PITUITARY HORMONES INFLUENCE POLYPHOSPHOINOSITIDE METABOLISM IN RAT BRAIN

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1. Introduction

In a variety of tissues an enhanced metabolism of phosphatidyl inositol (PI) is observed after receptor activation by hormones or neurotransmitters which utilize calcium as second messenger [1,2]. The enhancement is manifested by an increased rate of incorporation of P_i into PI and is frequently accompanied by an increase in labelling of phosphatidic acid (PA) [1]. It is thought to reflect an increased breakdown of PI to diacylglycerol within the plasma membrane, which is then rapidly phosphorylated to PA and consequently transformed into PI in the endoplasmic reticulum.

With respect to nervous tissue, the rapid metabolism of the polyphosphoinositides (DPI, phosphatidyl-myoinositol 4-phosphate; TPI, phosphatidyl-myoinositol 4,5-diphosphate) especially suggests an important effect in membrane function. In contrast to the low quantities of DPI and TPI in membranes from a variety of cell types, brain cell plasma membranes contain appreciable amounts of these polyphosphoinositides [3]. It appears that they play a role in the Ca^{2+} influx into the cell [1,2,4].

In peripheral tissues and cells it was demonstrated that peptides (vasopressin, insulin, substance P) may regulate the metabolism of cell membrane phosphatidyl inositides [5–8]. In view of these findings and our recent results on the regulatory role of peptide hormones and fragments of peptide hormones on synaptic plasma membrane protein phosphorylation [9,10], it was of interest to investigate whether such peptides influence brain membrane phospholipid metabolism. The experiments reported here demonstrate that ACTH and vasopressin affect the metabolism of phosphatidyl inositides and phosphatidic acid.

2. Materials and methods

2.1. *Chemicals*

Phospholipid standards including DPI and TPI were from Sigma Chemical Co. Synthetic $ACTH_{1-24}$ and lysine vasopressin (LVP) were obtained from Organon Int. BV, Oss. [^{32}P]orthophosphate was from New England Nuclear Corp., Boston, MA. All other chemicals were of the highest grade commercially available.

2.2. *Preparation of crude mitochondrial fraction*

Male rats of an inbred Wistar strain (TNO, Zeist) were used. To prevent the rapid postmortem hydrolysis of diphosphoinositide and triphosphoinositide [11], the rats were killed by whole body immersion into liquid nitrogen for 10 s, followed by rapid dissection of the cold but not frozen brain, at 0–4°C. The limbic system (hippocampus, septum, basal ganglia, diencephalon, pyriform cortex) was dissected as in [12]. Crude mitochondrial preparation was according to [9]. The limbic system was homogenized in 10 vol. 0.32 M sucrose by 10 up/down strokes of a Potter-Elvehjem Teflon/Glass homogenizer with a radial clearance of 0.125 mm rotating at 700 rev./min. The homogenate was centrifuged at 1000 × *g* for 10 min. The resulting supernatant was centrifuged at 10 000 × *g* for 10 min. The pellet (crude mitochon-

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drial/synaptosomal fraction) was resuspended in 10 vol. incubation medium (~5 mg protein/ml).

2.3. *Incubation and extraction*

The incubation was carried out at 37°C in small conical glass centrifuge tubes. Protein (~50 μg) as determined by the method of Lowry [13] was pre-incubated for 13 min at 37°C in 23 μl Krebs-Ringer solution (pH 7.4) containing in mM: 128 NaCl, 5 KCl, 2.7 $CaCl_2$, 1.2 $MgSO_4$, 25 Tris–HCl and 40 μCi $^{32}P_i$, carrier free. Subsequently the incubation was started by addition of 2 μl Krebs-Ringer solution with or without peptide hormone. The reaction was stopped by addition of 2 ml ice-cold mixture of chloroform:methanol: 13 N HCl (200:100:0.75, by vol.) [14]. Carrier polyphosphoinositides (10 μg P) were added, isolated as in [14]. The mixture was allowed to stand for 15 min at 4°C and by adding 0.4 ml 0.6 N HCl a biphasic system was formed. The upper phase was removed and the lower phase washed 3 times with 0.4 vol. chloroform:methanol:0.6 N HCl (3:48:47, by vol.). The resulting lower phase was transferred to another tube and dried under nitrogen flow at 30°C. It was redissolved in 100 μl chloroform:methanol:water (75:25:2, v/v/v). Three aliquots of 5 μl were taken for the determination of radioactivity incorporated into the total phospholipid fraction. The remaining part was again dried under nitrogen flow at 30°C and redissolved in 20 μl chloroform:methanol:water (75:25:2, by vol).

