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CALCIUM-DEPENDENT TURNOVER OF BRAIN POLYPHOSPHOINOSITIDES IN VITRO AFTER PRELABELLING IN VIVO

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Rat brain phospholipids were labelled in vivo by an intraventricular injection of ^{32}P. The radioactivity was found to accumulate predominantly in limbic structures, particularly hippocampus and diencephalon. A rapid and high specific labelling of the inositol phospholipids and phosphatidic acid was observed. The rate of incorporation into a crude myelin fraction was similar to that into a mitochondrial/synaptosomal fraction although phosphatidyl-*myo*-inositol 4,5-diphosphate was especially enriched in myelin. Upon incubation in vitro of the brain fractions after 2 h prelabelling in vivo, both phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate rapidly lost their radioactivity. Half of the labile fraction of the incorporated ^{32}P was removed within 2 min. None of the other phospholipids changed in the 30 min in vitro incubation period. The metabolism of the polyphosphoinositide proceeded at a lower rate when the temperature was lowered, and was Ca^{2+}-dependent. Further subcellular fractionation revealed that purified synaptosomes and myelin contained highly labelled phosphatidyl-*myo*-inositol 4,5-diphosphate. Mitochondria contained highly labelled phosphatidyl-*myo*-inositol but no phosphatidyl-*myo*-inositol 4-phosphate or phosphatidyl-*myo*-inositol 4,5-diphosphate. $ACTH_{1-24}$ did not inhibit the in vitro dephosphorylation of prelabelled polyphosphoinositide, confirming previous findings that the peptide affects the polyphosphoinositide kinases and not the respective phosphatases.

Introduction

The inositol phospholipids have been implicated in the regulation of membrane permeability and synaptic transmission in neurons [1,2]. An enhanced metabolism of phosphatidylinositol was observed in various tissues after receptor activation by hormones and neurotransmitters that utilize Ca^{2+} as their intracellular second messenger [1]. Its phosphorylated derivatives (phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-diphosphate) are quantitatively minor membrane components in most mammalian tissues, but relatively high concentrations of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate are found in nervous tissue [2]. The brain polyphosphoinositides are principally components of the myelin sheath or a contiguous structure, but a metabolically very active pool of polyphosphoinositide is present at non-myelin sites [3,4], presumably at synaptosomal or glial plasma membranes. The very rapid turnover of their monoesterified phosphate group [5] suggests that they have an important physiological role. They are implicated in the metabolism of Ca^{2+} at the cell membrane and their breakdown has been observed after the influx of this ion into the cell [6–8].

It has been found recently that the metabolism of phosphatidyl-*myo*-inositol 4,5-diphosphate, phosphatidyl-*myo*-inositol 4-phosphate and phosphatidic acid

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; ACTH, adrenocorticotropin.

is influenced by neuropeptides derived from ACTH [9] and by β-endorphin [10]. These peptide effects were obtained in a lysed membrane fraction from rat brain that was incubated for a short period (10 s) with [γ-^{32}P]ATP. Similar findings were done in purified phosphatidyl-*myo*-inositol 4-phosphate kinase preparations [10,11]. Furthermore, it appeared that ACTH acted on the lipid kinases and not on the respective phosphatases [9]. These findings prompted us to study the influence of ACTH and Ca^{2+} on the metabolism of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate in situations in which only radioactive dephosphorylation can be monitored.

We report here experiments in which the metabolism of these lipids was studied in vitro after prelabelling of the rat brain in vivo.

Materials and Methods

Animals and brain dissection

Male rat (150 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). They were supplied with a permanent brain cannula ending in the interventricular foramen [12]. After 1 week of recovery they received an intracerebroventricular injection of 3 μl saline containing 50 μCi $^{32}P_i$ (carrier-free, type NEX-054, New England Nuclear, Boston, MA, U.S.A.). The in vivo labelling period was 2 h unless stated otherwise. The rats were killed after this time interval by whole body immersion into liquid nitrogen for 8 s, followed by rapid dissection of the cold but not frozen brain at 0–4°C. The limbic system (hippocampus, septum, basal ganglia, pyriform cortex, diencephalon, mesencephalon) was dissected as described previously [13].

Subcellular fractionation

The dissected material from one rat (0.3 g) was homogenized in 10 ml 0.32 M sucrose by 10 up-and-down strokes of a Potter-Elvehjem teflon glass homogenizer with a radial clearance of 0.125 mm, rotating at 700 rev./min. The subcellular fractionation was based on the method of Gray and Whittaker [14]. Briefly, the homogenate was spun for 5 min at 700 × *g* (Sorvall SS34). After centrifugation of the supernatant for 10 min at 1000 × *g* the pellet was taken as the crude myelin fraction. The supernatant was spun for 10 min at 10 000 × *g* and the resulting pellet (P2) was taken as the crude mitochondrial/synaptosomal fraction.

