

Enhanced Hippocampal CA1 LTP but Normal Spatial Learning in Inositol 1,4,5-trisphosphate 3-kinase(A)-Deficient Mice

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Abstract

To define the physiological role of IP₃3-kinase(A) *in vivo*, we have generated a mouse strain with a null mutation of the IP₃3-kinase(A) locus by gene targeting. Homozygous mutant mice were fully viable, fertile, apparently normal, and did not show any morphological anomaly in brain sections. In the mutant brain, the IP₄ level was significantly decreased whereas the IP₃ level did not change, demonstrating a major role of IP₃3-kinase(A) in the generation of IP₄. Nevertheless, no significant difference was detected in the hippocampal neuronal cells of the wild-type and the mutant mice in the kinetics of Ca²⁺ regulation after glutamate stimulation. Electrophysiological

analyses carried out in hippocampal slices showed that the mutation significantly enhanced the LTP in the hippocampal CA1 region, but had no effect on the LTP in dentate gyrus (DG). No difference was noted, however, between the mutant and the wild-type mice in the Morris water maze task. Our results indicate that IP₃3-kinase(A) may play an important role in the regulation of LTP in hippocampal CA1 region through the generation of IP₄, but the enhanced LTP in the hippocampal CA1 does not affect spatial learning and memory.

Introduction

External signals arriving at cell-surface receptors induce second messengers in the cell to transfer information to the final effector system. Inositol 1,4,5-trisphosphate (IP₃) is one of the second messengers that is produced when phospholipase C (PLC) hydrolyzes phosphatidyl inositol bisphos-

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phate (PIP₂; Rhee et al. 1989). IP₃ produced by PLC binds to IP₃ receptor on the intracellular Ca²⁺ stores and induces calcium release into the cytosol (Irvine 1990; Furuichi and Mikoshiba 1995). IP₃ is then metabolized to inositol 1,3,4,5-tetrakisphosphate (IP₄) by IP₃3-kinase or to inositol 1,4-bisphosphate (IP₂) by 5-phosphatase. Two isotypes of IP₃3-kinase, A and B, each encoded by a distinct gene, were cloned from rat and human (Choi et al. 1990; Maillieux et al. 1992). IP₃3-kinase(A) is expressed in testis and in neuronal cells in brain (Vanweyenberg et al. 1995). In brain, the IP₃3-kinase(A) gene is highly expressed in pyramidal cells in the hippocampal CA1 region and Purkinje cells in cerebellum (Maillieux et al. 1991b; Yamada et al. 1992). These cells have often been studied with respect to long-term potentiation (LTP) and long-term depression (LTD), which are believed to be involved in learning and memory (Artola and Singer 1993; Malenka 1994). Therefore, it has been assumed that IP₃3-kinase(A) has a role in the memory process (Maillieux et al. 1991a). IP₃3-kinase(B) is widely distributed in various organs including heart, lung, thymus, and astrocytes in brain (Vanweyenberg et al. 1995). Until now, the IP₃3-kinase reaction was believed to be the only known pathway to produce IP₄ (Communi et al. 1995). However, the physiological role(s) of IP₃3-kinase(A) is not known yet.

There has been much effort over the last few years to envisage the possible physiological role(s) of IP₄. As IP₄ is rapidly produced in response to activation of PLC-coupled receptors in various cell types, it is believed that IP₄ has a second messenger role in the cell (Batty et al. 1985; Challiss and Nahorski 1991). Experimental results suggested a possible role of IP₄ in calcium homeostasis (Irvine 1991). IP₄ was also implicated to be involved in the neurotransmitter release by binding the C2B domain of synaptotagmin II (Fukuda et al. 1995). Recently, high-affinity IP₄-binding proteins were characterized to be GTPase-activating protein (GAP; Cullen et al. 1995; Fukuda and Mikoshiba 1996). However, the exact physiological role(s) of IP₄ is not clear yet.

LTP is an activity-dependent modification of the synaptic efficacy (Bliss and Collingridge 1993). Induction of LTP requires activation of postsynaptic glutamate receptors by depolarization of postsynaptic neurons, which is generated by high-frequency afferent stimulation (Madison et al. 1991; Bliss and Collingridge 1993). Two types of receptors, ionotropic receptors and metabotropic recep-

tors, mediate the increase of intracellular calcium concentration in neuronal cells and are believed to be necessary for induction of LTP (Malenka and Nicoll 1993; Bortolotto et al. 1994). Although NMDA receptors increase the intracellular Ca²⁺ concentration by opening their own channels, metabotropic glutamate receptors increase the intracellular Ca²⁺ concentration through activation of PLC enzymes, an event that produces IP₃ and diacylglycerol (DAG) (Regher and Tank 1990; Nakanishi 1992). Many reports have shown that metabotropic glutamate receptors are necessary for synaptic function and induction of LTP or LTD (Bashir et al. 1993; Hayashi et al. 1993; Linden 1994). It is generally believed that LTP in hippocampus is a synaptic substrate of memory (Bliss and Collingridge 1993; Eichenbaum 1996). Gene targeting strategies have been successfully used for studying molecular and cellular mechanisms of learning, especially for understanding the relationship between LTP and spatial learning (Mayford et al. 1995). In some cases, an association between LTP and learning and memory was demonstrated (Grant et al. 1992; Silva et al. 1992a,b; Aiba et al. 1994; Sakimura et al. 1995). In other cases, a dissociation was observed between LTP and learning and memory (Abeliovich et al. 1993a,b; Huang et al. 1996).

Here, we have defined the role of IP₃3-kinase(A) in vivo by generating and analyzing a null mutation of the IP₃3-kinase(A) locus. Focus was given to the role of this enzyme in Ca²⁺ regulation, LTP, and learning and memory.

Materials and Methods

ANIMALS

All the mice used for analysis of the mutants were F₂ progeny derived from matings of the heterozygotes in the F₁ (129/Sv × C57BL/6J) genetic background. The mice had ad lib access to food and water. Mice were housed in standard plastic cages (length × width × height, 25 cm × 18 cm × 13 cm) in an animal facility and kept on a 14:10-hr light-dark cycle with the light onset at 6 a.m. All animal handling was done following the guidelines of the Animal Care and Use Committee.

