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Phospholipase C beta 1 deficiency is associated with early-onset epileptic encephalopathy

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The epileptic encephalopathies of infancy and childhood are a collection of epilepsy disorders characterized by refractory, severe seizures and poor neurological outcome, in which the mechanism of disease is poorly understood. We report the clinical presentation and evolution of epileptic encephalopathy in a patient, associated with a loss-of-function mutation in the phospholipase C-β 1 gene. We ascertained a consanguineous family containing a male infant who presented with early-onset epileptic encephalopathy for detailed clinical phenotyping and molecular genetic investigation. In addition, a cohort of 12 consanguineous families of children with infantile spasms were analysed for linkage to the phospholipase C-β 1 gene locus. The male infant presented with tonic seizures in early infancy and subsequently developed infantile spasms. Over time, he developed drug-resistant epilepsy associated with severe neurological regression and failure to thrive. Molecular genetic investigation revealed a homozygous loss-of-function 0.5-Mb deletion, encompassing the promoter element and exons 1, 2 and 3 of phospholipase C-β 1 in the index case. Linkage to the phospholipase C-β 1 locus was excluded in the 12 other consanguineous families, consistent with genetic heterogeneity in this disorder. Although phospholipase C-β 1 deficiency has not previously been reported in humans, the *Plcb1* homozygote knockout mouse displays early-onset severe tonic seizures and growth retardation, thus recapitulating the human phenotype. Phospholipase C-β 1 has important functions in both hippocampal muscarinic

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acetylcholine receptor signalling and in cortical development. Thus, the discovery of a phospholipase C-β 1 mutation allows us to propose a novel potential underlying mechanism in early-onset epileptic encephalopathy.

Keywords: epileptic encephalopathy; infantile spasms; phospholipase C-β 1; PLCB1; hippocampus; muscarinic acetylcholine receptor

Abbreviations: IP_3 = 1,4,5-trisphosphate; PCR = polymerase chain reaction; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLCB1 = phospholipase C-β 1

Introduction

The epileptic encephalopathies are characterized by drug-resistant seizures, psychomotor retardation and, in the majority of cases, a poor neurological outcome. A number of epileptic encephalopathies may present in early life of which the most common disorder is infantile spasms (West syndrome; Hrachovy *et al.*, 2003). It is postulated that the infantile brain may be particularly susceptible to epileptic encephalopathy because the active and dynamic processes of synaptogenesis, apoptosis and progressive myelination occur in an environment of relative neuronal excitation (Ben-Ari *et al.*, 1997; Vallano, 1998; Simeone *et al.*, 2004). If a sustained, diffuse epileptic encephalopathy occurs in the midst of this reorganization, developmental milestones of brain maturation may not be achieved. Aberrant synaptogenesis and apoptosis may cause the brain to become inappropriately 'hardwired', thus increasing susceptibility to excitation (Rice *et al.*, 2000; Velisek *et al.*, 2001; Kalinichenko *et al.*, 2008; Zupanc, 2009). The result is a severe, early-onset epileptic encephalopathy with chronic seizures and concomitant cognitive and motor impairment. Although epileptic encephalopathies of infancy and childhood are often associated with structural abnormalities, inborn errors of metabolism and genetic defects, the underlying basis of many cases remains to be elucidated. In order to further investigate these disorders, we performed molecular genetic investigations in a consanguineous family with an affected child who presented with seizures in early infancy (and subsequently developed infantile spasms) and identified a pathogenic deletion of phospholipase C-β 1 (*PLCB1*) (MIM 607120).

Materials and methods

Index family and other subjects

The index case was clinically assessed independently by two paediatric neurologists. In addition, we studied DNA samples of children from 12 consanguineous families with infantile spasms (age of onset <1 year) of undetermined aetiology. Patients were ascertained through the United Kingdom Infantile Spasms Study (UKISS) group, the British Paediatric Neurology Surveillance Unit (BPNSU) reporting service and from a cohort of patients with infantile spasms from the Epilepsy Research Centre, Melbourne, Australia. For all patients, written informed consent was provided and study approval was obtained from local ethics committees.

Molecular genetic studies

A detailed description of the molecular genetic techniques is provided in the online Supplementary material. In brief, a genome-wide scan was undertaken using the Affymetrix 250K single nucleotide polymorphism array. Polymerase chain reaction (PCR) amplification and direct sequencing of *PLCB1* was undertaken for both genomic and complementary DNA. Long-range PCR techniques were used to characterize the putative deletion. Microsatellite marker analysis was undertaken in the 12 other consanguineous families to determine linkage to the *PLCB1* locus.