Purified synaptosomes and mitochondria were prepared according to the method of Booth and Clark [15]. Briefly, the dissected material from one rat (0.3 g) was homogenized in 10 vol. isolation medium (0.32 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.4). The homogenate was diluted to 10 ml with isolation medium and centrifuged at 1300 × *g* for 3 min. The supernatant was collected and after centrifugation at 17 000 × *g* for 10 min the resulting pellet was resuspended in 1.5 ml isolation medium. This suspension was diluted to 9 ml with 12% Ficoll/sucrose medium (12% (w/w) Ficoll/0.32 M sucrose/50 μM EDTA, pH 7.4) and gently homogenized by hand in a Potter-Elvehjem teflon glass homogenizer with a radial clearance of 0.375 mm. This suspension was introduced into a centrifuge tube and above this 4.5 ml of a 7.5% Ficoll/sucrose medium was layered (7.5% (w/w) Ficoll/0.32 M sucrose/50 μM EDTA, pH 7.4). Finally, on top of this 7.5% medium, 3.5 ml of isolation medium was layered. The tubes were centrifuged at 100 000 × *g* for 30 min (a Beckman SW 27.1 rotor in a Sorvall OTD-2 ultracentrifuge). Myelin and synaptosomes banded at the first and second interphase, respectively, with the free mitochondria being pelleted at the bottom [15]. The fractions were carefully removed by suction with a Pasteur's pipette and diluted with isolation medium to 10 ml. After centrifugation for 10 min at 10 000 × *g* the pellets were taken up in incubation medium in a final volume of 10 ml. In enzyme contamination studies [15] it was found that these synaptosomes are contaminated by 'free' mitochondria on a protein basis by 1–4%; they have a free membrane contamination below 1%.

Lipids from the various subcellular fractions were provisionally identified by two-dimensional thin-layer chromatography [16]. The presence of cerebrosides and sulphatides was taken as a measure of myelin contamination. Protein was determined according to the method of Lowry et al. [17].

Incubation in vitro

The in vitro metabolism of ^{32}P-labelled phospholipids was assayed in incubation medium [18] containing (final concentrations): 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgCl_2$, 0.75 mM

$CaCl_2$, 1.6 mM cytidine, 1.6 mM *myo*-inositol, 10 mM glucose, 20 mM Tris-HCl, pH 7.4, in a final volume of 25 μl at 37°C unless stated otherwise. The fractions obtained by subcellular fractionation were suspended in incubation buffer and kept at 0°C. Preincubation with synthetic $ACTH_{1-24}$ (Organon Oss Int. BV, Oss, The Netherlands) was performed for 5 min. The incubation was started by placing the incubation vessel in a water bath at the indicated temperature and was terminated by the addition of 2 ml ice-cold chloroform/methanol/13 M HCl (200 : 100 : 0.75, by vol) [19].

Lipid extraction and thin-layer chromatography

The procedure used is described in detail elsewhere [9]. Briefly, carrier polyphosphoinositides were added, and a biphasic system was obtained by the addition of 0.375 ml 0.6 M HCl. The upper phase was removed and the lower phase washed with 1 ml chloroform/methanol/0.6 M HCl (3 : 48 : 47, by vol). The resulting lower phase was dried under N_2 and redissolved in chloroform/methanol/water (75 : 25 : 2, by vol). Samples were taken for the determination of radioactivity incorporated into total phospholipids.

Phospholipids were separated by high-performance thin-layer chromatography (HPTLC; layer thickness 25 μm, Merck). Before use, the plates were impregnated with potassium oxalate as described [9] and activated for 15 min at 110°C. The extract was applied to the plate and the lipids were separated with chloroform/acetone/methanol/glacial acetic acid/water (40 : 15 : 13 : 12 : 8, by vol). On each plate 18 samples were run simultaneously. This procedure yielded quantitative separation of labelled lipids. They were visualized with iodine vapour and ^{32}P-labelled spots were detected by autoradiography on Kodak Royal X-Omat film (10–20 h). The spots were scraped from the plate and counted for radioactivity in a liquid scintillation spectrometer [9]. All operations (lipid prelabelling in vivo, subcellular fractionation, incubation, lipid extraction and thin-layer chromatography) were performed on the same day.

Results

The distribution of radioactivity over the brain after intracerebroventricular injection: time curve

To determine the distribution of injected [^{32}P]-orthophosphate at different time periods after the intracerebroventricular injection, five groups of animals were used. They were killed at 0.25, 0.5, 1, 2 and 4 h after the injection, and the total amount of label in the dissected brain parts was determined. The right hippocampus, right neocortex and the diencephalon contained the major part of the recovered radioactivity. The relative contribution of limbic structures (especially hippocampus) increased with increasing labelling time (data not shown). The structures from the right hemisphere contained more label than those from the left hemisphere, as was to be expected in view of the placement of the cannula above the right hemisphere [12].

In vivo incorporation of ^{32}P into individual phospholipids: time curve

In view of the data presented in the previous section and in previous studies on polyphosphoinositide metabolism [9,20], the limbic system was chosen for further study. In view of the reported high metabolic activity of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate [5] care was taken to avoid postmortem breakdown of these lipids. Control experiments showed that no loss of incorporated radioactivity occurred during the dissection of the cold but not frozen brain at 0–4°C. In these experiments, whole brain was taken out from the skull after immersion of the animal in liquid nitrogen. The brain was either immediately extracted with acidified chloroform/methanol as described (Materials and Methods) or after 1, 5 or 10 min at 0–4°C. There were no differences in amounts and ratios of radioactively labelled phospholipids. Furthermore, no breakdown of labelled lipid took place during homogenisation and subcellular fractionation (data not shown; see also Fig. 3).

The in vivo incorporation of ^{32}P into the different lipid classes was assessed in a synaptosomal/mitochondrial fraction (P2 fraction) and in a crude myelin fraction. Various in vivo labelling periods were compared (0.5, 1, 2, 4 h). The inositol lipids and phosphatidic acid were labelled very rapidly (Fig. 1): these lipids contained 85–90% of the incorporated radioactivity after 30 min. The contribution of other phospholipids (particularly phosphatidylglycerol, cardiolipin, phosphatidylethanolamine and phosphatidylcholine) increased with increasing incubation time.

