Measurement of *myo*-Inositol Turnover in Phosphatidylinositol: Description of a Model and Mass Spectrometric Method for Cultured Cortical Neurons

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ABSTRACT: Rates of *myo*-inositol (Ins) incorporation and turnover in phosphatidylinositol (PtdIns) were determined in cultured mouse cortical neurons. Cells were incubated with deuterium-labeled *myo*-inositol (Ins*) in culture medium free of unlabeled Ins. The time-dependent changes in the specific activity of cytosolic Ins* and membrane PtdIns* were measured by mass spectrometry. PtdIns turnover was modeled incorporating values for Ins* flux, cytosolic dilution, PtdIns concentration, and rate of incorporation into PtdIns. Recycled Ins diluted the labeled precursor pool, and a time course was obtained for this cytosolic process. The specific activity of the precursor pool at the plateau of the time-course curve was 0.43 ± 0.04 (mean ± SD). The incorporation of the tracer into PtdIns was linear between 4 and 10 h incubation of the neurons. After factoring in the extent of dilution of the tracer in the precursor pool, the rate of Ins incorporation into PtdIns was found to be 315 ± 51 nmol (g of protein)$^{-1}$ h^{-1}. The half-life of Ins in PtdIns was calculated for each point on the linear incorporation curve and then corrected for the tracer reincorporation. The half-life of Ins in PtdIns was 6.7 ± 0.2 h, which translates into a basal turnover rate of 10.3%/h in this in vitro system. The mathematical model and the stable isotope method described here should allow assessment of the dynamics of PtdIns signaling altered in certain diseases or by agents.

myo-Inositol (Ins)[1] is a highly dynamic component of phosphoinositides, a class of phospholipids that release second messengers in response to cell-surface receptor activation (*1, 2*). The signaling pathway involving Ins is cyclic, requiring continuous incorporation of Ins into membrane phosphatidylinositol (PtdIns) pools. The Ins moiety of PtdIns is phosphorylated by site-specific phosphoinositide kinases, and the polyphosphoinositide products are utilized for cellular processes (*3*). The most abundant polyphosphoinositide in neural tissues is phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (*4*). Receptor-mediated activation of phospholipase C causes the hydrolysis of PtdIns(4,5)P_2 and generates inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol. InsP_3, a potent second messenger, is rapidly dephosphorylated in the cytosol, and regenerated Ins is made available for PtdIns synthesis. A number of neurotransmitters, hormones, growth factors, and other agonists have been shown to influence PtdIns turnover during membrane signaling (*1, 2*).

The dynamics of Ins turnover may be altered in certain pathophysiological conditions. These include diabetes, where the Ins pool utilized for PtdIns synthesis is depleted in peripheral neurons (*5, 6*). An excessive accumulation of Ins in the brain is a phenotype of Down syndrome (trisomy 21) and of animal models of this disorder (*7*−*10*). Metabolite profiling by magnetic resonance spectroscopy indicates elevated concentrations of Ins and Ins metabolites in the Alzheimer disease brain (*11, 12*). Also, fibroblasts from Alzheimer disease patients show augmented InsP_3-mediated Ca^{2+} signaling (*13*), and the Alzheimer presenilin-1 and presenilin-2 mutations are reported to potentiate this signaling (*14, 15*). Certain therapeutic agents may alter PtdIns turnover and signaling. For instance, lithium inhibits inositol monophosphatase at therapeutic levels used in the treatment of bipolar disorder (*16*), and reduces brain Ins in patients with bipolar disorder (*17*). Ins depletion leading to decreased PtdIns turnover has been proposed as the mechanism of action of this ion (*18*).

A method to quantitate Ins turnover (in PtdIns) would be useful in investigating pathophysiological conditions or agents that modulate this dynamic process. In this paper, we describe a stable isotope procedure to measure Ins turnover in cultured cortical neurons under steady-state experimental conditions. The method involves incubation of cells in a culture medium where Ins is replaced entirely with deuterium-labeled *myo*-inositol (Ins*). A mass spectrometric technique is used to measure the time-dependent changes in specific activities of the tracer in cytosol and in PtdIns. PtdIns

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[1] Abbreviations: Ins, *myo*-inositol; Ins*, *myo*-[$^2H_6$]inositol; PtdIns, phosphatidylinositol; PtdIns*, phosphatidyl[$^2H_6$]inositol; PtdIns(4,5)-P_2, phosphatidylinositol 4,5-bisphosphate; InsP_3, inositol 1,4,5-trisphosphate; BHT, butylated hydroxytoluene.

