-strand cDNA synthesis was made using random primers and AMV Reverse Transcriptase. For each preparation of cDNA, a negative control was made without reverse transcriptase in the reaction mixture.

Primers were designed (St. George's Oligonucleotide Synthesis Service) for individual PKC isoforms (Table 1, [8–11]). Amplifications were done with a Perkin-Elmer thermal cycler set for 45 cycles. After

*Corresponding author. Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK. Fax: (44) (171) 380-7349.
E-mail: k.jones@ucl.ac.uk

*Abbreviations:* DAG, diacylglycerol; GV, germinal vesicle; hCG, human chorionic gonadotrophin; $IP_3$, inositol trisphosphate; M2, metaphase II; PBS, phosphate-buffered saline; PKC, protein kinase C; PVA, polyvinyl alcohol


*PII* S0014-5793(97)00782-5

an initial denaturation step 94°C for 3 min, the cycle consisted of 58°C annealing for 1 min, 72°C extension for 2 min, 94°C denaturation for 30 s with a final extension step for 10 min. In addition to primers for individual isoforms primers were designed for the ATP-binding site and the cysteine-rich region in C2 of the PKC sequence. For these primers annealing was performed at 50°C for 0.5 min with a 72°C extension for 1.5 min. Other conditions were identical to isoform primers. For each isoform the PCR reactions were repeated at least three times and the average number of repeats was five. PCR fragments were analysed by electrophoresis on a 1.5% agarose gel and DNA was visualised by ethidium bromide staining.

*2.3. Cloning of PCR products and DNA sequencing*

PCR products were ligated in to a linearized PCR 2.1 vector supplied in a TA Cloning Kit (Invitrogen) and transformed into One Shot competent cells of *Escherichia coli*. Positive clones were selected and grown overnight in L-broth containing 50 μg/ml ampicillin. DNA was extracted using a Wizard Minipreps DNA Purification Kit (Promega). The insertions were analysed by a restriction analysis using *Eco*RI. Sequencing reactions were carried out from the DNA having correct insertions by using a $T^7$ Sequencing Kit (Pharmacia P-L Bio Chemicals).

*2.4. Western blotting*

SDS-PAGE was performed on 10% polyacrylamide BioRad minigels [12] and the separated proteins were transferred onto immobilon-P membrane. About 1 μg of oocyte protein was loaded per lane, which corresponded to about 400 oocytes. Immobilin membranes were blocked with 3% BSA in 20 mM Tris-HCl, pH 7.6, 136 mM NaCl with 0.1% Tween-20 before overnight incubation with 0.2 μg/ml anti-PKC Mabs (Transduction Laboratories, Kentucky) at 4°C. Protein was detected using an Amersham ECL system following incubation with a horseradish peroxidase-conjugated secondary antibody (1 : 1000 dilution, supplied with the ECL Kit, Amersham). Positive controls were run for each experiment: for PKC-δ, mouse macrophage cells (1 μg protein per lane, Transduction Laboratories); whole-rat brain (1 μg, Transduction Laboratories) and whole-mouse ovary (1 μg); for PKC-λ, HeLa cell lysate (2 μg, Transduction Laboratories) and mouse ovary (1 μg). Images were then scanned into Aldus Photostyler version 2.0 using a deskscan.

## 3. Results and discussion

rtPCR was used to establish which isoforms of PKC were present in mouse oocytes. Primers were designed in order to amplify individual members of the PKC family (Table 1) for cPKC-α, -β, -γ, nPKC-δ, -ε, -η, -θ, and aPKC-λ, -ζ. All primers gave PCR products of the predicted size using mouse brain (data not shown). GV stage oocytes were used initially, denuded of any contaminating cumulus cells and with their zonae pellucidae removed. This was in preference to mature M2 oocytes because almost half of the total oocyte mRNA is degraded during maturation [13]. Using rtPCR on GV stage oocytes only mRNA for PKC-δ and -λ were detected (Fig. 1Aa′) while no PCR products for cPKCs (Fig. 1Aa′) or PKC-ε, -η, θ and -ζ (data not shown) were found by this technique.