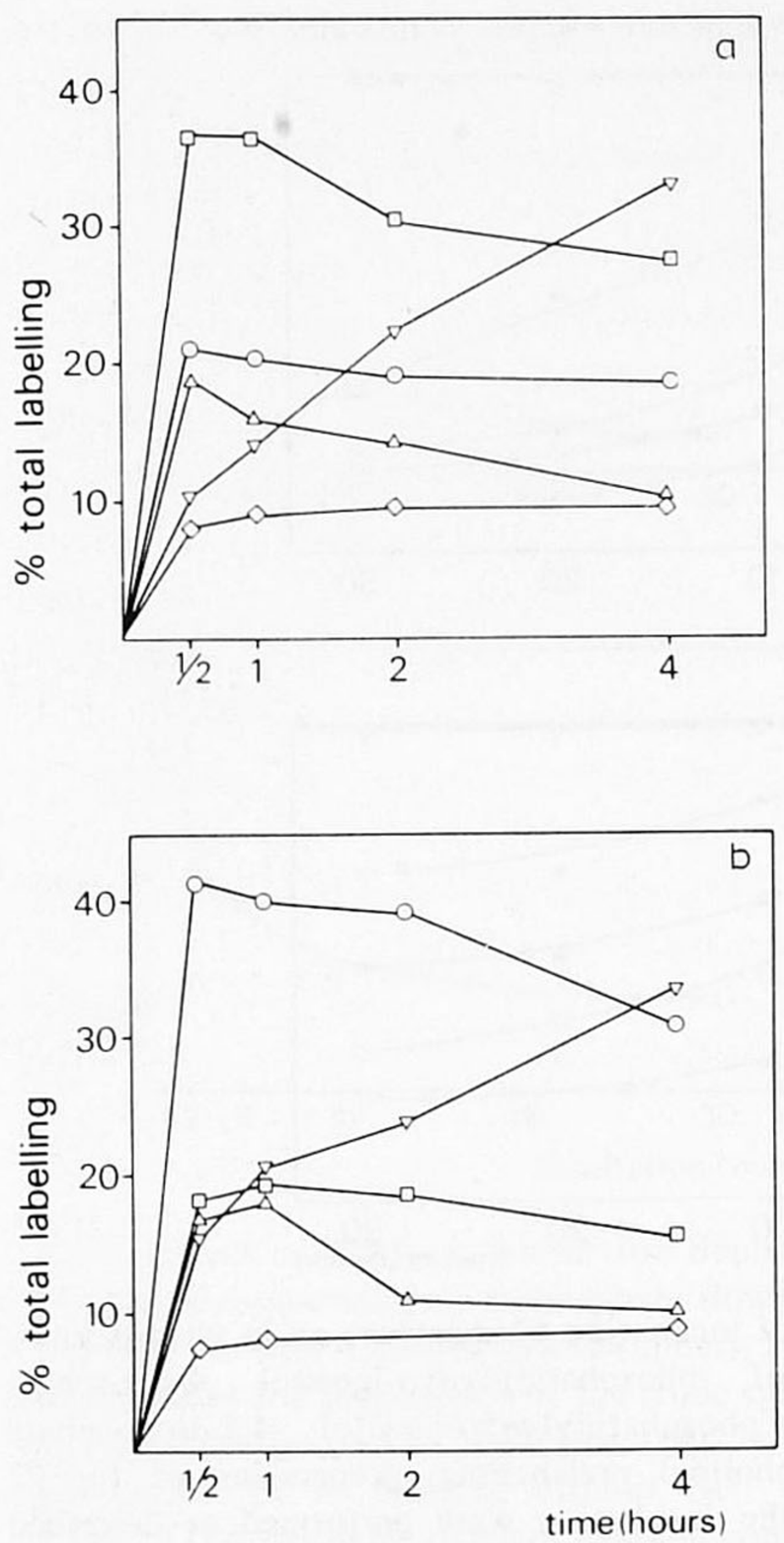

Fig. 1. Time curve of the in vivo incorporation of ^{32}P into individual phospholipids. Four groups of rats were taken. The animals received an intracerebroventricular injection of $^{32}P_i$ and were killed after 0.5, 1, 2 or 4 h. The limbic system was dissected; a crude mitochondrial/synaptosomal fraction (P2; Fig. 1a) and a crude myelin fraction (Fig. 1b) were obtained. The radioactive incorporation into membrane-phospholipids was determined as described. The incorporation of label into individual phospholipids is expressed as a percentage of the radioactivity incorporated into total phospholipids [21] to reduce variation due to differences in injection efficiency. This variation was manifest in the total amount of incorporation but not in the incorporation pattern. The mean of two different experiments ($n = 4$) is given. □, Phosphatidyl-*myo*-inositol; △, phosphatidyl-*myo*-inositol 4-phosphate; ○, phosphatidyl-*myo*-inositol 4,5-diphosphate; ◇, phosphatidic acid; ▽, rest.

These lipids contained 32% of the incorporated ^{32}P after a 4 h labelling period, and these values increased to 85% after 1 day (data not shown).

