Loss of Murine Na\textsuperscript{+}/myo-Inositol Cotransporter Leads to Brain myo-Inositol Depletion and Central Apnea*  

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myo-Inositol (Ins) and its polyphosphoinositide derivatives that are important in membrane signaling have long been held to play a special role in brain metabolism. As polyphosphoinositides turn over rapidly and are exceptionally abundant in nervous tissue, high Ins levels in the range of 2–15 mM that have been observed in brain may be necessary to maintain the rates of phosphoinositide synthesis in diverse membrane locations within neurons. Cellular concentration gradients of this magnitude indicate a dependence on active Ins transport, especially at the time of growth and differentiation. The Na\textsuperscript{+}/myo-inositol cotransporter (SMIT1 or SLC5A3) gene is highly expressed prenatally in the central nervous system and placenta. To gain more insight into brain Ins metabolism, while ascertaining the importance of SMIT1 as a transporter, we generated mice with a homozygous targeted deletion of this gene. Newborn SMIT1(-/-) animals have no evidence of SMIT1 mRNA, a 92% reduction in the level of brain Ins, an 84% reduction in whole body Ins, and expire shortly after birth due to hypoventilation. Gross pathologic and light microscopic examinations of each organ, as well as the placenta, of embryonic day 18.5 fetuses at near term gestation were normal. Based on \[^{3}H\]acetate incorporation into phospholipids of lung tissue explants, immunostaining of lung tissue for surfactant protein A, B, and C, and electron microscopic examination of alveolar cells, there was no evidence of abnormal pulmonary surfactant production by type 2 pneumocytes in lung. Although no histologic lesions were detected in the nervous system, electrophysiological studies of the brainstem pre-Bötzinger respiratory control center demonstrated an abnormal rhythm discharge with periods of central apnea. The cause of death can be explained by the regulatory defect in brainstem control of ventilation. This model demonstrates the critical importance of SMIT1 in the developing nervous system. The high affinity SMIT1 transporter is responsible for the Ins concentration gradient in the murine fetal-placental unit.

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§ The abbreviations used are: Ins, myo-inositol; PtdIns, phosphatidylinositol; SMIT1, Na\textsuperscript{+}/myo-inositol cotransporter 1; GC/MS, gas chromatography/mass spectrometry; SP, surfactant protein; PL, phospholipid; PBS, phosphate-buffered saline; TMS, trimethylsilylation; PGK, phosphoglycerokinase; BSTFA, N,O-bis(trimethylsilyl)triﬂuoroacetamide; TMCS, trimethylchlorosilane; E, embryonic day.
membrane proteins may depend on dynamic remodeling of membrane-bound inositol phosphate headgroups (26). In terms of phosphoinositide mass, it is likely that protein-phosphoinositide binding, along with PtdIns-anchoring of proteins to membrane bilayers (27), is the predominant occupation of phosphoinositides within a cell, especially the neuron with its rich cytoskeletal network and vesicle motor units.

The trapping detectable by 2-15 mit levels of Ins within a neuron through active transport, restricted efflux, and relatively high extracellular Ins levels as in cerebrospinal fluid may be essential to its homeostasis (4). Concentration gradients of this magnitude indicate a dependence on active Ins transport, especially at the time of growth and differentiation (28). The Na+/myo-Inositol cotransporter1 (SMIT1 or SLC5A3) is highly expressed prenatally in central nervous system and placenta (5, 29). To gain more insight into brain Ins metabolism, while ascertaining the importance of SMIT1 as a transporter, we generated mice with a homozygous targeted deletion of this gene and examined the importance of SMIT1 as a transporter, we generated mice with a homozygous targeted deletion of this gene and

Experimental Procedures

Materials

The murine 129svJ genomic library was from Stratagene (La Jolla, CA). The restriction endonucleases were from Promega (Madison, WI). The PGK-neomycin cassette was a gift from Dr. Nancy Cooke. The HSV-tk-DtE1 subunit cassette was a gift from Dr. Barbara Knowles. The myo-inositol and N.O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/trimethylchlorosilane (TMCS) mixture was from Sigma. The hexadeuterated myo-inositol ([d6]Ins) was from CDN Isotopes (Quebec, Canada). The Waymouth’s MB 752/1 medium and phosphate-buffered saline (PBS) were purchased from Invitrogen. The paraformaldehyde and glutaraldehyde were from Electron Microscopy Sciences ( Ft. Washington, PA). The Triton X-100 was from Roche Molecular Biochemicals. The [3H]acetate (100 μCi/ml), myo-[2-3H]inositol (22.3 Ci/mmol), and [32P]dCTP (3000 Ci/mmol) were from PerkinElmer Life Sciences. The halothane was from Abbott Laboratories, Inc. The sodium borohydride, Superfrost glass slides and Tissue Freezing Medium were from Fisher. All other chemicals were from Sigma.

