Journal of Assisted Reproduction and Genetics, Vol. 18, No. 5, 2001

ANIMAL EXPERIMENTATION

The Biological Significance of Phospholipase C $\beta 1$ Gene Mutation in Mouse Sperm in the Acrosome Reaction, Fertilization, and Embryo Development[1]

DOOSEOK CHOI,[2,4] EUNYOUNG LEE,[2] SEONGSOO HWANG,[2] KISUN JUN,[3] DAESOO KIM,[3] BYUNG-KOO YOON,[2] HEE-SUP SHIN,[3] and JE-HO LEE[2]

Submitted: April 11, 2000
Accepted: December 26, 2000

Purpose: *We carried out this study to evaluate the biological significance of phospholipase C β1 gene mutation in mouse sperm in the acrosome reaction, fertilization, and embryo development.*

Methods: *Study subjects were divided into two groups according to the sperm [intact phospholipase C (PLC) β1 and PLC $\beta 1^{-/-}$ C57BL/6J × CBA F_1 mouse sperm] used. The positive acrosome reaction rate labeled with fluorescein isothiocyanate–*Pisum sativum *agglutinin, the fertilization rate, and the rate of embryos developed to the stage of morula or blastocyst in the two groups were compared.*

Results: *The mouse sperm null for the PLC β1 gene showed a lower acrosome reaction rate than control sperm (69.2 vs 50.9%,* $P < 0.05$*). And the fertilization rate and the rate of embryos developed to the stage of morula or blastocyst were also lower in the group using PLC $\beta 1^{-/-}$ mouse sperm compared to the intact group (* $P < 0.05$*; 73.5 vs 51.8% and 15.7 vs 4.3%, respectively).*

Conclusions: *Mutation of the PLC β1 gene in the mouse sperm reduces the acrosome reaction rate, fertilization rate, and embryo development rate, which may be the etiologic factors responsible for the low reproductive rate of PLC $\beta 1^{-/-}$ mouse.*

KEY WORDS: phospholipase C β1 gene mutation; acrosome reaction; fertilization; embryo development.

INTRODUCTION

The acrosome reaction (AR) is an exocytotic event that releases hydrolytic enzymes from acrosome to facilitate sperm penetration through the zona pellucida (ZP) and is essential for the further proceeding of fertilization (1). The AR has been shown to be a calcium-dependent process and also to require extracellular calcium (2,3). The signaling cascade that leads to calcium influx into the sperm head is initiated by binding with the ZP and is a prerequisite for the AR (1,4). Acrosome exocytosis is induced by the increase in calcium in the sperm head. The increase in intracellular calcium is mediated by two major pathways; one is initiated by a family of G protein-linked receptors and the other is started by receptors linked by tyrosine kinases in the sperm plasma membrane (5–7). Phospholipase C (PLC) isozymes are known to play key roles in both pathways. Walensky and Snyder (7) reported that phosphoinositide-specific PLC activity catalyzed the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the second messenger molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate increased by PLC bound to the IP_3 receptor (IP_3R)-gated calcium

[1] Partly presented at the 54th Annual Meeting of the American Society for Reproduction Medicine, October 4–9, 1998, San Francisco, California.

[2] Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, IVF Clinic, Samsung Medical Center, Seoul, Korea.

[3] Department of Life Science, Pohang University of Science and Technology, Pohang, Korea.

[4] To whom correspondence should be addressed.

1058-0468/01/0500-0305$19.50/0

channel located on the acrosome membrane, which then activated the calcium efflux from the acrosome. Also, inositol 1,4,5-trisphosphate generated by the PLC pathway is known to play a role in oocyte activation (6–9). Among the PLC isozymes, PLC β and PLC γ are thought to play pivotal roles in intracellular calcium influx (7). Although the PLC isozymes are considered to be essential for the AR, the functions and significance of individual PLC isozymes in sperm has not been well studied. Using a mouse null for PLC β1, we carried out this study to evaluate the biological significance of the PLC β1 gene in sperm AR, fertilization, and embryo development.