2.4. *Thin layer chromatography (TLC) of phospholipids*

Phospholipids were separated by one-dimensional TLC on silica gel high-performance (HP) thin layer plates (layer thickness 25 μm, Merck). Before use, the plates were impregnated in 1% K-oxalate solution [15]. To make sure this oxalate was present on the plate homogeneously, the wet plate was lowered into a tray containing the solution. After 10 min the plate was removed, dried in air at room temperature and activated at 110°C for 10 min. The plates were developed, using chloroform:acetone:methanol:glacial acetic acid:water (40:15:13:12:8, by vol.) [16]. This procedure yielded quantitative separation of PA, PI, DPI and TPI. Lipids were located by exposure of plates to iodine vapour. Recovery of labelled lipids after TLC was > 97%.

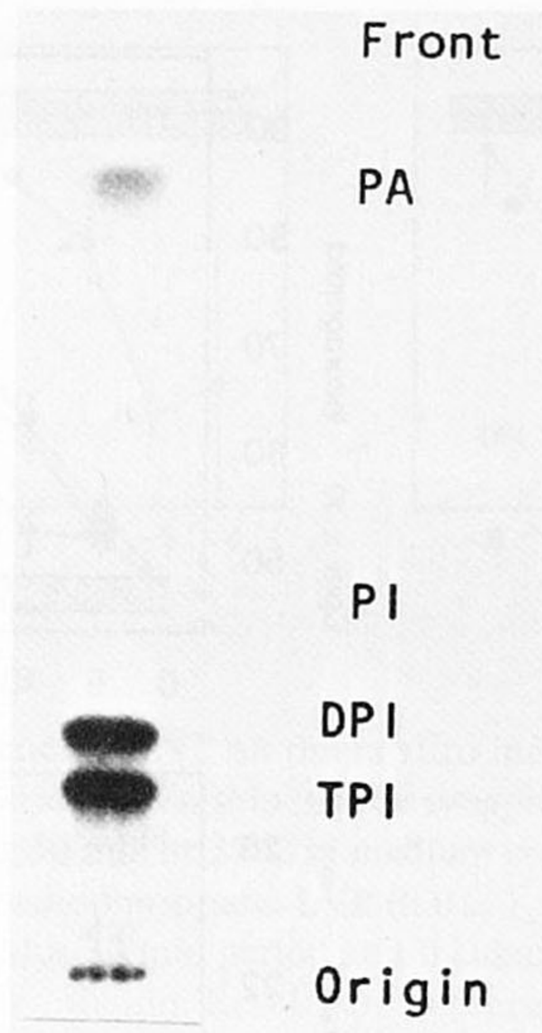

Fig.1. Autoradiogram after thin-layer chromatography of phospholipids on oxalate-impregnated silica gel H-HP TLC. TPI, triphosphoinositide; DPI, diphosphoinositide; PI, monophosphoinositide; PA, phosphatidic acid. Solvent: chloroform: acetone:methanol:glacial acetic acid:water (40:15:13:12:8, by vol.).

^{32}P-labelled spots were detected by autoradiography on Kodak Royal X-Omat X-Ray-film (10–20 h). The spots were scraped from the plates and counted for radioactivity in a liquid scintillation spectrometer with 3.6 ml of a scintillation fluid (a 7:23, v/v, mixture of Triton X-100 and a toxylene solution of 2.5 diphenyloxazole (PPO) and 1.4-bis (5-phenyloxazolyl-2)-benzene (POPOP)). Radioactivity was measured in a Mark II scintillation counter (Searle). Counting efficiency was ~70%.

2.5. *Statistics*

Differences between groups were tested by Student's *t*-test [17].