GENERATION OF IP₃3-KINASE(A)-DEFICIENT MICE

The strategy for vector construction was as described previously (Kim et al. 1997). Genomic

clones containing an exon that encodes the 5' NTR and the nucleotide sequence 1-456 from the initiation codon of cDNA were isolated by use of the rat brain cDNA (pKK 233-2/IP₃K, Choi et al. 1990) as a probe, mapped by various enzymes, and used to make the targeting vector. The *neo* cassette [PGK promoter-*neo*-PGK poly(A)] from pPNT (Tybulewicz et al. 1991) was inserted into nucleotide sequence 389 from the translation start codon for gene disruption and positive selection. The TK cassette from pPNT was attached to the end of the 3' homology region for negative selection. The final targeting construct had a 3.7-kb 5' homology and a 6.2-kb 3' homology to the genomic locus (Fig. 1A). The targeting vector was linearized at the 5' end and transfected into J1 ES cells (gift of E. Li, Massachusetts General Hospital/Harvard Medical School, Boston) at 270 V and 500 μF in the gene pulser (Bio-Rad). The transfected cells were plated on feeder layers of mitomycin C-treated (Sigma) embryonic fibroblast cells derived from 13.5-day embryos carrying the targeted mutation of β2-microglobulin (gift of R. Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, MA). Selection with 0.3 mg/ml G418 (GIBCO) and 2 μM Gancyclovir (Syntax, CA) was started 48 hr after

transfection and sustained for 10 days. Resistant colonies were picked and analyzed by Southern blotting. Three targeted ES cell clones were injected into C57BL/6J blastocysts to make chimeric mice. Male chimeras were crossed to C57BL/6J females to obtain the germ-line-transmitted offspring. The progeny were genotyped by Southern blotting.

SOUTHERN BLOT ANALYSIS

Ten micrograms of DNA isolated from each tail tip was digested with *SacI*, electrophoresed through 0.6% agarose gel, transferred with 0.4 N NaOH onto a nylon membrane, and hybridized at 65°C with ³²P-labeled probe, 1-kb *XbaI/BstBI* fragment that is 5' to and not included in the targeting vector. Blots were washed in 0.1× SSC and 0.1% SDS at 65°C for 10 min and exposed to autoradiography.

NORTHERN BLOT ANALYSIS

Brain total RNA was isolated from adult mice, 9 weeks old, by use of the Trireagent (MRC). Ten

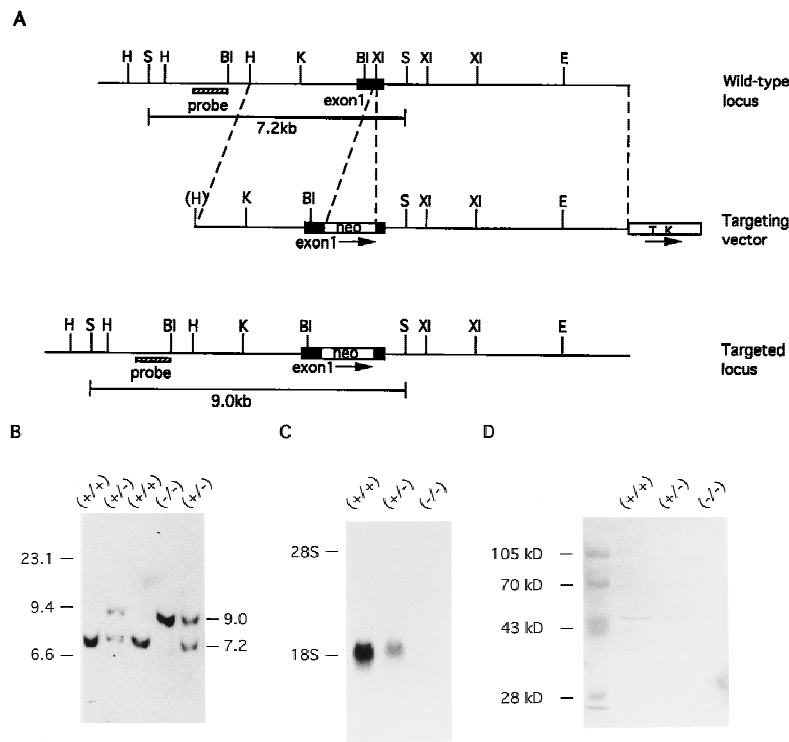


Figure 1: Disruption of the mouse IP₃-kinase(A) locus by gene targeting. (A) Structure of the wild type IP₃-kinase(A) gene locus, the targeting vector, and the targeted IP₃-kinase(A) gene locus. Rules under the wild-type and the targeted loci represent expected fragments when digested with *SacI* and hybridized with the probe (hatched bar). (BI) *BstBI*; (E) *EcoRI*; (H) *HindIII*; (K) *KpnI*; (S) *SacI*; (XI) *XhoI*. (Horizontal arrows) Transcriptional direction of the pgk-*neo* and the pgk-*tk* cassettes. (B) Southern blot analysis of offspring derived from intercrosses of IP₃-kinase (A) heterozygous parents. The 7.2-kb fragment is the wild-type allele and the 9.0-kb fragment, the targeted allele. (C) Northern blot analysis of the brain tissue. Ten micrograms of total brain RNA was isolated from a mouse of each genotype and hybridized with ³²P-labeled rat IP₃-kinase(A) cDNA. (D) Western blot analysis of brain tissue. Total brain protein was isolated from a mouse of each genotype and immunoblotted with anti-rat-IP₃-kinase(A) monoclonal antibodies. (+/+) Wild type; (+/-), heterozygote; (-/-), homozygote mutant.

micrograms of total RNA from each genotype was electrophoresed through 1% formaldehyde agarose gel, transferred onto a nylon membrane, and hybridized at 65°C with ³²P-labeled rat IP₃3-kinase(A) cDNA probe. Blots were washed in 0.1× SSC and 0.1% SDS at 65°C for 10 min and exposed to autoradiography.

WESTERN BLOT ANALYSIS

Total protein (150 μg) derived from 9-week-old wild-type and mutant brain was separated by 10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane overnight at 0.5 A in TGM buffer [25 mM Tris-Cl at pH 8.3, 192 mM glycine, 20% (vol/vol) methanol]. Equal loading of protein per lane was confirmed by Coomassie staining of a duplicated gel. The blotted membrane was incubated with 1% BSA in TBST (50 mM Tris-Cl at pH 7.5, 0.15 M NaCl, 0.2% Tween 20) at room temperature and then probed with a mixture of three kinds of mouse anti-rat IP₃3-kinase(A) monoclonal antibodies for 1 hr (Lee et al. 1990). After washing three times with TBST for 10 min, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (diluted 1:7500 in TBS, Promega) at 4°C for 1.5 hr and then washed three times with TBST. Finally, the membrane was incubated with NBT/BCIP substrate of alkaline phosphatase to develop the color.