MATERIALS AND METHODS

Study subjects were divided into two groups according to the sperm (intact PLC β1 and PLC $\beta 1^{-/-}$ mouse sperm) used. The sperm AR patterns were assessed by the fluorescein isothiocyanate–*Pisum sativum* agglutinin (FITC-PSA; L-0770; Sigma, St. Louis, MO) staining method, and fertilization and embryo development were evaluated using an in vitro fertilization (IVF) and in vitro culture system.

Animal

Three male mice null for PLC β1 (C57BL/6J $\times$ 129/sv) and three control (C57BL/6J $\times$ CBA, intact PLC β1) male mice were used. The mouse null for PLC β1 was generated by the gene targeting procedure as in the previous report (10).

Sperm Preparation

The caudal epididymides of three adult control and three PLC $\beta^{-/-}$ male mice were removed and their connective tissues were carefully dissected away separately. The caudae were washed twice in M16 medium and immersed in M16 medium without bovine serum albumin (BSA; fraction V; Sigma, ST. Louis, MO). Under a dissecting microscope, the tubules were pierced using a 27-gauge needle. Sperm were gently agitated to diffuse away from the tubules at room temperature for 2 min, until the medium became turbid. The caudae were removed and the sperm suspension was incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 20 min. Four hundred microliters of sperm suspension was transferred to 2 ml fresh M16 medium with 0.4% BSA. One milliliter of the sperm suspension in each male mouse was used for IVF and was reincubated for capacitation for 20 min. The other 1-ml sperm suspension was used for AR assay.

Acrosome Reaction Assay

Mouse sperm AR assay was conducted as described previouly (7) using the supplemented M16 medium. Mouse sperm isolated from the cauda were capacitated for 1 hr in a 37°C incubator. Only samples displaying vigorous motility, with more than 80% motile sperm, were used for further evaluation. Capacitated sperm were spun for 5 sec in a tabletop microfuge at 1000*g*. Supernatants were removed and the pellets were resuspended in Dulbecco's phosphate-buffered saline (PBS, Gibco-BRL, USA). Sperm concentration was determined using a hematocytometer and set at 1×10^7/ml. Fifty microliters of capacitated sperm was treated with 10 μM calcium ionophore A23187 (Sigma), while a control sample was treated with 1% dimethyl sulfoxide (DMSO; Sigma). After a 1 hr incubation, the samples were spun in a tabletop microfuge at 1000*g*. The pellets were resuspended in 50 μl PBS and the samples were subsequently mounted on slides and air-dried. The slides were fixed with 96% ethanol (absolute; Merck, Germany) at 4°C for 15 min and then air-dried. The acrosome-reacted sperm were labeled with 100 μl FITC-PSA in a humidity chamber at 4°C for 15 min and then washed by gently dipping in PBS, 20 times. The slides were coverslipped with 30% glycerol in PBS, air-dried, and stored in a dark space until scoring. Scoring of the slides was done within 48 hr of staining. One thousand sperm (250 sperm per slide) from each male mouse were evaluated for acrosome status under an oil immersion microscope at 100 $\times$ using Normarski optics (Olympus BX50-Flu.). Sperm heads that were completely dark or more than 50% dark and patchy in acrosome status were counted as positive, and those that were bright and or less than 50% dark and patchy were counted as negative (Fig. 1).

In Vitro Fertilization and Culture

Fifteen 8-week-old hybrid F_1 (C57BL/6J $\times$ CBA) female mice were superovulated by injection of 7 IU of pregnant mare's serum gonadotropin (PMSG; Sigma), followed by injection of 7 IU of human chorionc gonadotropin (hCG; Sigma) 45–48 hr later. Oviducts were excised from unmated females, 16–18 hr after hCG injection, and ovulated oocytes were released from the ampullae and collected into