Targeted SMIT1 Gene Deletion Construct

Previously, we cloned a genomic fragment that contained the intron-free coding region of the mouse SMIT1 homologue (33) from a 129svJ genomic library. The fragment is ~15 kb and exists in a λ bacteriophage vector. From this clone, an 11.0-kb XbaI fragment was subcloned and used to construct a targeting vector for homologous recombination by positive-negative selection. A 1.6-kb EcoRI-NcoI fragment within the SMIT1 coding region was replaced with a 1.9-kb PGK-neomycin cassette (see Fig. 1). A HSVtk-Dtα subunit cassette was ligated to the 3′-end of the vector to allow negative selection of the non-targeting events.

Homologous Recombination in Embryonic Stem Cells

Embryonic stem cells (129svJ) were electroporated, and G418-resistant clones were isolated in the laboratories of GenomeSystems, St. Louis, MO. In the laboratory of Dr. Gerard T. Berry, a 1-kb SacI fragment 5′ to the homologue contained in the targeting vector was used as a probe in Southern blot analyses to identify homologous recombination events. HindIII digestion of the wild-type locus generates a 10.4-kb fragment, whereas the correctly targeted locus yields a 10-kb fragment (Fig. 1). Twelve correctly targeted clones were identified among 192 resistant clones, yielding a targeting frequency of 1 in 16.

Generation of Chimeric Mice and Breeding of SMIT1 (+/−) Mice

In the Transgenic Animal Facility of the University of Pennsylvania, one embryonic stem clone was selected for blastocyst injection; of eight chimeric mice obtained, four transmitted embryonic stem cell DNA through the germ line and generated heterozygous offspring. Germline transmission was demonstrated by Southern blot analysis of tail DNA.

SMIT1 Gene Expression in SMIT1 (−/−) Mice

The content of SMIT1 transcript in adult, fetal, and placental tissues was determined by Northern blot analyses.

Lethal Brain myo-Inositol Depletion Due to Absent SMIT1

myo-Inositol Analysis in Brain Tissue and the Whole Embryo/Fetus

To maximize the sensitivity and specifically of Ins detection, we employed GC/MS using hexadeuterated [6,6]Ins and in an isotope dilution analysis. We employed trimethylsilylation (TMS) and an Hewlett-Packard 5890/5972 analyzer equipped with electron ionization in the selected ion monitoring mode (3). The selected ion monitoring fragments of interest are 217 for TMS-Ins and 220 for TMS-[d6]Ins. We generated a linear standard curve using 1000 pmol of [d6]Ins per derivatization vial, with Ins varying from 200 to 2 μmol (Fig. 1). The derivatization volume containing BSTFA/TMCS with standards was 300 μl, and 1 μl was used for each GC/MS injection. Based upon our results, this method will permit picomolar amounts of Ins to be reliably assayed.

The isotope dilution analysis with [d6]Ins was used to measure Ins levels in whole embryonic day (E) 10.5, E14.5, and E18.5 fetuses from transgenic mice and in amniotic fluid from one of the E16.5 sets. The fetuses were quickly frozen in liquid nitrogen after removal from the uterine sacs. The yolk sac DNA was used for genotyping. A 5-μl sample of amniotic fluid was frozen and lyophilized. The whole fetus was homogenized following addition of [d6]Ins. Aliquots were taken for protein assay. Following lyophilization of fetal extracts, the TMS-derivatized samples were analyzed by GC-MS.

