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12. An artificial seawater medium (0 ± 1 μM C) was prepared using precombusted salts (NaCl, KCl, $MgSO_4$, $CaCl_2$) with $NaHCO_3$ and organic-free, deionized water (Milli-RO and Milli-Q UV Plus). The filtrate (<0.7 μm pore size GF/F) from seawater samples was used as a natural inoculum (2% v/v) of marine bacteria. Based on the DOC content in the Gulf of Mexico seawater inoculum, the final concentration of DOC in the inoculated medium prior to the addition of glucose was 1 ± 1 μM. Based on the DOC content in the Sagami Bay seawater inoculum, the final concentration of DOC in the inoculated medium prior to the addition of glutamate was 2 ± 1 μM.
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17. THNS is the sum of seven neutral sugars (glucose, galactose, fucose, rhamnose, mannose, arabinose, and xylose), and THAS is the sum of three amino sugars [galactosamine (GalN), glucosamine (GlcN), and muramic acid (MA)], which were measured by HPLC after HCl hydrolysis (3 M, 5 hours, 100°C) (*13*, *28*). No samples had measurable mannosamine. MA was not detected after 1 year. THAA is the sum of 17 amino acids determined by HPLC (*14*) after vapor-phase hydrolysis (*36*). THAA composition is presented in Table 2 as four families of amino acids: acidic (aspartic acid, glutamic acid), basic (arginine, histidine, lysine), neutral (serine, glycine, threonine, tyrosine), and hydrophobic (β-alanine, alanine, γ-aminobutyric acid, methionine, phenylalanine, valine, isoleucine, leucine). The concentrations of individual amino acids are available as supplementary Web tables at www.sciencemag.org/cgi/content/full/292/5518/917/DC1
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REPORTS

Requirement of Phospholipase Cδ4 for the Zona Pellucida–Induced Acrosome Reaction

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Several phospholipase C (PLC) isoforms have been found in male and female mammalian gametes, and splicing isoforms of PLCδ4 are predominantly expressed in testis. Here we report that male mice in which the *PLCδ4* gene had been disrupted either produced few small litters or were sterile. In vitro fertilization studies showed that insemination with PLCδ4$^{-/-}$ sperm resulted in significantly fewer eggs becoming activated and that the calcium transients associated with fertilization were absent or delayed. PLCδ4$^{-/-}$ sperm were unable to initiate the acrosome reaction, an exocytotic event required for fertilization and induced by interaction with the egg coat, the zona pellucida. These data demonstrate that PLCδ4 functions in the acrosome reaction that is induced by the zona pellucida during mammalian fertilization.

Spatial and temporal activation of phosphoinositide turnover enables eukaryotic cells to induce various functions such as cell signaling, cytoskeletal remodeling, phagocytosis, membrane traffic, and ion channel activity (*1*, *2*). Phospholipase C (PLC) plays a crucial role in this turnover by hydrolyzing phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$) to generate two second messengers, inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] and diacylglycerol. Eleven PLC cDNAs have been cloned from mammalian cells, and they can be divided into four types—β, γ, δ, and ε—on the basis of sequence homology and activation mechanisms (*3*). The delta type of PLC is thought to be evolutionarily the oldest form in the mammalian PLC family, and its pleckstrin homology (PH) domain is known to function in membrane association through $PtdInsP_2$. However, the physiological role and mechanism(s) of regulation of PLC delta types has remained unclear.