Although the absolute amount of incorporation was similar in both subcellular fractions, the incorporation pattern was different: the ratio of ^{32}P-labelled phosphatidyl-*myo*-inositol 4,5-diphosphate over ^{32}P-labelled phosphatidyl-*myo*-inositol was about 2 in crude myelin, and 0.6–0.8 in the P2 fraction. On the basis of these data we decided to use a 2 h labelling period for further study, thus compromising the need for a high specific labelling of the phosphatidyl-*myo*-inositol, phosphatidyl-*myo*-inositol 4-phosphate, phosphatidyl-*myo*-inositol 4,5-diphosphate and phosphatidic acid, with a sufficient amount of incorporation.

In vitro metabolism of prelabelled phospholipids

A P2 fraction and a crude myelin fraction were obtained as described (see Materials and Methods) and suspended in the incubation medium described by Bradford [18]. The suspensions were incubated for a period of time ranging from 15 to 30 min, and the radioactivity in the individual phospholipids was determined. It appeared that the amount of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate decreased rapidly with incubation time. The radioactivity recovered from phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate reached an asymptote at 50 and 35% of the original level, respectively (Fig. 2). Half of this labile ^{32}P was removed within 2 min after the start of the incubation. Phosphatidyl-*myo*-inositol, phosphatidic acid and the other phospholipids were not changed in the 30 min incubation period. The results obtained with both subcellular fractions were similar.

When the P2 fraction was incubated at lower temperature to slow down the enzymic activity, it appeared that a significant dephosphorylation of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate occurred at 30, 24 and 18°C, but no breakdown was found at 0°C (Fig. 3).

The influence of extracellular Ca^{2+} and $ACTH_{1-24}$ on the dephosphorylation of phosphatidyl-myo-inositol 4-phosphate and phosphatidyl-myo-inositol 4,5-diphosphate

In view of the important role played by Ca^{2+} in the metabolism of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate [5–7,9] it was decided to test the dephosphorylation

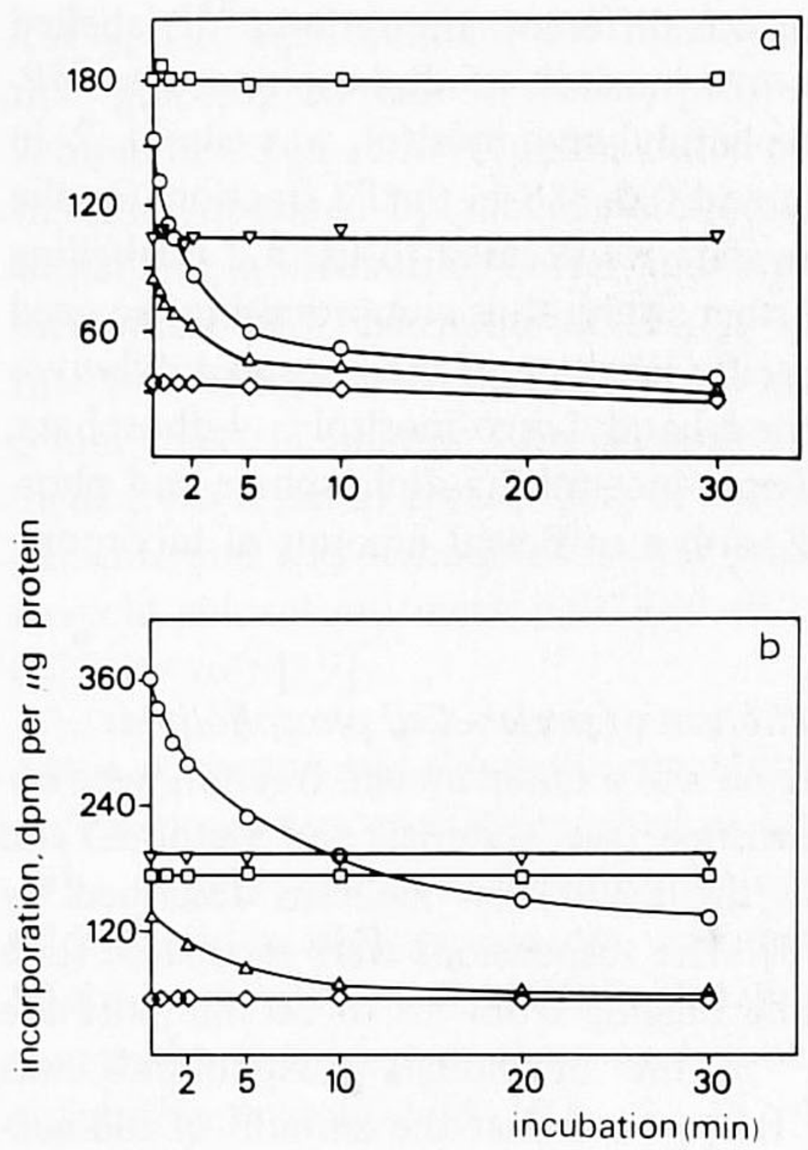

Fig. 2. In vitro dephosphorylation of polyphosphoinositide after prelabelling in vivo. Brain phospholipids were labelled in vivo for 2 h. Subcellular fractions were obtained and incubated in vitro (37°C) for varying periods of time. The results are expressed in dpm per µg incubated protein. a, P2 fraction; b, crude myelin fraction. □, Phosphatidyl-*myo*-inositol △, phosphatidyl-*myo*-inositol 4-phosphate; ○, phosphatidyl-*myo*-inositol 4,5-diphosphate; ◇, phosphatidic acid; ▽, rest.