3. Results

3.1. *The effects of peptides on the incorporation of [^{32}P]orthophosphate into phospholipids in vitro: effect of incubation time*

When a crude mitochondrial preparation was incubated in Krebs medium in the presence of

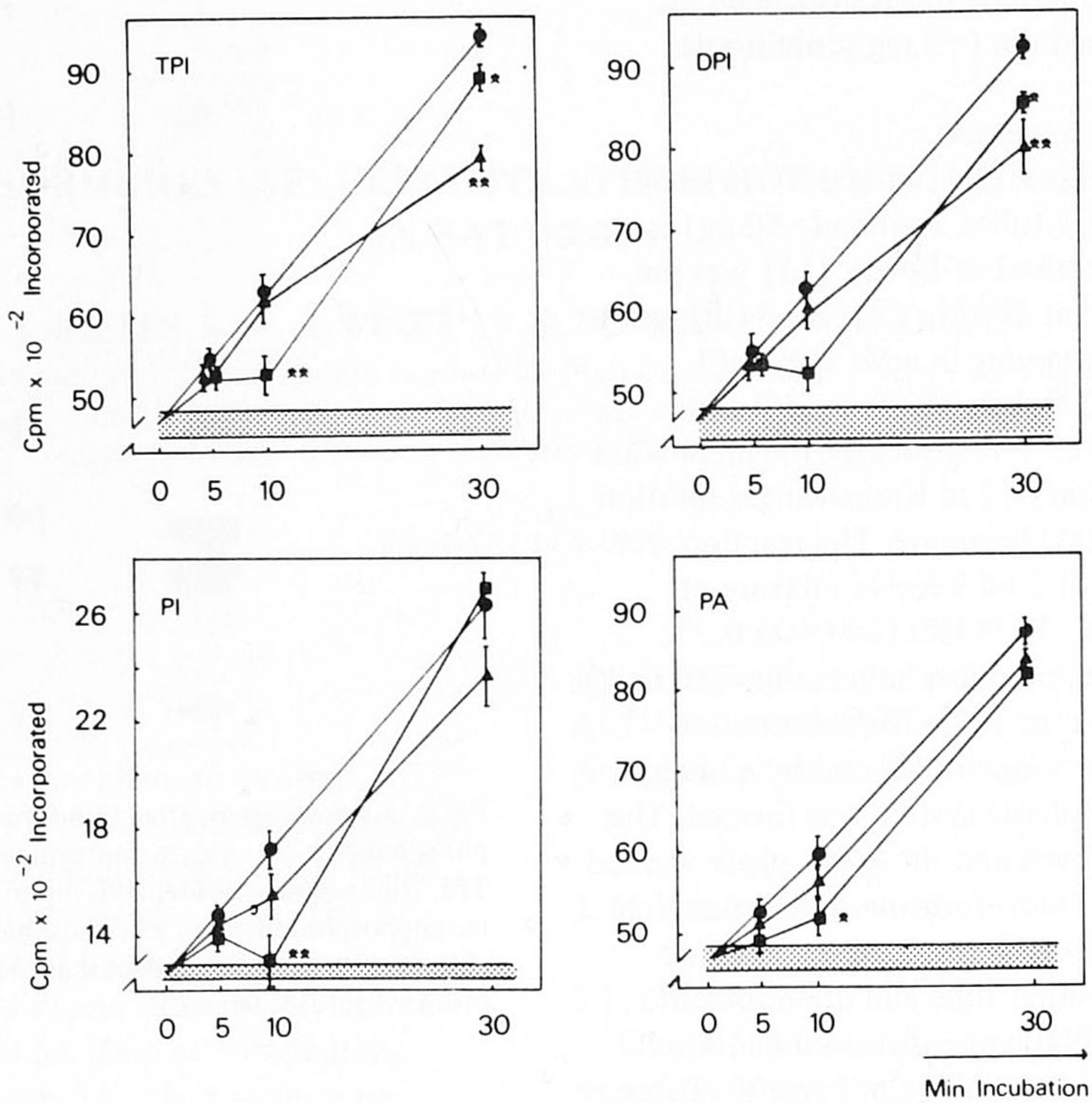

Fig.2. The influence of ACTH and LVP on the in vitro incorporation of ^{32}P: time curve. Crude synaptosomes were preincubated for 30 min at 37°C in medium containing 30 μCi of [^{32}P]orthophosphate as in section 2. ACTH or LVP were added after this period and incubation was continued for another 5, 10 or 30 min. Results are expressed as mean ± SEM (N = 12). Symbols: (●) control, no addition; (■) 10 μM $ACTH_{1-24}$ added; (▲) 10 μM LVP added. * $2p < 0.05$. ** $2p < 0.01$.