Results

Pedigree and index case

A male infant presented in early infancy with seizures. He was the first child of consanguineous (first cousin) healthy parents from Bangladesh (Supplementary Fig. 1). His younger brother (currently 16 months old) was fit and well. There was no family history of epilepsy or other progressive neurological disorders. Initially, the pregnancy followed a normal course with no history of abnormal fetal movements. However, during the third trimester there were concerns regarding moderate intrauterine growth retardation. Labour was induced at 38 weeks of gestation and the child was born by normal vaginal delivery. Birth weight was 2.44 kg (2nd centile) and head circumference was 32 cm (0.4th centile). His early neonatal course was uneventful.

The infant's clinical course is summarized in Fig. 1. Seizures heralded disease onset at 10 weeks of age. Focal seizures were characterized by eye rolling, lip smacking, drooling and peri-oral cyanosis followed by tonic stiffening and flexion of arms and legs. Each seizure was short, lasting between 5 and 20 s. Sometimes, clusters of 10–20 seizures would occur several times in a 24-h period. On clinical examination, the child had mild axial hypotonia and his head circumference was on the 0.4th centile, but otherwise detailed neurological examination revealed no abnormalities. At 10 weeks, neurodevelopmental assessment was age-appropriate. At this stage, awake and sleep EEG was normal for age. Clinical seizures were not observed during the EEG recording. Phenobarbitone therapy was subsequently instigated. There were no further seizures until 6 months of age. At this stage, the recurrence of seizures (which were identical in semiology to the previous seizures) was managed by a further increment of the phenobarbitone dosage. The patient was seizure free again for a further 2 months. At 8 months, he developed the clinical and EEG features of West syndrome. Clinical spasms were