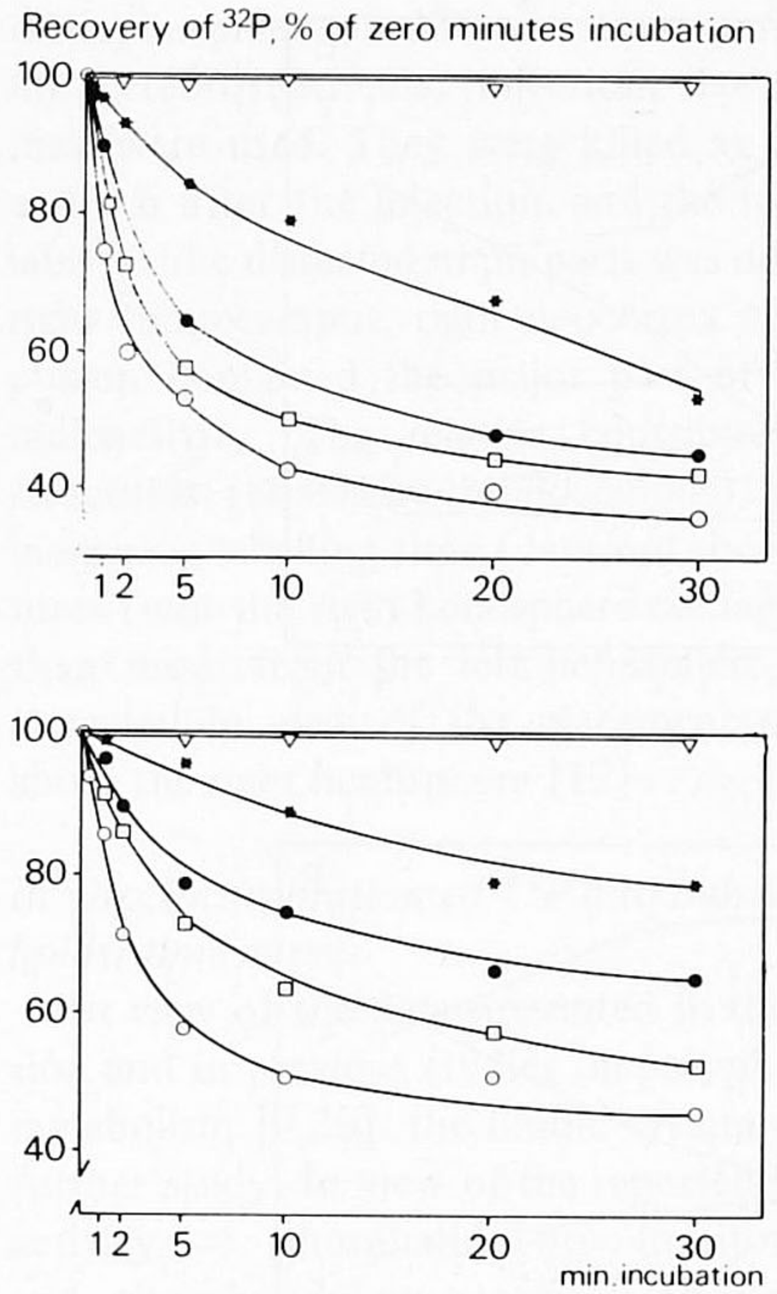

Fig. 3. Effect of incubation temperature on in vitro dephosphorylation of phosphatidyl-*myo*-inositol 4-phosphate (lower) and phosphatidyl-*myo*-inositol 4,5-diphosphate (upper). Phospholipid prelabelling, preparation of the P2 fraction and the incubation were performed as described (Fig. 2). The results are expressed as a percentage of the amount of radioactivity at the start of the incubation. ▽, 0°C; ★, 18°C; ●, 24°C; □, 30°C; ○, 37°C.

of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate in Ca^{2+}-free medium containing EGTA. It appeared that in this medium only 20 or 10% of the radioactive phosphatidyl-*myo*-inositol 4,5-diphosphate or phosphatidyl-*myo*-inositol 4-phosphate was broken down after a 30 min incubation period (Fig. 4).

The effect of $ACTH_{1-24}$ was tested because this peptide has been found to stimulate the formation of phosphatidyl-*myo*-inositol 4,5-diphosphate in a lysed synaptosomal fraction in vitro in a Ca^{2+}-sensitive way [9]. However, $ACTH_{1-24}$ did not affect the radioactive dephosphorylation in crude myelin or in the P2 fraction at any incubation temperature tested (18, 24, 30, 37°C). Likewise, no effects were found under Ca^{2+}-free conditions or with normal (0.75 mM) or high (2.7 mM) Ca^{2+} concentrations (data not shown).

Prelabelled phospholipids in purified synaptosomes, mitochondria and myelin

In order to obtain more information on the exact location of the prelabelled phospholipids, synaptosomes were purified according to the method of Booth and Clark [15]. The synaptosomes prepared by this method are only slightly contaminated by free mitochondria (1–4%). Also mitochondria were purified by this method and a myelin-enriched fraction was also obtained. Analysis of the lipid composition of the three fractions revealed that the synaptosomes and the mitochondria contained no cerebrosides and sulphatides (see Materials and Methods), indicating that they were devoid of myelin. As shown in Fig. 5, the phospholipid labelling pattern in myelin, synaptosomes and mitochondria was very different. The