Table 1
Primers used for rt-PCR

| PKC | Primers | Position | EMBL accession [a] | Fragment Size (bases) | Ref |
|---|---|---|---|---|---|
| α | TGAATCCTCAGTGGAATGAGT | 818-838 | X52685 | 324 | [8] |
| | GGTTGCTTTCTGTCTTCTGAA | 1122-1142 | | | |
| β | CCCGAAGGAAGCGAGGGCAATGAAG | 910-934 [b] | X59274 [b] | 227 | [9] |
| | AGTTCATCTGTACCCTTCCGCTCTG | 1112-1136 | | | |
| γ | CGGGCTCCTACGTCGGATGAG | 832-852 | L28035 | 543 | [8] |
| | GCAGGCGTCCTGGGCTGGCACC | 1354-1375 | | | |
| δ | CACCATCTTCCAGAAAGAACG | 883-903 | M69042 | 351 | [8] |
| | CTTGCCATAGGTCCAGTTGTTG | 1213-1234 | | | |
| ε | CATCGATCTCTCGGGATCATCG | 577-598 | M18331 | 732 | [8] |
| | CGGTTGTCAAATGACAAGGCC | 1288-1308 | | | |
| η | AGCTAGCCGTCTTCCACGAGACGC | 307-330 | M62980 | 383 | [c] |
| | GGACGACGCAGGTGCACACTTGG | 668-690 | | | |
| θ | TTGATCTTTCCAGAGCCACG | 1515-1534 | D11061 | 178 | [10] |
| | CTTCGCATCTCCTAGCATG | 1675-1693 | | | |
| λ | CGTTGGGAGCTCTGACAATC | 1371-1390 | D28577 | 239 | [c] |
| | ACCTGCTTTTGCTCCATCATG | 1590-1610 | | | |
| ζ | CGATGGGGTGGATGGGATCAAAA | 728-750 | M94632 | 680 | [8] |
| | GTGTTCATGTCAGGGTTGTCCG | 1387-1408 | | | |
| cPKC and nPKC | | | | | |
| Primer 1 | ACCTTCTGTGACCACTGT | | | cPKC 720-760 | [11] |
| | CTTGCCAAAGCTGCCTTT | | | nPKC 300-500 | |
| Primer 2 | ACCTTCTGCGACCACTGT | | | cPKC 720-760 | [11] |
| | CTTCCCAAAACTGCCTTT | | | nPKC 300-500 | |

a. all accession numbers refer to those on the EMBL database, and are sequenced from mouse, except for PKC-ε which is rat.
b. Position and EMBL accession number refer to PKC-β1. The size of the PCR product is identical for PKC-β2.
c. Designed by authors.

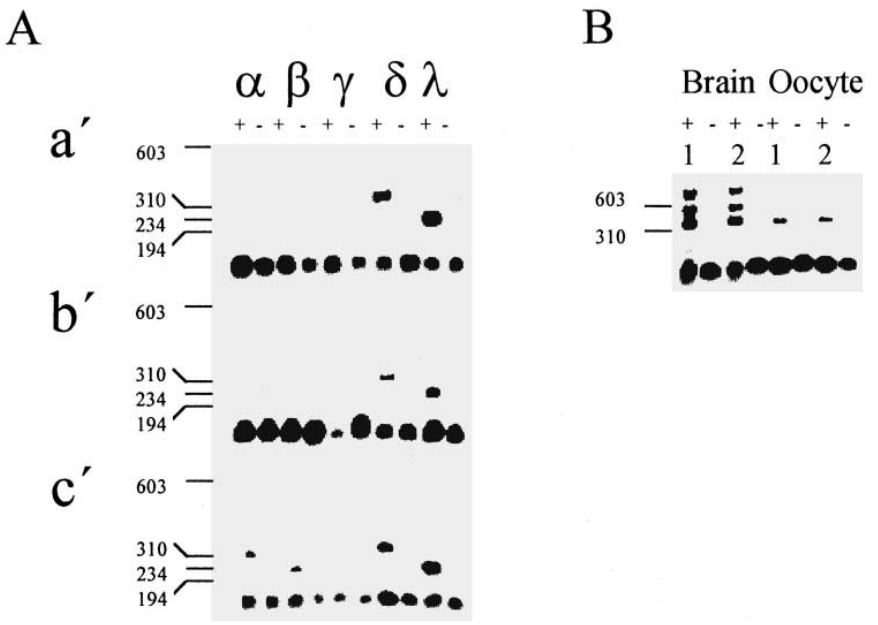


Fig. 1. PKC in mouse oocytes using rtPCR- and isoform-specific primers. (A) PKC-α, -β, -γ, -δ and -λ expression in: (a′) GV stage immature oocytes; (b′) mature M2-arrested oocytes; (c′) whole ovary. Only PKC-δ and -λ were detected in oocytes, while PKC-α and -β were in whole ovaries. (B) Two pairs of PCR primers (1 and 2) to the calcium-binding domain and the ATP-binding site give both high- (cPKCs) and low-weight (nPKCs) PCR products in mouse brain but in oocytes give only low-weight PCR products (assumed to be PKC-δ). (+) with reverse transcriptase, (−) without reverse transcriptase in the preparation of cDNA. Each lane represents the results of at least three separate cDNA preparations.