Phospholipid (PL) and Surfactant Protein (SP) Analyses in Lung Tissue and Electron Microscopy of Type 2 Pneumocytes

On day 18.5 of gestation, the uterus with fetuses intact was removed and placed in sterile PBS on ice, and each fetal lung was dissected out. Of the three lobes on the right side, one was fixed for EM, one was fixed for immunohistostaining (largest) and was then [3H]myo-[2-3H]inositol for labeling of PtdIns. Of the two lobes on the left side, one was frozen (~70 °C) for later use, and one (larger) was cultured for [3H]acetate incorporation into all phospholipids. Lobes were placed into individual wells of 24-well culture dishes with the precursor (250 μl of Waymouth’s media with either [3H]acetate (20 μCi/ml, 1 μat total acetate) or myo-[2-3H]inositol (10 μCi/ml, 7 μat Ins) and rocked for 5 h at three cycles/min in a 37 °C humidified incubator. Concentration of acetate was high (1 mM) to ensure rapid equilibration of the endogenous pools and eliminate pool effects. After incubation, the tissues were harvested into cold PBS, and then sonicated in saline and assayed for protein. To determine distribution of incorporation into newly synthesized phospholipids, total lipid extracts were separated by thin-layer chromatography (TLC), and the amount of tritium label in each PL was determined as described previously (34).

To prepare frozen tissue sections for immunofluorescence, lobes were fixed in 1% paraformaldehyde in PBS overnight at 4 °C, washed with PBS for 5 min, and embedded in Tissue Freezing Medium (Fisher). Cut sections were washed (3 min) with sodium bicarbonate (0.15% in PBS) to reduce autofluorescence and rinsed twice with PBS (5 min). Sections were incubated (30 min, 25 °C) in PBS containing 0.3% Triton X-100 + 5% bovine serum albumin + 10% normal goat serum to block nonspecific binding and permeabilize cells followed by a 5-min wash with PBS + Triton X-100. Coverslips were incubated with primary antibodies overnight at 4 °C. Antibodies used were: rabbit anti-human SP-A (polyclonal), anti-bovine SP-B (polycyonal prepared against SP-B extracted from bovine surfactant), and anti-rat SP-C (polyclonal which recognizes precursor forms) as described previously (35). To remove excess antibody, slides were incubated with PBS + Triton X-100 for 5 min.

Primary antibodies were detected by addition of secondary antibody (goat anti-rabbit IgG conjugated to Cy3; 200) for 1 h at 25 °C. Excess secondary antibody was removed by 2-min washes (twice each) with PBS + 0.3% Triton X-100, and then with PBS + 0.075% Triton X-100, and finally with PBS. Coverslips were air-dried and mounted with Mowiol (Calbiochem). Fluorescence was examined with an Olympus IX70 microscope and Metamorph imaging system.

For electron microscopy, lobes were fixed in 2.5% glutaraldehyde, 0.1 m sodium cacodylate (pH 7.2) for 3 h at 4 °C, washed, and postfixed with 1% osmium tetroxide. The tissue was embedded in epoxy resin, and ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL CX100II transmission electron microscope operated at 80 kV. Cells from two lungs of each group were examined (four sections/sample).

In Vitro Embryonic Mouse Preparation

The screening of respiratory motor pattern was double-blinded as neither the genotype of the fetus was known at the time of this recording nor were the results of the electrophysiological testing known by the individual performing the Southern blot.

18298

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Brainstem/Spinal-diaphragm Preparations—Fetuses (E18.5) were delivered from timed-pregnant mice anesthetized with halothane (1.25–1.5% delivered in 95% O₂ and 5% CO₂) and maintained at 37 °C by radiant heat. The timing of pregnancies of dams was determined from the appearance of sperm plugs in the breeding cages. Embryos were immediately decerebrated, and the brainstem/spinal cord with the ribcage and diaphragm muscles attached was dissected following procedures similar to those established for perinatal rats (36, 37). The neuraxis was continuously perfused at 27 ± 1 °C (perfusion rate of 5 ml/min, chamber volume of 1.5 ml) with Kreb’s solution that contained: 128 mM NaCl, 3.0 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgSO₄, 24 mM NaHCO₃, 0.5 mM NaH₂PO₄, and 30 mM D-glucose equilibrated with 95% O₂, 5% CO₂ at 27 °C (pH = 7.4).

