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Studies on Polyphosphoinositides in Developing Rat Brain

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Abstract: Polyphosphoinositides in rat brain exist in two forms: the metabolically active form that is readily attacked by the polyphosphoinositide phosphohydrolases, and the inert form that is attacked by the enzymes at a slower rate. The two pools continue to increase even during the postweaning period, suggesting a role in glial as well as myelin development apart from their role in neurons. **Key Words:** Polyphosphoinositides—Rat brain. **Uma S. and Ramakrishnan C. V.** Studies on polyphosphoinositides in developing rat brain. *J. Neurochem.* **40**, 914–916 (1983).

Polyphosphoinositides have been detected in myelin and nonmyelin structures (Eichberg and Dawson, 1965; Eichberg et al., 1971; Hauser et al., 1971; Eichberg and Hauser, 1973; Deshmukh et al., 1980). Rapid postmortem degradation of these compounds, during which the metabolically active fraction of polyphosphoinositides is lost, has been reported (Eichberg and Hauser, 1967; Hauser et al., 1971; Soukup et al., 1978). Studies on their levels and postmortem loss in the whole brain of rats and guinea pigs (Sheltawy and Dawson, 1969) and in different regions of the rat brain (Hauser et al., 1971) suggest the presence of metabolically active and inert pools of polyphosphoinositides. Gonzalez-Sastre and Folch (1971) have also confirmed this concept by carrying out studies using labeled phosphorus.

It was thought of interest to study the maturation of inert and metabolically active pools of polyphosphoinositides during the development of rat brain. Studies were therefore carried out to estimate the concentrations of polyphosphoinositides and their postmortem losses in the rat brain at different ages. The data obtained are reported in this paper.

MATERIALS AND METHODS

Albino rats of the Charles-Foster strain taken from the animal colony of this department were used for these studies. For preliminary experiments, animals of two ages (21 and 56 days old) were taken, and the postmortem losses in the levels of phosphatidylinositol 4',5'-phosphate (PhIpp) and phosphatidylinositol 4'-phosphate (PhIp) with time were determined. The heads of the animals were dropped into liquid nitrogen at different times after decapitation, i.e., 2 s, 1, 10, 20, 30, 40, and 70 min. The frozen heads were taken out from liquid nitrogen and allowed to thaw (time taken varied from 1–3 min depending on the age of the animal); the brains were removed and used for extraction and estimation of polyphosphoinositides by the method of Hauser and Eichberg (1973) as modified by Sharma et al. (1980).

One gram of the tissue sample was homogenized with 15 volumes of chloroform:methanol (1:1 vol:vol) containing 60 μmol of $CaCl_2$ using a pestle and mortar. This was filtered, and the residue was washed twice with about 3 volumes of chloroform:methanol (2:1 vol:vol). The damp tissue residue from the neutral solvent extraction was extracted with 24 volumes of chloroform:methanol (2:1 vol:vol) containing 0.5% concentrated HCl by shaking it in a rotary shaker at 150 rpm for 1 h. The extract after filtration was shaken with 0.2 volumes of 1 *M* HCl. The lower phase was successively washed with two-thirds its volume of chloroform:methanol:1*M* HCl (3:48:47 vol:vol:vol) and chloroform:methanol:0.01 *M* HCl (3:48:47 vol:vol:vol). The washed lower phase was made to one phase with methanol, and sufficient 15 *M* NH_4OH (approximately 1 drop) was added to adjust the pH to 7–8. PhIpp and PhIp spots were separated by thin-layer chromatography according to the method described by Gonzalez-Sastre and Folch (1968). Clean glass plates (20 × 20 cm) were coated to a thickness of 0.30 mm with silica gel 'H'. A slurry of 30 g of silica gel 'H' in 60 ml of 0.3–2% potassium oxalate solution was used for five plates. The concentration of potassium oxalate used var-

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Abbreviations used: PhIp, Phosphatidylinositol 4'-phosphate; PhIpp, Phosphatidylinositol 4',5'-phosphate.

