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Microdetermination of Phosphoinositides in a Single Extract

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A method that allows the quantification of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (DPI), and phosphatidylinositol 4,5-biphosphate (TPI) on a nanomolar scale is presented. The method is based on the simultaneous separation of lipids on high-performance thin-layer chromatography plates, followed by a microassay for phosphorus of PI spots and a densitometric assay of DPI and TPI.

The new procedure allows the determination of the phospholipids in small amounts (100 μg protein) of synaptosomes and synaptic plasma membranes, and in homogenates of microwave-fixed brain tissue (1 mg wet wt). The usefulness of the method is illustrated by showing the effect of Ca^{2+} on the breakdown of DPI and TPI in synaptosomal plasma membranes. © 1985 Academic Press, Inc.

KEY WORDS: thin-layer chromatography; phosphate assay; phospholipids.

Phosphoinositide lipids are implicated in the receptor-controlled mediation of cell responses to various stimuli (1,2). Although this class of lipids represents only a minor part of the total cell lipids, they have been intensively studied because of their specific biochemical properties and their fast turnover. In the majority of the published studies their metabolism was investigated by analysis of radioactivity incorporated in these phospholipids (1–3) or by measuring radiolabeled degradation products, for instance, the inositol phosphates (4,5). The assumption is made that changes in the amount of radioactivity are a reflection of changes in the total absolute amount of these phospholipids; however, measurements of concentration would be a more direct approach. Due to their relative low concentrations, estimation of phosphatidylinositol 4-phosphate (DPI)[1] and phosphatidylinositol 4,5-biphosphate (TPI) levels was difficult to obtain thus far, although a few papers describe determinations in the 10–100 nmol range (6–8). Here we describe a more sensitive method, allowing the simultaneous separation and determination of phosphatidylinositol (PI, 0.6–6 nmol), and DPI and TPI (0.1–1 nmol). In intact synaptosomes, synaptic plasma membranes (SPM), and homogenates from small dissected brain areas absolute levels of PI, DPI, and TPI were determined, as well as the effect of Ca^{2+} on the breakdown of polyphosphoinositides in SPM.

MATERIALS AND METHODS

Preparation of synaptosomes, SPM, and microwave-fixed brain tissue. Male rats (150 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). Synaptosomes were prepared from whole brain minus cerebellum according to Booth and Clark (9) and SPM were prepared according to Kristjansson *et al.* (10). Microwave fixation of

[1] Abbreviations used: DPI, phosphatidylinositol 4-phosphate; TPI, phosphatidylinositol 4,5-biphosphate; PI, phosphatidylinositol; SPM, synaptic plasma membranes; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetic acid; HPTLC, high-performance thin-layer chromatography.

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brain tissue *in situ* was performed as described by Wiegant *et al.* (11). Dissection of brain areas was carried out as described by Gispen *et al.* (12).

Incubation of SPM. The enriched SPM pellet was suspended in buffer A, containing 10 mM Na-acetate, 10 mM Mg-acetate, and 1 mM EGTA (pH 6.5). Aliquots of 100 μl of SPM (containing 100 μg protein) were incubated at 30°C. After 5 min 100 μl buffer A plus or minus 4 mM Ca^{2+} was added. After various times the incubation was terminated by adding 2 ml chloroform/methanol/12 N HCl (200:100:0.75 by vol, solution A).

Extraction and separation of lipids. Lipid extraction and separation was performed as described by Jolles *et al.* (3). Two milliliters of solution A was added to 200-μl aliquots (containing 100 μg of protein) of synaptosomes, SPM, or homogenates of microwave-fixed brain tissue, or to variable amounts of a standard mixture of the phosphoinositides (obtained from Sigma, St. Louis, Mo.). After the addition of 0.2 ml 1.2 N HCl a biphasic system was obtained. The upper phase was removed and the lower phase which contained the lipids was transferred to another glass tube and dried under N_2 at 30°C. The lipids, attached to the wall of the tube, were collected in 0.1 ml solution B (chloroform/methanol/H_2O, 75:25:2 by vol). After drying under N_2 again, the tubes were put in ice. The lipids were redissolved in 15 μl cold solution B and, for quantitative analysis, 5 μl was applied with a micropipet as a spot to silica gel high-performance thin-layer chromatography plates (HPTLC plates, layer thickness 25 μm, Merck, Darmstadt, FRG). Prior to use the silica gel plates were impregnated with K-oxalate (1%, w/v in methanol/H_2O, 2:3 by vol) and activated at 110°C for at least 15 min. Lipids were separated by ascending chromatography in a paper-lined chromatography chamber with chloroform/acetone/methanol/glacial acetic acid/H_2O (40:15:13:12:7 by vol) for 50 min at room temperature. After development, lipids were visualized with iodine vapor.