Medullary Slice Preparations—Details of the preparation have been described previously (38). Briefly, the brainstem/spinal cords isolated from the E18.5 fetuses were pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome (Leica, VT1000S). The brainstem was serially sectioned in the transverse plane starting from the rostral medulla to within 7.4). The medullary slice was continuously perfused in physiological solution similar to that used for the brainstem/spinal cord preparation except for the potassium concentration, which was increased to 9 mM to stimulate the spontaneous rhythmic respiratory motor discharge in the medullary slice.

Recording and Analysis—Recordings of diaphragm electromyography (see Fig. 4), hypoglossal (XII) cranial nerve root (see Fig. 5), and population neuronal discharge within the pre-Bötzinger complex (see Fig. 5) were made with suction electrodes. Signals were amplified, rectified, low-pass filtered, and recorded on computer using an analog-digital converter (Digidata 1200, Axon Instruments). Mean values relative to control for the period and peak integrated amplitude of respiratory motoneuron discharge were calculated.

RESULTS

Generation of SMIT1 Mutants—Using a genomic clone containing the entire coding region of the murine SMIT1 gene (33), we prepared a targeted deletion construct of the murine SMIT1 gene (Fig. 1) and generated a homozygous deletion model. To obtain homozygous SMIT1 mutant (−/−) mice, heterozygous F1 females and males were mated, and genotypes of their F2 offspring were analyzed at postnatal day 21. Of 170 F2 animals collected, 63 of them were SMIT1(+/+), and 107 were SMIT1(+/-). However, no SMIT1(−/−) mice were detected.

We subsequently determined that the SMIT1(−/−) mice die shortly after birth. To determine the time of death of the SMIT1(−/−) offspring of (+/−) × (+/−) matings, cesarean sections were performed on E18.5 pregnant females. To confirm that the fetuses were alive at the time of Cesarean section, each fetus in the litter was stimulated by a gentle pinch with blunt forceps before removal of the uterus. All responded to this physical stimulation by moving and extending extremities. By 20 min after the Cesarean birth, (+/+) and the (−/−) fetuses had begun to breath, move, squeal, and turn pink, whereas (−/−) pups became motionless and cyanotic following an interval characterized by irregular gasps of breath. All of the knockout animals that were observed died within 20 min after birth.

Pathology of SMIT1(−/−) Fetus—Body weight and external features of the newborn (−/−) fetuses were normal. In pathological examinations, there were no gross or microscopic malformations. Routine hematoxylin/eosin-stained sections of brain, spinal cord, dorsal root ganglia, heart, lungs, liver, kidneys, esophagus, stomach, intestines, adrenal, thyroid glands, and placenta appeared normal by light microscopy (data not shown).

SMIT1 Expression in SMIT1 Mutants—The deletion of the bulk of the SMIT1 coding region was confirmed by absent levels of SMIT1 transcript, demonstrated by Northern blot analyses of total RNA from fetal and placental samples (Fig. 2). In a survey of total RNA from placenta and adult mouse tissues, the primary 11-kb SMIT1 transcript was most abundant in kidney, placenta, and brain with weak expression in thymus, lung, bladder, and testes (data not shown). In a survey of poly(A) RNA-enriched samples from adult brain and kidney and placental and embryo tissues, only an 11-kb transcript was detected (data not shown). The SMIT1 11-kb transcript, demonstrated by Northern blot analyses of total RNA from adult brain, was reduced in the heterozygous (+/−) mice as compared with wild-type (+/+) (Fig. 2).

myo-Inositol Quantitation in SMIT1 Mutants—Using stable isotope dilution gas chromatography/mass spectrometry with hexadeuterated Ins ([2H₆]Ins) in an isotope dilution analysis, we measured the levels of Ins in fetal brain, as well as whole embryos or fetuses obtained by cesarean section. At E10.5, E14.5, and E18.5, the total body Ins content in SMIT1(+/+) controls was 2.96 ± 0.45 (n = 8), 1.50 ± 0.13 (n = 3), and 1.16 ± 0.32 (n = 9) μmol gram of wet weight, respectively (Fig. 3A). In

![Figure 1. Diagram of the SMIT1 targeting vector and the wild-type and the mutant alleles.](image-url)

**Fig. 1.** Diagram of the SMIT1 targeting vector and the wild-type and the mutant alleles. *PGK-neo*, neomycin-resistant gene driven by phosphoglycerokinase gene promoter; *Dta*, diphertheria toxin α-chain gene; *ORF*, open reading frame.
Lethal Brain myo-Inositol Depletion Due to Absent SMIT1