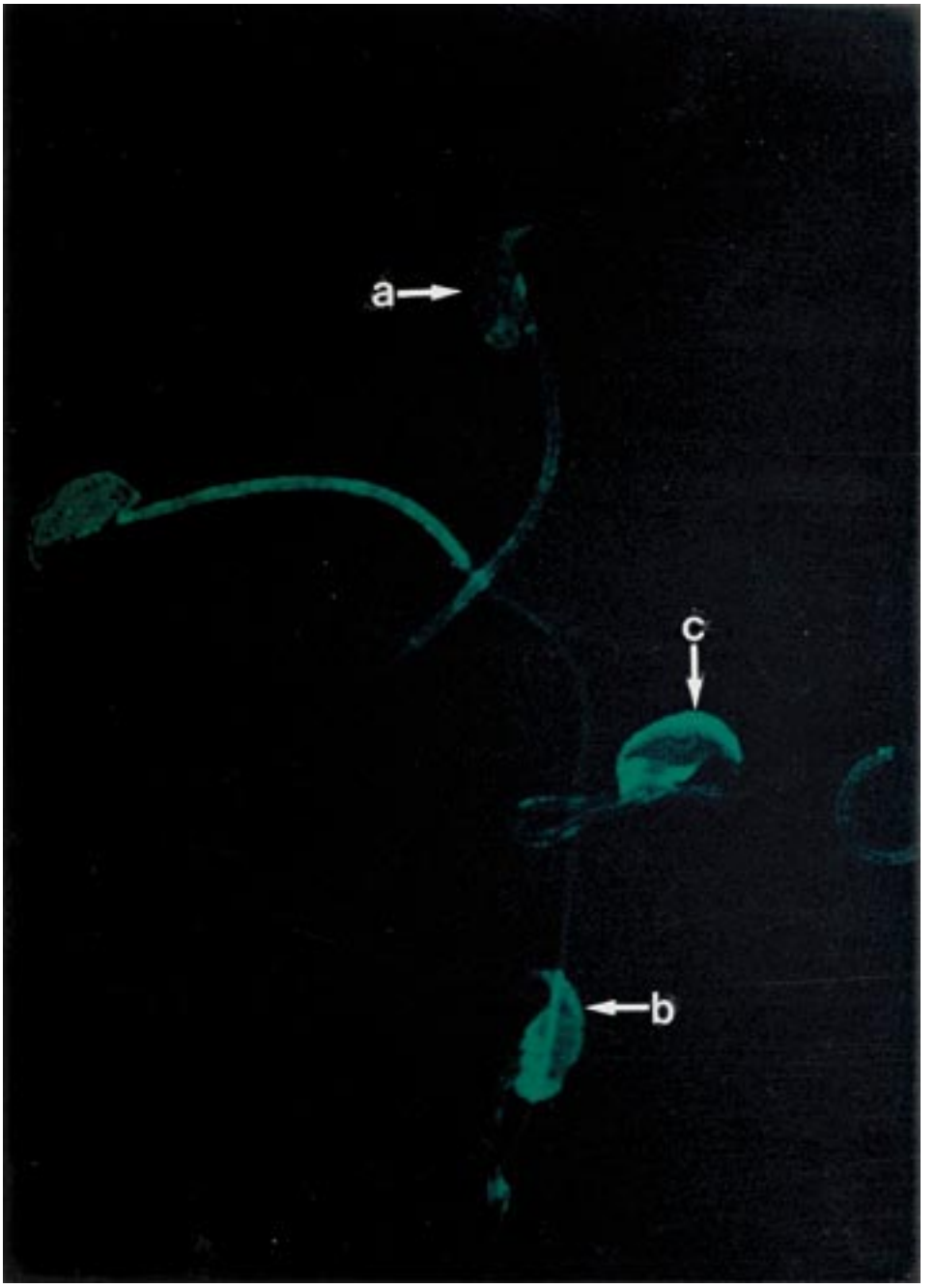

Fig. 1. Pattern of sperm acrosome reaction after treatment with 1% DMSO or 10 μM A23187 stained with fluorescein isothiocyanate–*Pisum sativum* agglutinin (FITC-PSA): (a) dark, 100% reacted; (b) patchy, $\geq$50% (c) bright, not reacted.