To understand the physiological importance of one of four isoforms of PLCδ, PLCδ4, we inactivated this gene by targeted disruption of exons 2 to 5, which encode the ATG translation start site (Fig. 1A) and the PH domain. Correct gene targeting of the mutant mice was verified by Southern blot analysis (Fig. 1B). Western-blot analysis of brain tissue revealed that neither 85-kD PLCδ4 nor fragments of the protein were expressed in homozygous mutant mice (Fig. 1C). Heterozygous PLCδ4$^{+/-}$ mice appeared phenotypically normal and fertile, and intercrosses between heterozygous mice produced pups with a Mendelian genetic distribution, indicating that disruption of this gene did not result in embryonic lethality. Homozygous adult PLCδ4$^{-/-}$ mice appeared healthy, and histological examination showed no obvious abnormalities in the brain, liver, kidney, or heart. However, fertility of PLCδ4 homozygous mutant males was severely impaired, whereas females seemed to be fertile. About 90.0% of intercrossed PLCδ4$^{+/-}$ females were fertile, and their average litter size was 7.6 pups. Similarly, 77.8% of PLCδ4$^{-/-}$ females became pregnant when mated with PLCδ4$^{+/-}$ males, and their average litter size was 5.8 pups. Conversely, when PLCδ4$^{-/-}$ females were mated with PLCδ4$^{-/-}$ males, only 35.2% of matings resulted in pregnancy, and the average litter size was 1.2 pups. Therefore, PLCδ4 affects primarily male fertility.

Histochemical analysis of testicular sections revealed that in PLCδ4$^{-/-}$ mice, spermatogenesis proceeded normally as indicated by the presence and production of mature sperm (Fig. 2, E and F). In wild-type testis, PLCδ4 was localized by using a specific antibody (*4*) to the outermost layer where the spermatogonia reside (Fig. 2, A and C), and it was also detected as a crescent-shaped domain in the developing

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acrosome in round spermatids (Fig. 2E). Furthermore, immunofluorescent staining of PLCδ4$^{+/+}$ sperm revealed that PLCδ4 was highly concentrated in the anterior acrosomal region (Fig. 2G). The PLCδ4 protein was not expressed in the testis of PLCδ4$^{-/-}$ mice (Fig. 2, B, D, and F). These results suggest that PLCδ4 may have a role in the formation or function of the acrosome.

To determine whether or not ablation of the *PLCδ4* gene affected the expression of other PLC isoforms, we examined by Western blotting the expression of PLCδ4 and of several other PLC isozymes in the testis, epididymis, and sperm (Fig. 2H). PLCδ4 was expressed in sperm, epididymis, and testis, and the molecular mass of PLCδ4 in these organs was approximately 90 kD, suggesting that this is a testis-specific PLCδ4, splice variant ALT II, which we had previously reported (*5*, *6*). Because sperm are present in the epididymis, the presence of a band in this tissue may reflect the presence of the protein in both the sperm and the epididymis. The expression of PLCγ2, PLCβ1, PLCβ3, and PLCδ2 was not modified by the absence of PLCδ4. We scarcely detected PLCγ1 in testis and epididymis and could not detect PLCβ4, although it was detectable in brain. Thus, the function of PLCδ4 may be very specific, because, despite the presence of multiple PLC isoforms in male reproductive tissues, PLCδ4 mutant males exhibit severe infertility.

To ascertain whether PLCδ4 was involved in fertilization, we conducted in vitro fertilization experiments (Fig. 3A). Epididymal sperm from PLCδ4$^{-/-}$ mice were similar in number, viability, morphology, and motility to sperm from wild-type males by microscope observation. We first examined eggs 6 hours after insemination and 97.7% of PLCδ4$^{+/+}$ eggs inseminated with wild-type sperm had extruded the second polar body, whereas only 7.5% of wild eggs inseminated with mutant sperm had done so ($P < 0.05$; chi-square test). When eggs were observed at 24 hours, 92.3% of PLCδ4$^{+/+}$ eggs inseminated with wild sperm had divided into two-cell embryos, whereas only 56.9% of eggs inseminated with PLCδ4-null sperm had cleaved ($P < 0.05$). Moreover, most of the PLCδ4$^{-/-}$ eggs fertilized with PLCδ4$^{-/-}$ sperm failed to develop to the blastocyst stage, although the few blastocysts that resulted developed normally when transferred into surrogate mothers (*7*), demonstrating that the deficiency of PLCδ4 affects the very early stages of fertilization and not the developmental processes that follow.