IMMUNOHISTOCHEMISTRY

Eight-week old mice were anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. Brain sections with thickness of 50 μm were obtained by use of a Vibratome. Mouse anti-rat IP₃3-kinase(A) monoclonal antibody (1:100 dilution) was used as a primary antibody. Sections were incubated for 2 hr at room temperature in goat anti-mouse biotinylated secondary antibody (1:200) in PBS plus 3% goat serum. Sections were incubated with the avidin-biotin complex for 40 min at room temperature and developed with a diaminobenzidine solution (Vector).

RENATURATION OF IP₃3-KINASE AFTER SDS-PAGE

The crude IP₃3-kinase from 9-week-old mouse brain homogenates was denatured in the presence of 3% SDS and 5% 2-mercaptoethanol without boiling and was immediately separated in a 10% SDS-polyacrylamide slab gel at 4°C. After electrophore-

sis, the gel was cut into 3-mm slices and each slice was homogenized in 400 μl of a solution containing 84 mM HEPES/NaOH (pH 7.5), 0.1% Triton X-100, 12 mM 2-mercaptoethanol, and 25% sucrose. After 12 hr of incubation at 4°C, fractions were centrifuged (12,000g at 4°C for 2 min) and the supernatants were assayed for IP₃3-kinase activity in the presence of 0.1% Chaps. Twenty microliters of the supernatant was used in the assay with a reaction time of 1 hr.

MEASUREMENT OF IP₃

Freshly isolated mouse brains, 7–11 weeks old, were frozen rapidly in dry ice. Samples were weighed and sonicated in 1 ml of 6% trichloroacetic acid (TCA). The samples were left on ice for 30 min to extract the water-soluble inositol phosphates. After centrifugation of the samples at 12,000g at 4°C for 10 min, the supernatant was extracted with diethyl ether to remove the TCA. Then, this extract was neutralized with 200 mM Trizma base and used as the IP₃ source. The protein concentration of the tissue pellet was determined for each sample to normalize the assay. IP₃ levels were determined by a competition assay (Challiss and Nahorski 1991). Assays were performed at 4°C in a final volume of 80 μl. A 20-μl aliquot of each sample was added to 20 μl of a buffer solution containing 100 mM Tris-HCl, 4 mM EDTA (pH 8.0) and 20 μl of [³H]IP₃ (0.15 μCi/ml). The binding assay was initiated by adding to each sample 300 μg of bovine adrenal cortex membrane preparation containing IP₃-binding proteins, and the mixtures were vortex-mixed intermittently for 15 min. Bound and free [³H]IP₃ was separated by centrifugation (12,000g, 4°C for 10 min), followed by aspiration of the supernatant. Water (100 μl) and 1 ml of scintillation cocktail were added to the pellet for the measurement of the radioactivity. A standard curve obtained from using varying amounts of IP₃ (0.15–1.5 pmole) was used for the calibration of the measurements in the binding assay. Student's *t*-test was used for data analysis.

METAL DYE DETECTION HPLC ANALYSIS OF INOSITOL PHOSPHATES

The whole procedure for metal dye detection HPLC is described in detail (Mayr 1988) and HPLC conditions and detection procedure were as described (Guse et al. 1995). Briefly, brains from mice, 9–11 weeks old, killed rapidly by cervical

dislocation, were removed and dissected within 2 min into cortex, cerebellum, midbrain, hippocampus, and brain stem. They were frozen in liquid nitrogen and weighed prior to extraction. Samples up to 150 mg wet weight were added to 1 ml of ice-cold 8% TCA (wt/vol) containing 15 μ l of EDTA (0.2 M) and 15 μ l of NaF (0.1 M). After homogenization [four times with a Mini-Ultra-Turrax (tubes and rotor were precooled in ice) at 13,500 rpm for 10 sec] the samples were frozen and thawed two times. The precipitates containing acid-labile phosphoinositides were removed by centrifugation at 14,000g for 5 min at 4°C, and then the supernatant was incubated for 30 min at 37°C to destroy creatine phosphate. Then, TCA was removed by extraction with 3 ml of ice-cold diethyl ether three times. Extracts were adjusted to pH 6.0 with 1 M triethanolamine and subsequently charcoal treated by addition of 50 μ l of a 20% (wt/vol) suspension of Norit A in 0.1 M NaCl and 50 mM NaOAc (pH 4.0) per sample corresponding to 100 mg of brain tissue. Each charcoal pellet was washed with 100 μ l of 0.1 M NaCl, 25 mM NaF, and 0.5 mM EDTA at pH 7.0, which subsequently combined with the supernatant. The supernatants were concentrated to 1 ml by lyophilization in a vacuum centrifuge and applied to a column of Mini Q3,2/3 for HPLC with metal dye detection. Student *t*-test was used for data analysis.

ELECTROPHYSIOLOGY

Mouse brains, 7–9 weeks old, were separated and placed in an ice-cold (4°C) artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 4.4 mM KCl, 25 mM NaHCO₃, 1 mM Na₂H₂PO₄, 3 mM CaCl₂, 3 mM MgSO₄, and 10 mM glucose. Hippocampal slices (400 μ m thick) were prepared by use of the McIlwain chopper (Mickle Laboratory Engineering) and transferred to a submersion recording chamber containing ACSF with 1 μ M bicuculine methiodide (Sigma). The temperature in the recording chamber was maintained at 30°C. Electrophysiological experiments were begun after waiting at least 2 hr for the slice to recover. A bipolar stimulating electrode (Master-8, AMPI) was used to stimulate Schaffer collateral and commissural fibers in the stratum radiatum of the CA1 region. To stimulate the perforant pathway, a bipolar stimulating electrode was placed along the margin of the hippocampal fissure between the dentate molecular layer and the subiculum. In field potential recordings, synaptic responses were elicited at 0.03

Hz and recorded with a low-resistance glass microelectrode filled with ACSF. Tetanus-induced LTP was elicited with trains (1 sec) of 100 Hz stimulation delivered three times with an intertrain interval of 20 sec. Field potential was recorded with the DAM 80 differential amplifier (WPI). Student *t*-test was used for data analysis.

