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Male Mice That Do Not Express Group VIA Phospholipase A_2 Produce Spermatozoa with Impaired Motility and Have Greatly Reduced Fertility*

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Abstract

The Group VIA Phospholipase A_2 (iPLA$_2\beta$) is the first recognized cytosolic Ca^{2+}-independent PLA_2 and has been proposed to participate in arachidonic acid (20:4) incorporation into glycerophosphocholine lipids, cell proliferation, exocytosis, apoptosis, and other processes. To study iPLA$_2\beta$ functions, we disrupted its gene by homologous recombination to generate mice that do not express iPLA$_2\beta$. Heterozygous iPLA$_2\beta^{+/-}$ breeding pairs yield a Mendelian 1:2:1 ratio of iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, and iPLA$_2\beta^{-/-}$ pups and a 1:1 male:female gender distribution of iPLA$_2\beta^{-/-}$ pups. Several tissues of wild-type mice express iPLA$_2\beta$ mRNA, immunoreactive protein, and activity, and testes express the highest levels. Testes or other tissues of iPLA$_2\beta^{-/-}$ mice express no iPLA$_2\beta$ mRNA or protein, but iPLA$_2\beta^{-/-}$ testes are not deficient in 20:4-containing glycerophosphocholine lipids, indicating that iPLA$_2\beta$ does not play an obligatory role in formation of such lipids in that tissue. Spermatozoa from iPLA$_2\beta^{-/-}$ mice have reduced motility and impaired ability to fertilize mouse oocytes *in vitro* and *in vivo*, and inhibiting iPLA$_2\beta$ with a bromoenol lactone suicide substrate reduces motility of wild-type spermatozoa in a time- and concentration-dependent manner.Mating iPLA$_2\beta^{-/-}$ male mice with iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, or iPLA$_2\beta^{-/-}$ female mice yields only about 7% of the number of pups produced by mating pairs with an iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{+/-}$ male, but iPLA$_2\beta^{-/-}$ female mice have nearly normal fertility. These findings indicate that iPLA$_2\beta$ plays an important functional role in spermatozoa, suggest a target for developing male contraceptive drugs, and complement reports that disruption of the Group IVA PLA_2 (cPLA$_2\alpha$) gene impairs female reproductive ability.

Phospholipases A_2 (PLA_2s)[1] catalyze hydrolysis of the *sn*-2 fatty acid substituent from glycerophospholipid substrates to yield a free fatty acid, *e.g.* arachidonic acid, and a 2-lysophospholipid that can initiate synthesis of lipid mediators (1, 2). Arachidonic acid

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(20:4), for example, is converted by various oxygenases to prostaglandins, leukotrienes, epoxy-trienes, and other mediators, and acetylation of 2-lysoplasmanylcholine yields the mediator platelet-activating factor (3). Both 20:4 and 2-lysophospholipids also have intrinsic mediator activities (4, 5).

Of mammalian PLA_2s so far cloned, secretory PLA_2s are low molecular weight enzymes that require millimolar Ca^{2+} concentrations for catalysis and affect eicosanoid generation, inflammation, and other processes (1). The platelet-activating factor-acetylhydrolase PLA_2 family exhibits substrate specificity for platelet-activating factor and oxidized phospholipids. Of Group IV cytosolic PLA_2 ($cPLA_2$) family members (1), $cPLA_2\alpha$ was the first identified and prefers substrates with *sn*-2 20:4 residues, catalyzes 20:4 release for subsequent metabolism, associates with its substrates in membranes upon rises in cytosolic [Ca^{2+}] in stimulated cells, and is also regulated by phosphorylation (6).

The Group VI PLA_2 ($iPLA_2$) enzymes (1, 2, 7, 8) do not require Ca^{2+} for catalysis and are inhibited by a bromoenol lactone (BEL) suicide substrate that does not inhibit secretory PLA_2 or $cPLA_2$ at similar concentrations (9, 10). The Group VIA PLA_2 ($iPLA_2\beta$) resides mainly in the cytoplasm of resting cells, but the Group VIB PLA_2 ($iPLA_2\gamma$) contains a peroxisomal targeting sequence and is membrane-associated (11).

Many cells express multiple distinct PLA_2s, and this might reflect redundancy or specific functions of an individual PLA_2. Physiological roles for PLA_2s can be studied with genetic gain-or loss-of-function manipulations. Overexpressing $iPLA_2\beta$ in insulinoma cells, for example, provides evidence for its participation in exocytosis, cell proliferation, and apoptosis (12–14), and $cPLA_2\alpha$ gene disruption by homologous recombination has produced $cPLA_2\alpha$-null mice that reveal a role for $cPLA_2\alpha$ in parturition, allergic responses, and post-ischemic brain injury (15, 16).

We have used homologous recombination to generate $iPLA_2\beta$-null mice. Among various tissues, testes of wild-type mice express the highest $iPLA_2\beta$ levels, and male $iPLA_2\beta^{-/-}$ mice produce spermatozoa with reduced motility and impaired ability to fertilize mouse oocytes *in vitro* and *in vivo*. Male $iPLA_2\beta^{-/-}$ mice are also much less fertile than wild-type males, but female $iPLA_2\beta^{-/-}$ mouse fertility is not markedly impaired. Our findings indicate that $iPLA_2\beta^{-/-}$ plays an important functional role in spermatozoa.

EXPERIMENTAL PROCEDURES

Generating $iPLA_2^{-/-}$ Knockout Mice

To prepare a knockout construct, we obtained a P1 clone with an $iPLA_2\beta$ gene fragment by screening a 129/SvJ mouse genomic DNA library with rat $iPLA_2\beta$ cDNA (8). The 7.8-kb EcoRV-BglII fragment containing exons 7–10 was sub-cloned into pBluescript SK-. A single XhoI site mapped to exon 9 near sequence encoding the ^{463}GTSTG^{467} lipase motif. A pGK-neo-poly(A) cassette with a neomycin resistance gene (neo) was inserted at this site to disrupt $iPLA_2\beta$ coding sequence and provide a positive selection marker. A pGK-thymidine kinase gene was inserted into the BglII site of the genomic fragment as a negative selection marker. This yielded a vector with 4.1 and 3.7 kb of the 5′ and 3′ sequences, respectively, homologous to the native gene for recombination.

[1]The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Group VI PLA_2; BEL, bromoenol lactone; ESI, electrospray ionization; MS, mass spectrometry; GPC, glycerophosphocholine; TAG, triacylglycerol; hCG, human chorionic gonadotropin; LPC, 2-lysophosphatidylcholine; ER, endoplasmic reticulum; SOC, store-operated channel

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The targeting fragment was excised with EcoRV and BglII and introduced into 129/SvJ mouse embryonic stem cells by electroporation. Clones resistant to G418 and ganciclovir were isolated and screened for homologous recombination by Southern blotting of genomic DNA digested with EcoRV. Six embryonic stem clones contained 6.7-kb fragments characteristic of iPLA$_2\beta$ gene disruption and, as expected, 8.7-kb fragments from the wild-type allele. The clones were injected into C57BL/6 mouse blastocysts, which were implanted for gestation to yield chimeras that were mated with wild-type mice to yield heterozygotes. Mating iPLA$_2\beta^{+/-}$ mice with each other gave iPLA$_2\beta^{-/-}$, iPLA$_2\beta^{-/+}$, and iPLA$_2\beta^{+/+}$ pups.