[^{32}P]orthophosphate, there was a linear incorporation of label into phospholipids for ⩽ 60 min (results not shown). The phosphoinositides and PA were found to contain > 98% of the incorporated radioactivity; TPI, DPI, PI and PA contained 30%, 30%, 8% and 30% of the radioactivity, respectively.

Figure 1 shows the separation of the labelled phospholipids on oxalate-impregnated precoated HP TLC plates; [^{32}P]orthophosphate present as contamination remained at the origin. Chromatography in the acetic solvent yielded optimal results: a quantitative separation of PA, PS and PI was achieved, and the phosphoinositides were more stable when developed under acetic conditions than under basic conditions. This made it unnecessary to convert the phosphoinositides into the more stable Ca^{2+}-salts [18].

In our hormone studies the membrane preparation was routinely preincubated for 30 min in the presence of [^{32}P]orthophosphate to allow radioactive phosphate to equilibrate with the organic phosphate pools. At the conclusion of this 30 min period, incubation was started by the addition of buffer with or without hormone and the incorporation of [^{32}P]orthophosphate into the phospholipids followed with time.

Figure 2 shows the effect of ACTH and LVP on incorporation of ^{32}P into TPI, DPI, PI and PA, after 5, 10 and 30 min incubation. As compared to the control the amount of label incorporated into all 4 lipids after 10 min incubation is greatly reduced upon addition of ACTH (10 μM). After 30 min only TPI and DPI are significantly reduced. LVP (10 μM) has no effect after 10 min but significantly reduces