CELL CULTURE AND CALCIUM IMAGING ANALYSIS

The hippocampi were dissected from newborn pups (1 day old; F₂ progeny from 129/Sv \times C57BL/6J background) and stored in DMKM solution containing 82 mM Na₂SO₄, 30 mM K₂SO₄, 16 mM MgCl₂, 0.25 mM CaCl₂, 1 mM kynurenic acid, 20 mM glucose, 0.001% phenol red, and 1.5 mM HEPES in ddH₂O at pH 7.4. The hippocampi were digested at 37°C for 20 min with papain solution containing 10 units of papain (Worthington) and 0.45 mg of cysteine (Sigma) in 1 ml of DMKM. The supernatant was discarded and the tissues were washed with DMKM three times. Then, the tissues were triturated with DMKM by pipetting up and down. After trituration, the supernatant was plated onto a laminin-coated (0.5 μ g/cm², GIBCO) and lysine-coated (100 μ g/ml, Collaborative Biomedical Product) coverslip and incubated at 37°C with 5% CO₂. After 1–2 hr, the media were replaced with growth media consisting of Neurobasal Medium (GIBCO) supplemented with 1 \times B27 (GIBCO), 1% penicillin/streptomycin, and 0.5 mM glutamine. These cultures were incubated for 7–10 days and then used for calcium imaging. The calcium imaging was carried out as described previously with some modifications (Wayman et al. 1995). Before imaging, cells were incubated for 30 min at room temperature in the dark with 5 μ M fura-2 acetoxy-methylester (Molecular Probes) in a calcium imaging buffer (140 mM NaCl, 10 mM HEPES, 5 mM KCl, 0.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose in ddH₂O at pH 7.4). Cells were then washed with the imaging buffer and allowed to rest in the original culture media in a CO₂ incubator for 1 hr before imaging. Excitation light was provided by a 75-W xenon lamp and was attenuated by 75% to minimize photobleaching. A G.W. Ellis fiber optic light scrambler (Technical Video Ltd.) was used to transmit the light to the microscope. Excitation, emission, and neutral density filters were purchased from Omega Optical. The objective used was a Nikon Fluor 20/1.3 NA. The images were intensified with the GenIIsys image intensifier (Dage-MTI) and acquired with the Dage-MTI CCD-

72 series camera. All image acquisitions were obtained at 1-sec intervals and recorded by use of the FLUOR software (Universal Imaging, West Chester, PA). Images were viewed on the Sony Trinitron color video monitor (PVM-1343MD) and printed.

MORRIS WATER MAZE TEST

The water maze was a circular plastic pool 120 cm in diameter and 50 cm in height. The pool was centrally located in a room (2.5 × 2.5 m) in which four different distal cues were hung on the four walls high enough to be seen in the pool. The water temperature was maintained at 24°C–26°C. Nontoxic water soluble paint was added to make the water opaque. A 10-cm diam. escape platform was submerged 1 cm below the water surface at a fixed location during the test. The day before training, each mouse was allowed to swim freely for 60 sec, allowed to climb the platform three times, and rest on the platform for 30 sec. On each trial, the mouse was placed facing along the edge of the wall and randomly released at four different start points. Each trial was ended when either the mouse had climbed onto the platform or 60 sec had passed before the mouse located the platform. After locating the platform, each mouse was allowed to rest for 30 sec on the platform. Mice were given one block of three trials a day for 9 consecutive days with a 1-hr intertrial interval each day. During each trial, the time elapsed for locating the platform was determined. A single probe test was carried out either 3 or 7 days after the last trial. Mice were placed and released at the location opposite the site where the platform had been located and allowed to swim for 60 sec. Probe trials were recorded by a video cassette recorder that was fixed on the ceiling where the entire surface area of the pool could be viewed. The time spent in each quadrant and the number of crossings of the platform were analyzed by double-blind test. Both male and female mice, aged 7–11 weeks, were used for the Morris water maze task. No significant gender difference was detected. The data were analyzed by use of one-way analysis of variance (ANOVA) and Newman–Keuls post hoc analysis.

Results

CHARACTERIZATION OF THE IP₃3-KINASE(A) NULL MUTATION

Mice heterozygous for the IP₃3-kinase(A) deletion showed no apparent abnormality. These het-

erozygous mice were intercrossed, and the mutant offspring were analyzed by Southern blotting (Fig. 1B). Of the 135 offspring analyzed, 37 (27%) were wild type, 63 (47%) were heterozygous, and 35 (26%) were homozygous for the mutation, indicating a normal development of IP₃3-kinase(A) mutant embryos. The mutation of the IP₃3-kinase(A) gene in the targeted mice was verified by Northern analysis and Western blotting. No RNA or protein for IP₃3-kinase(A) was detectable in the mutant brain (Fig. 1C,D). Immunohistochemical analysis also confirmed that the mutant homozygotes did not express any IP₃3-kinase(A) protein in the brain (Fig. 2A–D). The IP₃3-kinase(A)-deficient mice were fully viable and fertile, and showed no overt behavioral or motor abnormality. The gross or histological examination of the mutant brain did not reveal any abnormality (data not shown).

BIOCHEMICAL ANALYSIS OF IP₃3-KINASE(A)-DEFICIENT MICE

The extent and selectivity of the IP₃3-kinase(A) deficiency in the IP₃3-kinase(A) mutant mice was examined biochemically. Crude extracts of the whole brain from the wild-type and the mutant mice were used as the enzyme source. Enzymatic assay using [³H]IP₃ as a substrate showed a >80% decrease in IP₃3-kinase activity in the mutant mice (data not shown). As IP₃3-kinase can be easily re-natured after SDS-PAGE, we have used this technique to identify the nature of the residual IP₃3-kinase activity in the IP₃3-kinase(A) mutant mice. Two peaks of the IP₃3-kinase activity were identified in the SDS-gel analysis of the wild-type brain: A small peak, IP₃3-kinase(B) migrating at 70–80 kD, and a large peak, IP₃3-kinase(A) migrating at 53 kD (Fig. 3A). In the mutant brain samples, the IP₃3-kinase(A) peak was totally absent, whereas the IP₃3-kinase(B) peak was intact, indicating that the residual enzyme activity in the mutant was attributable to the IP₃3-kinase(B) isotype, which was known to be expressed in astrocytes.