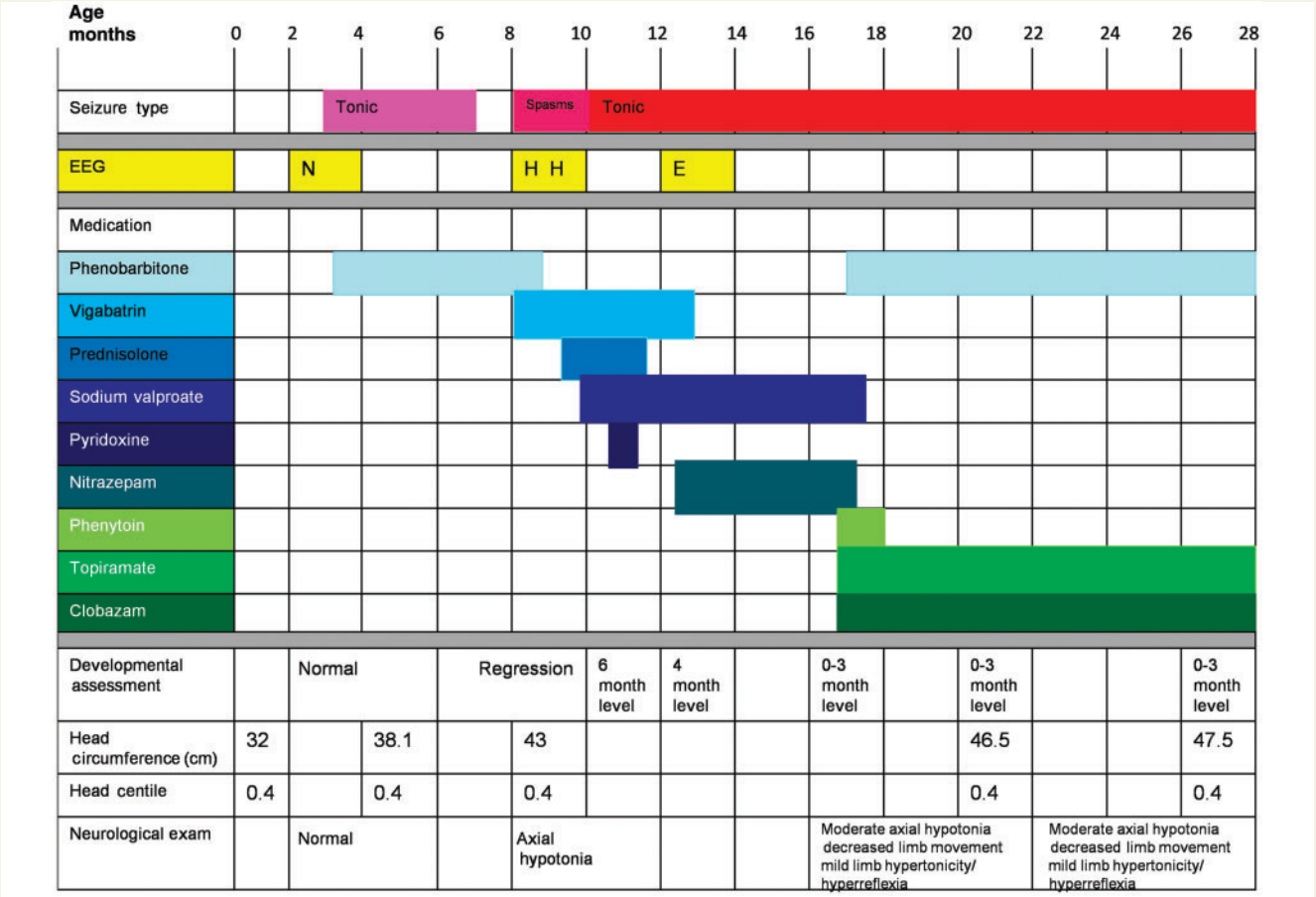


Figure 1 Patient clinical course. Schematic representation of the evolution of the index case's clinical course over the first 28 months of his life (prior to his death at 2.9 years). N = normal EEG; H = hypsarrhythmic pattern on EEG; E = encephalopathic pattern on EEG—diffuse generalized slowing.

characterized by sudden symmetrical whole-body flexion. Episodes lasted 1–2 s, followed by a pause for 5–10 s, then followed by a further spasm. Over a 24-h period, he had 2–5 such clusters of spasms (each lasting 10–60 min). The onset of spasms was associated with regression in all areas of development. Hypsarrhythmia was evident on EEG (Supplementary Fig. 2A), which persisted despite 2 weeks of vigabatrin therapy. Cessation of spasms was subsequently achieved by a course of prednisolone therapy. At 10 months, he developed seizures of different semiology. Seizure onset was heralded by an arrest of activity followed by staring, irregular breathing and bilateral peri-oral twitching. Over the next 2 years, the patient developed recurrent tonic and generalized tonic-clonic seizures. By 13 months, the EEG showed features of a diffuse encephalopathic process characterized by generalized slowing (Supplementary Fig. 2B). Sustained seizure control was not achieved despite the use of multiple anti-epileptic agents (Fig. 1). The parents declined a trial of adrenocorticotropic hormone therapy.

His head circumference continued to track along the 0.4th centile (body weight, 2nd centile). Following the onset of clinical spasms at 8 months, there was progressive developmental regression. By 2.5 years, he was functioning at the 0–3 month level, such that he could not lift his head when prone or roll over. Visual fixing and following was inconsistent. Over time, he developed severe head lag, axial hypotonia and a spastic quadriparesis.

MRI brain scans (at ages of 5 and 13 months) were normal but no further imaging studies were undertaken during the course of his life. Visual evoked responses and electroretinogram (at age 6 months) were normal. Wood's light examination and extensive neurometabolic investigations were also normal.

At 2.9 years of age, the patient developed adenovirus pneumonitis (confirmed by blood PCR analysis) followed by a secondary bacterial respiratory infection. Ventilator support was required (with high-frequency oscillation and nitric oxide therapy) for progressive respiratory difficulties. He failed to clinically respond to anti-viral drugs, multiple antibiotic agents, inotropic support and a trial of steroids. He died secondary to cardiorespiratory failure. Post-mortem examination of the brain was not performed.

Molecular genetic studies

Using the Affymetrix 250K single nucleotide polymorphism array, a genome-wide scan was undertaken in the index case. On detailed analysis, a 0.5-Mb region containing 23 sequential,

absent single nucleotide polymorphism calls was identified between single nucleotide polymorphism rs6118078 (8 048 714 bp) and rs6086520 (8 507 651 bp). This 0.5-Mb region (from ~8.04–8.50 Mb) was located within an extended region of homozygosity on chromosome 20 (with single nucleotide polymorphism homozygosity evident from 5.26–10.26 Mb). Subsequent copy-number analysis for this region indicated a homozygous deletion on chromosome 20 involving the *PLCB1* gene and no other coding genes (Supplementary Fig. 3). Specific PCR primers flanking *PLCB1* exons 1, 2 and 3 (predicted to be contained within the deletion) failed to amplify on repeated attempts (Supplementary Fig. 4) but all other *PLCB1* coding exons were amplified and sequencing demonstrated no abnormality.