2 ml of washing medium. After collection, all oocytes were mixed up for equal experimental conditions. The insemination droplets were 50 μl in volume and were covered with mineral oil (light form; Sigma) in a 60-mm culture dish (Falcon 3002; Becton-Dickinson, Lincoln Park, NJ). Approximately 10 oocytes were allocated to each insemination droplet, and sperm suspension (final concentration, about 2×10^6/ml) was also added to the droplet. Eighteen hours after insemination, the fertilized oocytes were washed in culture

medium and then transferred to the culture droplets (5–10 zygotes/droplet). M16 medium was used for washing and M16 medium supplemented with 10% FBS was used for insemination and culture. Then the embryos were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 3 more days. Change of the culture medium and observation of embryo development were performed daily. Formation of two pronuclei (2PN) or two cells at 18 hr after insemination was regarded as fertilization. Embryo development to the morula or blastocyst stage after 96 hr of culture was considered positive.

Statistical Analysis

The rate of AR, fertilization, and embryo development between the two groups was compared by Mann–Whitney test. $P < 0.05$ was considered statistically significant.

RESULTS

The AR-positive rate of PLC $\beta 1^{-/-}$ sperm was lower than that of control sperm after treatment with 1% DMSO ($P < 0.05$; Table I). Also, PLC $\beta^{1-/-}$ mouse sperm showed a consistently lower rate of AR than control sperm after treatment with 10 μM calcium ionophore A23187 ($P < 0.05$; Table I). The fertilization rate confirmed by formation of 2PN or two cells at 18 hr after insemination was higher in the control group than in the PLC $\beta 1^{-/-}$ group ($P < 0.05$; Table II). And the percentage embryo development to the morula or blastocyst stage in the control group was also higher than in the PLC $\beta 1^{-/-}$ group at 96 hr after insemination ($P < 0.05$; Table II).

Table I. Acrosome Reaction Patterns of C57BL/6J Mouse Epididymal Sperm After Treatment with DMSO and A23187 Assessed by FITC-PSA Staining[a]

	AR positive[b] (mean% ± SD)*	
Treatment	Control	PLC $\beta 1^{-/-}$
DMSO	69.2 ± 2.1^a	50.9 ± 2.7^a
A23187	82.2 ± 3.2^b	67.0 ± 4.6^b

[a] One thousand sperm (250 sperm/slide) were counted in each male mouse and three male mice were analyzed in each group. Control: C57BL/6J × CBA F_1 male, PLC $\beta 1$-intact sperm. DMSO: 1% dimethyl sulfoxide. A23187: 10 μM calcium ionophore. FITC-PSA: fluorescein isothiocyanate–*Pisum sativum* agglutinin.

[b] Sperm acrosome region stained completely dark or more than 50% dark and patchy.

* a vs b, $P < 0.05$.