The effect of the IP₃3-kinase(A) deficiency on the level of IP₃ and IP₄ was examined. We predicted that the level of IP₃ in the mutant mice would be higher than that in the wild-type mice on the basis of a previous suggestion that IP₃3-kinase(A) was involved in the regulation of the IP₃ level in the brain (Communi et al. 1995). But the whole brain of the wild-type and the mutant mice contained the same quantity of IP₃ [wild type (*n* = 7 independent measurements from three

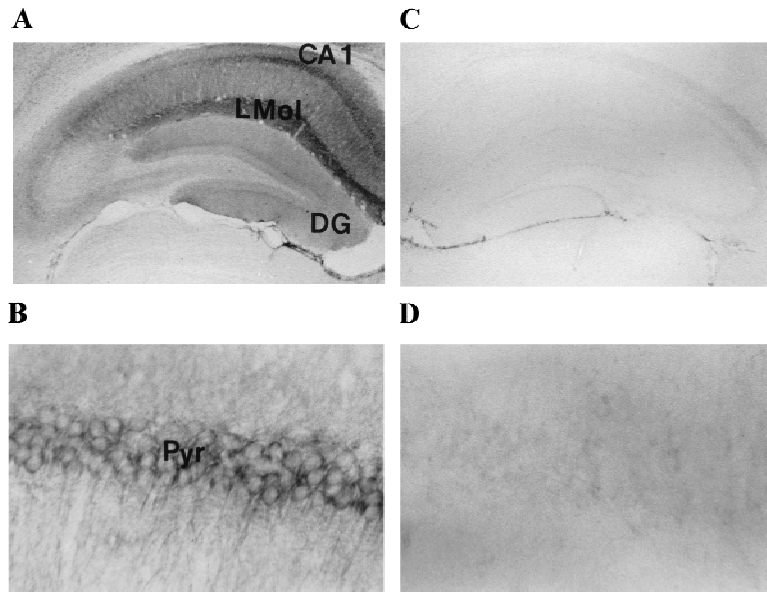


Figure 2: Distribution of IP₃-kinase(A) in the hippocampus of wild-type and mutant mouse. (A) Coronal section of the hippocampus of a wild-type mouse stained with anti-rat-IP₃-kinase(A) monoclonal antibodies. Magnification, 40x. (B) CA1 area of the hippocampus of a wild-type mouse. Magnification, 400x. (C) Coronal section of the hippocampus of a mutant mouse stained with anti-rat-IP₃-kinase(A) monoclonal antibodies. Magnification, 40x. (D) CA1 area of the hippocampus of a mutant mouse. Magnification, 400x. (CA1) CA1 area of hippocampus; (DG) dentate gyrus of hippocampus; (Pyr) pyramidal cells; (LMol) Stratum lacunosum-moleculare.

mice), 43.9 ± 4.8 pmoles/mg; mutant ($n = 7$ independent measurements from three mice), 45.8 ± 5.7 pmoles/mg; Student *t*-test, $P = 0.47$] when determined by the radioreceptor assay as described in Materials and Methods. Next, the level of IP₄ in the hippocampus was measured by HPLC analysis of brain homogenates as explained in Materials and Methods. As expected, the IP₄ level was significantly reduced in the mutant brain compared to that of wild-type [wild type ($n = 7$ mice), 0.617 ± 0.159 nmole/gram wet weight; mutant ($n = 7$ mice), 0.187 ± 0.088 nmole/gram wet

weight; Student *t*-test, $P = 0.04$] (Fig. 3B). As IP₃ can be dephosphorylated by IP₃-phosphatase to become IP₂, we examined the IP₃-phosphatase activity. The IP₃-phosphatase activity was not up-regulated in the mutant mice (data not shown).

LTP

In an attempt to study the role of IP₃-kinase(A) in synaptic plasticity, LTP was examined in the CA1 and the dentate gyrus (DG) area of the hippocampal slices from wild-type and mutant

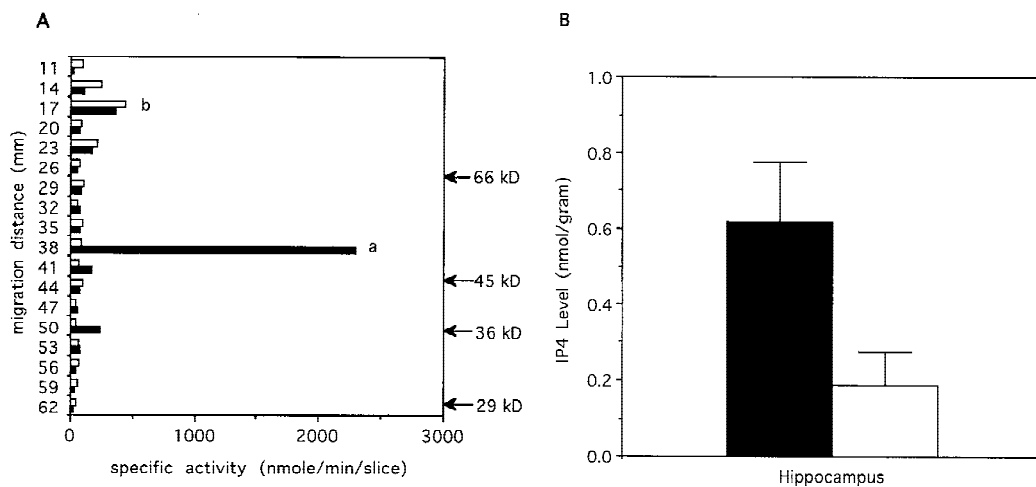


Figure 3: (A) Characterization of the residual IP₃-kinase activity after renaturation from SDS-PAGE. (a) IP₃-kinase(A) activity; (b) IP₃-kinase(B) activity. (Solid bars) +/+, wild-type; (open bars) -/-, mutant. (B) Comparison of the basal levels of IP₄ in wild-type and mutant hippocampi ($n = 6$ mice).

mice at 5–9 weeks of age. Because the immunostaining with anti-IP₃-kinase(A) antibodies revealed that IP₃-kinase(A) was highly localized in the CA1 pyramidal cell layer with a weak staining in the DG area, we thought it would be informative to compare the LTP in CA1 with that of the DG. We used seven mice (five males and two females) for each genotype, mutant and wild-type. When tetanic stimulation was delivered to the Schaffer collaterals in the presence of bicuculline, LTP was observed in the CA1 area in the slices from wild-type and mutant mice. The normalized fEPSPs slope for the wild type ($n = 7$ slices, seven mice) at 60 min after tetanus was $141 \pm 11\%$ and that of the mutant ($n = 7$ slices, seven mice) at 60 min after tetanus was $309 \pm 35\%$, which was a twofold enhancement from that of the wild type (Student's *t*-test, $P = 0.0002$; Fig. 4A). Contrary to the increased LTP in the CA1 of the mutant mice, however, no difference in the degree of LTP was observed in the DG area between wild-type and mutant mice (Fig. 4A). The increase of the fEPSPs slope at 60 min after tetanus was $169 \pm 13\%$ and $160 \pm 25\%$ in wild type ($n = 7$ slices, seven mice) and mutant ($n = 7$ slices, seven mice), respectively (Student's *t*-test, $P = 0.7783$).