The PCR products for PKC-δ and -λ, which were of the correct size as predicted by the PKC isoform base sequence, were confirmed to be PKC-δ and -λ by sequence analysis of inserts when cloned in *E. coli*. The cloned fragments of PKC-δ and PKC-λ both showed a 98% homology to their published respective sequence (EMBL database). These small differences were presumably due to Taq polymerase misreading.

It is feasible that mRNA for the PKC isoforms not detected in GV stage oocytes are transcribed only during the latter stages of maturation, shortly before fertilization even though there is a net decline in total poly(A) RNA. The PKC profile was examined therefore in mature M2 oocytes by rtPCR to determine if there was any change. However, the PKC profile obtained was the same as for GV stage oocytes with respect to the presence of PKC-δ and -λ but not cPKCs (Fig. 1Ab′) and also the absence of PKC-ε, -η, -θ; -ζ (data not shown).

The fact that no cPKCs were detected in oocytes allowed the oocyte preparations to be easily assessed for contamination from other cell types, notably ovarian follicular cells. Mouse ovary contained PKC-α and -β but not -γ (Fig. 1Ac′). This confirmed that the oocyte preparations were free from somatic contaminants such as follicular cells. It is also in agreement with the finding of PKC-α and -β in rat ovarian tissue [14].

The results suggest that oocytes do not have cPKCs, a property that appears different from somatic cells which generally have at least one type of cPKC. As a consequence it was necessary to corroborate this observation by using an alternative strategy. Two pairs of PCR primers were designed to the cysteine-rich zinc finger domain and the ATP-binding domain of PKC (Table 1). All cPKCs and nPKCs contain these regions and therefore should be detected by this technique. cPKCs contain the C2 $Ca^{2+}$-binding domain which is lacking in nPKCs making cPKC PCR products larger (720–760 bases) than those of nPKCs (300–500 bases). This technique has been used successfully in cardiac cells to discriminate cPKCs and nPKCs [11]. Using the two pairs of PCR primers both the larger cPKC and smaller nPKC PCR products were observed in brain (Fig. 1B). However, in oocytes only one band of 350 bases was seen. This is most probably PKC-δ which is predicted to give a 356 base PCR product [11].

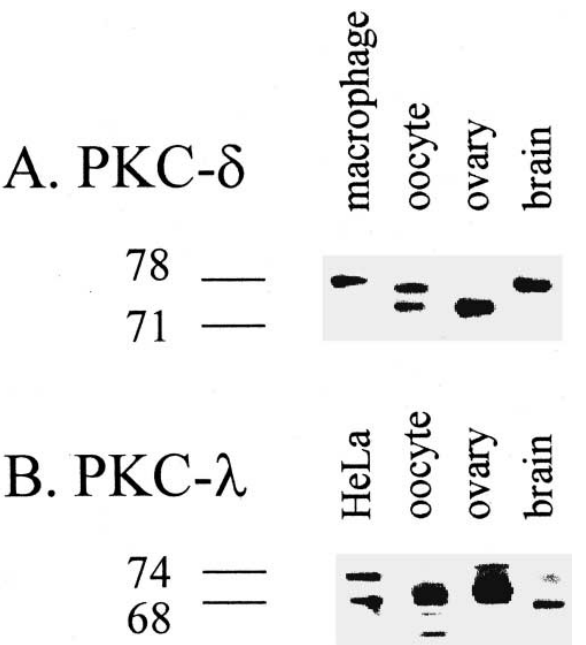


Fig. 2. PKC-δ and -λ protein in mouse oocytes. (A) PKC-δ was seen as a doublet in GV stage oocytes at 74 and 76 kDa. (B) PKC-λ appeared as a single major band at 71 kDa.

PKC-λ, being an aPKC, is not amplified by these primers, since it lacks the correct sequence in the zinc finger domain.

Having shown that oocytes only contain detectable amounts of mRNA for PKC-δ and -λ, these isoforms at the protein level were examined. For both immature oocytes (Fig. 2) and mature oocytes (not shown) PKC-δ and -λ proteins were observed. PKC-δ appeared as a doublet at 74 kDa and 76 kDa while PKC-λ was a 71 kDa protein. Both monoclonal antibodies to PKC-δ and -λ detected lower molecular weight proteins. These were assumed to be a products of PKC degradation as has been observed in other studies by Western blotting [15,16], but these smaller proteins were not examined further. Protein for PKC-α, -β and -γ was not detected in oocytes, using Westerns based on up to 1000 oocytes (not shown).