Quantification of PI. PI was scraped from the plates and, together with the silica, transferred to small Pyrex glass tubes (14 × 38 mm). Phosphorus was determined according to a modification of the method described by Hess and Derr (13). In short, 120 μl of a solution containing 10 N H_2SO_4 and 70% perchloric acid (1:6 by vol, solution C) was added to the samples and PI was digested for 30 min at 180–200°C. After cooling 90 μl 0.02 N H_2SO_4 was added and the silica was removed by centrifugation. The phosphorus color reagent consisted of 3 vol of malachite green (0.045% w/v in H_2O) and 1 vol ammonium molybdate (4.2% w/v in 4 N HCl), stirred for 30 min and filtered through Whatman No. 5 filter paper immediately before use. The supernatant containing inorganic phosphorus (30 μl) was mixed with the color reagent (150 μl) and the absorption at 660 nm was measured after 10 min in a Zeiss spectrophotometer equipped with a microcuvette holder.

Recovery of PI was determined by comparison with an inorganic phosphorus standard consisting of KH_2PO_4 (dried 4 h at 110°C) as a stock solution in 0.02 N H_2SO_4. Dilutions were made so that 40-μl portions contained 0–6 nmol phosphorus. After drying 40 μl of the standard solutions in the Pyrex glass tubes at 180–200°C 40 μl solution C was added and the tubes were heated for 30 min at 180–200°C. Subsequently, 30 μl 0.02 N H_2SO_4 was added, followed by 150 μl of the phosphorus color reagent. The absorption was read as described above.

Quantification of DPI and TPI. The method of Hedegaard and Jensen (14) was used for the determination of DPI and TPI. In short, the HPTLC plates previously stained with iodine were sprayed to transparency with 3% (w/v) Cu^{2+}-acetate in 8% (w/v) aqueous H_3PO_4. They were charred at 180°C for 10 min. The brown spots were subjected to densitometric scanning at 450 nm with a Zeiss PM-Q II spectrophotometer with a KM_3 chromatography attachment. The area under the peak was determined by the use

of a digitizer connected to a microcomputer (Hewlett–Packard, Type 9864A). The amounts of DPI and TPI were estimated by interpolation of curves obtained from standard amounts of DPI and TPI, which were run through the same extraction procedure and were separated on the same HPTLC plates as the biological samples.

Determination of protein. Amounts of proteins were determined by the method of Lowry *et al.* (15).

RESULTS AND DISCUSSION

The estimated amounts of PI in brain tissue reported are at least 10 times higher than those of DPI and TPI (16). Therefore, two different methods were combined to measure PI as well as DPI and TPI in the same sample of lipids after a one-step separation procedure. Figure 1 shows the standard curve of PI obtained after the phosphorus assay. The optical density at 660 nm was linear in the range 0–6 nmol PI. The smallest amount of PI that gave a significant difference from a blank value was 0.6 nmol PI. The values shown in Fig. 1 were obtained with only $\frac{1}{9}$ part of the original sample, since quantitative extraction of the lipids from the original samples and from silica plates necessitated the use of larger volumes than required for subsequent steps. It might be possible to adapt the procedure to higher proportions of the original samples, but in our assay the levels of DPI and TPI, rather than those of PI, determined the minimal amount of tissue to be used. The overall reproducibility of the phosphorus assay was estimated from quadruplicate measurements. The standard error of the mean (SE) was usually about 5 to 10%. The recovery of PI through extraction, chromatography and phosphorus assay was calculated by comparison with inorganic phosphorus standards and was 50–60%. This value was also reported by Farese *et al.* (17) for the determination of DPI and TPI on a 10- to 100-nmol scale.

