SUMMARY

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Phosphoinositides represent 5-10% of total phospholipids in most mammalian tissues, the highest concentrations occurring in the nervous tissue. Although present in trace quantities, they are functionally important components by virtue of their unique properties viz., binding to cations and proteins, high lability post-mortem, high content of arachidonic acid and high turnover rates. Considerable evidence exists to indicate that these lipids have a central role to play in receptor function in a wide variety of tissues. Receptor mediated hydrolysis of PtdIns(4,5)P_2 results in the formation of two products i.e., Ins(1,4,5)P_3 and diacylglycerol which have been shown to function as "secondary messengers" to activate two independent but parallel signal pathways. The two signal pathways appear to function in a synergistic manner to stimulate a wide variety of cellular processes. Studies have also shown that this PolyPI signalling system may be connected to cellular proliferation and differentiation. Certain oncogene products have been shown to act as inositol lipid kinases phosphorylating PtdIns and Ptdins4P. Recently, two groups of researchers have indicated a role for PtdIns(4,5)P_2 and its hydrolysis product Ins(1,4,5)P_3 in visual transduction. Apart from these functions, PolyPI are also implicated in myelin structure, axonal conduction, synaptic

transmission, attachment and activation of several enzyme systems, several kidney functions and prostaglandin biosynthesis.

Several authors have hypothesized that PolyPI may exist in the form of two pools in brain. The pool lost rapidly post-mortem and associated with gray matter structures possibly represents the metabolically active one and the other lost gradually post-mortem located in white matter structures represents the relatively stable pool. Recent studies on myelin and its subfractions indicate that an active pool of PolyPI also exists in this structure with the biosynthetic and hydrolytic enzymes being present in the heavy myelin fraction. The rapidly disappearing pool of PolyPI may thus have a functional role in both non-myelin and myelin membranes.

Quantitative extraction of PolyPI from tissues presented a number of difficulties and the methods used for tissue fixation to preserve these compounds varied widely. This lead to a wide range of values in literature. Few reports on the developmental changes in brain PolyPI indicated that maximum deposition of PtdIns4P occurs in the pre-weaning period while that of PtdIns(4,5)P_2 occurs both during pre- and post-weaning periods. However, no systematic study using improved methods of extraction has focussed on determining the levels of PolyPI pools during the development of rat brain.

Several studies on undernourished rat brain have shown that nutritional stress during the suckling period may have adverse effects on the maturation of neurons, glia and myelin. Also, the concentration of different lipids are significantly reduced in whole brain, gray matter, white matter, myelin and other subcellular membranes. Though altered metabolism of PolyPI has been documented in several diseased states, no investigations have been apparently carried out on these metabolically active and functionally important lipids in relation to the nutritional status during different stages of development.

The aim has therefore been to study the developmental pattern and assess the effects of varying degrees of undernutrition during different stages of development on phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol 4-phosphate (PtdIns4P) pools in rat whole brain and in brain regions.

Preliminary studies were first carried out on the post-mortem changes with time in young and adult rat brain PolyPI levels. Further, studies were extended to estimate PolyPI levels at different stages of the developing rat brain at two time points ("0 min" and 1 min representing 2 and 60 sec post-mortem values respectively). Studies were also carried to find out the effects of pre- and/or post-weaning undernutrition and post-weaning nutritional rehabilitation on PolyPI levels at "0 min"

and 1 min after decapitation of the animal. Isotope experiments using ^{32}Pi have also been carried out under selected conditions. Since the candidate got an opportunity to work in Prof. Hauser's laboratory at Harvard it was possible to extend these studies on the effects of pre- and post-weaning nutritional rehabilitation on PolyPI levels in brain regions enriched either in neuronal structures (cerebral cortex and cerebellum) or in glial cells and myelin (brain stem). To compare the effects on neural tissues with non-neural ones, kidney PolyPI have also been investigated.