Mammalian fertilization is accompanied by the initiation of persistent calcium oscillations that are responsible for triggering all events of egg activation (*8*, *9*). Thus, we investigated whether calcium responses were present in eggs inseminated with PLCδ4$^{-/-}$ sperm (Fig. 3B). The addition of capacitated PLCδ4$^{+/+}$ sperm to zona-free PLCδ4$^{+/+}$ eggs resulted in calcium oscillations after a delay of 1 to 15 min. Of 65 PLCδ4$^{+/+}$ eggs, 51 (78.5%) presented a typical pattern as shown in Fig. 3B (upper panel), and only nine eggs (13.8%) failed to show oscillations. Addition of PLCδ4$^{-/-}$ sperm to PLCδ4$^{+/+}$ eggs did not elicit calcium oscillations in most experiments. Of 60 eggs, 52 (86.7%) did not display calcium oscillations, as shown

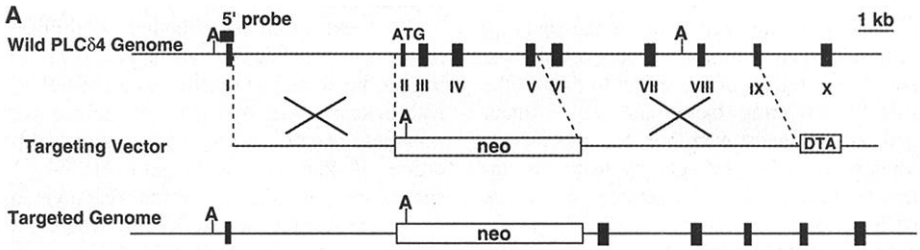

Fig. 1. Targeted disruption of *PLCδ4* gene (*21*). (**A**) Gene-targeting strategy. Exons II, III, IV, and V were replaced by the *neo* gene (neo). Diphtheria toxin A gene (DTA) was inserted at the 3′-end of the *PLCδ4* gene in the targeting vector. ApaLI (A) restriction enzyme sites are indicated. (**B**) Southern blot analysis of mouse genomic DNA. The expected fragments generated by ApaLI are 13.5 kilobase pairs (kbp) for the wild-type, and 5.6 kbp for the mutant alleles. The designation of +/+, +/−, and −/− indicates wild-type, heterozygous, and homozygous mutant mice, respectively. (**C**) Detection of PLCδ4 protein in brain nuclear extracts from PLCδ4$^{+/+}$, PLCδ4$^{+/-}$, and PLCδ4$^{-/-}$ mice.