In principle, the phosphorus assay would also be applicable to determine amounts of DPI and TPI. However, the low levels of these lipids compared to those of PI make it impossible to separate sufficient amounts of DPI and TPI without disturbing the separation pattern by the excess of PI and other phospholipids. Therefore, a more sensitive method was used to determine amounts of DPI and TPI. The method of Hedegaard and Jensen (14) relates the absorption of charred spots to that of standards. In Fig. 2A the chromatogram of increasing amounts of a standard mixture of PI, DPI, and TPI is shown. The amount of phospholipids in the standard mixture was calculated by weighing the lipids and by measuring the phosphorus by the microphosphorus assay as described above. The amounts of the lipids obtained after weighing and after using the phosphorus assay were in agreement. The smallest amount that gives detectable charring was about 0.1 nmol PI, DPI, or TPI. The values of the peak area obtained after densitometric scanning showed linearity in the range 0–1 nmol (Fig. 2B). Quadruplicate measurements of the standards resulted in a SE between 5 and 10%.

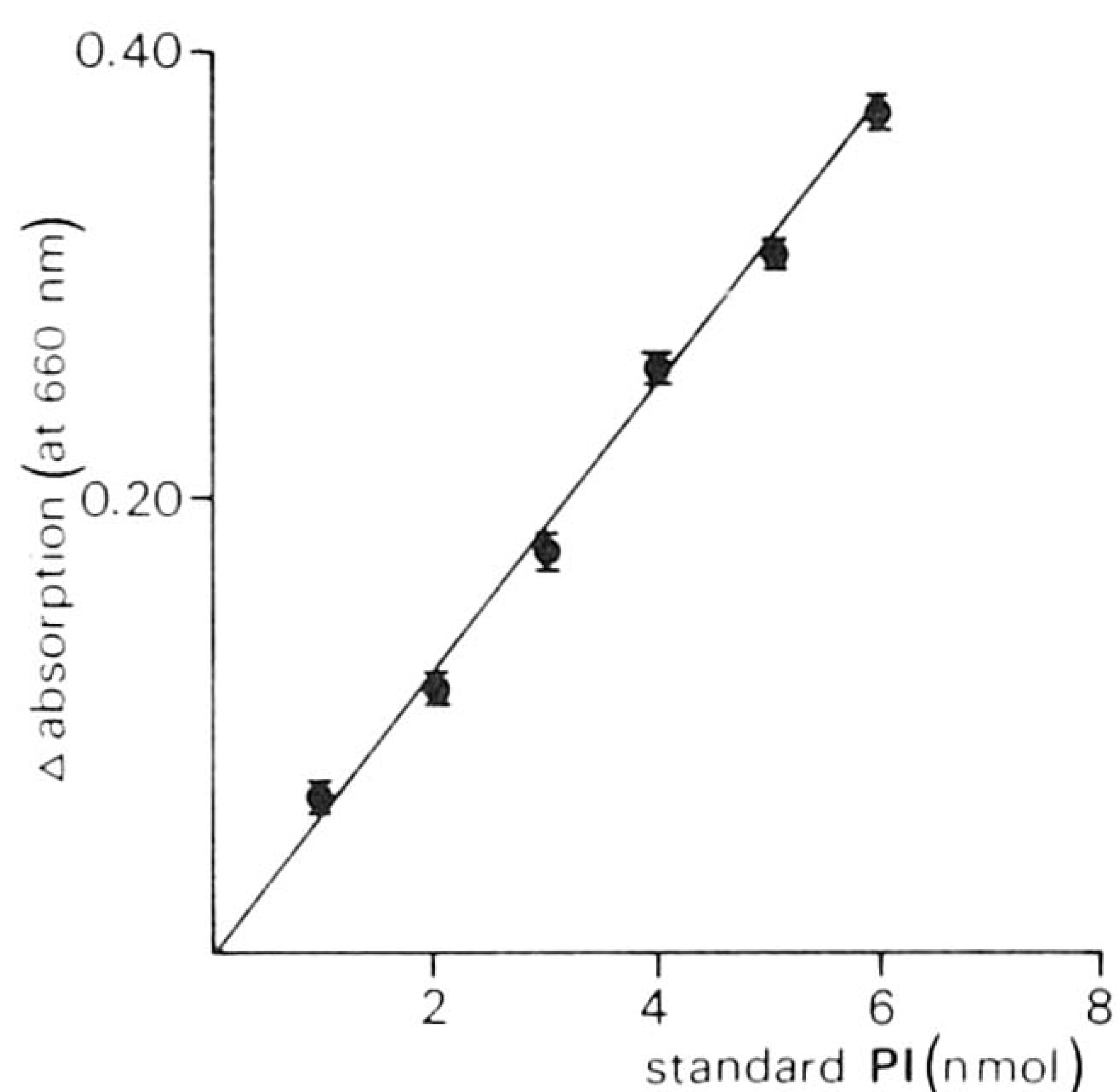

FIG. 1. Standard curve of PI. Standard PI was applied on a silica gel HPTLC plate. After the plate was developed, the amounts of phosphorus of the PI spots were determined as described under Materials and Methods. The amounts of PI on the ordinate are one-third of the amounts of PI applied on the silica gel plate. The absorption of blank areas from the silica gel plate was subtracted from the sample values. Each point is the mean ± SE for quadruplicate determinations.