Mice were genotyped with Southern blots of tail clipping genomic DNA digested with EcoRV using a ^{32}P-labeled probe prepared by PCR amplification or restriction endonuclease digestion with EcoRV and BglII to yield an 0.95-kb fragment located downstream of the targeting sequence. This probe hybridizes with an 8.7-kb DNA fragment in iPLA$_2\beta^{+/+}$ mice, with a 6.7-kb fragment in iPLA$_2\beta^{-/-}$ mice, and with both in iPLA$_2\beta^{+/-}$ mice (see Fig. 1*D*). The iPLA$_2\beta$ gene-targeting sequence inserts a new EcoRV site in genomic DNA to yield a fragment that hybridizes with the probe that is shorter than that from wild-type genomic DNA.

Northern and Western Blot Analyses

As described (8), tissue total RNA analyzed by electrophoresis was transferred to nylon membranes that were hybridized with iPLA$_2\beta$ cDNA probes labeled by random priming. The iPLA$_2\beta$ cDNA probe was amplified using reverse transcription-PCR (sense primer, 5′-TGTGACGTGGACAGCACTAGC; antisense primer, 5′-CCCCAGAGAAACGACTATGGA), which hybridizes to both short and long isoforms of iPLA$_2\beta$ (2). This region of cDNA represents the sequence that encodes amino acid residues 307–552 of the short isoform of iPLA$_2\beta$. A final stringency wash was followed by autoradiography, and the filters were then stripped and hybridized with cDNA probes to rat glyceraldehyde-3-phosphate dehydrogenase to mark RNA load (8). Western blotting was performed as described with tissue homogenates and iPLA$_2\beta$ antibody that had been raised in rabbits (12).

Ca^{2+}-independent Phospholipase A_2 Activity Assay

Tissue Ca^{2+}-independent PLA$_2$ specific activity was determined as described (8) in cytosol by monitoring hydrolysis of 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine in assay buffer (40 mM Tris, pH 7.5, 5 mM EGTA) to [^{14}C]linoleate as measured by TLC and liquid scintillation spectrometry. Specific activity was calculated from released [^{14}C]disintegrations/min and protein content.

Electrospray Ionization Mass Spectrometry of Lipids

Tissue lipids were extracted and infused into the ESI source of a triple stage quadrupole mass spectrometer in $CHCl_3/CH_3OH$ containing LiOH, as described (17–19). Glycerophosphocholine (GPC) lipids and triacylglycerols (TAGs) were analyzed as $[M+Li]^+$ ions. Seminolipid and glycerol-, inositol-, and ethanolamine-glycerophospholipids and phosphatidic acids were analyzed as $[M-H]^-$ ions. Tandem spectra were obtained by accelerating selected ions into a collision cell to induce dissociation, and product ions were analyzed in the third quadrupole. Internal standards, *e.g.* 14:0/14:0-GPC, were used for quantitation.

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Analyses of Motility of Spermatozoa and Effect of the iPLA$_2$β Inhibitor BEL

Male mice 8–10 weeks old were euthanized with pentobarbital in a protocol approved by our Animal Studies Committee. Caudae epididymides, and the vasa deferentia were removed and placed in prewarmed and pregassed human tubal fluid medium (Irvine Scientific, Irvine, CA). Morphology and viability of spermatozoa were assessed as described (20–22), did not differ between iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice, and were unaffected by BEL. Sperm suspension was placed in an incubation chamber (37 °C), and motility was quantified using CEROS computer-assisted semen analyses (version 10; Hamilton Thorne Research, Beverly, MA), as discussed (23). Total and progressive motilities were analyzed in about 10^4 spermatozoa from each genotype. A swim-up motility assay was also used that involved centrifugation, incubation (1 h, 37 °C), and counting spermatozoa that migrated into supernatant, as described (22). Spermatozoa were treated in some cases with various concentrations of BEL (0–20 μM), and motility was analyzed after various periods (0–30 min).

In Vitro Fertilization Assay

Female mice about 30 days old were injected with pregnant mare serum gonadotropin (7.5 IU intraperitoneally) and 48 h later were injected with human chorionic gonadotropin (hCG; 7.5 IU intraperitoneally), as discussed (24). Oviducts were collected 13 h later, and the oocyte cumulus complexes were removed. One or two complexes were placed in a culture dish (100 mm, 50 μl minimum essential medium, 25 mM $NaHCO_3$, 1% fatty acid-free bovine serum albumin; Sigma), and droplets were covered by embryo-tested mineral oil (Sigma). Spermatozoa collected from cauda epididymis were allowed to swim into minimum essential medium (10 min, 37 °C), aspirated, incubated (37 °C, 60 min, 5% CO_2, 4×10^6/ml) to permit capacitation, diluted, and added to oocyte droplets to achieve a concentration of 10^5 or 10^6 spermatozoa/ml. Spermatozoa and oocytes were co-incubated (5–24 h, 37 °C, 5% CO_2). Oocytes and zygotes were passed through a pipette to remove cumulus cells, fixed (1% paraformaldehyde), placed on a microscope slide with affixed coverslip, treated with acetic acid/ethanol (1/3 v/v, 2 min), stained (1% lacmoid/45% acetic acid), destained (45% acetic acid), and examined for oocyte fertilization reflected by a second polar body and two pronuclei with one near a sperm tail (24).

In Vivo Fertilization Assay

Superovulation of 6–8-week-old female mice was induced by injecting pregnant mare serum gonadotropin (10 IU, intraperitoneally; Sigma) and, after 48 h, hCG (10 IU, intraperitoneal, Sigma), as described (21–24). Those mice were then mated with iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{-/-}$ males overnight, and, after 48 h, the mice were euthanized with pentobarbital according to a protocol approved by our Animal Studies Committee. Dissected uterine horns were flushed with human tubal fluid medium containing 0.25% bovine serum albumin (Sigma) to retrieve one- and two-cell structures, which were then counted and cultured (human tubal fluid with 0.25% bovine serum albumin media microdroplets under mineral oil, 37 °C, 5% CO_2) for 72 h. The blastocyst embryos were then counted.

Fertility Tests

Male mice were placed with females for 6 weeks and then removed, and the pups were counted, as described (21). Females mated with iPLA$_2\beta^{-/-}$ males had a normal frequency of vaginal semen plugs, determined as described (25).

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Statistical Analyses

Comparisons between two groups or among three or more groups were performed with Student's *t* test or with analysis of variance using posthoc analysis (Statview 4.51, Abacus), respectively.

RESULTS

Generation of iPLA$_2$β-null Mice

Fig. 1 illustrates our scheme to generate mice with a disrupted iPLA$_2\beta$ gene and to determine their genotypes. An iPLA$_2\beta$ gene-targeting construct was introduced into mouse embryonic stem cells, and those that incorporated it by homologous recombination, which disrupts the iPLA$_2\beta$ gene coding sequence (Fig. 1*B*), were introduced into mouse blastocysts that were then implanted into pseudo-pregnant female mice. Progeny included chimeras, which were mated with wild-type mice, and litters included iPLA$_2\beta^{+/-}$ mice, reflecting iPLA$_2\beta^{-}$ allele germ-line transmission. Mating pairs of iPLA$_2\beta^{+/-}$ mice yielded iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, and iPLA$_2\beta^{-/-}$ pups in a nearly Mendelian 1:2: 1 distribution with a 1:1 male/female gender distribution (Table I).