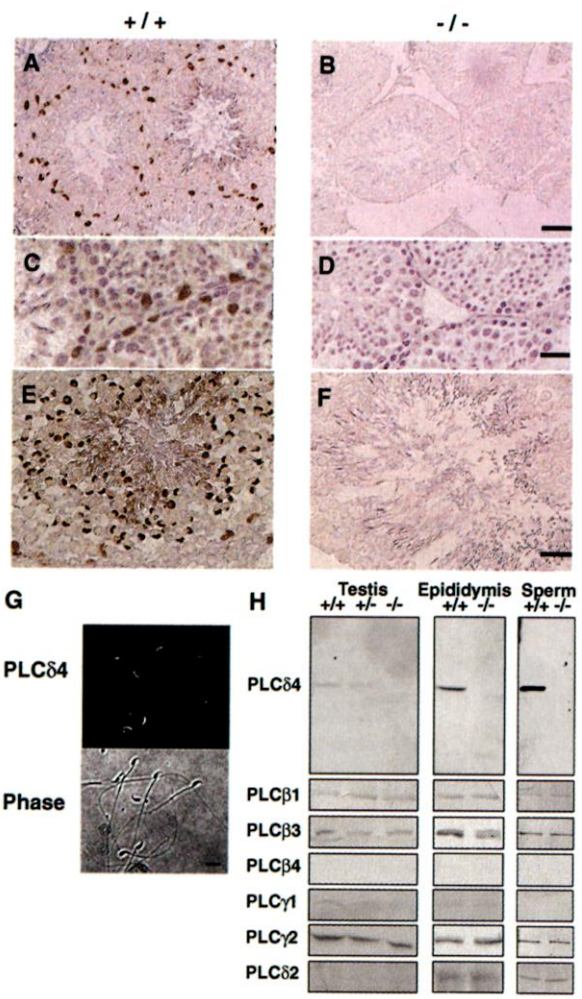

Fig. 2. Immunological detection of PLCδ4 in male reproductive tissues (*22*). Immunohistochemical staining was performed on testicular sections of wild-type (**A**, **C**, and **E**) and PLCδ4$^{-/-}$ (**B**, **D**, and **F**) males at 10 weeks of age by using a polyclonal antibody against PLCδ4. HE counterstaining was carried out in (C) and (D). (**G**) Localization of PLCδ4 in sperm. Sperm were incubated with polyclonal antibody against PLCδ4, and then treated with fluorescein isothiocyanate–conjugated antibody against rabbit. Scale bar: 50 μm (A and B), 20 μm (C to F), 10 μm (G). (**H**) Presence of PLC isozymes in male reproductive tissues. Western blot analysis of PLC isozymes from testis, epididymis, and sperm was carried out by using antibodies against a variety of PLC isoforms as shown.

in the upper tracing of the lower panel of Fig. 3B, whereas six eggs (10.0%) showed transients after a delay of 24 to 40 min (lower tracing), and two eggs (3.3%) exhibited calcium oscillations within 15 min (*10*). These results show that PLCδ4 in sperm plays an essential role in an early step or steps of fertilization.

We next examined whether the inability of mutant sperm to initiate timely oscillations was due to failure of the sperm to release the calcium-activating factor (*11*, *12*). Mutant and control sperm were injected into the cytoplasm of wild-type eggs by using the intracytoplasmic sperm injection procedure (ICSI). Both PLCδ4$^{+/+}$ and PLCδ4$^{-/-}$ sperm initiated calcium spikes at similar times after injection and with intervals of 23.7 ± 8.7 min and 22.8 ± 12.4 min, respectively (Fig. 4A). In addition, eggs subjected to ICSI by using PLCδ4$^{+/+}$ or PLCδ4$^{-/-}$ sperm extruded the second polar body and cleaved at approximately the same rates (92% or 80%, and 68% or 65%, respectively). Thus, PLCδ4$^{-/-}$ sperm are capable of inducing activation if delivered into the cytoplasm of eggs. Furthermore, when zona-free eggs were preloaded with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) to assess sperm fusion, almost all eggs failed to incorporate sperm when inseminated with PLCδ4$^{-/-}$ sperm (4.8%) (*10*), whereas most of the eggs inseminated with wild-type sperm were penetrated within 40 min of insemination (88.0%). Therefore, PLCδ4 is an essential protein for events preceding, or leading to, sperm fusion during mammalian fertilization.

We next examined whether solubilized mouse zona pellucida, which is known to trigger the acrosome reaction in a physiological manner (*13*), was able to induce the acrosome reaction in PLCδ4$^{-/-}$ sperm. Addition of zona pellucida into PLCδ4$^{+/+}$ sperm induced the acrosome reaction in 45.3% of these sperm after 15 min, whereas it induced only 16.3% of PLCδ4$^{-/-}$ sperm to undergo the reaction (Fig. 4B). Acrosome reaction induced by A23187 or thapsigargin (*14*) was not inhibited in PLCδ4$^{-/-}$ sperm. These data show an important role of the PLCδ4 protein in mediating the zona pellucida–induced acrosome reaction. The delay in egg activation by the PLCδ4$^{-/-}$ sperm was more evident when examined within 40 min of insemination and, as time after insemination increased, mutant sperm appeared to gain ability to fertilize eggs in vitro (Fig. 3A), indicating that mutant sperm might also be able to undergo spontaneous acrosome reaction, a phenomenon whose occurrence is known to increase in a time-dependent manner (*14*).