It remains possible that cPKCs, not detected by rtPCR or Western blotting techniques are present in oocytes, at a level below the sensitivity of the assays. Their detection in brain and in ovary by rtPCR could be due to these tissues having a larger amount of mRNA for these isoforms, although it was not possible to detect cPKCs using a technique distinguishing them from nPKCs, which were readily discernible. The number of 400–1000 oocytes used here for Western blotting is greater than the 50 needed for detection of cdc2, cyclin B1, cdc25 and wee1 in mouse oocytes by ECL [17] and the 80 needed for the $IP_3$ receptor in hamster oocytes [18] but less than the 5000 needed for detection of the ryanodine receptor [19]. It is therefore plausible that oocytes do contain cPKCs at a very level below that of the predominant isoforms of PKC-δ and -λ.

In conclusion it has been shown that the only two PKCs capable of detection in mouse oocytes, both immature GV stage and mature M2 arrested, are PKC-δ and PKC-λ. It now needs to be determined what are their respective roles at fertilization. In mouse oocytes phorbol ester addition mimics addition of $Ca^{2+}$ ionophore in the pattern of proteins phosphorylated very early after activation [20]. The present results suggest that this rise in cytosolic $Ca^{2+}$ must activate PKC indirectly since both PKC-δ and -λ have no $Ca^{2+}$-binding domain. This may be through switching on of phosphatidylcholine-specific phospholipase C and phospholipase D which have dependency on $Ca^{2+}$ for activity and generate DAG in a wide number of cell types [21].

*Acknowledgements:* The authors would like to thank Mr. D. Gilbert for his expert technical assistance and Professor D.G. Whittingham for his support. This work was funded by the Medical Research Council, UK.

## References

[1] Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 168–173.
[2] Foltz, K.R. and Shilling, F.M. (1993) Zygote 1, 276–279.
[3] Colonna, R. and Tatone, C. (1993) Zygote 1, 243–256.
[4] Gallicano, G.I., Schwarz, S.M., McGaughey, R.W. and Capco, D.G. (1993) Dev. Biol. 156, 94–106.
[5] Ducibella, T., Kurasawa, S., Duffy, P., Kopf, G.S. and Schultz, R.M. (1993) Biol. Reprod. 48, 1251–1257.
[6] Moore, G.D., Kopf, G.S. and Schultz, R.M. (1995) Dev. Biol. 170, 519–530.
[7] Fulton, B.P. and Whittingham, D.G. (1978) Nature 273, 149–151.
[8] Freire-Moar, J., Cherwinski, H., Hwang, F., Ransom, J. and Webb, D. (1991) J. Immunol. 147, 405–409.
[9] Ponzoni, M., Lucarelli, E., Corrias, M.V. and Cornaglia-Ferraris, P. (1993) FEBS Lett. 322, 120–124.
[10] Pessino, A., Sparatore, B., Patrone, M., Passalacqua, M., Melloni, E. and Pontremoli, S. (1994) Biochem. Biophys. Res. Commun. 204, 461–467.
[11] Kohout, T.A. and Rogers, T.B. (1993) Am. J. Physiol. 264, C1350–1359.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Bachvarova, R., DeLeon, V., Johnson, A., Kaplan, G. and Paynton, B.V. (1985) Dev. Biol. 108, 325–331.
[14] Cutler Jr., R.E., Maizels, E.T. and Hunzicker-Dunn, M. (1994) Endocrinology 135, 1669–1678.
[15] Croquet, F., Brehier, A., Gil, S., Davy, J. and Feger, J. (1996) Biochim. Biophys. Acta 1315, 163–168.
[16] Fujihara, M., Connolly, N., Ito, N. and Suzuki, T. (1994) J. Immunol. 152, 1898–1906.
[17] Mitra, J. and Schultz, R.M. (1996) J. Cell Sci. 109, 2407–2415.
[18] Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. (1992) Science 257, 251–255.
[19] Ayabe, T., Kopf, G.S. and Schultz, R.M. (1995) Development 121, 2233–2244.
[20] Colonna, R., Tatone, C., Malgaroli, A., Eusebi, F. and Mangia, F. (1989) Gamete Res. 24, 171–183.
[21] Billah, M.M. and Anthes, J.C. (1990) Biochem. J. 269, 281–291.