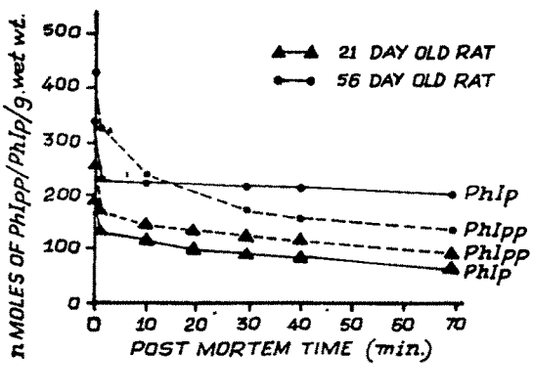

FIG. 1. Phosphoinositide levels in rat brain at various times postmortem.

ied depending on the amount of calcium present in the gel. Samples containing 3–10 μg of polyphosphoinositide phosphorus were evaporated in a warm water bath maintained at 60°C. They were dissolved in minimum quantities of chloroform:methanol:H_2O (7.5:2.5:0.2 vol:vol:vol) solution and then applied to the activated plates quantitatively. The plates were developed at 30°C to a height of 18 cm in a tank saturated for 45–60 min with the solvent system containing *n*-propanol and 4 *M* NH_4OH in the ratio of 2:1 (vol:vol). Polyphosphoinositide phosphorus was estimated by the method of Bartlett (1959) after PhIpp and PhIp spots were scraped from the thin-layer chromatograms.

For studying the postmortem losses at different ages, 0-, 7-, 14-, 21-, 34-, and 63-day-old rats were used. The heads of one set of rats were frozen immediately in liquid nitrogen after decapitation, and in the second set the heads were frozen 1 min after decapitation. The brains removed from the frozen heads were used for extraction and estimation of polyphosphoinositides.

RESULTS AND DISCUSSION

The data on the postmortem losses in PhIpp and PhIp in rat brain at different times after decapitation are given in Fig. 1. Significant losses were observed in PhIp and PhIpp, which were maximum in the first minute after decapitation. The pattern of decline was found to be similar in 21- and 56-day-old rats, suggesting that it may not be age dependent. The high postmortem losses during the first minute may indicate the level of metabolically active phosphoinositides that are present in a form that can be easily attacked by polyphosphoinositide phosphohydrolases (Nijjar and Hawthorne, 1977).

The data on the concentration of rat brain polyphosphoinositides at 0 and 1 min after decapitation in 0-, 7-, 14-, 21-, 34-, and 63-day-old rats are given in Table 1. Certain assumptions have been made to discuss the data obtained. The values for 1-min postmortem are assumed to give an idea of the level of metabolically inert or structural polyphosphoinositides. The loss in the 1-min postmortem value for PhIp may also include some amount of PhIp formed by the degradation of PhIpp.

The concentrations of total PhIpp and PhIp increased from birth to 63 and 34 days, respectively. In this connection, our values obtained for PhIp were higher than those reported by Eichberg and Hauser (1967) and Soukup et al. (1978). This may be due to the inclusion of $CaCl_2$ in the neutral solvent extraction step, as this has been shown to improve PhIp recovery (Eichberg and Hauser, 1973). In the case of PhIpp, there was a peak increase between 21 and 34 days, whereas in the case of PhIp it was between 14 and 21 days. In this connection, Salway

TABLE 1. *Polyphosphoinositides in developing rat brain*

Age (days)	0	7	14	21	34	63
	$n_0 = 2$ $n_1 = 2$	$n_0 = 4$ $n_1 = 4$	$n_0 = 8$ $n_1 = 5$	$n_0 = 9$ $n_1 = 9$	$n_0 = 7$ $n_1 = 7$	$n_0 = 4$ $n_1 = 4$
PhIpp						
0 min (A)[a]	108 ± 7	164 ± 24	213 ± 6	269 ± 17	433 ± 6	476 ± 9
1 min (B)	95 ± 4	102 ± 13	156 ± 15	199 ± 8	262 ± 11	368 ± 30
A − B	13	62	57	70	171	108
Significance between (A) and (B):p <	NS	0.01	0.01	0.01	0.001	0.05
PhIp						
0 min (C)	84 ± 18	105 ± 6	132 ± 12	206 ± 13	234 ± 7	222 ± 21
1 min (D)	83 ± 5	101 ± 19	105 ± 14	171 ± 12	203 ± 5	166 ± 10
C − D	1	4	27	35	31	56
Significance between (C) and (D):p <	NS	NS	NS	0.1	0.01	0.1

Brains of four rats were pooled for each sample in the case of 0- and 7-day-old animals. At all other ages, only one brain was used for each sample. Values are nmol/g wet weight; means ± SE.

n_0, number of samples used for determination of polyphosphoinositide level at 0 min; n_1, number of samples used for determination of polyphosphoinositide level at 1 min.

[a] Two-second postmortem value is taken as 0-min value.

et al. (1968) have reported that the kinases responsible for PhIp and PhIpp synthesis are increased in activity at 5 and 14–24 days, respectively. Myelination also takes place at 10–25 days of age. These data suggest that perhaps PhIp matures during the preweaning period, whereas PhIpp matures during the postweaning period.