The interaction of the sperm with the zona pellucida leads to a persistent calcium increase in the sperm, which is critical in order for the acrosome reaction to proceed (*15*, *16*). Furthermore, this calcium elevation appears to be mediated by calcium influx through a store depletion–operated pathway (*17*). Our results suggest that PLCδ4 may be involved in the generation of Ins(1,4,5)P_3 and this, in turn, leads to persistently elevated intracellular calcium concentrations in the sperm with emptying of the calcium stores, which may be the stimulus that triggers and sustains the capacitative calcium influx. PLCδ4 may also alter intracellular levels of PtdInsP_2, and this may lead to changes in membrane stability that facilitates exocytosis (*2*). Furthermore, an abnormal phosphoinositide turnover might affect cholesterol metabolism, the depletion of which from the sperm outer membrane is required for the acrosome reaction (*18*, *19*). Last, PLCδ4 may directly promote calcium influx by regulating calcium channels coupled to Ins(1,4,5)P_3 receptor–PtdInsP_2 (*20*).

Our knockout experiments demonstrate that PLCδ4 plays an essential role in mediating the zona pellucida–induced acrosome reaction, which is required for fertilization. Continued efforts to analyze the function of PLCδ4 will assist in elucidating the molecular mechanisms underlying mammalian fertilization and may have implications in the field of contraception.

A

Egg	Sperm	Second polar body extrusion (6 h)	Two-cell stage (24 h)
+/+	+/+	169 / 173 (97.7 %)	157 / 170 (92.3 %)
+/+	-/-	12 / 160 (7.5 %)	111 / 195 (56.9 %)

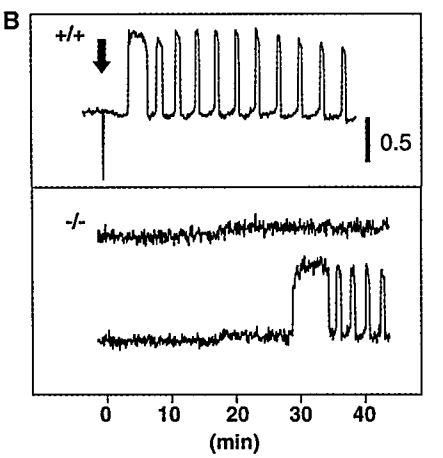

Fig. 3. In vitro fertilization studies using PLCδ4-deficient eggs and sperm (**A**) (*23*). Zona-intact eggs collected from PLCδ4$^{+/+}$ mice were inseminated with either PLCδ4$^{+/+}$ or PLCδ4$^{-/-}$ sperm. Figure shows the number of eggs that extruded second polar body (6 hours) or proceeded to the two-cell stage (24 hours). (**B**) Calcium oscillations are abolished/delayed after insemination with PLCδ4$^{-/-}$ sperm. Typical patterns of calcium oscillations in zona-free eggs inseminated with PLCδ4$^{+/+}$ (upper panel) or PLCδ4$^{-/-}$ sperm (lower panel) are shown. The arrow denotes the time of sperm addition. Scale bar: 0.5 $\Delta F/F$.

