Purification of polyphosphoinositides by chromatography on immobilized neomycin

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Summary The binding of polyphosphoinositides (phosphatidylinositol phosphate and phosphatidylinositol bisphosphate) to the antibiotic neomycin is utilized for the purification of these lipids. Neomycin is reductively coupled to reactive glass beads (Glycophase-CPG) and serves as the stationary phase in column chromatography. A total lipid extract is prepared from tissues with chloroform–methanol–KCl or chloroform–methanol–HCl and washed once with acidified methanol–water. After the addition of an equal volume of methanolic 200 mM ammonium acetate, the extract is directly applied to the column. All lipids but the polyphosphoinositides are removed from the column by rinsing with 150 mM ammonium acetate in chloroform–methanol–water. Increasing the salt concentration to 600 mM elutes phosphatidylinositol phosphate. While further increases in ionic strength are not sufficient for a quantitative removal of phosphatidylinositol bisphosphate, the lipid is completely eluted by the addition of either ammonia or HCl to the solvent. The column can be recycled and used repeatedly.

Supplementary key words affinity chromatography · phosphatidylinositol phosphate, bisphosphate · di-, triphosphoinositide

The polyphosphoinositides, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate,[1] are quantitatively minor constituents of most eukaryotic tissues. They are of special biochemical interest because of the rapid turnover of their monoesterified phosphate groups, which appears to be responsive to physiological stimuli applied to the tissues (1–5). A metabolically less active pool has been reported present in myelin (6).

The polyphosphoinositides were first isolated from brain and characterized by Folch (7), and procedures of extraction and purification have since been repeatedly improved (8–10). Nevertheless, presently available methods for the isolation of these lipids are tedious and time-consuming. We report here a rapid and quantitative purification of the two lipids based on the specific affinity of the aminoglycosidic antibiotic neomycin to the polyphosphoinositides.

We have previously observed effects of neomycin on the metabolism of polyphosphoinositides in kidney (11) and the inner ear (12, 13) in vivo. In vitro studies with subcellular fractions from brain (14) and with monomolecular films of polyphosphoinositides (15, 16) suggested a direct interaction between the antibiotic and the lipids. These interactions were specific for the polyphosphoinositides and were not seen with other lipids, including phosphatidylinositol. By using immobilized neomycin as the stationary phase in column chromatography, this affinity can be utilized for the purification of the polyphosphoinositides.

Methods

Labeling and extraction of polyphosphoinositides. Albino guinea pigs (ca. 300 g) were injected with 25 μl of neutralized [^{32}P]orthophosphate in saline (100 μCi, carrier-free) into each cerebral hemisphere. One hr later the animals were killed by decapitation and the brain was quickly excised and homogenized in 10 ml of chloroform–methanol 1:2 (v/v) with a Polytron (Brinkman Instruments, Westbury, NY).

Aliquots of the homogenate were extracted by two different procedures. Per ml of homogenate were added: (A) 0.6 ml of 1 M KCl and 0.6 ml of chloroform (adapted from (8)); or (B) 0.6 ml of 1.2 N HCl and 0.6 ml of chloroform (adapted from (9)). After centrifugation, the lower phase was collected, and the

[1] 1-(3-*sn*-Phosphatidyl)-D-myo-inositol 4-phosphate (1-phosphatidylinositol 4-phosphate) and 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4,5-bis(phosphate) (1-phosphatidylinositol 4,5-bisphosphate).

interface and upper phase were extracted twice more with 1 ml of chloroform each. The pooled lipid extracts were washed once with methanol–1 N HCl 1:1 (v/v). An equal volume of 200 mM ammonium acetate in methanol was added to the extract which was then applied to the chromatographic column (see below). If the lipids were to be stored instead, the washed extract was dried under a stream of nitrogen at a water bath temperature of 35°C, and the dried lipids were kept at −20°C. Under these conditions, degradation of the lipids seemed minimal.

For thin-layer chromatography, dried lipids were taken up in chloroform–methanol 2:1 (v/v) and applied to pre-coated thin-layer plates of Silica Gel 60 (EM Lab, Elmsford, NY) and developed in chloroform–methanol–15 N NH_4OH–water 90:90:7:22 (by vol.). Radioactive lipids were located by radioautography on Kodak NO-Screen X-ray film, scraped off the plates, and counted in a liquid scintillation counter.