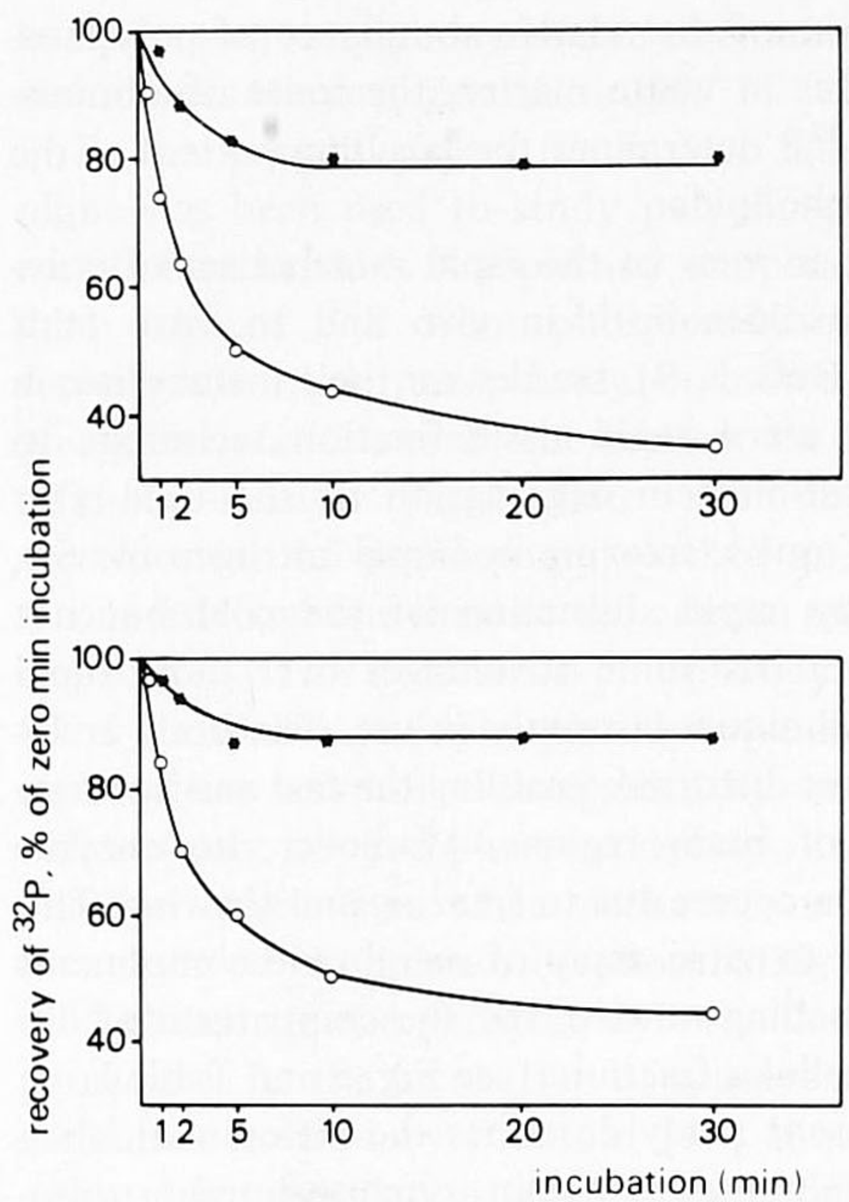

Fig. 4. Effect of Ca^{2+} on the in vitro dephosphorylation of phosphatidyl-*myo*-inositol 4-phosphate (lower) and phosphatidyl-*myo*-inositol 4,5-diphosphate (upper). Phospholipid prelabelling and the preparation of the crude synaptosomal fraction were performed as described (Fig. 2). The incubation was performed in medium without Ca^{2+} and with 1 mM added EGTA (★) or in standard medium containing 0.75 mM Ca^{2+} (○). The results are expressed as a percentage of the amount of radioactivity at the start of the incubation.