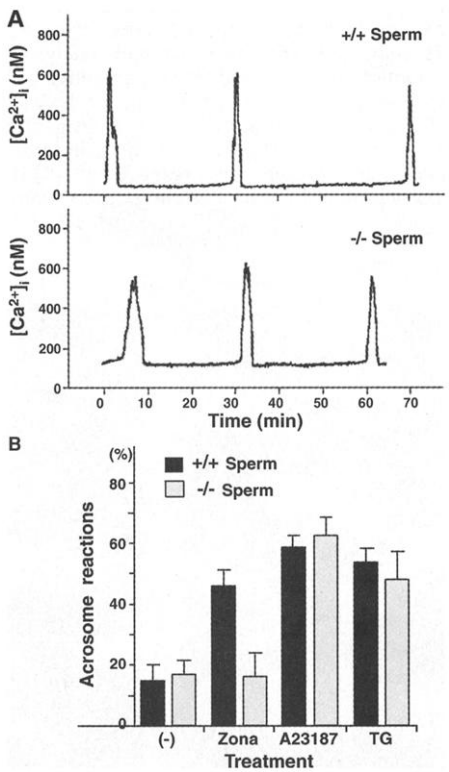

Fig. 4. PLCδ4 plays an essential role in the zona pellucida–induced acrosome reaction. (**A**) Calcium oscillations after ICSI (*24*). Calcium monitoring started within 10 min of the injection, and five eggs injected with PLCδ4$^{+/+}$ sperm and eight eggs injected with PLCδ4$^{-/-}$ sperm were monitored. (**B**) Acrosome reaction is deficient in PLCδ4$^{-/-}$ sperm. Sperm capacitated for 1 hour were treated with 0.3 ZP/μl solubilized mouse zona pellucida (ZP), 5 μM A23187, or 2.5 μM thapsigargin (TG) for 15 min at 37°C, and then stained with 0.04% Coomassie Brilliant Blue G-250 to monitor the occurrence of acrosome reaction. Mouse zonae were isolated from mouse eggs under an inverted microscope, and soluble extracts were prepared as previously described (*25*). Data represent the results of three independent experiments in which 150 to 200 sperm were counted in each group.

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21. Phage clones containing the first coding exon of PLCδ4 were isolated from a FIXII mouse genomic library (strain 129SV/J). The linearized targeting vector was electroporated into 129SV/J ES cells. Chimeric males were bred to C57BL/6J females and DNA samples from the tail of agouti offspring were analyzed by Southern blotting.
22. For immunohistochemistry, adult testes from PLCδ4$^{+/+}$ or PLCδ4$^{-/-}$ mice were fixed in 4.0% formaldehyde, embedded in paraffin, and thin sections were treated with a procedure described in the Technical Bulletin of the TSA immunodetection kit (NEN). For Western blot analysis, 30 μg of homogenates of testis, epididymis, or sperm were subjected to SDS–polyacrylamide gel electrophoresis and electrically transferred onto a polyvinylidene difluoride membrane. The membranes were then incubated with antibodies against PLC isozymes (Santa Cruz Biotechnology). For immunostaining, sperm were fixed with 3.7% formaldehyde for 1 hour, permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100 for 5 min, and then incubated with antibody against PLCδ4 for 1 hour.
23. Fresh cauda epididymal sperm were capacitated for 1 hour in modified Krebs-Ringer (TYH) medium. A drop of sperm suspension (0.1 to 0.2 × 10^6 sperm per milliliter) was added to the medium containing eggs from wild-type mice. Embryo development was followed by microscopic observation. For measuring calcium oscillation, cumulus cells were removed by brief exposure to hyaluronidase, and the zona pellucida was removed by exposure to acid tyrode's solution (pH 2.5). Eggs were loaded at 37°C for 30 min with 3 μM calcium green I-AM (Molecular Probes). Fertilization-induced changes in intracellular calcium concentrations were measured by using confocal microscopy at 37°C.
24. Sperm were collected into PBS and frozen in liquid nitrogen. After thawing, a single sperm head was injected per egg. ICSI was performed on eggs kept in 50-μl drops of tyrode lactate–Hepes + 5% fetal bovine serum at 18° to 19°C as described before (*11*). Following ICSI, eggs were subjected to calcium monitoring.
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Control of Glutamate Clearance and Synaptic Efficacy by Glial Coverage of Neurons

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Analysis of excitatory synaptic transmission in the rat hypothalamic supraoptic nucleus revealed that glutamate clearance and, as a consequence, glutamate concentration and diffusion in the extracellular space, is associated with the degree of astrocytic coverage of its neurons. Reduction in glutamate clearance, whether induced pharmacologically or associated with a relative decrease of glial coverage in the vicinity of synapses, affected transmitter release through modulation of presynaptic metabotropic glutamate receptors. Astrocytic wrapping of neurons, therefore, contributes to the regulation of synaptic efficacy in the central nervous system.