Northern blots revealed that wild-type mouse testes iPLA$_2\beta$ mRNA content exceeds that of muscle, pancreas, kidney, liver, brain, heart, adipose, and epididymis (Fig. 2*A*). No iPLA$_2\beta$ mRNA was detected in testes or other tissues of iPLA$_2\beta^{-/-}$ mice. Western blots also revealed high iPLA$_2\beta$ protein expression in wild-type testes, but no iPLA$_2\beta$ protein was detected in iPLA$_2\beta^{-/-}$ testes or spermatozoa (Fig. 2*B*).

The highest level of Ca^{2+}-independent PLA$_2$ activity is also observed in the testes of wild-type mice (Fig. 3*A*), and, as characteristic of iPLA$_2\beta$ (10–12), it is stimulated by ATP and inhibited by a BEL suicide substrate (Fig. 3*B*). Testes and other tissues of iPLA$_2\beta^{-/-}$ mice exhibit much less total Ca^{2+}-independent PLA$_2$ activity than wild-type mouse tissues (Fig. 3*A*), suggesting that iPLA$_2\beta$ is ordinarily the major tissue Ca^{2+-}independent PLA$_2$ and that there is little up-regulation of other Ca^{2+}-independent PLA$_2$s, such as cPLA$_2\gamma$ and iPLA$_2\gamma$ (1–2), to compensate for the loss of iPLA$_2\beta$.

Effect of iPLA$_2$β Gene Disruption on Tissue Content of Arachidonic Acid-containing Phosphatidylcholine and Other Lipids

Studies of [$^3H_8$]arachidonic acid incorporation into P388D1 tumor cells have been taken to imply that the function of iPLA$_2\beta$ is to generate lysophosphatidylcholine acceptors for arachidonic acid (20:4) incorporation into GPC lipids (26), and it might thus be expected that iPLA$_2\beta^{-/-}$ mouse tissues would be deficient in 20:4-containing GPC lipids. The lipids were extracted from wild-type and iPLA$_2\beta^{-/-}$ mouse testes and analyzed by ESI/MS in the presence of LiOH. Fig. 4*A* is the ESI/MS positive total ion current spectrum for a lipid extract from wild-type mouse testes, and it contains ions with even *m*/*z* values for Li^+ adducts (17) of internal standard (*m*/*z* 684) dimyristoyl-GPC (14:0/14:0-GPC) and of endogenous 16:0/18:1-GPC (*m*/*z* 766) and the 20:4-containing species 16:0/20:4-GPC (*m*/*z* 788) and 18:1/20:4-GPC (*m*/*z* 814).

The spectrum also contains odd *m*/*z* value ions for Li^+ adducts of sphingomyelins (*e.g.* 16:0-sphingomyelin at *m*/*z* 709) and of TAGs (18) with various fatty acid substituents (*e.g. m*/*z* 837, 863, and 889). Fig. 4*C* illustrates the tandem spectrum that identifies the species represented by the ion of *m*/*z* 788 as 16:0/20:4-GPC (20), and it contains ions for losses of trimethylamine (*m*/*z* 729), of phosphocholine (*m*/*z* 605), of Li^+ phosphocholine (*m*/*z* 599), of palmitic acid (16:0, *m*/*z* 532), of Li^+ 16:0 (*m*/*z* 526), of 20:4 (*m*/*z* 484), of Li^+ 20:4 (*m*/*z* 478), of 16:0 plus trimethylamine (*m*/*z* 473), of the ketene of 20:4 plus Li^+ phosphocholine

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(*m*/*z* 313), and 16:0 acylium ion (*m*/*z* 239). Fig. 4*D* illustrates tandem spectra that identify TAG Li^+ adducts (18), such as that of the ion of *m*/*z* 863 for 16:0/18:1/18:2-TAGLi^+ that contains ions reflecting losses of 16:0 (*m*/*z* 607), of Li^+ 16:0 (*m*/*z* 601), of oleic acid (18:1, *m*/*z* 581), of Li^+ 18:1 (*m*/*z* 575), of linoleic acid (18:2, *m*/*z* 583), and of Li^+ 18:2 (*m*/*z* 577). Incomplete exchange of Li^+ for Na^+ on occasion resulted in satellite peaks in some TAG spectra (*e.g.* *m*/*z* 853, 879, and 905 in Fig. 4*B*) that did not occur in others (Fig. 4*A*).

The ESI/MS spectrum for Li^+ adducts of iPLA$_2\beta^{-/-}$ mouse testes lipids (Fig. 4*B*) is nearly identical to that for iPLA$_2\beta^{+/+}$ mice (Fig. 4*A*), and abundances of ions for 20:4-containing GPC lipids (*m*/*z* 788 and *m*/*z* 814) relative to the internal standard (*m*/*z* 684) are virtually identical in *panels A* and *B* of Fig. 4, indicating that iPLA$_2\beta^{-/-}$ mouse testes are not deficient in 20:4-containing GPC lipids. Negative ion ESI/MS analyses (19) of testes lipids reveal 20:4-containing glycerophosphoethanolamine and glycerophosphoinositol lipids, *e.g.* 18:0/20:4-glycerophosphoethanolamine and 18:0/20:4-glycerophosphoinositol, and other lipid species. Negative ion ESI/MS spectra for iPLA$_2\beta^{-/-}$ and iPLA$_2\beta^{+/+}$ testes lipids are also nearly identical (not shown). Lack of iPLA$_2\beta$ thus has little effect on testes phospholipid composition or 20:4-content, and iPLA$_2\beta^{-/-}$ and iPLA$_2\beta^{+/+}$ testes also do not differ in gross or microscopic anatomy or weight.

Motility of Spermatozoa from Wild-type and iPLA$_2\beta^{-/-}$ Mice

Although there is a modest reduction in number of spermatozoa produced by iPLA$_2\beta^{-/-}$ mice ($1.5 \pm 0.4 \times 10^7$/mouse) compared with wild-type mice ($3.4 \pm 0.7 \times 10^7$/mouse), there is a marked reduction in motility of iPLA$_2\beta^{-/-}$ mouse spermatozoa. Fig. 5*A* summarizes swim-up motility analyses and illustrates that motility of spermatozoa from iPLA$_2\beta^{-/-}$ mice is less than 5% of that of spermatozoa from iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{+/-}$ mice. Fig. 5*B* illustrates computer-assisted spermatozoa analyses of motility and also demonstrates reduced motility of spermatozoa from iPLA$_2\beta^{-/-}$ compared with iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{+/-}$ mice.

Effect of Inhibiting iPLA$_2\beta$ Activity with a BEL Suicide Substrate on Motility of Spermatozoa

To examine further the role of iPLA$_2\beta$ in motility, suspensions of spermatozoa were treated with varied concentrations of the iPLA$_2\beta$ inhibitor BEL (9–10) for various intervals, and motility of spermatozoa was determined. BEL reduced motility of spermatozoa in a concentration- and time-dependent manner at [BEL] as low as 5 μM (Fig. 6*A*) and at times as early as 5 min (Fig. 6*B*).