PAIRED PULSE FACILITATION

To test for any modification of the presynaptic function, we investigated the difference in the paired pulse facilitation (PPF) in the CA1 and the DG when two identical stimuli were delivered in three different interstimulus intervals (ISI) to the Schaffer collaterals and the perforant pathway, respectively. PPF is the presynaptic form of the short-term plasticity in which the synaptic response to the second paired stimulus in rapid succession is increased because of residual Ca²⁺ in the presynaptic terminal from the first stimulus. Therefore, the extent of facilitation is increased with shorter ISI. We could not observe any significant difference in the extent of the PPF over three ISIs (50, 100, and 200 msec) between the slices obtained from wild type ($n = 5$ slices, three male mice) and mutant ($n = 5$ slices, three male mice) in the CA1 and the DG region (CA1, 50-msec delay: Wild type, $177 \pm 5.6\%$; mutant, $173 \pm 9.3\%$; Student's *t*-test, $P = 0.9825$; DG, 50-msec delay: wild type, $149 \pm 6.1\%$; mutant, $151 \pm 13.4\%$; Student *t*-test, $P = 0.9024$; Fig. 4B). These results suggest that IP₃-kinase(A) may not be essential in the mobili-

zation of Ca²⁺ and presynaptic neurotransmitter release.

CALCIUM IMAGING ANALYSIS

As the IP₄ was suggested to be involved in calcium homeostasis in cells and the hippocampal CA1 LTP is significantly increased in IP₃-kinase(A)-mutant mice, we analyzed the intracellular calcium change in hippocampal neuron cell cultures of wild-type and mutant mice. Unexpectedly, the relative calcium change, when treated with 50 μM glutamate followed by a 5-min perfusion with the calcium imaging buffer, showed no significant difference between wild-type and mutant mice (Fig. 5). Therefore, the increase of the hippocampal CA1 LTP in IP₃-kinase(A)-mutant mice is not coupled to a global intracellular Ca²⁺ increase induced by glutamate.

WATER MAZE TEST

To test the spatial learning ability of the IP₃-kinase(A)-mutant mice, we used the Morris water maze task as described in Materials and Methods. For the hidden-platform test, mice were trained by one block of three trials for 9 consecutive days. During the 9-day test, both wild type ($n = 15$ mice) and mutant ($n = 17$ mice) showed a significant reduction in the time taken to find the hidden platform, and the two groups did not show any difference in this test. No difference between wild-type and mutant mice was detected [$F(1,30) = 0.03$, $P = 0.8618$, one-way ANOVA; Fig. 6A]. In the probe test carried out 3 days after the last training block, both wild-type and mutant mice spent significantly more time in the quadrant where the platform had been located during training than in the other quadrants. Wild-type mice spent more time in the trained quadrant than in the other quadrants [$F(3,56) = 185.61$, $P < 0.0001$, one-way ANOVA; Newman-Keuls post hoc comparison: trained greater than all other quadrants, $P < 0.01$]. Similarly, the mutant mice spent more time in the trained quadrant than in the other quadrants [$F(3,64) = 126.32$, $P < 0.0001$, one-way ANOVA; Newman-Keuls post hoc comparison: trained greater than all other quadrants, $P < 0.01$; Fig. 6B]. In addition, both wild-type and mutant mice crossed the exact position where the platform had been located more frequently than any other corresponding position of other quadrants. Wild-type

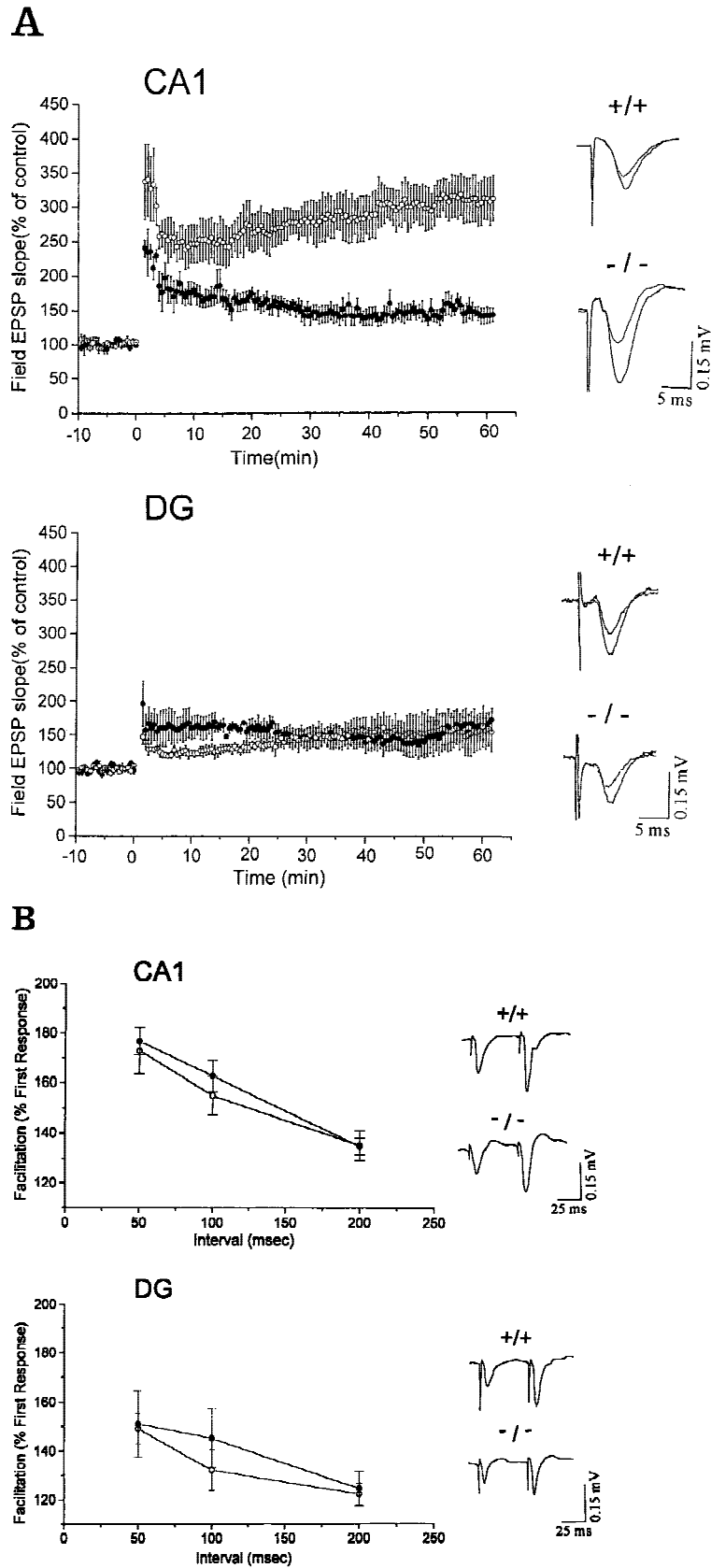


Figure 4: Synaptic responses of wild-type and mutant mice in the hippocampal CA1 area and the dentate gyrus. (A) LTP in wild-type and mutant mice. LTP was expressed as the percentage of the mean \pm S.E.M. of the value before tetanic stimulation. (CA1) Hippocampal CA1 area; (DG) dentate gyrus. Representative recordings are shown at *right*. (B) Paired pulse facilitation in wild-type and mutant mice. The size of the peak field potential for the second pulse of a pair as a function of the interpulse interval (○) -/-; (●) +/+.