Preparation of immobilized neomycin. The supporting matrix was Glycophase-CPG (Pierce, Rockford, IL). Two types were used with essentially similar results: 120/200 mesh with 550 Å pore diameter, and 200/400 mesh with 250 Å pore diameter. Approximately 20 g of beads were oxidized to the aldehyde form with 500 ml of 6 mM $NaIO_4$ at room temperature for 1 hr (17). The suspension was agitated on a shaker and vacuum was frequently applied to remove gas bubbles trapped within the matrix. The oxidized beads were washed three times with 500-ml portions of distilled water.

One liter of 60 mM neomycin sulfate (USP grade), adjusted to pH 9 with NaOH, was added to the oxidized beads. At 20 min and 40 min after this addition, approx 200 mg of sodium borohydride was added and vacuum was applied frequently. The reaction was terminated at 60 min and the glass–neomycin adduct was washed with 500 ml each of distilled water, 1 mM HCl, distilled water, and methanol–water 1:1 (v/v). The product may be stored at room temperature in methanol–water or may be equilibrated with the chromatography solvent, 150 mM ammonium acetate in chloroform–methanol–water 3:6:1 (by vol.).

Neomycin is a polyamine with six primary amino groups. By providing a large excess of neomycin in the coupling procedure, crosslinking should be minimized. The high pH facilitates Schiff base formation and should also assure that coupling occurs randomly over the amino groups unless steric factors intervene. This may be important since the specific configuration of amino groups required for the lipid binding is yet unknown.

Thorough reduction of the neomycin adduct is essential, since otherwise amino group-containing phospholipids can engage in Schiff base formation with the aldehydic matrix.

Results

Column chromatography. The two extraction procedures were found equally effective in extracting phospholipids, including polyphosphoinositides, from brain and kidney. The KCl procedure was preferred because it extracted less colored (nonphospholipid) material. Radioactive labeling of the lipids by $^{32}P_i$ was utilized to visualize the polyphosphoinositides after separation on thin-layer chromatograms. They are difficult to detect with chemical methods since they comprise only 1–2% of the total phospholipids. The rapid turnover of their monoesterified phosphate groups in brain and kidney, however, provides for a high level of radioactivity in these lipids. (**Fig. 1,** *S*).

The kinetics of polyphosphoinositide binding to the neomycin adduct appears to be sufficiently fast to permit high flow rates. Routinely, 10–20 column volumes of solvent were applied per hr, but occasionally flow rates of up to 60 column volumes/hr were used without apparent detriment to the separation obtained.

Preliminary experiments had indicated that phospholipids other than polyphosphoinositides can be displaced from neomycin at rather low ionic strength of the solvent (40–100 mM ammonium acetate). Indeed, most phospholipids do not bind to the neomycin adduct under the conditions under which the lipid extract is applied to the column. In Fig. 1, fractions 1 and 2 are column eluates collected while the original extract was loaded. Samples 1 and 1′ are different aliquots of the same eluate, demonstrating the dependency of the R_f values on the amount of lipid separated. Washing the column with 150 mM ammonium acetate in chloroform–methanol–water 3:6:1 (by vol.) will elute all other phospholipids without removing the polyphosphoinositides (Fig. 1, fractions 3 and 4). In fractions 3 and 4, five and three column volumes, respectively, were collected. Fraction 4 contained less than 2% of the radioactivity eluted in fractions 1–4. Treating the chromatographic plates with phosphate-detecting and amino group-detecting sprays confirmed what appeared evident from the pattern of radioactivity, i.e., no lipids other than the polyphosphoinositides were detectable in eluates subsequent to fraction 4.

Elution of phosphatidylinositol phosphate begins at a concentration of about 350 mM ammonium acetate, but for elution in a smaller volume, 600 mM should be applied. At this higher concentration of ammonium acetate in chloroform–methanol–water 3:6:1 (by vol),