In Vitro Fertilization of Mouse Oocytes

The reduced motility of iPLA$_2\beta^{-/-}$ spermatozoa suggested that they might be impaired functionally. Fig. 7*A* illustrates that wild-type spermatozoa exhibit a concentration-dependent ability to fertilize oocytes from wild-type female mice *in vitro*, but spermatozoa from iPLA$_2\beta^{-/-}$ mice achieved fertilization only about 5% as often as wild-type spermatozoa at the highest concentration tested, indicating that iPLA$_2\beta^{-/-}$ spermatozoa are functionally impaired.

In Vivo Fertilization of Mouse Oocytes

Fertilization experiments *in vivo* were performed by inducing wild-type female mice to ovulate by treatment with injected pregnant mare serum gonadotropin and hCG. They were then mated with control male mice of normal reproductive competence or with iPLA$_2\beta^{-/-}$ male mice. Products from oocytes were recovered from the uterus, and it was determined whether fertilization had occurred and whether there had been subsequent development of

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blastocysts. Fig. 7*B* illustrates that when female mice were mated with control, reproductively competent male mice, 84 two-cell structures were observed by 48 h after hCG injection, and 86% of them later developed into blastocysts. In contrast, when female mice were mated with iPLA$_2\beta^{-/-}$ male mice, only 2 two-cell structures were observed by 48 h after hCG injection, and neither developed into a blastocyst (Fig. 7*B*). Both *in vitro* (Fig. 7*A*) and *in vivo* (Fig. 7*B*) fertilization experiments thus demonstrate functional impairment of spermatozoa from iPLA$_2\beta^{-/-}$ mice.

Fertility of iPLA$_2\beta^{-/-}$ Male Mice

The reduced motility of iPLA$_2\beta^{-/-}$ mouse spermatozoa and their impairment in fertilizing oocytes raised the question of whether these mice would exhibit reduced fertility. Male iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, and iPLA$_2\beta^{-/-}$ mice were thus mated with female iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, and iPLA$_2\beta^{-/-}$ mice. Ten breeding pairs were examined for each possible genotypic pairing. Each pair was allowed to mate for 6 weeks, and the number of pups each pair produced during this period was determined. Table II illustrates that with wild-type iPLA$_2\beta^{+/+}$ male partners, female iPLA$_2\beta^{+/+}$ mice bore 59 pups and female iPLA$_2\beta^{-/-}$ mice produced 70 pups, indicating that female iPLA$_2\beta^{-/-}$ mice exhibit no severe reproductive defect.

Similar numbers of pups were produced by pairs of either wild-type iPLA$_2\beta^{+/+}$ males or heterozygous iPLA$_2\beta^{+/-}$ males with female mice of all three genotypes. A total of 214 pups were sired by iPLA$_2\beta^{+/+}$ males, and 235 pups were sired by iPLA$_2\beta^{+/-}$ males (Table II), indicating that iPLA$_2\beta^{+/-}$ males are not reproductively impaired. In contrast, iPLA$_2\beta^{-/-}$ male mice sired only 16 pups or about 7% of the number sired by iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{+/-}$ males, reflecting severe reproductive impairment in iPLA$_2\beta^{-/-}$ males despite a normal frequency of vaginal semen plugs in females with which they were mated.

Male iPLA$_2\beta^{-/-}$ males were not completely infertile and sired more pups with wild-type iPLA$_2\beta^{+/+}$ female partners than with iPLA$_2\beta^{+/-}$ or iPLA$_2\beta^{-/-}$ female partners (Table II). No mechanistic explanation for that finding is obvious because iPLA$_2\beta^{+/-}$ females produced more pups (total of 183) than did iPLA$_2\beta^{+/+}$ females (total of 148 pups) when mated with iPLA$_2\beta^{+/+}$ or iPLA$_2\beta^{+/-}$ males. The severe impairment of fertility of male iPLA$_2\beta^{-/-}$ mice is reflected by the fact that they sired only a single pup with iPLA$_2\beta^{+/-}$ female partners under these conditions. The larger number of pups resulting from matings of iPLA$_2\beta^{+/+}$ females with iPLA$_2\beta^{-/-}$ males might reflect chance variation, although an occult mechanistic basis cannot be excluded.

DISCUSSION

Male iPLA$_2\beta^{-/-}$ knockout mice have greatly reduced fertility that would impose a selection bias against that genotype, although female iPLA$_2\beta^{-/-}$ mouse reproductive ability is not dramatically impaired. The low iPLA$_2\beta^{-/-}$ male fertility is associated with markedly reduced motility of spermatozoa. Inhibition of iPLA$_2\beta$ with BEL also reduces motility of wild-type spermatozoa in a concentration- and time-dependent manner that resembles the effect of eliminating iPLA$_2\beta$ by gene disruption. The iPLA$_2\beta$-null mouse thus joins a group of recently reported mouse models involving gene disruption that produce selective impairment of male (but not female) fertility associated with reduced motility of spermatozoa. Other such models include disruption of the genes for soluble adenylyl cyclase (20), for the voltage-gated cation channels Catsper1 and CatSper2 (27–29), and for plasma membrane Ca^{2+}-ATPase 4 (25).

Signals that regulate motility of spermatozoa include changes in cAMP and intracellular [Ca^{2+}] (27–33), and both parameters are affected by products of PLA$_2$ action, which include

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a free fatty acid and a 2-lysophospholipid. Mice deficient in soluble adenylyl cyclase activity are infertile because of a severe sperm motility defect (20), and both abnormalities also occur in mice null for the catalytic subunit of cAMP-dependent protein kinase A (33). Products of iPLA$_2\beta$ action affect downstream effects of cAMP, and iPLA$_2\beta$ overexpression in insulinoma cells amplifies secretion induced by glucose and agents that elevate [cAMP] (12). The iPLA$_2\beta$ reaction products 2-lysophosphatidylcholine (LPC) and 2-lysoplasmenylcholine activate cAMP-dependent protein kinase A and enhance phosphorylation of the cAMP response element-binding protein in cardiac myocytes (34), and LPC generated by iPLA$_2\beta$ also regulates cAMP- and cAMP-dependent protein kinase A-dependent events in macrophages and endothelial cells (35, 36).

The flagellar motion underlying sperm motility is cyclic and is associated with [Ca^{2+}] oscillations at the base of the flagellum that occur at the frequency of the flagellar beat (37). Hyperactivated motility involves Ca^{2+} release from intracellular sequestration sites, such as the redundant nuclear envelope that surrounds the axoneme at its origin in the flagellar base (30). The fact that motility of spermatozoa is impaired by knockout of channels that mediate Ca^{2+} entry (27–29) or a plasma membrane pump that extrudes Ca^{2+} (25) is consistent with a requirement for Ca^{2+} oscillations for flagellar motion.