Astrocytes contribute to the regulation of synaptic transmission by controlling glutamate diffusion and concentration in the extracellular space (*1–3*). Changes in the glial coverage of neurons in the vicinity of synapses may thus alter glutamate clearance and synaptic transmission (*4*). To examine the effect of changes in glial coverage on synaptic transmission, we recorded from the magnocellular nuclei of the hypothalamus, which undergo a well-documented anatomical neuroglial plasticity in response to intense stimulation, like lactation (*5, 6*). This results in a decreased coverage of neurons by astrocytic processes and a relative absence of these processes in the vicinity of the synapses. The changes are reversed with cessation of stimulation in postlactating animals.

To investigate such neuroglial interactions, we first examined the functional consequences of a glutamate clearance deficiency on excitatory synaptic transmission. We inhibited glutamate transporters in the rat supraoptic nucleus (SON) with either dihydrokainate (DHK)—a specific inhibitor of GLT-1 (*7, 8*), a glutamate transporter exclusively expressed in astrocytes (*9–11*)—or L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), a broad-spectrum glutamate transporter blocker (*8*). In virgin rats, where glial coverage is extensive (*5, 6*), whole-cell voltage-clamp recordings (*12*) of SON neurons revealed that evoked excitatory postsynaptic currents (EPSCs) were inhibited reversibly (Fig. 1, A and B) by 100 μM DHK (46.6 ± 8.0% of control values, $n = 9$) and 300 μM PDC (51.2 ± 6.8%, $n = 12$). That PDC and DHK depressed evoked EPSCs to the same extent suggests that, for the most part, their effect was mediated by inhibition of GLT-1 transporters, which are present only on astrocytes in this and other brain areas (*11*). The depression in EPSC amplitude induced by glutamate transporter antagonists was associated with an increase in the paired-pulse facilitation (PPF) ratio (*13*), from 1.2 ± 0.1 to 1.6 ± 0.2 (40-ms interval; $P < 0.05$, $n = 6$). Analysis of miniature EPSCs (mEPSCs) (Fig. 1, C to E) revealed that PDC decreased the frequency (−34.0 ± 6.1%; $P < 0.05$, $n = 6$), but not the size (−1.6 ± 4.3%; $P > 0.05$), of these events (Fig. 1F). This indicates that the decrease in excitatory synaptic transmission associated with blockade of glutamate transporters had a presynaptic origin.

These effects could result from a local build-up of glutamate that would lead to activation of presynaptic glutamate receptors controlling neurotransmitter release (*14, 15*). Group III metabotropic glutamate receptors (mGluRs) are known to induce a presynaptic inhibition in the SON (*16*). We thus investigated the action of PDC in the presence of L-2-amino-4-phosphonobutyric acid (L-AP4) and 2-amino-2-methyl-4-phosphonobutanoic acid (MAP4), an agonist and an antagonist of group III mGluRs, respectively (Fig. 2, A and C). Whereas L-AP4 (200 μM) impaired evoked EPSCs (19.6 ± 2.8%; $P < 0.05$, $n = 5$), subsequent addition of PDC had no further effect on EPSC amplitude (22.8 ± 3.0%; $P > 0.05$, $n = 5$). In contrast, 250 μM MAP4 increased EPSC amplitude (130.0 ± 10.0%; $P < 0.05$, $n = 9$), confirming the existence of a tonic activation of these receptors (*16*). MAP4 prevented the effects of PDC (131.2 ± 14.5%; $P > 0.05$, $n = 5$). These findings indicate that blockade of glutamate transporters in the SON modifies the concentration and/or diffusion of glutamate in the extracellular space, leading to activation of presynaptic group III mGluRs and inhibition of glutamate release.

To investigate whether similar changes in excitatory synaptic transmission occurred under physiological conditions that modify glial coverage of SON neurons, we examined hypothalamic slices obtained from lactating and postlactating animals. Glutamate transporter blockade was less effective in reducing

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