Long-range PCR techniques were employed to characterize the putative deletion. Primers were designed outside of the deletion boundaries suggested by the Affymetrix single nucleotide polymorphism data. A forward primer upstream of exon 1 (at 8 029 271 bp) and a reverse primer in intron 3 (at 8 522 598 bp) were utilized for long-range PCR. These two primers were ~0.5 Mb apart. Using these primers, an amplicon of ~7 kb (Supplementary Fig. 5) was obtained both in the index case (IV:1) and in parent (III:8).

To exclude the possibility that the deletion was a polymorphic variant, we analysed 660 ethnically matched control chromosomes for the presence of the deletion-specific PCR product and did not identify any chromosomes carrying the deletion (Supplementary Fig. 5).

Sequencing of this PCR fragment (in both IV:1 and III:8), using serial forward primers (located from 8 029 271 to 8 034 292 bp), allowed the genomic deletion to be characterized (Fig. 2A). The telomeric and centromeric genomic breakpoints were mapped to 8 034 442 to 8 034 510 bp and 8 520 654 to 8 520 722 bp, respectively. The precise location of the breakpoint could not be further defined, as there was a 68-bp sequence that showed 100% sequence homology for both the upstream and intron 3 sequence (Fig. 2A) defining a 486-kb deletion. Using the NCBI Basic Local Alignment Search tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi), we determined that the deletion occurred within a region of 96% sequence homology between the upstream sequence (8 029 511 and 8 035 562 bp) and intron 3 sequence (8 575 746 and 8 581 771 bp). A heterozygous deletion with similar breakpoints (8 002 182 to 8 595 665 bp) was found in 1/540 HapMap chromosomes (http://www.sanger.ac.uk/humgen/cnv/data/cnv_data/display/, http://genome.ucsc.edu/cgi-bin/hgc?hgsid=162444873&o=8002181&t=8595665&g=ct_WGTPCNVs_617&i=%2Ftrash%2Fct%2Fct_genome_773f_8dfd30.bed+819).

The identified deletion showed appropriate segregation with disease status in the parents (obligate carriers) and unaffected sibling. The parents showed normal PCR amplification and sequencing of exons 1, 2 and 3 of *PLCB1*. Both parents were also positive for the deletion-specific PCR amplicon.

To confirm that the homozygous 0.5-Mb deletion produced loss of *PLCB1* expression, *PLCB1* was sequenced in parental complementary DNA and genomic DNA samples. This revealed two heterozygous single nucleotide polymorphisms in parental genomic DNA (rs2076413 and rs2294597**)** but only mono-allelic *PLCB1* expression in parental complementary DNA (Fig. 2B). The expressed allele was the non-deleted allele that had not been inherited by the affected child. These results were consistent with loss of *PLCB1* expression from the deleted allele.