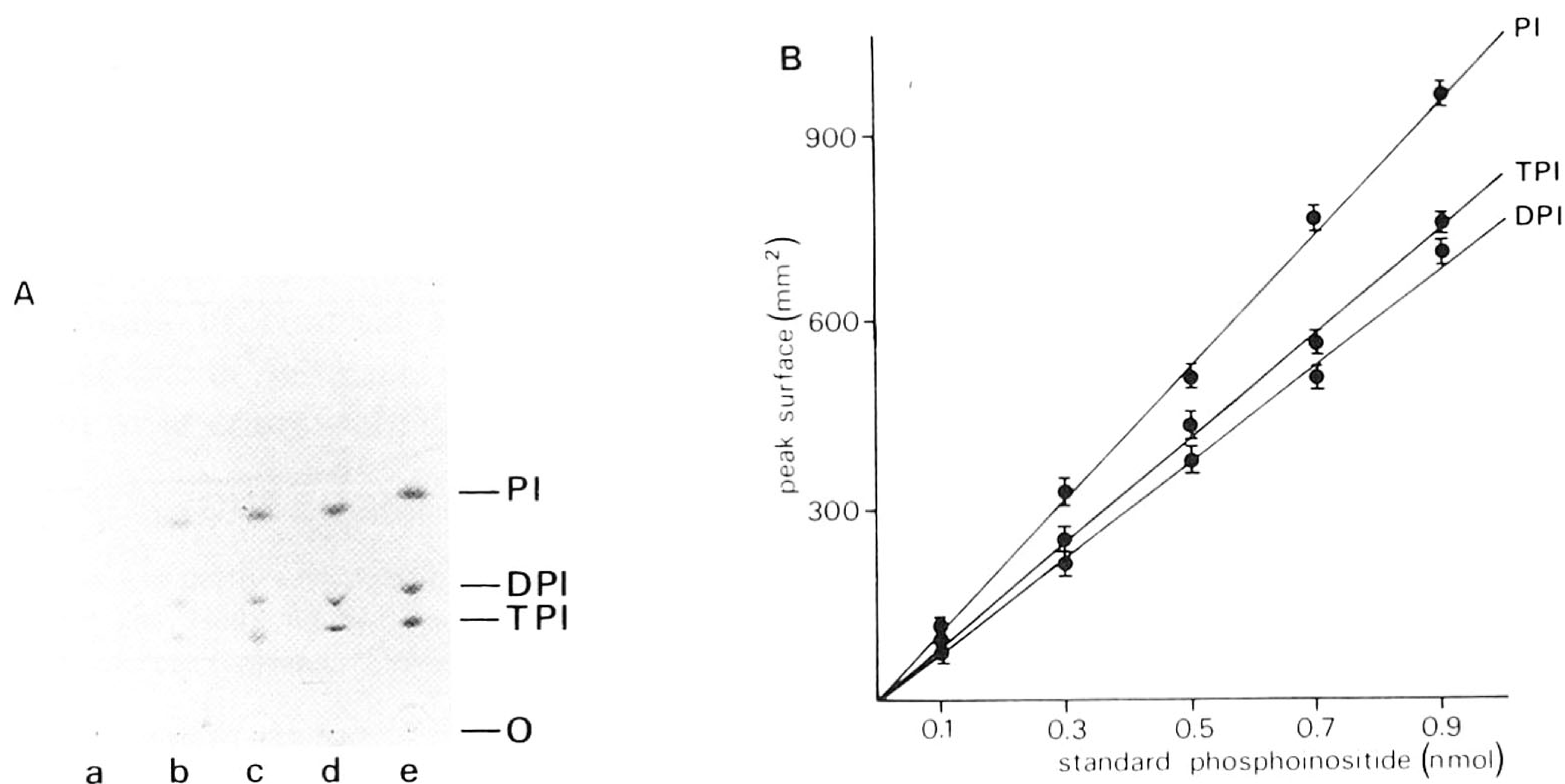

FIG. 2. (A) Chromatogram of standard PI, DPI, and TPI. Different amounts of standard phosphoinositides are applied on a silica gel HPTLC plate. After development the plate was sprayed with 3% Cu^{2+}-acetate in 8% aqueous H_3PO_4 and charred at 180°C for 10 min. Lanes a–e represent the patterns obtained when, respectively, 0.1, 0.3, 0.5, 0.7, and 0.9 nmol of the individual phospholipids were subjected to the HPTLC plates. O, origin. (B) Standard curves of PI, DPI, and TPI. The charred chromatogram of various amounts of PI, DPI, and TPI was subjected to densitometric scanning. Quantification of the peaks was performed by measuring the area under the peaks (mm^2). Each point is the mean ± SE for quadruplicate determinations.

The phosphorus assay and the densitometric assay were used to determine levels of the phosphoinositides in different biological samples. In the densitometric assay the degree of charring is related to fatty acid unsaturation (14). Therefore, the variations in the degree of unsaturation of phosphoinositides from rat brain and from bovine brain (source of the standards) may have resulted in a systematic error in the method, yet it may be too small to be of relevance (14).

Figure 3 shows the charred chromatogram of synaptosomal phospholipids. PI is one of the major phospholipids, whereas DPI and TPI are visible as minor spots (Fig. 3). Cardiolipin, presumably extracted from intrasynaptosomal mitochondria, was also present. Some poorly detectable spots near the position of DPI and TPI probably represent lysophosphoinositides.

In Table 1 the determined amounts of PI, DPI, and TPI in synaptosomes are shown. In addition, we determined the levels of phosphoinositides in SPM and in homogenates of hippocampus, septum and neostriatum of rats killed by microwave irradiation to prevent postmortem degradation (Table 1). An interesting difference was found between the levels of TPI in synaptosomes and SPM and in the levels found in the three homogenates. Rapid hydrolysis of TPI in synaptosomes and SPM (18) and variations in TPI-rich myelin (18,19) are likely causes for these differences. The levels of DPI and TPI reported here are similar to those reported by other investigators (1,2). However, the levels of PI (70–100 pmol/μg protein, Table 1) are high when compared with values reported by others (about 0.5–3.0 μmol/g tissue; (2,6,20)). Factors such as the method of preparing the tissue, extraction of PI, and subsequent separation may be responsible for this discrepancy. We employed another separation procedure (used by Farese *et al.* (17)) and a different separation system (used by Schacht *et al.* (21)), but we found the same values for the level of PI in the homogenates of microwave-irradiated rat brains.