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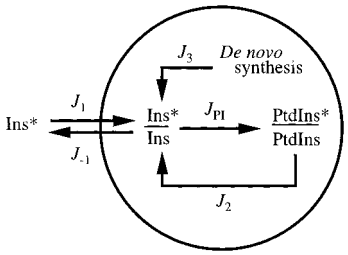

FIGURE 1: Model representing cell uptake of Ins* (J_1), dilution by cytosolic Ins (J_2 and J_3), and incorporation (J_{PI}) into PtdIns.

turnover (Figure 1) is determined in terms of the flux of Ins* into the cell (J_1), the rate of incorporation of Ins* into the lipid (J_{PI}), the concentration of Ins in the lipid, and the specific activity of the precursor pool (λ). The model takes into account tracer dilution due to recycling (J_2), but not de novo synthesis (J_3). However, the de novo synthesis of Ins has been shown to be negligible in the case of neurons (*19*, *20*).

EXPERIMENTAL PROCEDURES

Materials. C57BL/6 mice were procured at the Frederick Cancer Research Development Center, National Cancer Institute (Frederick, MD). GibcoBRL neurobasal medium (containing Ins or no Ins) was obtained from Life Technologies (Gaithersburg, MD). Ins, *scyllo*-inositol (internal standard), primulin, butylated hydroxytoluene (BHT), phospholipase C (*B. cereus*), and alkaline phosphatase were from Sigma Chemical Co. (St. Louis, MO). Protein was assayed by BCA reagent (bovine serum albumin as standard), available from Pierce Chemical Co. (Rockford, IL). Mixed-bed ion-exchange resin, AG 501X8 (D), was from Bio-Rad Laboratories (Hercules, CA). The tracer *myo*-[$^2H_6$]inositol (Ins*) was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Gas chromatographic/mass spectrometric (GC/MS) analysis indicated that the product was practically free of unlabeled Ins and >98% enriched in $^2H_6$-labeled species. Acetic anhydride and pyridine were from Alltech Associates Inc. (Deerfield, IL), and 4-(dimethylamino)-pyridine was from Fluka Chemical Corp. (New York, NY). Solvents used for the extraction and processing of analytes were from Burdick & Jackson (Muskegon, NY). The gas chromatograph/mass spectrometer was a Finnigan MAT ITS40 (San Jose, CA), and the capillary column (Rtx50) was obtained from Restek Corp. (Bellefonte, PA). TLC plates used for phospholipid separation were Kieselgel 60 (0.25 mm thickness) purchased from EM Science (Gibbstown, NJ). The probe sonicator was from Misonix Inc. (Farmingdale, NY), the evaporator was SpeedVac AES1010 from Savant Instruments Inc. (Farmingdale, NY), and the microplate reader was Labsystems Multiskan MCC/340 (Finland). GraphPad Prism software (San Diego, CA) was used to analyze the Ins* uptake and incorporation data.

Cultured Cortical Neurons. Pregnant mice were killed at embryonic day 16 using CO_2 asphyxiation followed by cervical dislocation. The fetuses were removed and killed by decapitation, and the brains were removed. Pieces of cerebral cortex were dissected, trypsinized (0.012% trypsin), and triturated by passing through a fire-polished glass pipet. The dissociated cells were plated, at a density of 5.0×10^5 cells/dish, onto polylysine-coated six-well plates. Serum-free neurobasal medium containing glutamine and B27 supplement was used for both plating and feeding. The cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and the medium was replenished every third or fourth day. Experiments were performed between 8 and 10 days in culture.

Uptake Experiment. The uptake medium was prepared by supplementing Ins-free neurobasal medium with 40 μM Ins*. Glutamine and B27 supplement were added to obtain the composition of the growth medium. The uptake medium was maintained at 37 °C. To start an experiment, the growth medium (containing 40 μM Ins) was removed from the cultured cortical neurons and immediately replaced with 500 μL of uptake medium. At the end of the incubation with Ins*, cells were washed 3 times with ice-cold neurobasal medium devoid of Ins. The cells were lysed with 500 μL of ice-cold methanol (containing 0.005% BHT) and scraped, and the extract was transferred to an Eppendorf tube. The wells were rinsed with 250 μL of methanol followed by 250 μL of KCl (10 mM). Both washes were combined with the original 500 μL of methanol extract.

Extraction. Cell extracts in the Eppendorf tubes were sonicated and transferred to tapered-bottom glass tubes. To recover residual cell extract, methanol (250 μL) was added to the Eppendorf tube and sonicated, and the solvent was combined with the extract in the glass tube. Into the tube were added 400 μL of KCl (10 mM), 100 μL of *scyllo*-inositol (1 ng/μL), and 2 mL of chloroform and mixed by vortexing (5 min). The extract was refrigerated (15 min) and centrifuged (2000*g*) at 5 °C for 5 min. The clear, top layer was transferred to an Eppendorf tube for the analysis of Ins and Ins*. The organic layer was quantitatively transferred to another glass tube (tapered), and the interfacing layer was saved for protein analysis. All the fractions were stored at −20 °C until analysis.