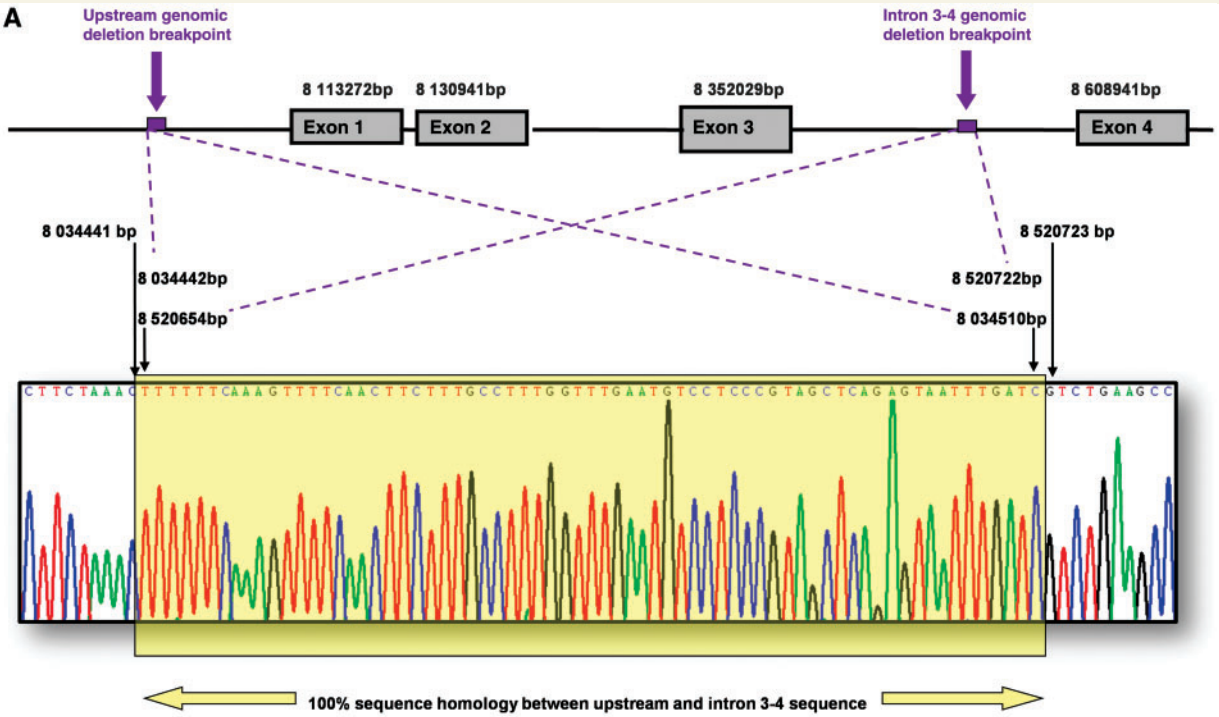
Microsatellite marker analysis undertaken in 12 further consanguineous families (using markers D20S905, D20S892, D20846, D20S448, D20S115, D20S879, D20S851, D20S175 and D20S160) excluded linkage at the *PLCB1* locus. Additionally, none of these families were positive for the deletion-specific PCR products.

Discussion

We report a novel association of infantile epileptic encephalopathy with the first homozygous loss-of-function *PLCB1* mutation described in humans. This identified deletion shows appropriate familial segregation with disease status and is absent in an extensive analysis of ethnically matched control chromosomes. The homozygous deletion in our patient resulted in loss of the predicted *PLCB1* promoter sequence (Peruzzi *et al.*, 2002) and the first three coding exons of the gene. No other coding genes are affected by the 0.5-Mb deletion. Analysis of parental genomic and complementary DNA for expressed single nucleotide polymorphisms in exons 24 and 31 demonstrated that the deleted allele is associated with complete loss of expression of *PLCB1* (although *PLCB1* transcripts are alternatively spliced, the promoter deletion was associated with silencing of *PLCB1* expression in all transcripts). The genomic deletion is likely to have originated from an abnormal recombination event between two highly homologous repetitive sequences located upstream of exon 1 and in intron 3.

PLCB1 is a member of a group of phosphoinositide-specific phospholipase C-ß enzymes that couple with guanine-nucleotide-binding G proteins to mediate a wide variety of extracellular signals transduced across the cell membrane. There are four known isoenzymes (β1–β4). *PLCB1* maps to chromosome 20p12.3 (Peruzzi *et al.*, 2000) at 8 112 824–8 949 003 bp and encodes several transcripts ranging in size from 288 bp (30 amino acids) to 6729 bp (1216 amino acids). PLCB1 is a post-synaptic receptor-activated, G protein-coupled phosphodiesterase. It catalyses the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (PIP_2). In turn, diacylglycerol activates the phosphorylating enzyme protein kinase C. IP_3 regulates the release of Ca^{2+} from the endoplasmic reticulum (Fig. 3) (De Camilli *et al.*, 1996; Hannan *et al.*, 2001). IP_3, PIP_2 and diacylglycerol all have direct effects on vesicular trafficking within the cell (Fisher *et al.*, 1992; Fabbri *et al.*, 1994; De Camilli *et al.*, 1996). PLCB1 thus plays a key role in the intracellular transduction of a large number of extracellular signals (involving neurotransmitters and hormones) modulating diverse developmental and functional aspects of the mammalian CNS.