The concentration of PhIpp after 1 min postmortem increased from birth to 63 days of age, and that of PhIp up to 34 days of age, thereby indicating that structural polyphosphoinositides are being formed both during preweaning and postweaning periods. The metabolically active polyphosphoinositides also increased both during pre- and postweaning periods, suggesting their importance in neuronal, glial, and myelin metabolism. Studies carried out on the subfractions of myelin have proved that there is a metabolically active pool of polyphosphoinositides even in myelin, and that the necessary synthetic and hydrolyzing enzymes are present in the "heavy" myelin fraction (Deshmukh et al., 1978; Hwan et al., 1981).

Although metabolically inert and active polyphosphoinositides continue to increase during preweaning and postweaning periods, the concentrations are found to be higher from 21 days onward, suggesting a role in glial and myelin metabolism apart from their role in neurons. It is necessary to carry out further studies either on isolated neuronal, glial, and myelin fractions from the brain or on neuronal and glial cells cultivated *in vitro* to obtain precise knowledge regarding the role of these pools in different types of nerve cells.

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Effects of Preweaning Undernutrition and Continued Postweaning Protein Deficiency or Nutritional Rehabilitation on Polyphosphoinositides in Rat Brain

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Abstract: Metabolically inert polyphosphoinositides seem to play an important role in the structural development of neurons, glia, and myelin. The metabolically active pool of PhIpp appears to be important for the functional development of glia and myelin during the postweaning period, whereas PhIp seems to be more important for the functional development of neurons during the preweaning period. Neonatal undernutrition reduces the concentrations of structural polyphosphoinositides and metabolic PhIp while metabolic PhIpp remains unaltered. These effects can be reversed by postweaning nutritional rehabilitation. A continued postweaning protein deficiency of neonatally undernourished rats affects structural PhIpp more than PhIp. Metabolically active PhIpp is drastically reduced. **Key Words:** Glia—Myelin—Neonatal undernutrition—Polyphosphoinositides—Postweaning—Preweaning—Protein. **Uma S. and Ramakrishnan C. V.** Effects of preweaning undernutrition and continued postweaning protein deficiency or nutritional rehabilitation on polyphosphoinositides in rat brain. *J. Neurochem.* **40**, 1026–1029 (1983).

Studies carried out by different investigators have suggested the presence of two pools of polyphosphoinositides in rat brain, namely, structural or metabolically inert and metabolically active polyphosphoinositides (Eichberg et al., 1971; Hauser et al., 1971; Hauser and Eichberg, 1973; Uma and Ramakrishnan, 1983). Inert polyphosphoinositides continue to be deposited, even during the postweaning period, suggesting their importance in the structural development of glia and myelin. Metabolically active phosphatidyl inositol 4′5′-phosphate (PhIpp) shows a peak rise during the postweaning period, suggesting its major role in glial and myelin metabolism. Metabolic phosphatidyl inositol 4′-phosphate (PhIp) shows a peak rise between 14 and 21 days of age, indicating its major role in neurons during synaptogenesis (Uma and Ramakrishnan, 1983).

The adverse effects of nutritional stress during the suckling period on the maturation of neurons (Cragg, 1972; Gambetti et al., 1974; Burns et al., 1975; Shoemaker and Bloom, 1976), glia (Siassi and Siassi, 1973; Krigman and Hogan, 1976), and myelin (Bass, et al., 1970*b*; Krigman and Hogan, 1976; Stewart et al., 1974; Griffin et al., 1977) are well documented. The concentrations of different lipids have been shown to be significantly reduced in neonatally undernourished rats (Culley and Mertz, 1965; Rajalakshmi and Nakhasi, 1974; Krigman and Hogan, 1976; Reddy and Sastry, 1978; Reddy et al., 1982).

Since metabolically inert and active pools of PhIpp and PhIp are found to be important for neuronal cells, which mature during the preweaning period, and glia and myelin, which continue to mature during the postweaning period, the question arises whether nutritional stress during preweaning and postweaning peroids will affect the concentrations of metabolically inert and active pools of polyphosphoinositides. Studies were therefore carried out to find the effects of preweaning undernutrition and continued postweaning protein deficiency or nutritional rehabilitation on the concentrations of polyphosphoinositide pools in rat brain. The results of these studies are reported in this paper.

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Abbreviations used: PhIp, Phosphatidyl inositol 4′-phosphate; PhIpp, Phosphatidyl inositol 4′5′-phosphate.