Isolation and Hydrolysis of PtdIns. The organic layer was evaporated (SpeedVac, 24 °C), and the lipid residue was reconstituted in 60 μL of chloroform/methanol (2:1; v/v) mixture and applied (2 cm wide band) onto a TLC plate. The plate was developed in a solvent system consisting of 30 mL of chloroform, 9 mL of methanol, 25 mL of 2-propanol, 6 mL of KCl (0.25%), and 18 mL of ethyl acetate (*21*). The PtdIns band was detected (under UV light following primulin spray), and silica gel scrapings were transferred to a glass tube. Silica gel was dispersed by sonication with 2 mL of chloroform/methanol (50:50; v/v) mixture containing 0.1% formic acid and 0.005% BHT. The suspension was filtered through glass wool (packed in a Pasteur pipet), and an additional 1 mL of the solvent mixture was added to the tube, sonicated, and filtered. The filtrate was mixed with 1.5 mL of chloroform and 1.1 mL of KCl (10 mM) and centrifuged, and the aqueous layer was discarded.

The organic layer was evaporated, and the residue was reconstituted in 500 μL of ether. Hydrolysis of the polar headgroup was started following the addition of 500 μL of Tris buffer (20 mM, pH 7.5) containing 10 units of phospholipase C. The reaction was completed by vortexing for 2 h at 24 °C. The internal standard, *scyllo*-inositol (100 μL; 1 ng/μL), was added, and the reaction mixture was evaporated. The residue was acidified with 100 μL of HCl (1 M), evaporated to complete dryness, and then treated with

500 μL of Tris buffer (50 mM, pH 9.0) containing phenol red indicator (6% ammonia solution was added if needed). The alkaline sample was mixed with 25 μL of $MgCl_2$ (10 mM) and 20 units of alkaline phosphatase, and incubated at 37 °C for 2 h. Mixed-bed ion-exchange resin was packed in 1 mL pipet tip (with loosely packed cotton at the pointed end) and washed with water. The sample was diluted with 1 mL of water and passed through the resin. Ins and Ins* were eluted with an additional 2 mL of water, collected in a glass tube, and dried (evaporation at 60 °C until most of water removed and then at 40 °C).

GC/MS Analysis. Ins, Ins*, and *scyllo*-inositol (internal standard) in the sample were converted to acetate derivatives and analyzed by GC/MS, as described previously (*22*, *23*). Briefly, the residue was heated with acetic anhydride and pyridine [containing 4-(dimethylamino)pyridine], dissolved in hexane/ethyl acetate mixture, and washed with $NaHCO_3$ solution. The organic layer was evaporated to dryness, and the residue was reconstituted in ethyl acetate. An aliquot of this solution was injected into GC/MS (ion trap mass spectrometer), and ions generated by chemical ionization, *m*/*z* 373 for Ins and *scyllo*-inositol and *m*/*z* 379 for Ins*, were monitored. The aqueous component of the cell extract (250 μL) was evaporated and similarly analyzed for free Ins and Ins*. PtdIns and PtdIns* concentrations were obtained from standard curves generated by the analysis of varying concentrations of Ins and Ins* (along with a fixed concentration of *scyllo*-inositol) mixture carried through the enzymatic hydrolysis steps. Concentrations of Ins and PtdIns, and their labeled analogues, were expressed as nanomoles per gram of protein. Protein was measured in a microplate reader following color development with BCA reagent.

Modeling and Calculation of PtdIns Turnover and Half-Life. The uptake experiment was started at $t = 0$ by replacing neurobasal growth medium with the same medium containing 40 μM Ins*. The neuronal uptake of medium Ins* is countered by the efflux of cytosolic Ins at the same rate (Figure 1). Thus, the total concentration of Ins (labeled and unlabeled, Ins* + Ins) in the medium as well as in the cell should remain unaltered throughout the experiment. Additionally, PtdIns concentration was not altered during the experiment (data not shown).

Figure 1 illustrates unidirectional fluxes of Ins across the cell membrane (J_1 and J_{-1}) between the bath and the cytoplasm, the flux of Ins from the PtdIns pool into the cytoplasm (J_2), and the rate of de novo synthesis of cytoplasmic Ins (J_3). Under the experimental conditions, medium Ins* is in large excess, and its dilution by Ins diffusing from cells (J_{-1}) is negligible. This can be seen from the following calculations. During incubation (5 min to 10 h), the *total* concentration of Ins and Ins* in the cultured neurons was 423 ± 78 pmol (mean ± SD; $n = 27$ plates). The cells were incubated in 0.5 mL of medium containing 20 nmol of Ins* (100%). Thus, if *all* of Ins diffused out of cells, the tracer in the bath would be diluted by a factor of *only* 2.1%. Accordingly, the tracer dilution was considered negligible in this experimental condition, and the specific activity of Ins could be taken as 1, with $[Ins_{total}]_{outside} = [Ins^*]_{outside} = 40$ μM at all times.