DISCUSSION

The AR in sperm is the breakdown and merging of the plasma membrane with the outer acrosome membrane. This reaction is facilitated by an increase in the intracellular calcium concentration in the sperm head. This process is the exocytosis of the enzyme contents of the acrosome. Acrosomal exocytosis requires extracellular calcium and zona binding of sperm initiates a signal cascade that leads to calcium influx (11). Progesterone also has been shown to induce acrosomal exocytosis (11–14). An increase in intracellular calcium (15), pH (16), cAMP (17), IP_3 (18,19), and DAG (14) has been associated with ligand-induced acrosome exocytosis in sperm. Although the specific signal transduction mechanism that triggers the AR has not been fully clarified, it is at least partly regulated by a second messenger, IP_3. IP_3 is produced by phosphoinositide-specific PLC and is known to control many cellular processes by generating internal calcium signals (5). The 10 mammalian PLC isoforms can be classified into three groups; PLC β, PLC δ, and PLC γ (20). There are four isoforms in PLC β, two in PLC γ, and four in PLC δ. All PLC isoforms contain a conserved catalytic domain, a plechstrin homology (PH) domain in the amino terminus of the protein, and additional regulatory sequences (21). The PLC γ family participates in the mitogenic response by directly coupling to both receptor and nonreceptor tyrosine kinase, whereas the PLC β family acts through G protein-dependent pathways. However, it is not clear what roles the individual isozymes in a given PLC family play *in vivo* or to what extent they determine the specificity of individual signaling pathways (10). IP_3 increased by PLC bound to the IP_3 receptor (IP_3R) of the gated calcium channel located on the anterior acrosome membrane, and IP_3-gated calcium release may be an important mechanism for activating the extracellular calcium influx required for the AR (7). Also, IP_3-gated calcium release is well known to be essential for egg activation (8). In this study, we evaluate the biological significance of PLC $\beta 1$ (localized on the sperm head) in mouse sperm AR, fertilization, and embryo development using mutant mouse sperm which lack the PLC $\beta 1$ gene. Mutation of the PLC $\beta 1$ gene in male mice resulted in a significant decrease, but not to the full extent, in the spontaneous sperm AR compared with that in the wild-type mouse sperm. This result suggests that PLC $\beta 1$ is involved in the AR but does not seem to play the pivotal role. Another possibility, although not elucidated in this study, is that PLC $\beta 1$ may be the main enzyme in the normal AR

Table II. Fertilization and Development of Embryos in C57BL/6J Mouse After In Vitro Fertilization (IVF) and Culture[a]

Group	Total No. of oocytes[b]	No. of embryos according to development stage (mean % ± SD)*		
		Fertilization	3- to 8-cell	MO–BL
Control	166	122 (73.5 ± 3.6)[a]	65 (39.2 ± 1.4)[b]	26 (15.7 ± 2.6)[c]
PLC $\beta1^{-/-}$	139	72 (51.8 ± 4.5)[a]	16 (11.5 ± 3.7)[b]	6 (4.3 ± 2.2)[c]

[a] Control: C57BL/6J × CBA F_1 male, PLC β1-intact sperm. MO–BL: morula to blastocyst stage of embryo. Fertilization: formation of 2PN or two cells at 18 hr after insemination was regarded as fertilization.
[b] A total of 305 oocytes from 15 egg donors was collected and mixed up for equal experimental conditions.
* a vs b vs c, $P < 0.05$.

process and have a compensatory mechanism for the loss of PLC β1 (21). The latter hypothesis is supported by reports that some of the biological effects of PLC β can be mediated by PLC γ, as certain G protein-coupled receptors can activate PLC γ, thus providing a potential compensatory mechanism for the loss of PLC β isoforms (21,22). Mouse heterozygotes for the PLC β1 mutation are known to be normal and fertile, whereas homozygotes display retarded growth, low viability after birth, and a low reproductive rate (10). Also, in our results, the fertilization rate and embryo development in the PLC $\beta1^{-/-}$ group decreased compared to those in the control group. This finding may be one explanation for the low reproductive potential of the mouse null for PLC β1.

CONCLUSION

Mutation of the PLC β1 gene in mouse sperm reduces the AR rate, the fertilization rate, and embryo development. The decreased rate of AR, fertilization, and embryo development in PLC $\beta1^{-/-}$ mouse sperm may be etiologic factors responsible for the low reproductive rate.

ACKNOWLEDGMENTS

This work was supported by KOSEF through SRC-MTRC.