MATERIALS AND METHODS

Albino rats of Charles-Foster strain taken from the stock colony of this department were used for these studies. Females weighing 200–250 g were fed a stock diet (18% protein) during gestation. After delivery the litter size was adjusted to eight, and the dams were divided into two groups, control (L^+) and experimental (L^-), and fed 20% and 5% protein diets, respectively. The composition of the diets is given in Table 1. Some of the pups from L^+ and L^- groups were decapitated at 3 weeks of age, and their brains were used for the estimation of polyphosphoinositides. The remaining pups in the L^+ group were fed a 20% protein diet (L^+P^+) for a period of 6 weeks, and those from the L^- group were further divided into two groups. One group was fed a 5% protein diet (L^-P^-) and the other a 20% protein diet (L^-P^+) *ad lib.* for 6 weeks. All animals were individually caged, and water was given *ad lib.* After 6 weeks of treatment they were decapitated, and the brains were used for the experiment. In all these experiments one set of heads was frozen immediately in liquid nitrogen after decapitation and the second set was frozen 1 min after decapitation. The brains removed from the frozen heads were used for extraction and estimation of polyphosphoinositides by the method of Hauser and Eichberg (1973) modified by Sharma et al. (1980). The details of the method have been described by Uma and Ramakrishnan (1983).

RESULTS AND DISCUSSION

The results given in Table 2 show that concentrations of total (0-min postmortem values) and structural polyphosphoinositides (1-min postmortem values) decrease in the brain of 21-day-old neonatally undernourished rats. The metabolically active pool of PhIpp does not seem to be affected, whereas the PhIp pool appears to decrease by 23%. There is no direct way to find out whether this decrease is statistically significant. The values for 0 and 1 min postmortem in each group, as well as values for L^+ and L^- groups at each period are statistically significant, and those for the metabolically active pool in L^+ are always greater than for L^-. It is therefore assumed that the metabolically active PhIp pool in the undernourished group is decreased significantly. The data suggest the possibility that the structure of neurons, glia, and myelin and perhaps the function of neurons may be affected during synaptogenesis. In this connection it is known that undernutrition affects maturation of neurons (Cragg, 1972; Gambetti et al., 1974; Burns et al., 1975; Shoemaker and Bloom, 1976), glia (Siassi and Siassi, 1973; Krigman and Hogan, 1976), and myelination (Bass et al., 1970*b*; Krigman and Hogan, 1976; Stewart et al., 1974; Griffin et al., 1977).

TABLE 1. *Composition of low and high protein diets*[a]

Ingredients	Diet	
	20% protein (g/100 g diet)	5% protein
Washed casein	24.0	6.0
Vitamin mixture	2.0	2.0
Salt mixture	4.0	4.0
Peanut oil	7.0	7.0
Sucrose	20.0	20.0
Sago (metroxylon sago)	43.0	61.0

Vitamin mixture composition (mg/kg diet): Thiamine hydrochloride, 4.0; riboflavin, 4.0; pyridoxine hydrochloride, 1.0; niacin, 15.0; calcium *d*-pantothenate, 10.0; inositol, 200.0; folic acid, 1.0; D-biotin, 0.001; choline chloride, 750; paraaminobenzoic acid, 10.0; sucrose, 19.0 g/kg; vitamin B_{12}, 5 mcg/kg; vitamin A acetate, 250 IU/g; vitamin D_3 (cholecalciferol), 9.37 IU/g.

Salt mixture composition (g/100 g salt mixture): $CaCO_3$, 6.85; $CaHPO_4 2H_2O$, 11.28; dicalcium tricitrate $4H_2O$, 30.82; K_2HPO_4, 21.87; KCl, 12.47; NaCl, 7.70; $MgSO_4$, 3.83; $3MgCO_3\ Mg(OH)_2\ 3H_2O$, 3.51; $Fe(NH_4)$ citrate, 1.53; $CuSO_4\ 5H_2O$, 0.10; NaF, 0.013; $MnSO_4$, 0.018; $KAl(SO_4)_2\ 12\ H_2O$, 0.009; KI, 0.004.

[a] Rajalakshmi and Nakhasi (1974).