The rate of incorporation of cytosolic Ins into PtdIns, J_{PI} [nmol (g of protein)$^{-1}$ h^{-1}], is proportional to its concentration in the cytosol and depends on the action of PtdIns synthase. The rate of incorporation of Ins* into PtdIns, equal to J^*_{PI} [nmol (g of protein)$^{-1}$ h^{-1}], also is proportional to its concentration in the cytosol. Thus, cytosolic Ins can be considered the "precursor pool" for incorporation. In view of the above relations, $J^*_{PI}(t)$, the rate of incorporation of tracer into PtdIns at any time t after the cells are exposed to the bath, equals

$$J^*_{PI}(t) = J_{PI}[Ins^*/Ins_{total}]_{inside}(t) \quad (1)$$

where $[Ins^*/Ins_{total}]_{inside}(t)$ is the specific activity of the precursor pool at time t. In this study, the mole fraction of Ins* or mole percentage of Ins* (percentage of Ins* in combined Ins and Ins* fractions) is defined as the specific activity of Ins*. As noted above, the specific activity of the bath (outside) always equals 1:

$$[Ins^*/Ins_{total}]_{outside} = 1 \quad (2)$$

The ratio of specific activities in the cytoplasm and the bath at time t after the cells are exposed to the bath is given as

$$Q(t)\lambda = \frac{[Ins^*/Ins_{total}]_{inside}(t)}{[Ins^*/Ins_{total}]_{outside}} = [Ins^*/Ins_{total}]_{inside}(t) \quad (3a)$$

where the bath specific activity is 1 (eq 2). $Q(t)$ is a time-dependent factor that represents the extent to which the specific activity of the precursor pool has approached steady state. Under our experimental conditions, $Q(0) = 0$ at $t = 0$. $Q(t)$ increases after $t = 0$ until the tracer has reached a steady-state concentration in the precursor pool, at which time $Q(t) = 1$. In this case, the precursor pool specific activity equals λ from eq 3a:

$$\lambda = [Ins^*/Ins_{total}]_{inside}(t = \text{steady state}) \quad (3b)$$

It can be appreciated from Figure 1 (when no tracer present) that λ represents the steady-state ratio of unlabeled Ins influx from the bath into the precursor pool, to the sum of unlabeled influx from the bath (J_1), release from PtdIns (J_2), and de novo biosynthesis (J_3) [see discussion by Robinson et al. (*24*) of similar use of a dilution factor in considering recycling of fatty acids within brain phospholipids]. However, as de novo synthesis of Ins is insignificant (*19*, *20*):

$$\lambda = \frac{J_1}{J_1 + J_2 + J_3} \cong \frac{J_1}{J_1 + J_2} \quad (4)$$

Combining eqs 1 and 3a gives

$$J^*_{PI}(t) = J_{PI}[Ins^*/Ins_{total}]_{inside}(t) = J_{PI}Q(t)\lambda \quad (5)$$

As $J^*_{PI}(t)$, $Q(t)$, and $[Ins^*/Ins_{total}]_{inside}(t)$ are time-dependent but J_{PI} and λ are not, eq 5 can easily be integrated and rearranged to give an "operational" equation for J_{PI}:

$$J_{PI} = \frac{PtdIns^*(T)}{\lambda \int_0^T Q(t)\, dt} \quad (6)$$

where PtdIns*(T), in nmol of PtdIns*/g of protein, is the amount of Ins* accumulated in phosphatidylinositol at time T due to flux $J^*_{PI}(t)$ of Ins* from the precursor pool

$$\text{PtdIns*}(T) = \int_0^T J^*_{\text{PI}}(t)\, dt \quad (7)$$

Equation 7 is valid assuming no loss of deuterium-containing material from the phosphatidylinositol pool between time 0 and T h.

To solve eq 6 for J_{PI}, the integral of $Q(t)$ is determined at different times from the measured time course of specific activity of cytosolic Ins (eq 3a), λ is taken as the steady-state cytosolic specific activity at $Q(t) = 1$ (see below, Figure 3), and PtdIns*(T) is determined analytically (see text).