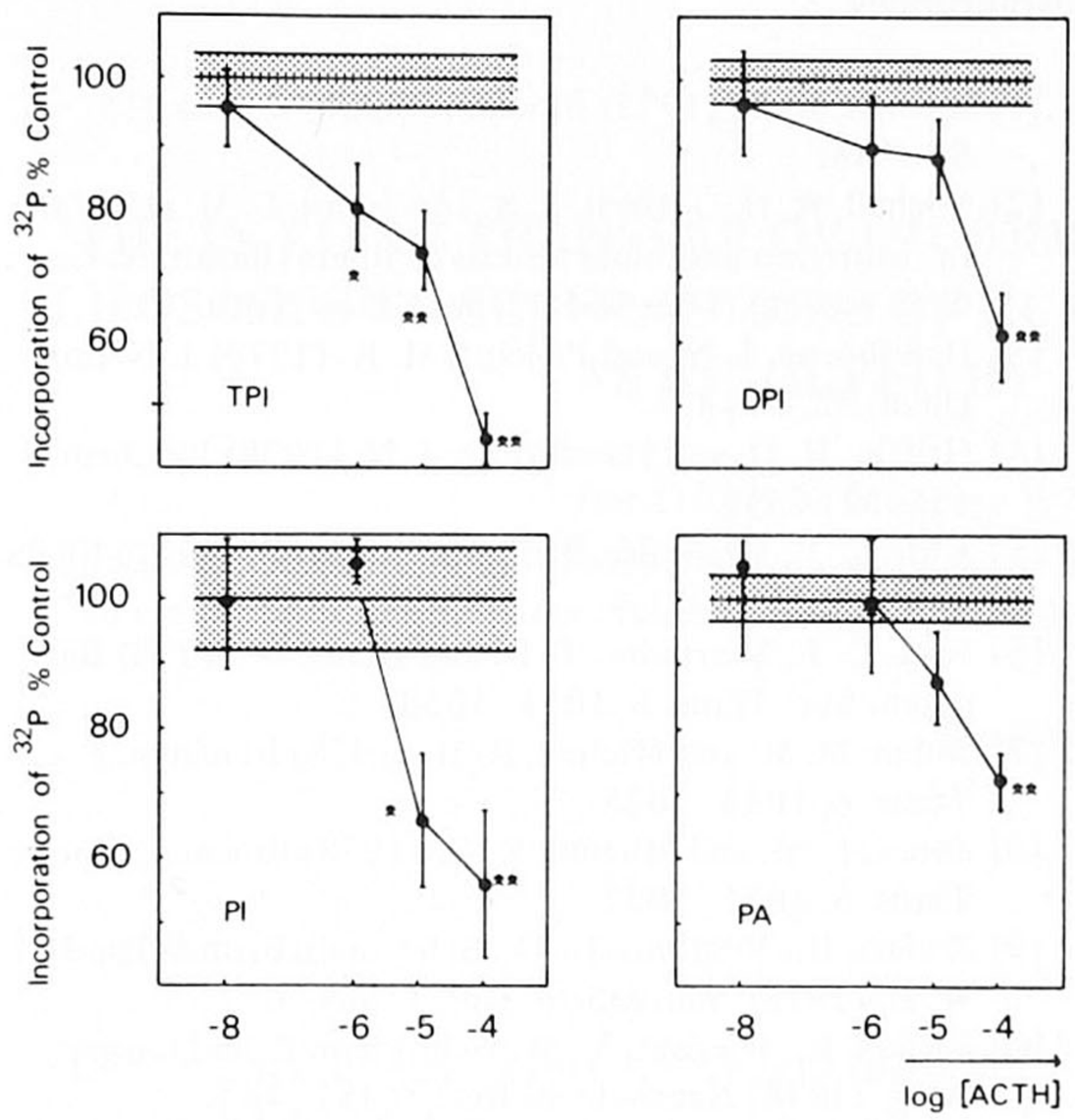

Fig.3. The influence of $ACTH_{1-24}$ on the in vitro incorporation of ^{32}P: dose–response relationship. Crude synaptosomes were incubated for 30 min at 37°C in medium containing 30 μCi of [^{32}P]orthophosphate. ACTH (0.01, 1, 10, 100 μM) was added after this 30 min period and incubation was continued for another 10 min. Results are expressed as the percentage of labelling in the 10 min incubation period (mean ± SEM, $N = 6$), * $2p < 0.05$. ** $2p < 0.01$.

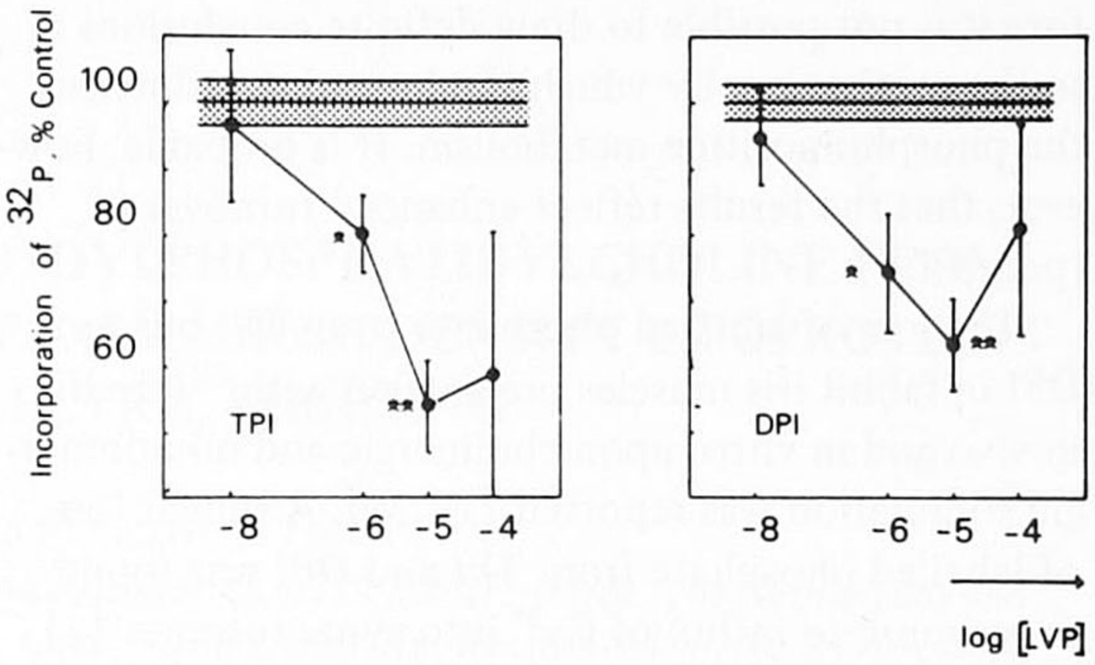

Fig.4. The influence of LVP on the in vitro incorporation of ^{32}P: dose–response relationship. Crude synaptosomes were preincubated for 30 min at 37°C in medium containing 30 μCi of [^{32}P]orthophosphate. LVP (0.01, 1, 10, 100 μM) was added after this 30 min period and incubation was continued for another 30 min. Results are expressed as the percentage of labelling in the 30 min incubation period (mean ± SEM, $N = 16$). * $2p < 0.05$. ** $2p < 0.02$.

the amount of label incorporated in TPI and DPI after 30 min incubation.