When neonatally undernourished rats were nutritionally rehabilitated for 6 weeks by feeding 20% protein diet during the postweaning period, the concentrations of total and inert polyphosphoinositides returned to normal. In fact the total and metabolically active PhIp levels were found to be higher than controls. The data on 1-min and 0–1-minute values for PhIp should be discussed with reservations, since the 1-min value may also represent some PhIp formed by the degradation of PhIpp. These data indicate that nutritional rehabilitation may reverse any of the structural or functional changes produced in neurons, glia, and myelin due to changes in polyphosphoinositide concentration during preweaning undernutrition. In this connection nutritional rehabilitation subsequent to deprivation in early life is not found to correct fully the deficits in other brain lipids such as cholesterol, galactolipids, and phospholipids (Culley and Linenberger, 1968; Dobbing, 1968; Dickerson and Jarvis, 1970; Geison and Waisman, 1970; Smart et al., 1973; Reddy and Sastry, 1978). Consistent with this is the observation that the deficit persists in the amount of myelin (Simons and Johnston, 1976; Reddy et al., 1979) and myelin lipids (Yusuf et al., 1981). Recent studies by Reddy et al. (1982) have shown that with nutritional rehabilitation of neonatally undernourished rats, the deficits in lipid concentrations in the grey matter are reversed, but not those in the white matter.

In the case of neonatally undernourished rats continued on low protein diet for 6 weeks during postweaning period, there was a decrease in total (0-min value) and inert polyphosphoinositides (1-min value). The decrease was more in the case of PhIpp compared to PhIp. In this connection it is reported that PhIpp increases much more than PhIp during postweaning period (Uma and Ramakrishnan, 1983). The effects of continued postweaning protein deficiency seem to be more severe on total PhIpp concentration compared to neonatal undernutrition (39% of control value in the L^-P^-

TABLE 2. *Effects of preweaning undernutrition, continued postweaning protein deficiency, and nutritional rehabilitation on rat brain polyphosphoinositides*

	L^+ $n_0 = 9$ $n_1 = 9$	L^- $n_0 = 7$ $n_1 = 5$	L^+P^+ $n_0 = 4$ $n_1 = 4$	L^-P^- $n_0 = 6$ $n_1 = 5$	L^-P^+ $n_0 = 4$ $n_1 = 4$
PhIpp					
0 min (A)	269 ± 17	158 ± 15 (59)[a]	470 ± 9	147 ± 10 (31)[b]	425 ± 35 (89)[d]
1 min (B)	199 ± 8	82 ± 5 (41)[a]	368 ± 30	138 ± 13 (38)[b]	350 ± 17 (95)[d]
A − B	70	76	108	9	75
Significance between A and B (P<)	.01	.001	.05	NS	0.1
PhIp					
0 min (C)	206 ± 13	135 ± 10 (66)[a]	222 ± 21	143 ± 11 (64)[c]	239 ± 32 (108)[d]
1 min (D)	171 ± 12	108 ± 9 (63)[a]	166 ± 11	109 ± 11 (66)[c]	143 ± 17 (86)[d]
C − D	35	27	56	34	96
Significance between C and D (P<)	.1	.05	0.1	0.1	.001

Values are given in nmol/g wet weight and are expressed as means ± SE. Numbers in parentheses are percentage differences between control and active groups.

n_0, Number of samples used for determination of polyphosphoinositide levels at 0 min; n_1, number of samples used for determination of polyphosphoinositide levels at 1 min.

[a] Significant difference between L^+ and L^-, $p < 0.001$.

[bc] Significant differences between L^+P^+ and L^-P^-: *b*, $p < 0.001$; *c*, $p < 0.01$.

[d] No significant difference between L^-P^+ and L^+P^+.

group and 59% in the L^- group). The deficits in PhIp during neonatal undernutrition do not seem to increase by continued postweaning protein deficiency. As far as metabolically active PhIpp is concerned, although preweaning undernutrition does not affect the concentration, continued postweaning protein deficiency seems to affect it drastically. In the control animals there is a 150% increase in this pool between 3 and 9 weeks, whereas continued protein deficiency reduces it to 10%. This suggests that the metabolic pool of PhIpp may be important for glial and myelin maturation in the postweaning period. The deficits in the metabolic pool of PhIp during neonatal undernutrition is not increased significantly by continued postweaning protein deficiency, thereby suggesting that this pool may be more important for neuronal maturation during the preweaning period.

Thus it appears from these studies that the inert pool of polyphosphoinositides which is presumed to play an important role in the structure of neurons, glia, and myelin is affected both by preweaning undernutrition and postweaning protein deficiency. The metabolically active pool of PhIpp seems to be severely affected by postweaning protein deficiency. Postweaning nutritional rehabilitation reverses the effects of [illegible] two pools of polyph[illegible]s underline the importance of nutritional status in the development of structural and functional polyphosphoinositides involved in the development and maturation of neurons, glia, and myelin.

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