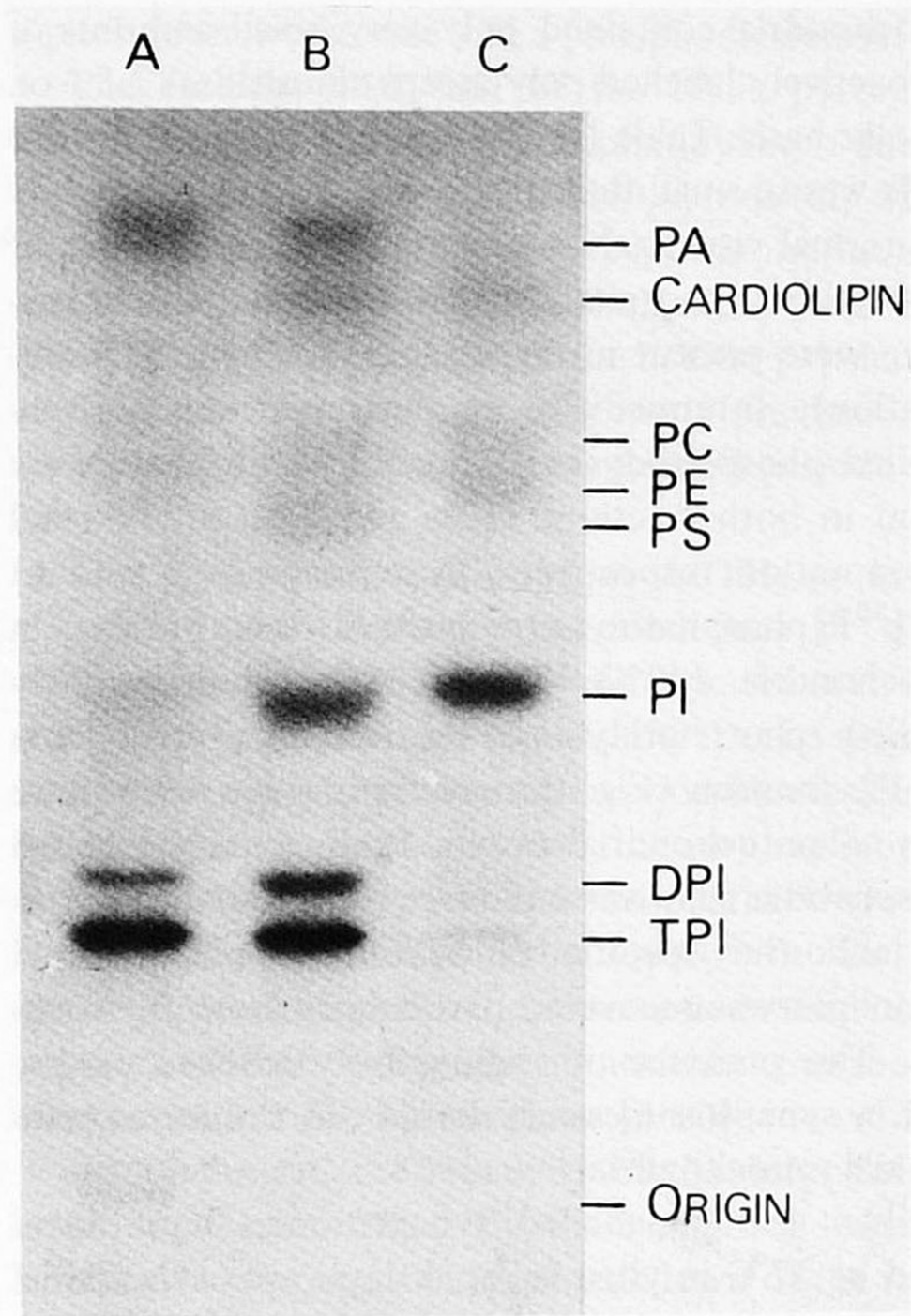

Fig. 5. Autoradiogram after extraction and thin-layer chromatography of phospholipids after 2 h in vivo labelling. a, myelin; b, synaptosomes; c, mitochondria. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, Phosphatidic acid; PI, phosphatidyl-*myo*-inositol; DPI, phosphatidyl-*myo*-inositol 4-phosphate; TPI, phosphatidyl-*myo*-inositol 4,5-diphosphate.

TABLE I

INCORPORATION INTO PHOSPHOLIPIDS OF MYELIN, SYNAPTOSOMES AND MITOCHONDRIA

Brain phospholipids were labelled in vivo for 2 h, and synaptosomes, mitochondria and myelin were purified as described in Materials and Methods. The incorporation is expressed as dpm per μg protein (mean ± S.E.). Three different experiments gave similar results.

Lipid class	Incorporation (dpm/μg protein)		
	Myelin	Synaptosomes	Mitochondria
Phosphatidyl-*myo*-inositol	73 ± 4 (6.1)	171 ± 13 (19.5)	262 ± 29 (48.8)
Phosphatidyl-*myo*-inositol 4-phosphate	149 ± 9 (12.3)	147 ± 9 (16.8)	17 ± 4 (3.2)
Phosphatidyl-*myo*-inositol 4,5-diphosphate	672 ± 40 (55.5)	283 ± 16 (32.3)	17 ± 2 (3.2)
Phosphatidic acid	135 ± 3 (11.1)	62 ± 5 (7.1)	28 ± 2 (5.2)
Cardiolipin	42 ± 5 (3.5)	66 ± 3 (7.5)	69 ± 4 (12.9)
Phosphatidylserine	20 ± 2 (1.7)	30 ± 4 (3.4)	9 ± 2 (1.7)
Phosphatidylethanolamine	41 ± 10 (3.4)	60 ± 14 (6.9)	70 ± 12 (13.0)
Phosphatidylcholine	36 ± 3 (3.0)	44 ± 4 (5.0)	60 ± 8 (11.2)
Unidentified	41 ± 5 (3.4)	12 ± 1 (1.4)	5 ± 1 (1.0)
Total incorporation	1 209 (100)	875 (100)	537 (100)

mitochondria contained only very small amounts of radioactively labelled polyphosphoinositides (2.5% on a molar basis; Table I); The absolute amount of these lipids was so small that they could not be detected by the normal visualization techniques. High amounts of ^{32}P-labelled phosphatidyl-*myo*-inositol 4,5-diphosphate were present in myelin (52% of the total incorporation); intermediate amounts of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate were found in both fractions (12% and 17% of the total incorporation, respectively). A strikingly high amount of [^{32}P]phosphatidyl-*myo*-inositol was present in mitochondria (49%). Therefore, the radioactively labelled phosphatidyl-*myo*-inositol recovered from the P2 fraction (Fig. 1) may for a major part have been of mitochondrial origin. Radioactively labelled phosphatidic acid was recovered especially from myelin, and the specific mitochondrial phospholipid cardiolipin was recovered particularly from that fraction. The presence of radioactively labelled cardiolipin in synaptosomes may be due to the intrasynaptosomal mitochondria.

When the prelabelled synaptosomes were incubated at 37°C in vitro, a rapid dephosphorylation of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate was observed that was similar to that found in the crude fractions. Likewise, no change in phosphatidyl-*myo*-inositol, phosphatidic acid and the other phospholipids was observed. Similarly, the effects of Ca^{2+} and the lack of effect of $ACTH_{1-24}$ were similar to those obtained in the P2 fraction (data not shown).