The consequences of bi-allelic loss of *PLCB1* are evident from a specific *Plcb1* homozygous knockout mouse model (Kim *et al.*, 1997) that presents with an epileptic phenotype. Kim *et al.* (1997) demonstrated that while mice heterozygous for the generated *Plcb1* null mutation were normal and fertile, the homozygote



B

Parent	Mother	Mother	Father
Exon	24	31	31
SNP	rs2076413 (G/A)	rs2294597 (C/T)	rs2294597 (C/T)
Genomic DNA	G/A	C/T	C/T
cDNA	A	T	T

Figure 2 Genomic DNA and complementary DNA sequencing results. (**A**) Definition of the genomic breakpoint of the deletion. The upstream genomic breakpoint was determined to be between 8 034 442 and 8 034 510 bp and the downstream genomic breakpoint was determined to be between 8 520 654 and 8 520 722 bp. A 68-bp sequence which showed 100% sequence homology for both the upstream and introns 3 and 4 sequence was identified (highlighted in the yellow box). (**B**) Sequencing analysis of genomic and complementary DNA in the index case's mother (III:7) and father (III:8) for *PLCB1* exons 24 and 31. Although there is bi-allelic expression of single nucleotide polymorphisms rs2076413 (G/A) and rs2294597 (C/T) in genomic DNA, there is mono-allelic expression of these single nucleotide polymorphisms rs2076413 (A) and rs2294597 (T) in cDNA.

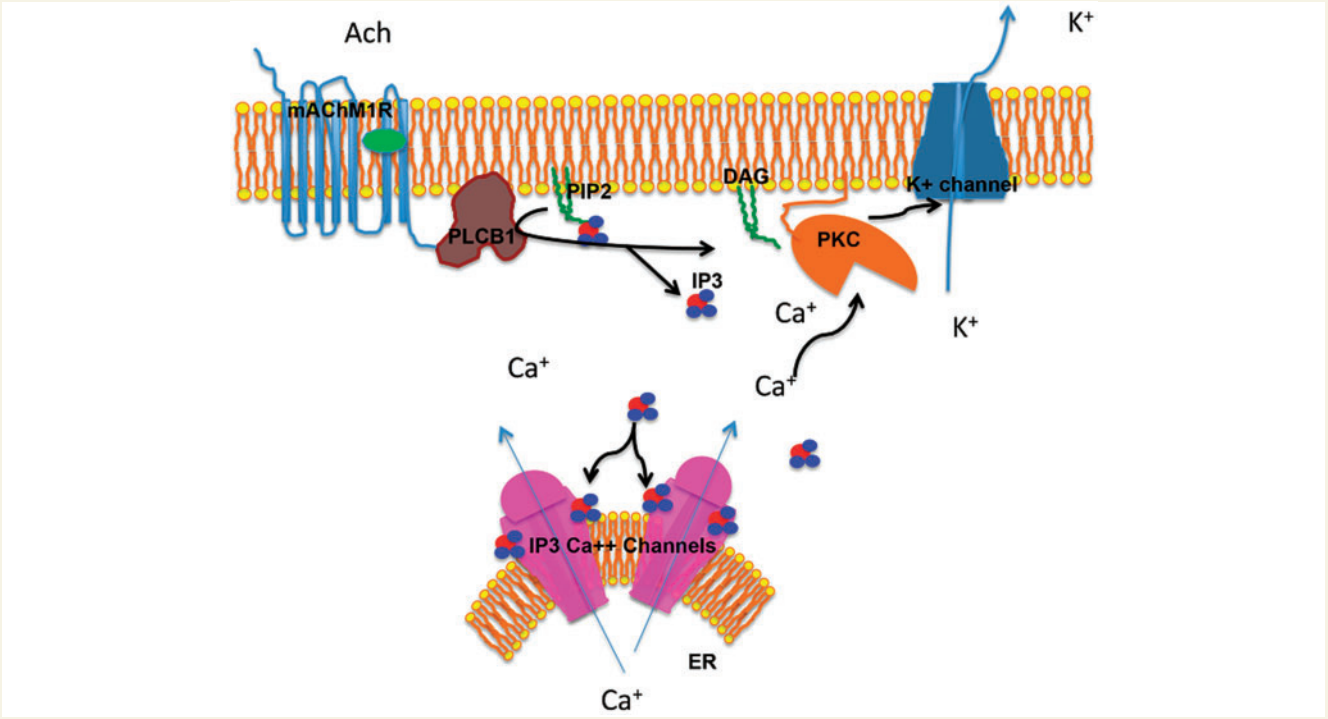


Figure 3 Proposed cellular function of PLCB1 enzyme (De Camilli *et al.* 1996; Hannan *et al.* 2001). The G protein-coupled muscarinic acetylcholine receptor activates PLCB1, which hydrolyses PIP_2 to diacylglycerol (DAG) and IP_3. Diacylglycerol directly activates protein kinase C (PKC), whereas IP_3 activates the IP_3-activated endoplasmic reticulum-Ca^{2+} channels. Ca^{2+} released from the endoplasmic reticulum (ER) also activates protein kinase C. Protein kinase C phosphorylates the potassium (K^+) channel, increasing K^+ efflux and causing depolarization. Ach = acetylcholine.