The basic animal paradigm was restricted to nutritional manipulations during two stages of development, the vulnerable pre-weaning period and six weeks after weaning. Dams were fed a diet containing either 4-5% or 20-22% protein during lactation (pre-weaning period). The litters were used at 3 weeks of age and designated L^- (undernourished) and L^+ (control), respectively. Other litters received either a low or normal protein diet for an additional 6 weeks resulting in groups L^+P^+ (control), L^+P^- (post-weaning protein deficient), L^-P^+ (pre-weaning undernourished and post-weaning rehabilitated) and L^-P^- (pre- and post-weaning undernutrition).

For studies on the time course of post-mortem losses in whole brain, rats of two ages (21 and 56 days old) were used. The heads of rats were immersed in liquid nitrogen at different

times after decapitation viz. 2 sec. 1, 10, 20, 30, 40 and 70 min and the brains from the frozen heads were used for extraction and analyses of PolyPI. Results showed a ≅ maximum depletion of PtdIns(4,5)P_2 and PtdIns4P in the first minute (≅ 27%) after decapitation followed by a steady decline to 70 minutes. The rate of decrease (nmoles of PolyPI lost/min) was found to be highest between 0-1 min which gradually dropped with increasing time post-mortem. The pattern of decline was similar in 21- and 56- day old rats, suggesting that it may not be age dependent. There may be two pools of PolyPI in brain, one that is easily accessible and rapidly attacked by the PolyPI phosphohydrolases and the other which undergoes slow degradation. Results also suggest the possible existence of more than two pools each one being relatively less active or more stable than the other and presumably located in different structures of the brain. It is postulated that the 1 min post-mortem value represents the sum of the relatively metabolically stable (pool A_1) and active (pool C) pool of PolyPI and that lost during 0-1 min represents a highly active (pool B) pool of PolyPI in brain. Similarly the 10 min post-mortem value is postulated to represent a sum of the metabolically stable (pool A_2- more stable than A_1) and active (pool D-less active than B and C) pool and that lost during 1-10 min to represent the relatively active (pool C-less active than pool B) pool of PolyPI in brain.

For further experiments on whole brain, two time points were chosen for the determination of PolyPI levels, "0 min" (2 sec post-mortem value representing total levels) and "1 min" (representing levels of relatively stable pool A_1 + C after decapitation. The difference between the two (0-1 min) has been assumed to represent the highly metabolically active pool (B) in brain. PtdIns4P being an intermediate in both biosynthetic and degradative pathways of PtdIns(4,5)P_2, the values obtained for the former are perhaps less revealing.

PolyPI pools are presumably located in different cell structures of the brain which mature during different stages of development. The levels of PolyPI pools during development were therefore investigated. For these studies, brains from 0, 7, 14, 21, 34 and 63-day-old rats were used. The concentrations of total PtdIns(4,5)P_2 and PtdIns4P were found to increase from birth to 63 and 34 days respectively. PtdIns(4,5)P_2 increased rapidly from birth to 21 and 34 days (after weaning), while a rapid increase in PtdIns4P was observed between 14 and 21 days (before weaning). The metabolically highly active pool (pool B) of PtdIns(4,5)P_2 is deposited rapidly after weaning during the period of glial cell maturation and continued myelination. It, therefore, seems to be more important in glial and myelin metabolism. Rapid deposition of this pool of PtdIns4P occurs before weaning during the period of active synaptogenesis and therefore may be important

in neuronal metabolism. On the other hand, the relatively inert pools (pools A_1 + C) of both lipids are deposited during pre- and post-weaning periods indicating their importance in glial and myelin metabolism apart from their role in neurons.

Observations on the developmental pattern of PolyPI pools lead to several questions : (a) what would be the effect of nutritional deprivations on the metabolically highly active pool of PtdIns(4,5)P_2 deposited rapidly after weaning (i) post-weaning protein deficiency alone (ii) pre-weaning undernutrition superimposed by post-weaning protein deficiency? (b) Would there be any damage to the metabolically highly active pool of PtdIns4P deposited before weaning by pre-weaning undernutrition? (c) What would be the influence of nutritional alterations during pre- and post-weaning periods on the metabolically relatively inert pools of PolyPI.