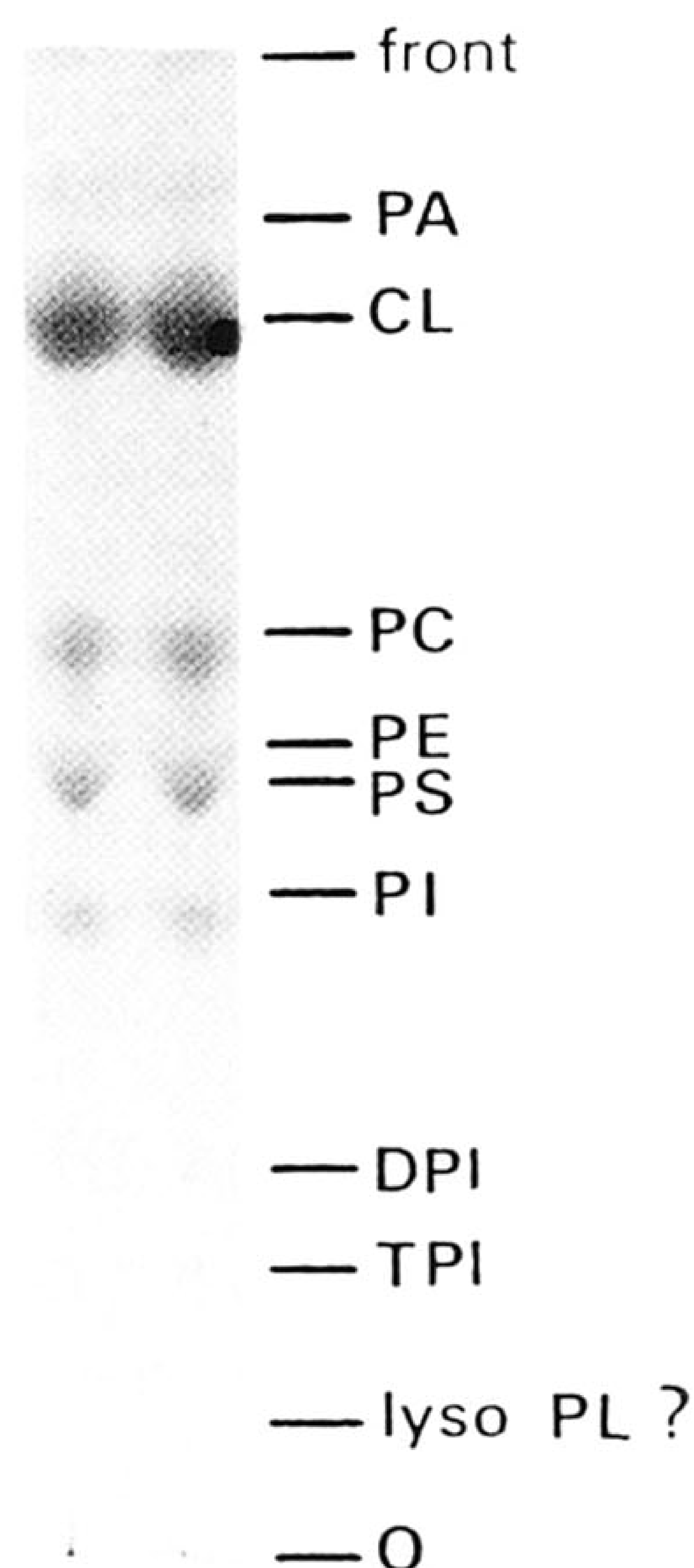

FIG. 3. Chromatogram of synaptosomal phospholipids. Phospholipids extracted from synaptosomes (100 μg protein) were subjected to a HPTLC plate. After development the plate was sprayed and charred as described in Fig. 2A. The identification of the spots was performed by comparison with pure, commercially available standards. O, origin; lyso PL, lysophospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid.