The turnover rate (τ) of Ins in $\text{PtdIns}_{\text{total}}$ is defined as (*24*)

$$\tau = \frac{J_{\text{PI}}}{[\text{PtdIns}_{\text{total}}]} \times 100\ (\%/\text{h}) \quad (8)$$

Therefore, the half-life $t_{1/2}$ (h) assuming linearity equals

$$t_{1/2} = \frac{[\text{PtdIns}_{\text{total}}] \times 0.693}{J_{\text{PI}}} \quad (9)$$

RESULTS

The GC/MS method enabled quantitation of endogenous cellular Ins and, simultaneously, medium Ins*, taken up by the neurons. Cell or medium components did not interfere in this sensitive and specific analytical technique, previously developed to measure Ins in biological samples (*22*, *23*). Following Ins* incorporation, PtdIns and PtdIns* in cell extracts were also quantitated by the mass spectrometric technique. Figure 2e shows detection of ions, *m*/*z* 373 for Ins and *m*/*z* 379 for Ins*, each formed by the loss of one acetate moiety from the corresponding hexaacetate derivative. *scyllo*-Inositol, a stereoisomer of Ins, also yields a *m*/*z* 373 ion which was monitored to quantitate Ins and Ins*. The mass spectrometric assay of PtdIns involved enzymatic release of the headgroup. Phospholipase C (from *Bacillus cereus*) is known to hydrolyze PtdIns to a mixture of inositol 1,2-cyclic phosphate and inositol 1-phosphate (Figure 2a−c) in almost equal proportions (*25*). Acidification converted inositol 1,2-cyclic phosphate to inositol 1-phosphate, and free Ins was recovered by alkaline phosphatase treatment. When PtdIns standard (Avanti Polar Lipids, Alabaster, AL) was hydrolyzed by this procedure, Ins was recovered in 104 ± 6% (n = 3) yield, consistent with previously reported, complete hydrolysis of PtdIns by bacterial phospholipase C (*26*, *27*). The recovery of radiolabeled PtdIns from TLC was 87 ± 2% (n = 3), and concentrations of PtdIns measured in cell extracts were corrected for the loss.

The intracellular Ins and Ins* concentrations were measured at nine different time points, beginning at 5 min and ending at 10 h, following exposure of the neurons to the tracer (Table 1). The concentration of combined Ins and Ins* pools was 423 ± 78 pmol/plate (n = 27). The concentration of Ins or Ins* per gram of protein was not determined since the calculation of turnover required precursor pool specific activity only. The combined concentration of PtdIns and PtdIns* was 1543 ± 286 pmol/plate (n = 12), obtained for the later time points, 4, 6, 8, and 10 h, when significant Ins* incorporation occurs. The protein concentration in neurons was measured for these time points, and the value was 384 ± 32 μg/plate (n = 12).

Figure 3a shows the time course of precursor pool specific activity in the cytosol of the cultured cortical neurons incubated in medium containing Ins*. The specific activity of Ins* reached a plateau between 4 and 10 h, and a true steady state was not achieved because of the tracer reincorporation (incorporation of Ins* released from PtdIns*). Consequently, a pseudo-steady-state for Ins* was assumed between 4 and 10 h, and the precursor pool specific activity λ was determined by fitting the data to the equation: mol % = λ × time/(time + constant), using GraphPad Prism software (Figure 3a). Accordingly, the time-course analysis of Ins* uptake provided a value for λ of 0.43 ± 0.04 (r = 0.83). Additionally, a similar value, 0.43 ± 0.05, for λ was obtained by averaging the specific activities between 4 and 10 h. Using the trapezoidal rule (*28*), the integrated value of $Q(t)dt$ from time 0 to T (eq 6) was determined for each time point t by dividing the area under the curve (AUC) from t_0 to that particular time point by λ. Table 1 shows results of these calculations. $Q(T)$ increased with time, attaining a value of ∼1 at a pseudo-steady-state of the precursor pool (Table 1). The $Q(T)$ values 0.9, 0.9, 1.1, and 1.1, determined at 4, 6, 8, and 10 h uptake, respectively, verify the appropriateness of the uptake data used to determine the λ value. Some deviation in the early time points (possibly reflecting the media-replacement effect) should not significantly alter the turnover data.

Cellular concentrations of PtdIns and PtdIns* were obtained at 4, 6, 8, and 10 h following Ins* exposure. The time-dependent changes in PtdIns* concentrations are shown in Table 1, and these data were used to determine J_{PI}. Figure 3b demonstrates a linear (r = 0.99) increase in the mole percent of PtdIns* between 4 and 10 h, resulting from continuous incorporation of Ins* into the lipid under the steady-state experimental condition. The incorporation rate J_{PI} was calculated using eq 6, and the value (mean ± SD) for each time point (n = 3) is shown in Table 2. Thus, J_{PI} for 12 determinations was 315 ± 51 nmol (g of protein)$^{-1}$ h^{-1}. Using J_{PI} data and the combined concentrations of PtdIns and PtdIns*, the $t_{1/2}$ of Ins in PtdIns was calculated (eq 9) for each time point (4−10 h). The results (Table 2) indicate a small but significant increment in $t_{1/2}$ as more of PtdIns* is formed. To correct for the tracer reincorporation (due to Ins* originating from PtdIns*), the incorporation time and $t_{1/2}$ data were plotted as shown in Figure 4. Extrapolation of the curve to time zero provided a $t_{1/2}$ of 6.7 ± 0.2 h. Thus, in cultured neurons, the rate of Ins turnover in PtdIns was 10.3%/h.