Fig. 2. SMIT1 expression in heterozygous and homozygous mutant tissues. Each lane contained 30 μg of total RNA. Hybridization was with [32P]-labeled SMIT1 antisense riboprobe. +/+; wild-type; +/−, heterozygous; −/−, homozygous mutants. A, adult brain samples. Exposure time was overnight. B, E14.5 placental samples. Exposure time was overnight.

age-matched SMIT1(−/−) mutants, the levels were reduced by 77% (n = 7), 64% (n = 6), and 84% (n = 3), respectively, whereas in SMIT1(+/−) heterozygotes, the levels were reduced by 52% (n = 5), 32% (n = 6), and 43% (n = 6), respectively. At E18.5, the Ins in isolated brain tissue was 7.80 ± 0.80 (n = 7), 5.98 ± 1.50 (n = 5), and 0.60 ± 0.40 (n = 3) μmol/grams of wet weight from SMIT1(+/+), (+/−), and (−/−), respectively (Fig. 3B). A similar trend was noted in amniotic fluid where the level of Ins was 480 nM (n = 5), 325 (n = 5), and 198 μM (n = 1) in the SMIT1(+/+), (+/−), and (−/−) samples, respectively. In tissues obtained from control adult mice and analyzed by GC/MS, the level of Ins in brain, kidney, and liver was 4.31 ± (n = 3), 4.26 ± 1.34 (n = 3) and 0.10 ± 0.03 μmol/grams of wet weight, respectively (mean ± S.D.). Thus, the whole SMIT1(−/−) near term fetus has an Ins level (≥186 μM) comparable with those measured in normal adult liver (≥100 μM) and in a SMIT1(−/−) amniotic fluid sample (198 μM).

Surfactant Phospholipid Studies in Lung Tissue—We considered that the failure of SMIT1(−/−) pups to initiate effective respiration could reflect abnormalities of pulmonary surfactant or type II cell morphology. Therefore, precursor incorporation and PL analysis in lung tissue was performed using E18.5 fetuses. There were no differences in PL composition or synthesis detected by [3H]acetate incorporation in the tissue from (−/−) fetuses as compared with (+/+) or (+/−) fetuses (data not shown). The acetate incorporation into total PL was 1.16 ± 0.09 (n = 7), 1.49 ± 0.15 (n = 11) and 1.34 ± 0.14 (n = 5) nmol/mg protein in lung tissue from the SMIT1(+/+), (+/−), and (−/−) fetuses, respectively. The relative myo-[2-3H]inositol incorporation into PtdIns expressed as Ins/acetate was not lower in the (−/−) lungs as compared with either wild-type (+/+) or (+/−) samples (data not shown). The uptake of myo-[2-3H]inositol into the aqueous fraction of lung explants was also not different in samples from SMIT1(−/−) fetuses as compared with (+/+) and (+/−) fetuses (data not shown). Morphology of type II cells was examined by both light and electron microscopy. By electron microscopy, the ultrastructure of the type II pneumocytes in the (−/−) fetuses showed no morphologic abnormalities, and the surfactant-containing lamellar bodies appeared normal in both number and structure (data not shown). By immunostaining, the relative density of type II cells appeared similar in all groups, and the type II cells had normal contents and subcellular distribution of the surfactant proteins, SP-A, SP-B, and SP-C (data not shown).

Electrophysiological Analyses of Rhythmic Respiratory Neuronal Activity—In view of the normal findings related to pulmonary surfactant in SMIT1(−/−) mice, it appeared likely that the primary hypoventilation was due to nervous system dysfunction. To test this hypothesis, recordings of inspiratory neural activity were obtained from multiple sites within the central neuraxis in E18.5 fetuses with different genotypes (Figs. 4 and 5). Diaphragm electromyography recordings from each of the SMIT1(−/−) fetuses had very irregular respiratory rhythm patterns that had several characteristic features (Fig. 4). First, there were 3–8 apneic episodes of 15–60-s duration per a 10-min period. Second, the periods of suppressed respiratory discharge were interspersed with bouts of rhythmic respiratory bursting of much higher frequency than observed in wild-type preparations. Third, there were regular occurrences of augmented breaths that were of larger amplitude and slightly longer duration. Fourth, the duration and amplitude of inspiratory bursts (other than augmented bursts) in preparations from SMIT1(−/−) fetuses were less than observed for the wild type. Abnormal respiratory discharge patterns were also observed in recordings from the hypoglossal
Lethal Brain myo-Inositol Depletion Due to Absent SMIT1