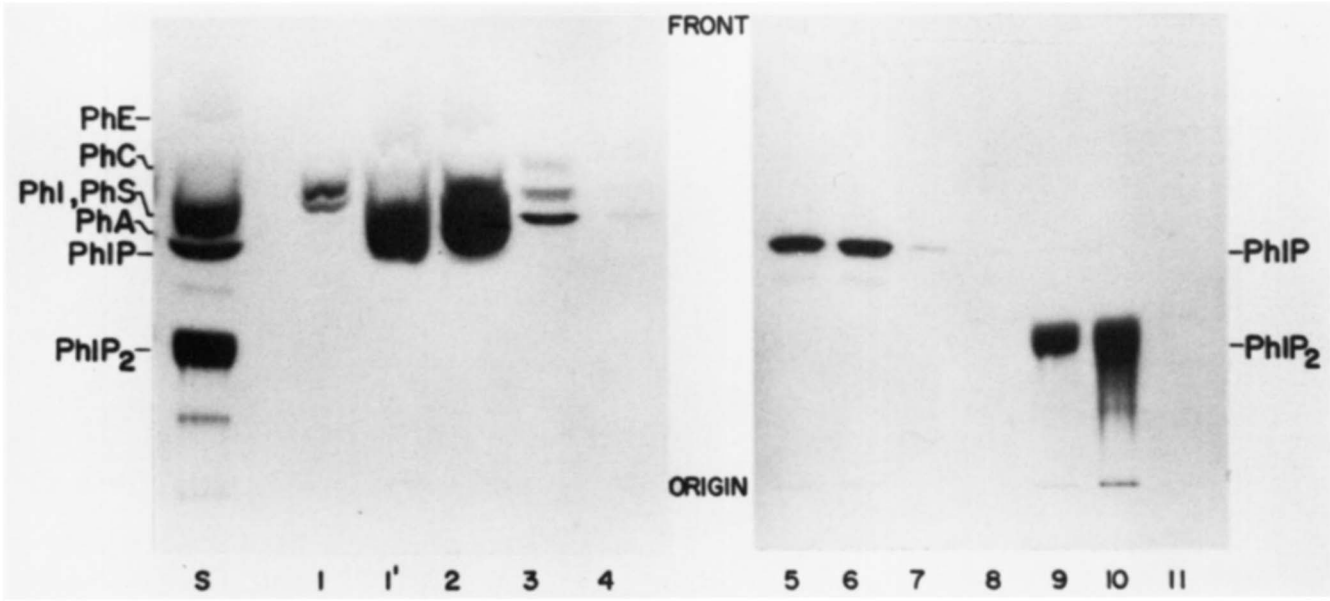

Fig. 1. Characterization by thin-layer chromatography of lipid fractions separated on immobilized neomycin. A total lipid extract from half a guinea pig brain, previously labeled with $^{32}P_i$, was applied to 0.6 ml of immobilized neomycin. Aliquots of the extract (*S*) and of the column fractions (1–11) were separated by thin-layer chromatography and the above radioautogram was obtained as described in Methods. Fractions 1 and 2 were collected while extract was applied to the column. Fractions 3 and 4 were eluted with 150 mM ammonium acetate in chloroform–methanol–water 3:6:1 (by vol.); volumes collected were 3 ml and 2 ml, respectively. Fractions 5–8 were eluted with solvent containing 600 mM ammonium acetate, fractions 9–11 with chloroform–methanol–15 N NH_4OH 3:6:1 (by vol.); elution volume, 3 ml each. Various aliquots of the column fractions were applied to the plate. From fraction 1, two different aliquots, 1% and 4% of the eluted volume, were spotted (samples 1 and 1′). Abbreviations: PhE, PhC, PhI, PhS—phosphatidylethanolamine, choline, inositol, serine; PhA—phosphatidic acid; PhIP, $PhIP_2$—phosphatidylinositol phosphate, bisphosphate.

over 90% of the lipid was eluted in 10 column volumes of solvent (Fig. 1, fractions 5 and 6). Phosphatidylinositol bisphosphate was not detected in the eluates, even after 30 column volumes of solvent.

By increasing the ammonium acetate concentration to 750 mM or 1 M, phosphatidylinositol bisphosphate could be displaced from the column. However, even at 1 m salt, displacement was inefficient, and in two experiments quantitative recovery was not achieved after elution with 30 column volumes of solvent (less than 80% of lipid eluted). Still higher salt concentrations increase the viscosity, resulting in impractical flow rates. Conveniently, phosphatidylinositol bisphosphate can be eluted with 10 column volumes of chloroform–methanol–15 N NH_4OH 3:6:1 (by vol) as shown in fractions 9 and 10, Fig. 1. Alternatively, the lipid can be displaced with chloroform–methanol–12 N HCl 3:6:1 (by vol). The use of neomycin as a displacing agent is unfeasible because of its very low solubility in organic solvents.