The results of shorter incubation times (5 min) are consistent in that decreased amounts of label were incorporated upon hormone addition. These findings were not statistically significant.

3.2. *Dose–response relationship*

The effect of different concentrations (0.01, 1, 10 and 100 μM) of both peptides on the labelling of the phospholipids was determined. As fig.3 shows, ACTH at 10 min incubation, has a dose-dependant effect on the incorporation of label into the phosphoinositides and PA. Maximal effects were obtained for 100 μM ACTH whereas 1 μM only affected TPI. LVP at 30 min incubation, significantly reduced the labelling of TPI and DPI; for PI and PA smaller and inconsistent results were obtained (fig.4). Results obtained with 100 μM of the peptide showed a large variation and 0.01 μM had no effect.

4. Discussion

This paper deals with the phosphorylation of brain membrane phospholipids in the presence of peptide hormones. [^{32}P]orthophosphate was used as precursor. Prior to addition of the hormone the membrane preparation was prelabelled for 30 min. From the data it was concluded that vasopressin and ACTH decrease the amount of label incorporated into the phosphoinositides and PA. TPI and DPI were most responsive to addition of the hormone. Our results were obtained with a crude mitochondrial fraction; it is very probable that the phosphorylation in this fraction is due to the synaptosomes present, as mitochondria do not contain polyphosphoinositides, and enzymes involved in the PI effect are localized in the plasma membrane [1].

The incorporation of ^{32}P into membrane phospholipids, as determined under our incubation conditions, is the result of phosphorylation of PI (DPI) kinases, dephosphorylation by TPI (DPI) phosphomonoesterases and by TPI (DPI, PI) phosphodiesterases, and exhaustion of radioactive ATP by ATPases. There-

fore it is not possible to draw definite conclusions as to the mechanism by which the hormone influences the phosphoinositide metabolism. It is probable, however, that the results reflect enhanced turnover of (poly)PI.

The loss of labelled phosphate from TPI but not DPI in rabbit iris muscles prelabelled with ^{32}P both in vivo and in vitro, upon cholinergic and noradrenergic stimulation was reported [19,20]. A similar loss of labelled phosphate from TPI and DPI was found in response to influx of Ca^{2+} into synaptosomes [4]. A relation has been suggested between the enhanced turnover of TPI and DPI, the PI effect and processes dependent on the influx of Ca^{2+} into the cell [1,2]. In studies on the PI effect in synaptosomes, acetylcholine was found to affect the PI metabolism at 10–1000 μM, maximal effects being obtained at 100 μM [21]. In our hands maximal effects were obtained at 10 μM LVP and 100 μM ACTH. ACTH at 1–100 μM was found to decrease the amount of label in phosphoinositides and PA. Peptide hormones have been claimed to have a PI effect in some peripheral tissues: vasopressin rapidly stimulated the incorporation of [^{32}P]orthophosphate into PI in isolated hepatocytes [5–7], insulin had a PI effect in adipose tissue [22] and substance P in rat parotid gland [8].

The effects of peptide hormones on phosphoinositide metabolism are of special interest in view of the influence of ACTH and related peptides on brain membrane protein phosphorylation [9,10]. With respect to phosphorylation of membrane proteins, changes in membrane permeability in nerve and peripheral tissue have been reported (see [23]). For nervous tissue such alterations are thought to influence neurotransmission. Recent evidence suggests a special role for Ca^{2+} as the phosphorylation of specific synaptosomal membrane proteins has a strict requirement for Ca^{2+} [24–26].

Further information on the interaction of peptides with nervous tissue is needed to reach a conclusion as to the mechanisms of peptide-induced changes in synaptosome phospholipid phosphorylation, and to relate this information to the known influence of peptides on brain protein phosphorylation.

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