Discussion

The present study describes the in vivo labelling of rat brain phospholipids and their metabolism in vitro. The data obtained with the in vivo prelabelling technique attract attention to the following points:

First, the site of injection is a major determinant of the distribution of radioactivity over the brain. Using an intracisternal injection, Friedel et al. [22, 23] found that the regional distribution was cerebellum > brain stem > cerebral hemispheres. In the present study, however, after intracerebroventricular injection the regional distribution was limbic system > cerebral cortex > myelencephalon > cerebellum. In view of the relative abundance of polyphosphoinositides in white matter, the route of administration of ^{32}P determines the labelling pattern of the brain phospholipids.

Second, in view of the rapid metabolism of polyphosphoinositides both in vivo and in vitro (this study and Refs. 5, 9), studies on their metabolism in vivo must use a rapid tissue-fixation technique to prevent post-mortem degradation of the lipid. Our method of quick freezing in liquid nitrogen for 8 s, followed by rapid dissection of the cold but not frozen brain has some advantages over those rapid fixation techniques currently in use. The brain architecture is not distorted, enabling the fast and accurate dissection of brain regions. Moreover, no enzyme denaturation occurs due to freezing and thawing. This enables the in vitro assay of membrane components after prelabelling in vivo and the comparison of different subcellular fractions (see Fig. 5 and Table I).

The present study describes the differential labelling of membranes of myelin, synaptosomal or mitochondrial origin (Fig. 5, Table I). The highly labelled phosphatidyl-*myo*-inositol 4-phosphate and especially phosphatidyl-*myo*-inositol 4,5-diphosphate that were found in myelin support findings by others [24–26]. Furthermore, highly labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate were present in synaptosomes that were apparently devoid of myelin contamination as they did not contain cerebrosides and sulphatides. More phosphatidyl-*myo*-inositol 4-phosphate was present in synaptosomal plasma membranes than myelin. This corroborates findings by others [3,4,27] and is in line with the findings that the phosphatidyl-*myo*-inositol kinase is preferentially located in the plasma membrane [28]. The fact that highly labelled phosphatidyl-*myo*-inositol was found in mitochondria may indicate that phosphatidyl-*myo*-inositol-exchanging proteins [29] are active in vivo, as mitochondria lack the capacity to synthesize phosphatidyl-*myo*-inositol [30]. Furthermore, the absence of significant amounts of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate corroborates findings by others [31] that mitochondria lack the appropriate kinase activity. The small amount of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate found in mitochondria

in the present study may be due to contamination by membranes of synaptosomal origin.

In the present paper, the in vivo labelling technique has been used to study polyphosphoinositide metabolism in vitro. A rapid and temperature-dependent loss of ^{32}P from phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate was observed: the breakdown of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate reached an asymptote at one-half and one-third of the original radioactivity, respectively. This decrease in phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate was not accompanied by an increase in radioactively labelled phosphatidyl-*myo*-inositol. The question arises as to what mechanism is involved in the observed breakdown of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate. As the incubation of synaptosomes in the medium described by Bradford [18] allows for oxydative phosphorylation, the presence of unlabelled ATP may result in the exchange of labelled monoesterified [^{32}P]phosphate for unlabelled phosphate. In that case the remaining radioactivity resides in the diesterified [^{32}P]phosphate. One would have expected to find a breakdown of phosphatidyl-*myo*-inositol by phosphodiesterase activity, as seen in the agonist-induced phosphatidyl-*myo*-inositol response. However, it may be that under non-stimulated conditions this enzyme is much less active, as would appear from Fig. 2. More research is required to describe the route by which the breakdown of radioactive label goes in more detail.

The presence of Ca^{2+} appeared of crucial importance as polyphosphoinositide breakdown was inhibited greatly in the absence of this ion and in the presence of EGTA (Fig. 4). However, although it has been reported that the phosphomonoesterases have an absolute requirement for Ca^{2+}, the phosphodiesterases are especially sensitive to this ion [32,33]. Other studies on polyphosphoinositide metabolism in vitro after prelabelling in vivo also stress the important role of Ca^{2+}. From experiments with neurotransmitters [7,8] and Ca^{2+} ionophore A23187 [6] it has been concluded that influx of Ca^{2+} stimulates the breakdown of polyphosphoinositide at the cytoplasmic side of the plasma membrane. In these studies, the breakdown was performed by the combined action of a phosphomonoesterase and a phosphodiesterase.

The findings that half of the labile portion of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate was removed within 2 min is another indication for the very rapid metabolism of these lipids [5,9]. These findings support the notion [9] that the conflicting evidence obtained in many previous studies may be due to the long incubation periods used (10–30 min).

The present observation that $ACTH_{1-24}$ is not effective under experimental conditions in which only radioactive dephosphorylation can be seen (this study) confirms previous reports [9] that its action is not on the polyphosphoinositide phosphatases but on the respective kinases. Likewise, the very rapid breakdown of radioactively labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate strengthens the observations that neuropeptides affect polyphosphoinositide metabolism after very short incubation times [9,10].

In conclusion, the reactivity of the polyphosphoinositides is manifested by their rapid and specific labelling in vivo and the fast breakdown of the radioactive label in vitro. The fact that highly labelled phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-diphosphate are found in purified synaptosomes may point to their importance for synaptic function.

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