In an attempt to provide answers to the above questions studies were carried out to see the effects of pre- and post-weaning undernutrition on the concentration of PolyPI pools in rat brain. Additional studies were made on the continuation of pre-weaning undernutrition during the post-weaning period and of the reversibility of the effects observed at weaning with dietary rehabilitation after weaning.

Pre-weaning undernutrition reduced the concentration of total (pools A + B) and metabolically relatively stable pools

(pools A_1 + C) of PtdIns(4,5)P_2 and PtdIns4P significantly while the more active pools of the two lipids were not affected. The deficits observed at weaning were reversed by nutritional rehabilitation during the post-weaning period. Protein deficiency during the post-weaning period alone as also continued feeding on a low protein diet after weaning decreased the metabolically highly active pool of PtdIns(4,5)P_2 suggesting a role of this component in the functional development of glial and myelin membranes which continues actively after weaning. Deficits, whenever observed, were greater in PtdIns(4,5)P_2 than in PtdIns4P indicating that the higher phosphorylated derivative is more sensitive to stress.

Reduction in the levels of PolyPI pools by undernutrition induced during the pre- and/or post-weaning periods could result from altered metabolism of these pools presumably located in different structures of the brain. In order to establish this, the incorporation of intraperitoneally injected $^{32}P_i$ into these compounds was studied. Pre-weaning undernutrition appeared to increase the metabolic activity of the pools of PtdIns(4,5)P_2 while that of PtdIns4P was found to be reduced. Post-weaning protein deficiency did not have any effect on either the levels or the specific radioactivity of the metabolically relatively inert pool (pools A_1 + C) of both lipids suggesting that the metabolic activity of this pool remains unaffected in contrast

to the effects produced by pre-weaning undernutrition. On the other hand, large reductions in the levels of the metabolically highly active pool (pool B) of PtdIns(4,5)P_2 were accompanied by no changes in the specific radioactivities suggesting decreased synthesis or increased catabolism in the post-weaning protein deficient brains. The physiological significance of these results remain unknown. Since several enzymatic reactions of phosphoinositide metabolism are regulated by Ca^{2+} ions, the availability of the ion may be a critical factor responsible for the metabolic activity of different pools of PolyPI localized in different cellular membranes.

To provide further insight into the fate of metabolic pools of PolyPI the nutritional studies on whole brain were extended to discrete brain regions, namely, cerebral cortex and cerebellum (enriched in neuronal cell bodies) and brain stem (enriched in myelin). In these studies the time required for dissection and freezing of brain regions was 0.75 to 1 min, since separation of brain regions, especially gray matter was not possible from heads frozen in liquid nitrogen within 2 sec after decapitation. The metabolically highly active pool (pool B) lost during the first minute could not be therefore determined in different brain regions. Data from tissues dissected at 0.75-1 min after decapitation and 10 minutes later were compared. The 10 min post-mortem value was assumed to represent the metabolically

relatively inert pool (pool A_1) presumably located in myelin and the 1-10 min post-mortem value to represent the active pool (pool C) presumably located in non-myelin membranes.

Levels of PolyPI in the "0 min" samples from all six groups (L^+, L^-, L^+P^+, L^+P^-, L^-P^+, and L^-P^-) followed the order brain stem > cerebellum > cerebral cortex, confirming previous observations that PolyPI are enriched in white matter structures, mainly in the myelin sheath.

The lability post-mortem of PolyPI paralleled the content of gray matter : cerebral cortex > cerebellum > brain stem in all groups regardless of diet. This suggests that the pool of PolyPI lost rapidly is located in gray matter structures rich in neuronal cells, and the relatively less active pool is located in white matter strictures rich in glial cells and myelin.

Pre-weaning undernutrition reduced the levels of metabolically active (pool C) and inert (pool A_1) pools ("0 min" values representing 1 min post-mortem value) of PtdIns(4,5)P_2 and PtdIns4P in all the three brain regions studied. The effects at weanling on both pools were reversed only partially on nutritional rehabilitation. However, which pool is less reversible (A_1 or C) is not clear, Post-weaning protein deficiency did not affect this pool of both lipids in any of the

three brain regions. Comparison of the data obtained on whole brain and brain regions indicated that 1 min post-mortem levels of PolyPI are not totally representative of the metabolically inert pool in myelin but a portion of it is also located in non-myelinated structures.