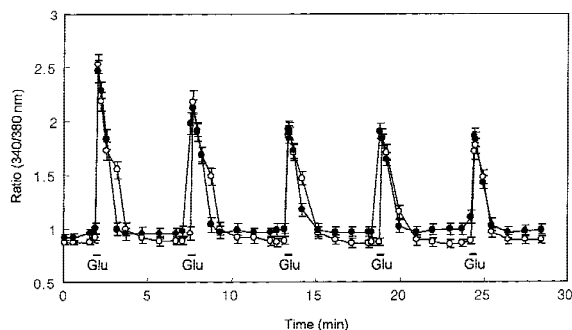


Figure 5: Glutamate-induced Ca^{2+} change in wild-type and mutant hippocampal neurons. Representative hippocampal neuronal cells from wild-type and mutant mice showing Ca^{2+} response. Glutamate ($50 \mu\text{M}$) was applied during the underlined time periods (30 sec) and washed off by perfusion with Ca^{2+} imaging buffer for 5 min. Each value represents the mean \pm S.E.M. (\bullet) +/+, wild type ($n = 27$); (\circ) -/-, mutant ($n = 29$). (Glu) Glutamate.

mice crossed the exact location of the platform in the trained quadrant more than the other alternative locations in other quadrants [$F(3,56) = 103.49$, $P < 0.0001$, one-way ANOVA; Newman-Keuls post hoc comparison: trained greater than all other quadrants, $P < 0.01$]. Similarly, mutant mice crossed the exact location of the platform in the trained quadrant more often than the other alternative locations in the other quadrants [$F(3,64) = 114.64$, $P < 0.0001$, one-way ANOVA; Newman-Keuls post hoc comparison: trained greater than all other quadrants, $P < 0.01$; Fig. 6C]. Taken together, these data indicate that the IP_3 -kinase(A)-mutant mice are not different from the wild-type mice in spatial learning and memory. As the hippocampal CA1 LTP in the mutant mice was much greater than in the wild-type mice, we also tested whether memory was maintained longer in the mutant mice. For this test, we have carried out the probe test 7 days after the last training block. Both wild type ($n = 11$ mice) and mutant ($n = 8$ mice) showed a slight decrease of memory retention, and no significant difference in memory retention was detected between wild-type and mutant mice (data not shown).

Discussion

IP_3 mediates various cellular functions by releasing Ca^{2+} from internal Ca^{2+} pools in response to various hormones or neurotransmitters in a variety of cell types (Berridge 1993). As IP_3 induces

various cellular responses, the amount of IP_3 was assumed to be tightly regulated by IP_3 -kinase and 5-phosphatase (Woodring and Garrison 1997). While IP_3 -5-phosphatase provides the inositol moiety for recycling, IP_3 -kinase produces IP_4 , which is thought to be involved in controlling Ca^{2+} homeostasis (Communi et al. 1995). Therefore, IP_3 -kinase has a crucial role in inositol metabolism. However, many aspects of inositol phosphate metabolism are not yet determined. In our IP_3 -kinase(A)-null mutant study, biochemical analysis revealed that the generation of IP_4 was severely impaired in the mutant brain, whereas the amount of IP_3 was comparable to that of wild-type mice. These data indicate that the IP_3 -kinase is not essential for the regulation of the IP_3 level but is important for generation of IP_4 .

Many IP_4 -binding proteins were purified and the binding motif was characterized, although the real IP_4 receptor has not been yet determined (Fukuda et al. 1994; Cullen et al. 1995). There are many suggestions concerning the possible role(s) of IP_4 . The first proposed role of IP_4 is calcium mobilization through binding to the IP_3 receptor or to the yet-unidentified IP_4 receptor (Wilcox et al. 1993; Irvine and Cullen 1993). Our Ca^{2+} imaging data suggest that the global calcium mobilization in response to glutamate was normal in the hippocampal neuronal cells of the IP_3 -kinase(A)-mutant mice. However, this result does not exclude the possibility that Ca^{2+} release from intracellular Ca^{2+} stores might be altered in the mutant when treated with metabotropic glutamate receptor specific agonists. The second possible role of IP_4 is the regulation of neurotransmitter release. It has been reported that binding of IP_4 to the C2B domain of synaptotagmin II blocks neurotransmitter release from the presynaptic terminal of giant axon of squids (Fukuda et al. 1995). Interestingly, our mutant mice, which have a reduced amount of IP_4 compared to that of wild-type mice, showed an increased hippocampal CA1 LTP as discussed below. However, the PPF measurement in the hippocampus of the IP_3 -kinase(A)-mutant mice was normal, suggesting that Ca^{2+} mobilization and presynaptic neurotransmitter release were normal in mutant mice. This finding does not exclude the possibility that changes in presynaptic function in the mutant could be altered during higher frequencies or longer durations of presynaptic fiber stimulation. Finally, the third possible role of IP_4 may be through the stimulation of one of the GAPs (Cullen et al. 1995). Recently, a targeted disruption of the