Insulin secretion by β-cells is oscillatory and associated with [Ca^{2+}] oscillations produced by a cyclic process that involves glucose-induced Ca^{2+} release from endoplasmic reticulum (ER) and resultant activation of a nonselective plasma membrane cation channels (38). Activation of these store-operated channels (SOC) depolarizes the plasma membrane and causes voltage-operated Ca^{2+} channels to mediate Ca^{2+} influx. This results in refilling of ER Ca^{2+} stores and inactivation of the depolarizing SOC (38). This cycle repeats itself in an oscillatory manner in the continued presence of stimulus.

Recently, iPLA$_2\beta$ has been found to participate in regulating SOC (39). Ca^{2+} store depletion-induced activation of depolarizing SOC and resultant activation of voltage-operated Ca^{2+} channels in smooth muscle cells involves production of Ca^{2+} influx factor by ER. Ca^{2+} influx factor then interacts with calmodulin so as to release it from and relieve its tonic inhibition of iPLA$_2\beta$, which then catalyzes phospholipid hydrolysis. LPC produced by iPLA$_2\beta$ then activates SOC (39). Pharmacologic and biochemical evidence supports operation of this pathway in vascular smooth muscle cells and β-cells (38–41). The operation of a similar pathway in spermatozoa could rationalize the requirement for Ca^{2+} store release (30), iPLA$_2\beta$ (this report), Ca^{2+} entry channels (27–29), and a Ca^{2+} extrusion pump (25) in the oscillatory flagellar motion that underlies the motility of spermatozoa.

The free fatty acid product of iPLA$_2\beta$ action could also participate in regulating [Ca^{2+}] in spermatozoa subcellular compartments. Arachidonic acid (20:4) facilitates Ca^{2+} entry from the extracellular space and Ca^{2+} release from ER (42), and 20:4-containing plasmenylethanolamine species are abundant in ER and are excellent iPLA$_2\beta$ substrates (43). Moreover, Ca^{2+} influx factor is an arachidonate oxygenation product (44), and its production could involve iPLA$_2\beta$ action.

The fact that spermatozoa from iPLA$_2\beta^{-/-}$ mice are defective in *in vitro* fertilization in which a high concentration of spermatozoa are placed in close proximity to oocytes suggests that these spermatozoa might be defective in properties in addition to motility. Ca^{2+} signaling in the tail of spermatozoa is involved in regulating flagellar motion, and [Ca^{2+}] in the head of spermatozoa is involved in the acrosomal reaction induced in spermatozoa by oocyte zona pellucida (45), a reaction in which iPLA$_2\beta$ could participate. A PLA$_2$ is activated during induction of the acrosomal reaction by zona pellucida and releases arachidonic acid and LPC from spermatozoa membrane phospholipids (46). LPC also

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induces the acrosome reaction in spermatozoa (30, 32), and one species of LPC (2-lysoplasmanylcholine) is the precursor of the platelet-activating factor, which is produced by spermatozoa and is an autocrine inducer of capacitation (47).

Our finding that homozygous iPLA$_2\beta$ gene disruption impairs male reproductive ability by causing production of spermatozoa with reduced motility complements reports that cPLA$_2\alpha$ gene disruption impairs female reproductive ability by preventing parturition (15–16). PLA$_2$ activities are thus involved in multiple steps of the reproductive process, and the reduced motility and fertilization competence of spermatozoa from male iPLA$_2\beta^{-/-}$ mice coupled with the reduction in motility of spermatozoa induced by inhibiting iPLA$_2\beta$ with BEL suggest that iPLA$_2\beta$ is a potential target for developing male contraceptive agents.

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References

1. Six D, Dennis E. Biochim Biophys Acta. 2000; 1488:1–19. [PubMed: 11080672]
2. Ma Z, Turk J. Prog Nucleic Acids Res Mol Biol. 2001; 67:1–33.
3. Murphy RC, Fitzpatrick FA. Methods Enzymol. 1990; 187:1–628.
4. Brash AR. J Clin Investig. 2001; 107:1339–1345. [PubMed: 11390413]
5. Radu CG, Yang LV, Riedinger M, Au M, Witten ON. Proc Natl Acad Sci U S A. 2004; 101:245–250. [PubMed: 14681556]
6. Gijon M, Spencer D, Kaiser A, Leslie C. J Cell Biol. 1999; 145:1219–1232. [PubMed: 10366595]
7. Tang J, Kriz RW, Wolfman N, Shaffer M, Seehra J, Jones SS. J Biol Chem. 1997; 272:8567–8575. [PubMed: 9079687]
8. Ma Z, Ramanadham S, Kempe K, Chi XS, Ladenson J, Turk J. J Biol Chem. 1997; 272:11118–11127. [PubMed: 9111008]
9. Hazen SL, Zupan LA, Weiss RH, Getman DP, Gross RW. J Biol Chem. 1991; 266:7227–7232. [PubMed: 2016324]
10. Ma Z, Ramanadham S, Hu Z, Turk J. Biochim Biophys Acta. 1998; 1391:384–400. [PubMed: 9555100]
11. Mancuso D, Jenkins CM, Gross RW. J Biol Chem. 2000; 275:9937–9945. [PubMed: 10744668]
12. Ma Z, Ramanadham S, Wohltmann M, Bohrer A, Hsu FF, Turk J. J Biol Chem. 2001; 276:13198–13208. [PubMed: 11278673]
13. Ma Z, Bohrer A, Wohltmann M, Ramanadham S, Hsu FF, Turk J. Lipids. 2001; 36:689–700. [PubMed: 11521967]
14. Ramanadham S, Hsu FF, Zhang S, Jin C, Bohrer A, Song H, Bao S, Ma Z, Turk J. Biochemistry. 2004; 43:918–930. [PubMed: 14744135]
15. Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, Sapirstein A. Nature. 1997; 390:622–625. [PubMed: 9403693]
16. Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, Komagata Y, Maki K, Ikuta K, Ouchi Y, Miyazaki J, Shimizu T. Nature. 1997; 390:618–622. [PubMed: 9403692]
17. Hsu FF, Bohrer A, Turk J. J Am Soc Mass Spectrom. 1998; 9:516–526. [PubMed: 9879366]
18. Hsu FF, Turk J. J Am Soc Mass Spectrom. 1999; 10:587–600. [PubMed: 10384723]
19. Ramanadham S, Hsu FF, Bohrer A, Nowatzke W, Ma Z, Turk J. Biochemistry. 1998; 37:4533–4567.
20. Esposito G, Jaiswal B, Xie F, Krajnc-Franken M, Robben T, Strik A, Kuil C, Philipsen R, van Duin M, Conti M, Gossen J. Proc Natl Acad Sci U S A. 2004; 101:2993–2998. [PubMed: 14976244]