The metabolically inert pool (10 min post-mortem value-pool A_1) of PtdIns(4,5)P_2 was moderately affected in brain stem and cerebellum and that of PtdIns4P in cerebral cortex and cerebellum by pre-weaning undernutrition. However, these effects were restored to normal upon subsequent rehabilitation during the post-weaning period. Protein deficiency after weaning did not affect this pool in any of the regions examined. A comparison of the results with those on morphology of glial cells and myelin suggests that the 10 min post-mortem values may represent the metabolically inert pool of PolyPI bearing a closer relationship to myelin and glial cells.

The metabolically active pool of PolyPI lost between 1 and 10 min post-mortem (pool C that is less active than the pool lost during the first minute post-mortem) was virtually eliminated in the cerebral cortex and cerebellum by pre-weaning undernutrition and these effects were not reversed on nutritional rehabilitation. Post-weaning protein deficiency decreased this pool of PtdIns4P in the cerebral cortex alone. These results are in contrast to

the studies on whole brain data where the metabolically highly active pool (pool B) lost between 0 and 1 min post-mortem was not affected in L^- whole brain but was decreased in L^+P^- (mainly PtdIns(4,5)P_2) brains. This differential effect of under-nutrition on the PolyPI pool lost between 0 and 1 min post-mortem (whole brain studies) and that lost between 1 and 10 min post-mortem (regional studies) suggest the existence of more than one metabolically active pool. It is possible that one pool might readily become exposed to the PolyPI. phosphohydrolases so as to be promptly hydrolysed while the other would initially be protected against hydrolytic cleavage.

Like the brain, rat kidney is also endowed with the ability to synthesize myo-inositol and its phospholipid derivatives i.e, PolyPI. These lipids are known to play an important role in several kidney functions and their metabolism has been reported to be altered in several diseased states. In view of this, levels of PolyPI pools in the kidney were also analyzed for possible differential effects of nutritional modifications in neural versus non-neural tissues. For this study levels of PolyPI pools from kidneys dissected at 0.75-1 minute after decapitation and 10 minutes later were compared.

Protein deprivation lowered PtdIns4P (1 and 10 min post-mortem) preferentially in kidney at weaning, and this effect was not reversed by rehabilitation indicating the importance of

PtdIns4P in kidney functions. Significant losses post-mortem (35-40%) could be detected only in PtdIns(4,5)P_2 in all the five (L^+, L^-, L^+P^+, L^+P^- and L^-P^+) groups of animals. Comparison of the results on kidney with brain show similar amount of deficits in the 1 min post-mortem levels of PtdIns4P at weaning. In contrast to the kidney deficits in PtdIns4P are partially reversed in brain on subsequent rehabilitation during the post-weaning period. A preferential decrease of PtdIns4P as compared with PtdIns(4,5)P_2 suggests that the lipid is not merely an intermediate in the synthesis and catabolism of PtdIns(4,5)P_2. Separate enzymes mediating the phosphodiesteratic cleavage of PtdIns4P and PtdIns(4,5)P_2 in kidney cortex could be responsible for the preferential decrease of PtdIns4P. What relation does the influence of nutritional status on PolyPI metabolism has to renal functions is not known.

In sum, nutritional influences seem to have a profound effect on the PolyPI pools in whole brain, brain regions and kidney. The differential effects of undernutrition on the rapidly lost active pool of PolyPI in whole brain (0-1 min post-mortem) and in brain regions (1-10 min post-mortem)suggests the possible existence of more than two pools, each one probably having a different orientation of substrate to the phospho-hydrolases in different membranes. It remains to be determined whether the rapid loss of PolyPI post-mortem is due to a

phosphodiesteratic or phosphomonoesteratic cleavage, or a combination of both, of the substrate molecule. Future investigations on inositol phosphates and g diglyceride levels post-mortem, phospholipase activities, subcellular distribution of the PolyPI pools and, especially, on elevated turnover in response to stimulation will help to clarify the function of these compounds. Undernutrition promises to be a useful tool in this field.