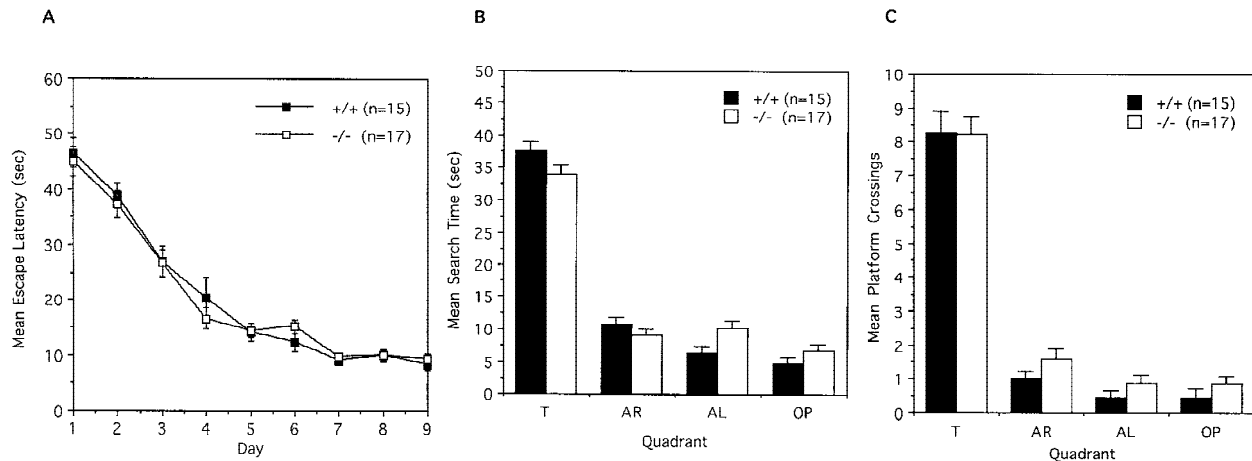


Figure 6: Behavioral analysis of wild-type and mutant mice in the Morris water maze task. (A) Mean escape latency during the hidden platform test. Mice were trained in three trials a day for 9 consecutive days. (■) +/+, $n = 15$; (□) -/-, $n = 17$. (B) The mean search time spent in each quadrant during the probe test 3 days after the last training. (C) The mean platform crossings in each quadrant during the probe test 3 days after the last training. (B,C) (Solid bars) +/+, $n = 15$; (open bars) -/-, $n = 17$. (T) Trained quadrant; (AL) adjacent left quadrant; (AR) adjacent right quadrant; (OP) opposite quadrant.

guanine nucleotide exchange factor (Ras-GRF), which activates Ras signaling, caused a significant decrease in amygdala LTP and an impairment of amygdala-dependent learning and memory without affecting hippocampal CA1 LTP or spatial learning and memory (Brambilla et al. 1997). Therefore, the investigators suggested that the Ras signaling pathway was involved in the synaptic transmission and amygdala-dependent long-term memory. As the Ras signaling is inactivated by GAP (Lowy and Willumsen 1993), which is activated by IP_4 (Cullen et al. 1995), it is plausible to speculate that the enhanced hippocampal CA1 LTP in the IP_3 -kinase(A) mutant mice might have been caused by the continuous presence of an active Ras signaling because GAP is not activated in the absence of IP_4 .

The hippocampus, which expresses LTP, is thought to be an important structure for contextual, spatial, and declarative learning and memory (Kim and Fanselow 1992; Eichenbaum 1997). However, the correlation of LTP and learning and memory is still controversial. Some mutant mice showed that an absence or decrease of hippocampal LTP impaired learning and memory. Studies with CaM kinase II-deficient mice revealed deficient hippocampal LTP and impaired spatial learning (Silva et al. 1992a,b). The NMDA receptor $\epsilon 1$ -deficient mice also showed a decrease of hippocampal CA1 LTP and reduced spatial learning and memory (Sakimura et al. 1995). Recently, a conditional deletion of the NMDAR1 revealed a close relationship between CA1 LTP and spatial learning

(Tsien et al. 1996). On the other hand, other mutant mice showed that an absence or decrease of hippocampal LTP was dissociated from learning and memory. Mice with the protein kinase C- γ null mutation showed an absence of hippocampal CA1 LTP but retained normal spatial learning and memory (Abeliovich et al. 1993a,b). Mice lacking tissue-type plasminogen activator (t-PA) also revealed that a reduction of L-LTP in the hippocampal CA1 and CA3 regions did not affect hippocampus-dependent learning and memory (Huang et al. 1996). Interestingly, targeted disruption of the AMPA receptor subunit GluR2 showed enhanced hippocampal CA1 LTP, which was caused by an increased Ca^{2+} permeability in the CA1 pyramidal neurons (Jia et al. 1996). Unfortunately, however, a behavioral test for learning and memory was not possible with these mutant mice because of high mortality and defective motor coordination. As the IP_3 -kinase(A)-deficient mice were healthy, showed no morphological abnormalities in the brain, and expressed a significantly enhanced LTP in the hippocampal CA1 region, these mutant mice provided an excellent opportunity to examine whether increased synaptic plasticity in the hippocampal CA1 affects spatial learning and memory. Perhaps contrary to our expectation, the IP_3 -kinase(A)-deficient mice showed no enhancement or other abnormality of learning and memory in the Morris water maze task. The dissociation of increased LTP and spatial learning and memory in the IP_3 -kinase(A)-deficient mice may be explained

as follows. First, the test paradigms that we used for learning and memory were not sensitive enough to quantify a minor change in learning and memory in the mutant. Second, hippocampal LTP is just an on/off switch system for learning and memory, and, therefore, the amplitude of LTP is not important for learning and memory (Bortolotto et al. 1994). Third, it is possible that low-frequency-stimulated LTP such as θ burst stimulation may be normal in mutant mice, and it could be the low-frequency-stimulated LTP that is more closely associated with spatial learning and memory. Finally, hippocampal LTP is actually dissociated from spatial learning and memory as in the case of PKC- γ - or t-PA-deficient mice (Abeliovich et al. 1993a,b; Huang et al. 1996).

The increase of intracellular Ca^{2+} concentration by both ionotropic and metabotropic receptors is an essential factor for LTP induction (Bliss and Collingridge 1993). In this study, the deficiency of IP₃-kinase(A) showed a significantly increased LTP in the hippocampal CA1 region, but not in the DG. However, our Ca^{2+} imaging analysis suggested that the increase in the hippocampal CA1 LTP of the IP₃-kinase(A)-mutant mice was not caused by a global increase of the intracellular Ca^{2+} in the neuronal cells induced by glutamate. The discrepancy here may be explained as follows. As the LTP measurements were carried out with slices derived from young mice (5–9 weeks), neuronal cell cultures derived from newborn mice (1 day old) might not represent the actual Ca^{2+} concentration change in the neurons of the young mice. In fact, the IP₃-kinase(A) activity from the rat brain is very low until 1 week of age, increases by 13-fold between 2 and 3 weeks after birth, and reaches a plateau at 13 weeks (Moon et al. 1989). To answer this question, it will be necessary to examine the Ca^{2+} regulation in hippocampal slices of the young mutant mice.

In conclusion, IP₃-kinase activity is the major pathway to generate IP₄, and IP₄ or IP₄-derived inositol phosphates may be necessary for induction and maintenance of hippocampal CA1 LTP, and the high-frequency-induced enhancement of hippocampal CA1 LTP does not affect spatial learning and memory.

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