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21. Hagaman J, Moyer J, Bachman E, Sigony S, Magyar P, Welch J, Smithies O, Krege J, O'Brien D. Proc Natl Acad Sci U S A. 1998; 95:2252–2257.
22. Schwartz D, Goldfinger N, Kam Z, Rotter V. Cell Growth & Differ. 1999; 10:665–675.
23. Moley KH, Vaughn WK, DeCherney AH, Diamond MP. J Reprod Fert. 1991; 93:325–332.
24. Miller D, Gong X, Decker G, Schur B. J Cell Biol. 1993; 123:1431–1440. [PubMed: 8253842]
25. Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch KP, Neyses L. J Biol Chem. 2004; 279:28220–28226. [PubMed: 15078889]
26. Balsinde J, Bianco I, Ackermann E, Conde-Frieboes K, Dennis E. Proc Natl Acad Sci U S A. 1995; 92:8527–8531. [PubMed: 7667324]
27. Carlson A, Westenbroek R, Quill T, Ren D, Clapham D, Hille B, Garbers DL, Babcock D. Proc Natl Acad Sci U S A. 2003; 100:14864–14868. [PubMed: 14657352]
28. Quill T, Ren D, Clapham D, Garbers DL. Proc Natl Acad Sci U S A. 2001; 98:12527–12531. [PubMed: 11675491]
29. Quill T, Sugden SA, Rossi K, Doolittle L, Hammer R, Garbers DL. Proc Natl Acad Sci U S A. 2003; 100:14869–14874. [PubMed: 14657366]
30. Ho HC, Suarez SS. Biol Reprod. 2001; 65:1606–1615. [PubMed: 11673282]
31. Ho HC, Suarez SS. Biol Reprod. 2003; 68:1590–1596. [PubMed: 12606347]
32. Marquez B, Suarez SS. Biol Reprod. 2004; 70:1626–1633. [PubMed: 14766720]
33. Skalhegg BS, Huang Y, Su T, Iddzerda RL, McKnight GS, Burton KA. Mol Endocrinol. 2002; 16:630–639. [PubMed: 11875122]
34. Williams S, Ford D. Am J Physiol. 2001; 281:H168–H176.
35. Maggi LB Jr, Moran JM, Scarim AL, Ford DA, Yoon JW, McHowat J, Buller RM, Corbett JA. J Biol Chem. 2002; 277:38449–38455. [PubMed: 12167650]
36. Martinson B, Albert C, Corbett J, Wysolmerski R, Ford DA. J Lipid Res. 2003; 44:1686–1691. [PubMed: 12810819]
37. Suarez SS, Varosi M, Dai X. Proc Natl Acad Sci U S A. 1993; 90:4660–4664. [PubMed: 8506314]
38. Roe MW, Worley JF, Qian F, Tamarina N, Mittal AA, Dralyuk F, Blair NT, Mertz RJ, Philipson LH, Dukes ID. J Biol Chem. 1998; 273:10402–10410. [PubMed: 9553098]
39. Smani T, Zakharov SI, Csutora P, Leno E, Trepakova E, Bolotina VM. Nature Cell Biol. 2004; 6:113–121. [PubMed: 14730314]
40. Wolf MJ, Wang J, Turk J, Gross RW. J Biol Chem. 1997; 272:1522–1526. [PubMed: 8999823]
41. Nowatzke W, Ramanadham S, Hsu FF, Ma Z, Bohrer A, Turk J. Endocrinology. 1998; 139:4073–4085. [PubMed: 9751485]
42. Ramanadham S, Gross RW, Turk J. Biochem Biophys Res Commun. 1992; 184:647–653. [PubMed: 1575739]
43. Ramanadham S, Bohrer A, Gross RW, Turk J. Biochemistry. 1993; 32:13499–13509. [PubMed: 8257685]
44. Rzigalinski BA, Willoughby KA, Hoffman SW, Falck JR, Ellis EF. J Biol Chem. 1999; 274:175–182. [PubMed: 9867827]
45. Talbot P, Schur B, Myles D. Biol Reprod. 2003; 68:1–9. [PubMed: 12493688]
46. Yuan Y, Chen W, Shi Q, Mao L, Yu Q, Fang X, Roldan E. Biol Reprod. 2003; 68:904–913. [PubMed: 12604641]
47. Wu C, Stojanov T, Cham O, Ishi S, Shimuzu T, Li A, O'Neill C. J Biol Chem. 2001; 276:26962–26968. [PubMed: 11350972]

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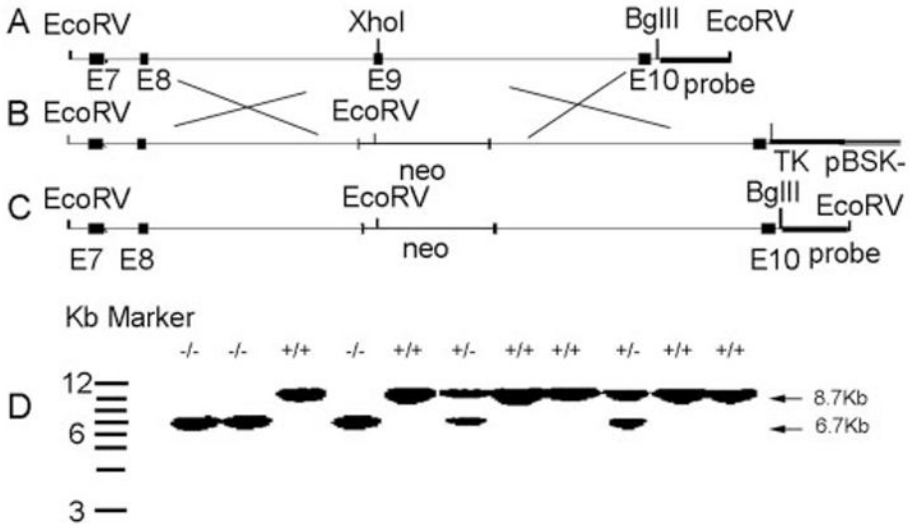

FIG. 1. Scheme for disrupting the iPLA$_2\beta$ gene in mice, identifying disrupted and wild-type iPLA$_2\beta$ alleles, and determining genotypes of progeny of mating male and female iPLA$_2\beta^{+/-}$ pairs

A–C, wild-type allele (*A*) and scheme for preparing the knockout construct and incorporating it into the iPLA$_2\beta$ gene (*B* and *C*). *D*, Southern blot identification of disrupted and wild-type iPLA$_2\beta$ alleles and genotypes of offspring from iPLA$_2\beta^{+/-}$ mating pairs.

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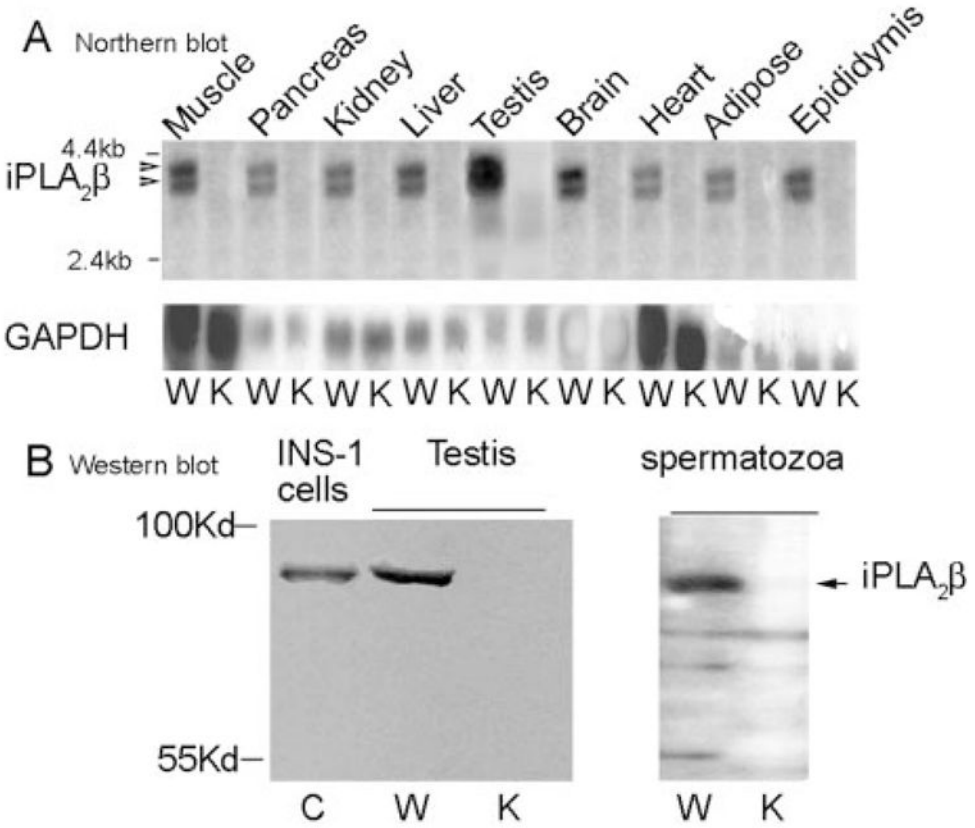