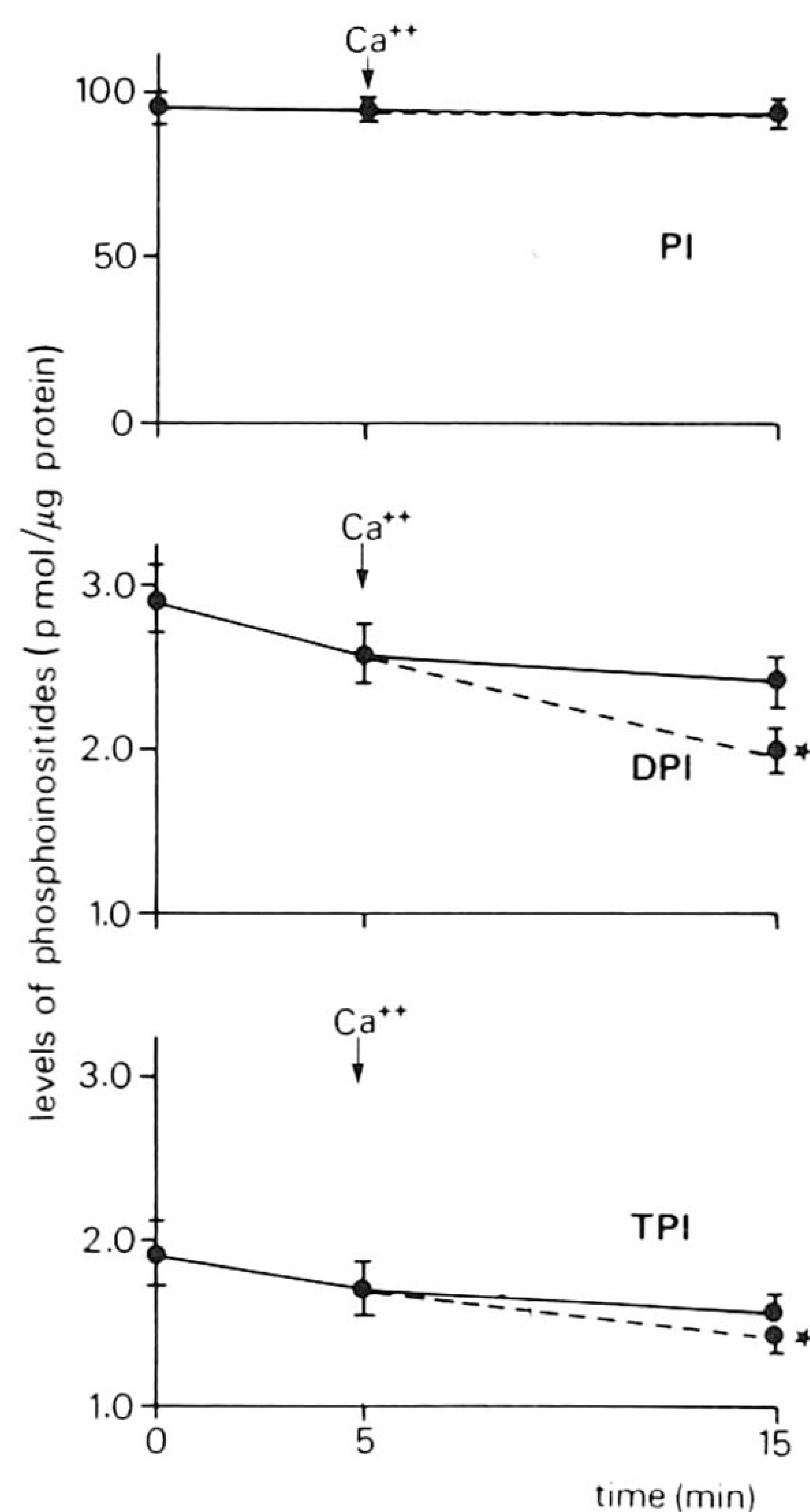

FIG. 4. Influence of Ca^{2+} on PI, DPI, and TPI levels in SPM. SPM was incubated at 30°C for 5 min. Subsequently, buffer solution plus (- - -) or minus (——) Ca^{2+} was added. The final Ca^{2+} concentration was 1 mM. After different time periods the incubation was terminated and the values of PI, DPI, and TPI were estimated (see Materials and Methods). Bars indicate mean ± SE, $n = 4$; * $P < 0.01$ (Student's t test).

Incubating SPM at 30°C resulted in a slight decrease of the amounts of DPI and TPI (Fig. 4). After 5 min about 10% of the total amounts of DPI and TPI disappeared, while after 15 min about 20% had disappeared. Incubating SPM in the presence of Ca^{2+} lowered significantly the amount of the polyphosphoinositides. Apparently, Ca^{2+} stimulated the breakdown of polyphosphoinositides, which is in agreement with studies employing radiolabeled lipids (1,2,5,22). In these studies it was found that polyphosphoinositide phosphodiesterase was stimulated by Ca^{2+} (5). The absence of an effect on PI may be due to the high basal level of this phospholipid. Changes in the same order of magnitude as observed for DPI and TPI (i.e., 0.2–0.4 pmol/μg protein) may not be detectable in PI (basal level about 91 pmol/μg protein).