DISCUSSION

In neurons, Ins is utilized for PtdIns synthesis and turnover. There is no evidence for other metabolic pathways where significant amounts of Ins are consumed. Accordingly, the turnover model assumes that Ins* taken up by the neurons enters the PtdIns cycle without being channeled to other metabolic compartments and that specific activities in all cytosolic cellular compartments are equal. Metabolites of Ins do not interfere in the mass spectrometric measurement, and thus the data for Ins and Ins* provided true precursor pool specific activities. A steady-state experimental condition was established by incubating cortical neurons in the culture medium in which 40 μM Ins was entirely in the form of Ins*. Thus, the tracer was taken up by the cell under the same environment as it was in the growth medium. Ins* is actively transported into neurons by the Na^+/Ins cotransporter

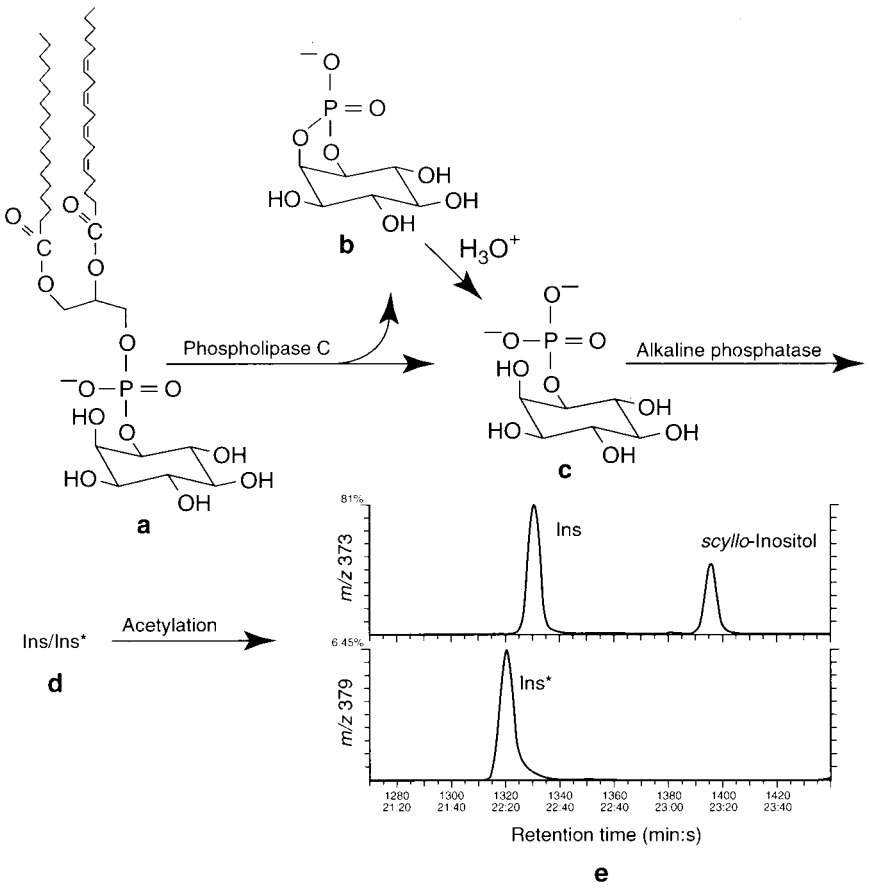

FIGURE 2: Analytical procedure used to measure Ins and Ins* in PtdIns/PtdIns*: phospholipase C-mediated cleavage of PtdIns (a) yielding a mixture of inositol 1,2-cyclic phosphate (b) and inositol 1-phosphate (c); dephosphorylation, recovering Ins and Ins* (d); and GC/MS detection (*m*/*z* 373 and 379 ions) of acetate derivatives of Ins, Ins*, and *scyllo*-inositol (internal standard) (e). The chromatogram is for the cells exposed to Ins* for 4 h.