FIG. 4. Recordings of rectified and integrated diaphragm electromyography (EMG) from in vitro preparations isolated from E18.5 fetuses. The drawing on the left depicts the brainstem/spinal cord preparation with the diaphragm connected via the phrenic nerve. As shown in A, the inspiratory rhythm was continuous and regular in wild-type preparations. As shown in B, in contrast, SMIT1(−/−) preparations all had very irregular respiratory rhythmic patterns that had several defining features. There were 3–8 apneas of 15–60 s duration per 10-min period. Overall, the interburst interval in SMIT1(−/−) preparations (7.8 ± 8.1 s) was significantly longer than in SMIT1(+/+) preparations (4.3 ± 2.4 s). As shown in C, the periods of suppressed respiratory rhythmic discharge were interspersed with bouts of rhythmic respiratory bursting of much higher frequency (interburst interval of 1.4 ± 0.5 s) than observed in wild-type preparations. Also, there were regular occurrences of augmented breaths that were of larger amplitude and slightly longer duration. As shown in D, the duration and amplitude of rectified and integrated inspiratory bursts (other than augmented bursts) in SMIT1(−/−) preparations were ~65% of those observed for (+/+) preparations.

FIG. 5. Recordings of integrated and rectified inspiratory neuronal discharge in medullary slice preparations isolated from E18.5 fetuses. As shown in A, the abnormal rhythmic patterns were also observed in recordings from the hypoglossal nerve roots of the medullary slice preparations from SMIT1(−/−) fetuses. PBC, pre-Boëtziinger complex. As shown in B, direct recordings of neuronal population discharge within the PBC demonstrated that the irregular rhythms were present in the putative respiratory rhythm generating center. As shown in C, as was the case in the brainstem/spinal cord preparation, the duration and amplitude of rectified and integrated motor discharge (other than augmented bursts) were decreased as compared with (+/+) preparation (recordings from XII motoneurons).

nerve rootlets of the medullary slice preparations (Fig. 5). Direct recordings of brainstem neuronal population discharge within the pre-Boëtziinger complex (38) demonstrated that the irregular rhythms were present in the putative respiratory rhythm generating center, rather than simply reflecting a failure of transmission of inspiratory drive to motoneuron populations (Fig. 5). There were no significant differences in the respiratory motor patterns generated in wild-type (+/+ versus carrier (+/−) fetal in vitro preparations.

DISCUSSION

Our study shows that the SMIT1 transporter is the primary murine transporter for the maintenance of an Ins concentration gradient during embryonic and fetal life. This high affinity active transport system is responsible for the Ins concentration gradient in the fetal-placental unit. As shown in Fig. 3, a SMIT1 gene dosage effect is also evident with heterozygotes having levels of Ins intermediate between wild-type and knockout fetuses. In the normal whole embryo and fetus, the Ins levels continue to drop as the organism approaches term; the levels are higher at E13.5 than at E14.5 or E18.5. This may be related to de novo synthesis of Ins in the mammalian fetus and placenta (14, 39–41) and reflected in the fetal plasma Ins concentration that is significantly higher than the maternal level at any time during gestation (39, 41–43). The plasma Ins level rapidly declines after birth (39, 42), perhaps demonstrating the ability of SMIT1 to concentrate, at the level of the placenta, the Ins produced by both placental and fetal tissues. There is little movement of Ins from mother to fetus or from fetus to mother (42, 43). The role of the low affinity SMIT2 transporter in the maintenance of a cellular Ins concentration gradient is unknown (44). It may be important during fetal life when extracellular fluid concentrations are at their peak. Based on the Ins concentrations in E18.5 fetuses and amniotic fluid, our data are most compatible with a total collapse of the cellular Ins concentration gradient at term gestation. This is the first model of severe Ins deficiency in a mammal. The lethal hypoventilation can be explained on the basis of central apnea and abnormal respiratory rhythmogenesis generated within the pre-Boëtziinger complex, the putative respiratory rhythm generating center in the brainstem (38).