REFERENCES

1. Breitbart H, Spungin B: The biochemistry of the acrosome reaction. Mol Hum Reprod 1997;3:195–202
2. Yanagimachi R, Usui N: Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res 1974;89:161–174
3. Fraser LR: Motility patterns in mouse spermatozoa before and after capacitation. J Exp Zool 1977;202:439–444
4. Kopf GS, Gerton GL: The mammalian sperm acrosome and the acrosome reaction. *In* Elements of Mammalian Fertilization, PM Wasserman (eds). Boca Raton, FL, CRC Press, 1991, pp 153–203
5. Spungin B, Margalit I, Breitbart H: Sperm exocytosis reconstructed in a cell-free system. J Cell Sci 1995;108 (Pt8):2525–2535
6. Dupont G, McGuinness OM, Johson MH, Berridge MJ, Borgese F: Phospholipase C in mouse oocytes: Characterization of β and γ isoforms and their possible involvement in sperm-induced Ca^{2+} spiking. Biochemistry 1996;316(2):583–591
7. Walensky LD, Snyder SH: Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosome of mammalian sperm. J Cell Biol 1995;130:857–869
8. Miyazaki S, Yuzaki M, Nakada K, *et al.*: Block of Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science (*Wash. DC*) 1992;257:251–255
9. Shilling FM, Carroll DJ, Muslin AJ, Escobedo JM, Williams LT, Jaffe LA: Evidence for tyrosine kinase and G protein-coupled pathways leading to starfish egg activation. Dev Biol 1994;162:590–599
10. Kim DS, Jun KS, Lee SB, *et al.*: Phospholipase C isozymes selectively coupled to specific neurotransmitter receptors. Nature 1997;389:290–293
11. Osman RA, Andris ML, Jones AD, Meizel S: Steroid induced exocytosis: The human sperm acrosome reaction. Biochem Biophy Res Commun 1989;160:828–833
12. Blackmore PF, Beebe SJ, Danforth DR, Alexander N: Progesterone and 17a-hydroxyprogesterone: Novel stimulators of calcium influx in human sperm. J Biol Chem 1990;265:1376–1380
13. Blackmore PF, Neulen J, Lattanzio F, Beebe SJ: Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. J Biol Chem 1991;266:18655–18659
14. Roldan ERS, Murase T, Shi QX: Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science 1994;266:1578–1581
15. Lee MA, Storey BT: Endpoint of first stage of zona pellucida-induced acrosome reaction in mouse spermatozoa characterized by acrosome H^+ and Ca^{2+} permeability. Gamete Res 1989;24:303–326
16. Florman HM, Tombes RM, First NL, Babcock DF: An adhesion-associated agonist from the zona pellucida activites G protein-promoted elevations of internal Ca^{2+} and pH that mediate mammalian sperm acrosome reaction. Dev Biol 1989;135:133–146
17. Hyne RV, Garbers DL: Calcium-dependent increase in adenosine 3′,5′-monophosphate and induction of the arosome

reaction in guinea pig spermatozoa. Proc Nal Acad Sci USA 1979;76:5699–5703
18. Domino SE, Garbers DL: The fucose-sulfate glycoconjugate that induces an acrosome reaction in spermatozoa stimulates inosotol 1,4,5-trisphosphate accumulation. J Biol Chem 1988;263:690–695
19. Thomas P, Meizel S: Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. Biochem J 1989;264:539–546
20. Lee SB, Rhee SG: Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Curr Opin Cell Biol 1995;7:183–189
21. Schlessinger J: Phospholipase Cγ activation and phosphoinositide hydrolysis are essential for embryonal development. Proc Natl Acad Sci USA 1997;94:2798–2799
22. Rhee SG, Bae YS: Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 1997;272:15045–15048
23. Bedford JM: Sperm capacitation and fertilization in mammals. Biol Reprod 1970;2 (Suppl 2):128–158
24. Roldan ERS, Harrison RAP: Polyphosphoinositide breakdown and subsequent exocytosis in the Ca^{2+}/ionophore-induced acrosome reaction of mammalian spermatozoa. Biochem J 1989;259:397–406
25. Rhee SG, Choi KD: Regulation of inositol phospholipid-specific phospholipase C isozymes. J Biol Chem 1992; 267:12393–12396
26. Spungin B, Breitbart H: Calcium mobilization and influx during sperm exocytosis. J Cell Sci 1996;109(7):1947–1955
27. Florman HM, First N: Regulation of acrosome exocytosis. Dev Biol 1988;128:464–473