Table 1: Mean ± SD Values of $Q(T)$ and PtdIns*(T)[a]

exposure time (T)	$Q(T)$	$\int_0^T Q(t)\,dt$ (h)	PtdIns*(T) (nmol/g of protein)
5 min	0.42 ± 0.02	0.017	
15 min	0.40 ± 0.07	0.086	
30 min	0.61 ± 0.09	0.214	
1 h	0.58 ± 0.04	0.513	
2 h	0.62 ± 0.04	1.116	
4 h	0.87 ± 0.06	2.606	366 ± 54
6 h	0.93 ± 0.07	4.409	561 ± 26
8 h	1.07 ± 0.07	6.416	992 ± 187
10 h	1.10 ± 0.04	8.588	1030 ± 140

[a] The integral of $Q(t)dt$ from time 0 to T was calculated using the trapezoidal rule from the time course for Ins* taken up by the cortical neurons (Figure 3a).

(*23*, *29*). The tracer, as it accumulates in the cytosol, is diluted by Ins at a certain rate as a result of the turnover of PtdIns (Figure 1). The neuronal PtdIns concentration was higher when compared with its precursor, Ins, and thus the specific activity of the tracer leveled off due to the turnover process. In addition, a near-steady-state of the tracer is supported by the $Q(T)$ data for the time points for which the incorporation and turnover rates were calculated (Table 1). A true steady-state for the cytosolic Ins* was not attained in this cell culture model because of the incorporation of Ins* released from PtdIns* (J_2 process). Thus, the precursor pool was considered to be in a pseudo-steady-state when the specific activity of the intracellular Ins* reached plateau.

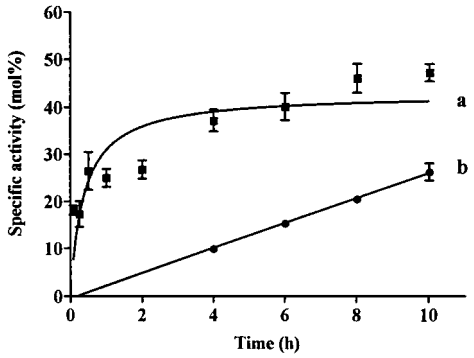

FIGURE 3: Time course of specific activity of Ins* in cultured cortical neurons following replacement of neurobasal medium with the same medium containing Ins* (a); time course of specific activity of PtdIns* showing linear incorporation of the tracer into lipid between 4 and 10 h (b). Data points represent mean ± SD values for triplicate samples. The equation for the Ins* uptake curve: mol % = λ × time/(time + constant), provided the λ value 0.43 ± 0.04.

The experimental data adequately fit into the PtdIns turnover model, and the mathematical formulas derived to calculate turnover are based on the concept of tracer dilution by the recycled Ins. A value of λ equal to 0.43 suggests that 43% of cytoplasmic Ins was derived from the bath by transport and that the remaining 57% was released from PtdIns, in these cultured cortical neurons. The dilution of the tracer (Ins*) by recycled Ins as well as the time course leading to a pseudo-steady-state condition were taken into account in calculating the rate of incorporation J_{PI}. The

Table 2: Incorporation Rate (J_{PI}) and Half-Life ($t_{1/2}$) Calculated for Each Time Point on the Time Course of Incorporation of Ins* into PtdIns

time (h)	J_{PI} [nmol (g of protein)$^{-1}$ h^{-1}] mean ± SD ($n = 3$)	$t_{1/2}$ (h) mean ± SD ($n = 3$)
4	326 ± 48	7.87 ± 0.16
6	296 ± 14	8.60 ± 0.14
8	359 ± 68	9.26 ± 0.04
10	280 ± 38	9.70 ± 0.64

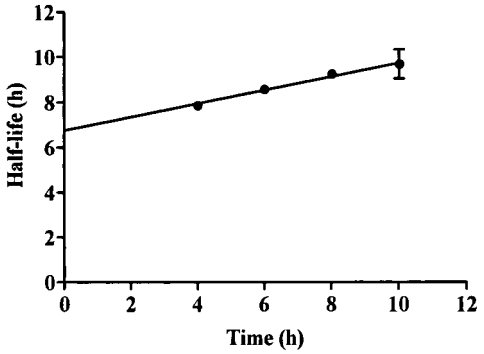

FIGURE 4: Plot of calculated $t_{1/2}$ of Ins in PtdIns for the time points 4−10 h and extrapolation of the curve to 0 h to correct the $t_{1/2}$ value for tracer reincorporation.

incorporation of the tracer into PtdIns was linear between 4 and 10 h, suggesting a near-steady-state nature of the precursor pool. J_{PI} (Table 2) did not differ significantly from one time point to the other, and thus the effect of the tracer reincorporation on these data was not detected. However, $t_{1/2}$ increased with time, and Ins* reincorporation was factored in for more accurate assessment of the PtdIns dynamics.