Plcb1$^{-/-}$ mice displayed retarded growth and low viability after birth. Most mice died suddenly, starting from Week 3 after birth. The death of these mice was preceded by status epilepticus or intractable intermittent seizures. The seizures were generalized in nature, either tonic events or generalized tonic-clonic episodes. Histochemical analysis of the *Plcb1*$^{-/-}$ hippocampus after spontaneous seizure revealed selective loss of somatostatin-containing interneurons in the hilus (Kim *et al.*, 1997). There is evidence that hilar interneurons containing somatostatin are selectively lost by electrical stimulation of adjacent granule cells of the hippocampus, thus indicating that seizures in *Plcb1*$^{-/-}$ are evoked through hippocampal hyperexcitability. *PLCB1* deficiency selectively impairs muscarinic acetylcholine receptor signalling (Fig. 3, Kim *et al.*, 1997) in the hippocampus as well as the temporal cortex and cerebellum. It may disrupt normal inhibitory neuronal circuitry, thus lowering the brain's threshold for seizures.

Accurate diagnosis and subsequent clinical management of the epileptic encephalopathies is established by careful clinical assessment of seizure semiology, disease evolution and findings on EEG. Identification of causative gene mutations can be helpful in refining the diagnosis but is possible in only some cases. Defining the genetic defects can, however, provide valuable insights into the pathophysiological mechanisms underlying this group of disorders. To date, epileptic encephalopathies (such as West syndrome) have been associated with mutations of *ARX* (Kutamura et al., 2002), controlling the development of gamma aminobutyric acid (GABA)-ergic interneurons (MIM 300382) and *CDKL5* (Weaving *et al.*, 2004), encoding a phosphorylated protein with protein kinase activity (MIM 300203). *De novo* heterozygous mutations in *STXBP1* [encoding syntaxin binding protein 1, essential for synaptic vesicle release (MIM 602926)] have been shown to cause Ohtahara syndrome (Saitsu *et al.*, 2008) and West syndrome. Interstitial deletions of 7q11.23-q21.11 involving *MAGI2* [encoding a synaptic scaffolding protein membrane associated guanylate kinase inverted-2, (MIM606382)] are also associated with West syndrome (Marshall *et al.*, 2008). More recently, mutations in *PNKP* [causing DNA repair defects (MIM 605610)] have been identified in patients with early-onset intractable seizures (Shen *et al.*, 2010).

In order to investigate the extent of genetic heterogeneity, we sought evidence for linkage to the *PLCB1* locus in a further 12 consanguineous families containing 2–4 children with infantile spasms (age of onset <1 year) of undetermined aetiology. In all families, microsatellite marker analysis excluded linkage to chromosome 20p12.3. It is important to note that the index case had final MRI of the brain at 13 months of age, because the evolution of focal seizures could potentially herald the development of a radiologically discernible focal brain abnormality (that may have been evident on only later scans following completion of brain myelination after 2 years of age). In order to establish the frequency of *PLCB1* mutations, we propose that future mutational screening should thus be undertaken in children with early-onset

epileptic encephalopathy, including those with focal brain abnormalities.

In conclusion, we report *PLCB1*-associated epileptic encephalopathy in a male infant originating from a consanguineous family. Identification of this homozygous *PLCB1* deletion (inherited in an autosomal recessive manner) has facilitated appropriate genetic counselling for this family. Although we have only identified a single case, future mutational analysis of further cases of infantile epileptic encephalopathy may identify more *PLCB1* cases, allowing enhanced phenotypic delineation of this disorder. The identification of a human model of *PLCB1* inactivation has also implicated a novel disease pathway (and further potential candidate genes), which can be further explored in the future to elucidate the molecular basis of other unresolved epileptic encephalopathies.

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Supplementary material

Supplementary material is available at *Brain* online.

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