FIG. 2. Expression of iPLA$_2\beta$ mRNA in tissues and of iPLA$_2\beta$ immunoreactive protein in testes and spermatozoa of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice

A, Northern blots of iPLA$_2\beta$ mRNA in tissues of wild-type (*W*) and iPLA$_2\beta^{-/-}$ (*K*) mice. *B*, Western blots of iPLA$_2\beta$ immunoreactive protein in INS-1 insulinoma cells (*C*, control) and in testes (*left panel*) and spermatozoa (*right panel*) of wild-type (*W*) and iPLA$_2\beta^{-/-}$ knockout (*K*) mice.

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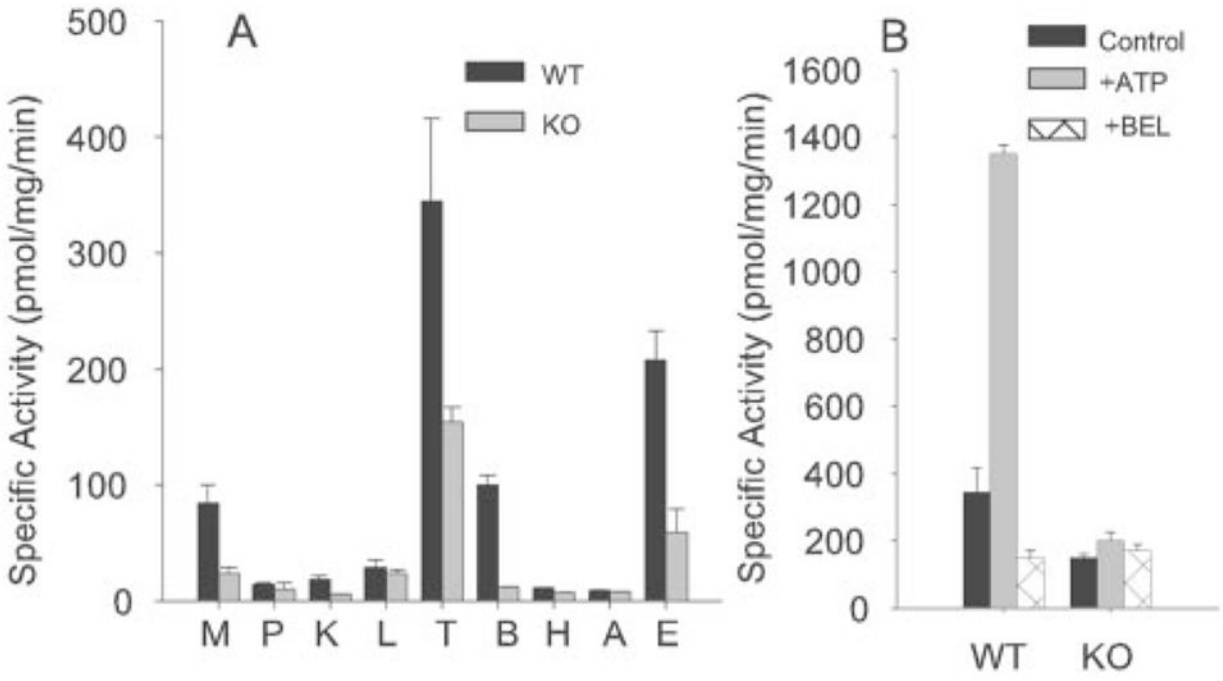

FIG. 3. Enzymatic activity of iPLA$_2\beta$ in tissues and effects of ATP and BEL on iPLA$_2\beta$ activity in testes of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice

A, tissue iPLA$_2\beta$ activities in wild-type (*WT*, *black bars*) and iPLA$_2\beta^{-/-}$ knockout (*KO*, *shaded bars*) mice determined as described under "Experimental Procedures" in muscle (*M*), pancreas (*P*), kidney (*K*), liver (*L*), testes (*T*), brain (*B*), heart (*H*), adipose (*A*), and epididymis (*E*). *B*, effect of ATP (1 mM) and the iPLA$_2\beta$ inhibitor BEL (10 μM) on testes Ca^{2+}-independent PLA$_2$ activity in wild-type (*WT*) and iPLA$_2\beta^{-/-}$ knockout (*KO*) mice.

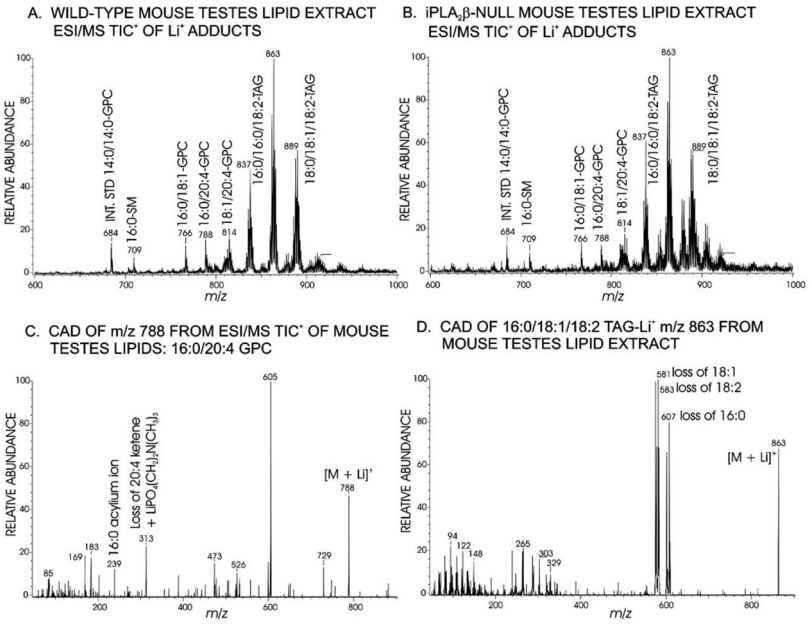

FIG. 4. ESI/MS analyses of glycerolipids from testes of iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice

ESI/MS positive total ion current (*TIC*) tracing of a lipid extract from wild-type (*A*) or iPLA$_2\beta^{-/-}$ knockout (*B*) mouse testes infused in LiOH solution. *C*, tandem mass spectrum from collisionally activated dissociation (*CAD*) of the ion of *m*/*z* 788 for 16:0/20:4-GPC-Li$^+$. *D*, tandem mass spectrum from collisionally activated dissociation of the ion of *m*/*z* 863 for 16:0/18:1/18:2-TAG-Li$^+$.