The turnover rate of 10.3%/h likely reflects the dynamics of conversion of PtdIns to PtdIns(4,5)P_2 and release of the second messenger, InsP_3. In addition, metabolism of other less abundant polyphosphoinositides (*30*−*32*) and diesteratic cleavage of PtdIns (*33*) contribute to this basal turnover rate. A discrete pool of PtdIns may be utilized for signal transduction (*34*), and this fraction of PtdIns may have a different turnover rate. However, the model assumes that PtdIns turns over as a single homogeneous pool and thus the rate of 10.3%/h is an average turnover rate for functional and structural PtdIns in the membrane. In neural tissues, the concentration of PtdIns(4,5)P_2 is 10−15% that of PtdIns (*35*, *36*), and therefore a severalfold higher turnover rate may be predicted for the Ins moiety in PtdIns(4,5)P_2. During a tracer incorporation study, PtdIns(4,5)P_2 is likely to exhibit the same specific activity as its precursor, PtdIns. The incorporation rate for PtdIns(4,5)P_2 should be the same, unless a separate pool of PtdIns is operative in the phosphorylation and second messenger release pathways. However, a higher turnover rate for Ins in PtdIns(4,5)P_2 is possible because of the lower abundance of this polyphosphoinositide relative to PtdIns (eq 8). The present study may be expanded to measure the specific activity of PtdIns(4,5)P_2 and thus the turnover rate for this second messenger precursor. In addition, the possible existence of a separate pool of PtdIns for signaling should be explored.

The PtdIns turnover rate reported here is for unstimulated cultured neurons and likely represents the lower limit of the turnover rate in vivo. A higher turnover rate is predicted for in vivo systems, particularly in organs such as brain and heart. In brain, the concentration of Ins is 5−6 mM, and that of phosphoinositides and inositol phosphates combined may approach 3 mM (*37*−*39*). The effect of alteration in the concentration of intracellular Ins on PtdIns turnover remains to be clarified, although the K_m for brain PtdIns synthase matches the brain cytosolic Ins concentration (*40*). It is probable that changes in Ins concentration affect certain discrete pools of PtdIns that turn over rapidly.

The present technique can be used to measure PtdIns turnover in cells from Down syndrome and diabetic animal models, where Ins homeostasis is reported to be altered (*5*, *9*, *10*). Appropriate cell preparations may be used to identify disease-related changes in the turnover. In addition, changes in the activity of PtdIns kinase, phospholipase C, or other enzymes involved in the PtdIns cycle may alter the turnover. Based on our in vitro cell culture model and procedure, an in vivo procedure also might be devised to measure PtdIns turnover in brain for probing animal models of certain diseases. The approach used for measuring fatty acid turnover in brain phospholipids of awake rats could be adapted in this case (*24*).

Inhibition of inositol monophosphatase by lithium is likely to reduce Ins recycling and thereby increase the precursor pool specific activity at the pseudo-steady-state. This stable isotope technique can thus be used to evaluate the effect of lithium-induced reductions of Ins recycling on the turnover of PtdIns in cultured cells and, possibly, in intact animals. Another possible application is in the evaluation of effects of agonists that bind specific receptors and activate PtdIns signaling. Effects of such agonists generally are determined from the amount of InsP_3 released when prelabeled cells are stimulated. However, it is difficult to assess the true amount of InsP_3 because of rapid inactivation by phosphatases (*41*). During this transmembrane signaling, depleted phosphoinositides are immediately replenished by the synthesis. Thus, increased incorporation and turnover of Ins in PtdIns is likely to reflect the dynamics of second messenger release.

The incorporation rate J_{PI} allows us to estimate the energy consumption in the turnover of Ins in PtdIns. The reaction of Ins and cytidine diphosphate diacylglycerol mediated by PtdIns synthase requires no ATP. The subsequent steps, phosphorylation of the Ins ring by phosphoinositide kinases leading to the formation of PtdIns(4,5)P_2, utilize two molecules of ATP. An additional three molecules of ATP are required for recycling of the second messenger diacylglycerol to cytidine diphosphate diacylglycerol via phosphatidic acid. Thus, a total of five ATP molecules are utilized in the complete turnover of PtdIns via the PtdIns(4,5)P_2 pathway. The Ins incorporation rate, 315 nmol (g of protein)$^{-1}$ h^{-1}, translates to an ATP consumption rate of 1.58 μmol (g of protein)$^{-1}$ h^{-1} (five ATP molecules for the incorporation of each Ins molecule) for PtdIns turnover. This value is expected to be higher in intact brain and in stimulated conditions in vivo and in vitro. The ATP consumption rate for PtdIns turnover in cultured neurons is negligible compared with the overall rate of ATP consumption by the brain of the awake rat, 7.49 mmol (g of protein)$^{-1}$ h^{-1} (*42*). This comparison may be inappropriate, however, because PtdIns cycle enzymes might conceivably be downregulated in cultured, chronically unstimulated neurons. Differences also exist in energy metabolism between cultured neurons and intact brain (*43*).

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