TABLE 1

LEVELS OF PHOSPHOINOSITIDES IN SYNAPTOSOMES, SPM, AND HOMOGENATES OF HIPPOCAMPUS, SEPTUM, AND NEOSTRIATUM. THE HOMOGENATES WERE OBTAINED FROM DISSECTED BRAIN AREAS OF RATS KILLED BY MICROWAVE IRRADIATION. RESULTS ARE EXPRESSED AS pmol/μg PROTEIN ± SE ($n = 3$).

	PI	DPI	TPI
Synaptosomes	88 ± 4	2.8 ± 0.2	2.0 ± 0.2
SPM	91 ± 5	2.9 ± 0.2	1.9 ± 0.2
Hippocampus	50 ± 4	2.9 ± 0.2	9.3 ± 0.3
Septum	58 ± 4	3.6 ± 0.3	10.2 ± 1.0
Neostriatum	78 ± 5	4.3 ± 0.3	14.1 ± 1.1

In conclusion, the method presented here allows the determination of absolute amounts

of the phosphoinositides in synaptosomes (100 μg protein), SPM (100 μg protein), and in small amounts of brain tissue (1 mg wet wt). In the study of the metabolism of phosphoinositide lipids, the presently described method allows the determination of the absolute amounts in contrast to the studies analyzing the fate of radiolabel of these lipids, as has been most commonly done to date.

ACKNOWLEDGMENT

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