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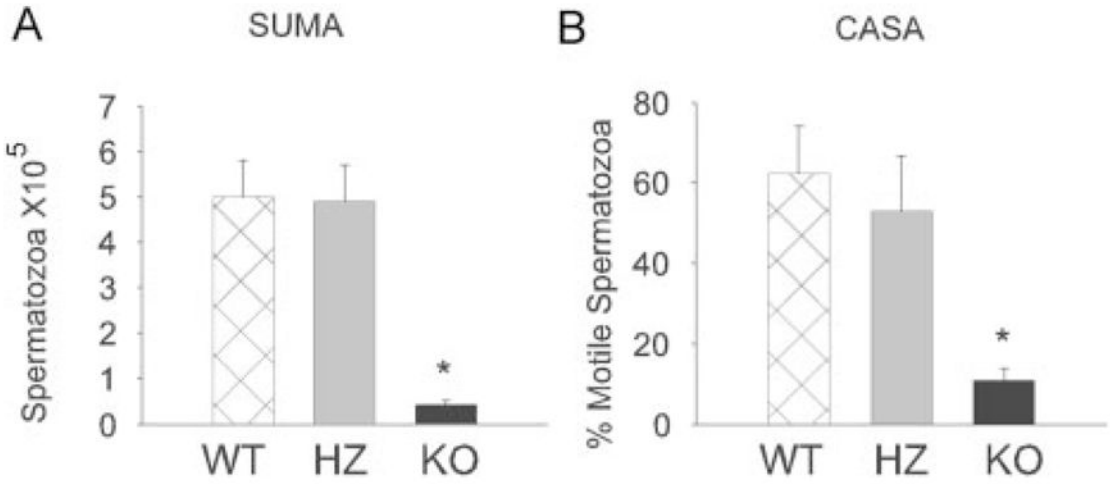

FIG. 5. Motility of spermatozoa from iPLA$_2\beta^{+/+}$, iPLA$_2\beta^{+/-}$, and iPLA$_2\beta^{-/-}$ mice

Motility of spermatozoa from wild-type (*WT*), heterozygous (*HZ*), and iPLA$_2\beta^{-/-}$ knockout (*KO*) mice examined by swim-up motility assay (*A*) or computer-assisted semen analyses (*B*) studies described under "Experimental Procedures." The *error bars* reflect S.E. ($n = 4$; *, $p < 0.001$).

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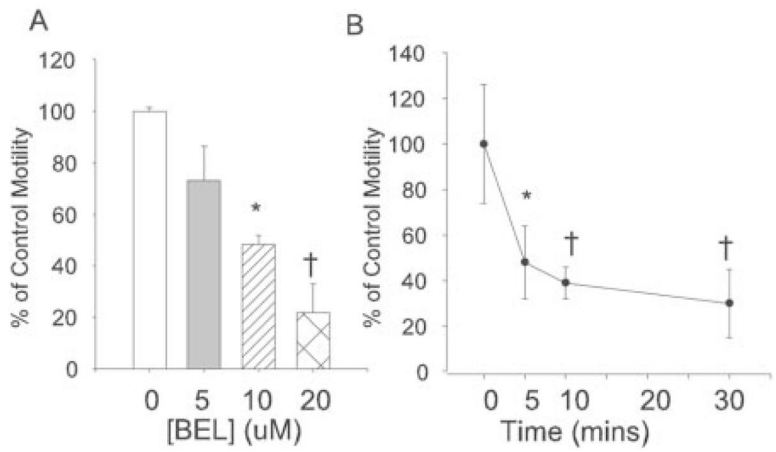

FIG. 6. Effect of the iPLA$_2\beta$ inhibitor BEL on motility of wild-type mouse spermatozoa

Motility of spermatozoa was examined with varied concentrations of BEL (*A*) at 60 min or with 20 μM BEL for various periods (*B*). The *error bars* reflect S.E. ($n = 7$; *, $p < 0.05$; †, $p < 0.01$).

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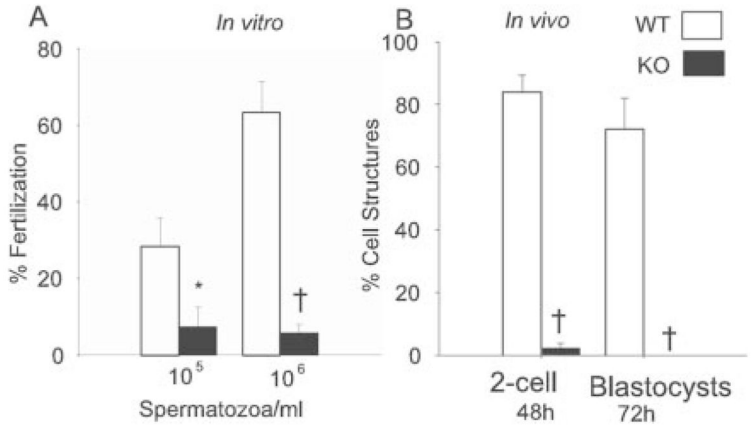

FIG. 7. Fertilization of mouse oocytes *in vitro* and *in vivo* by spermatozoa from iPLA$_2\beta^{+/+}$ and iPLA$_2\beta^{-/-}$ mice

A, fertilization of mouse oocytes *in vitro* by varied concentrations of spermatozoa from wild-type iPLA$_2\beta^{+/+}$ (*WT*, *shaded bars*) mice and of iPLA$_2\beta^{-/-}$ knockout (*KO*, *black bars*) mice determined as under "Experimental Procedures." *B*, fertilization of mouse oocytes *in vivo* by control male mice of normal reproductive competence (*shaded bars*) or iPLA$_2\beta^{-/-}$ knockout male (*black bars*) mice determined as under "Experimental Procedures" (*, $p < 0.05$; †, $p < 0.001$).

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Table I

Distribution of genotypes and genders in offspring from mating male iPLA$_2\beta^{+/-}$ heterozygous mice with female heterozygous iPLA$_2\beta^{+/-}$ mice

Genotype	Total no. of pups	Gender	No. of pups
iPLA$_{2\beta}^{+/+}$	31	Male	13
		Female	18
iPLA$_{2\beta}^{+/-}$	72	Male	35
		Female	37
iPLA$_{2\beta}^{-/-}$	35	Male	17
		Female	18

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Table II

Number of offspring from mating pairs of male and female mice of different genotypes

Male	Female	Pups in 6 weeks with 10 mating pairs
+/+	+/+	59
	+/−	85
	−/−	70
	Total	214
+/−	+/+	89
	+/−	98
	−/−	48
	Total	235
−/−	+/+	15
	+/−	1
	−/−	0
	Total	16