One of the hypotheses that may explain the biochemical mechanism of central nervous system dysfunction in the SMIT1(−/−) newborn is that the marked reduction in Ins levels limits the activity of PtdIns synthase and as a consequence retards the production of phosphoinositides. Potentially, this could lead to signaling abnormalities. Polyphosphoinositide binding to proteins might be affected, resulting in an altered regulation of synaptic vesicle trafficking (26). In that regard, it is interesting that the synaptotagmin-1 knock-out newborn pup with a selective deficiency of a phosphatidylinositol-4,5-P2-5′-phosphatase isofrom found in clathrin-coated vesicles of nerve terminals has a phenotype that bears many similarities to the SMIT1(−/−) newborn (26). This genetic model suggests that a potentially lethal perturbation in neurotransmission, as related to synaptic vesicle protein recycling, in the first day of life is largely without effect in prenatal life. Although a putative deficit in neuronal regulation in our model may be present throughout the neuraxis because the phosphoinositide deficiency is widespread, it is not surprising that the first recognizable manifestation in postnatal life would involve the respiratory control center pacemaker and present as abnormal respiratory rhythmogenesis. Still unexplained, however, is the reason why Ins levels in murine brain tissue are more than 6-fold higher (7.80 versus 1.16 μmol/grams of wet weight) than whole body levels at the time of birth. This may indicate a special dependence of brain cells on Ins to facilitate a process having levels of Ins intermediate between wild-type and knock-out fetuses. For example, millimolar levels are found in the adult nervous system, kidney, and testes (1, 3, 14, 43), whereas

Based on measurements of tissue extracts or cultured cells derived from such tissues, the apparent concentration of Ins in mammalian cells is in both the micromolar and millimolar range. For example, millimolar levels are found in the adult nervous system, kidney, and testes (1, 3, 14, 43), whereas

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micromolar levels have been detected in adult liver, skeletal muscle, and heart (1, 12, 14, 43, 45). Micromolar concentrations of Ins may be sufficient for de novo synthesis of PtdIns as the $K_m$ of phosphatidylinositol synthase for Ins has been reported to be in the micromolar range when the assay was carried out using microsomes from mammary gland and lung tissues (46, 47). Further support for the concept that low micromolar concentrations of Ins could be sufficient to allow for adequate PtdIns synthesis stems from work demonstrating that low levels of Ins could be sufficient to allow for adequate PtdIns synthesis as the de novo synthesis of PtdIns in cultured fetal endothelial cells from ~5 to 1.5 mM did not appear to lower the rate of PtdIns synthesis (50). Rate-limiting amounts of CDP-diacylglycerol in cellular membranes (51) may be more important than variations in Ins concentrations between 2 and 15 mM (4) in the regulation of PtdIns synthesis. Thus, the utilization of CDP-diacylglycerol for phosphatidylglycerol synthesis (49) has been reported to be more important than variations in Ins concentrations between 2 and 15 mM (4) in the regulation of PtdIns synthesis. We found that an experimentally induced reduction in the estimated intracellular Ins concentration in cultured type 2 pneumocytes (48). In a microsomal fraction of cultured cells derived from adult liver, skeletal muscle, and heart (1, 12, 14, 43, 45). Micromolar concentrations have been detected in adult liver, skeletal muscle, and heart (1, 12, 14, 43, 45). Micromolar concentrations of Ins may be sufficient for de novo synthesis of PtdIns as the $K_m$ of phosphatidylinositol synthase for Ins has been reported to be in the micromolar range when the assay was carried out using microsomes from mammary gland and lung tissues (46, 47). Further support for the concept that low micromolar concentrations of Ins could be sufficient to allow for adequate PtdIns synthesis stems from work demonstrating that low levels of Ins could be sufficient to allow for adequate PtdIns synthesis as the de novo synthesis of PtdIns in cultured fetal endothelial cells from ~5 to 1.5 mM did not appear to lower the rate of PtdIns synthesis (50). Rate-limiting amounts of CDP-diacylglycerol in cellular membranes (51) may be more important than variations in Ins concentrations between 2 and 15 mM (4) in the regulation of PtdIns synthesis. 

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