After elution, the fractions are acidified and the lipids washed free of salt. For each 10 ml of solvent containing 600 mM ammonium acetate, 3 ml of 2.4 N HCl are added; for each 10 ml of ammoniacal solvent, 3 ml of 6 N HCl are added. The resulting upper phase is removed, the lower phase is washed twice with an equal volume of methanol–1 N HCl 1:1 (v/v) and dried under nitrogen prior to storage.

Phospholipids should be exposed to acidic or basic solvents only briefly because hydrolysis may occur. If such precaution is taken, the concentrations of ammonia or HCl used here will not cause significant degradation of lipids.

The yields of polyphosphoinositides were calculated on the basis of their radioactivity in the starting sample (determined after thin-layer chromatographic separation of the total lipid extract) compared to that in the respective column eluate. In three experiments under similar conditions, yields were 91 ± 13% SD for phosphatidylinositol bisphosphate, and 88 ± 6% SD for phosphatidylinositol phosphate. In two preliminary experiments, the polyphosphoinositide content of guinea pig brain, as obtained under the conditions described in Methods, was determined. There were 380 nmol of phosphatidylinositol bisphosphate and 160 nmol of phosphatidylinositol phosphate per g wet brain. These values are similar to those reported for rat brain (9).

Identification of polyphosphoinositides. The fractions obtained cochromatographed with standards prepared by the procedure of Hendrickson and Ballou (10) in two solvent systems: the standard

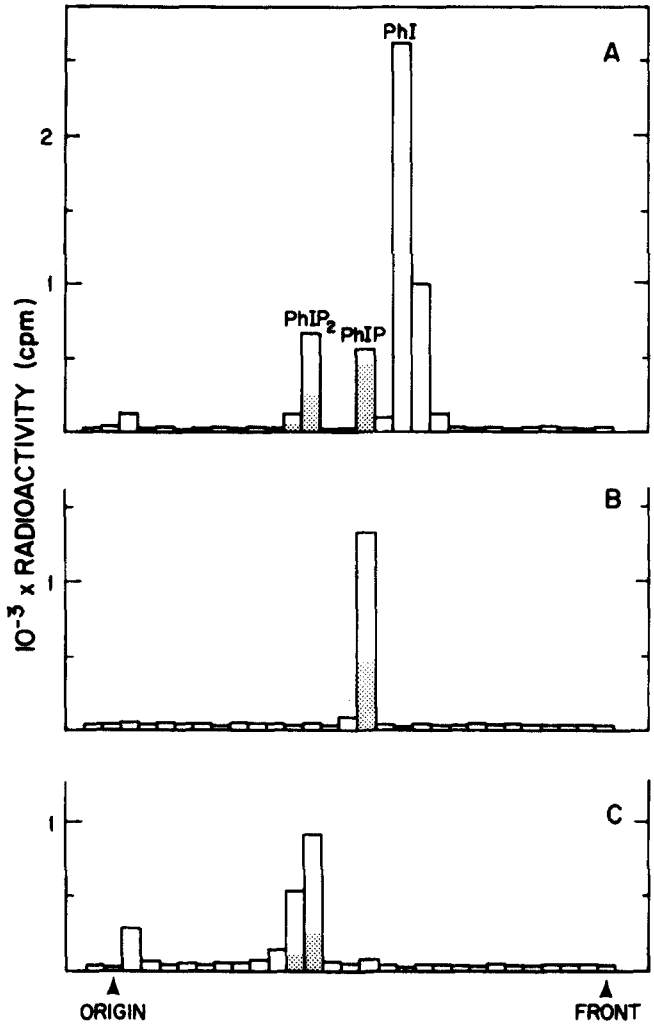

Fig. 2. Thin-layer chromatographic separation of lipid fractions labeled with [^{3}H]inositol. Three lipid fractions were separated by thin-layer chromatography as described in Methods. After separation, the chromatographic plate was divided into 5-mm sections which were scraped into vials and counted by liquid scintillation. *A*, total lipid extract; *B*, eluate from immobilized neomycin with solvent containing 620 mM ammonium acetate; *C*, eluate with ammoniacal solvent. Solid bars, ^{3}H-radioactivity; hatched bars, previously prepared ^{32}P-polyphosphoinositides cochromatographed with ^{3}H-labeled lipids. Abbreviations: PhI, PhIP, $PhIP_2$—phosphatidyl inositol, phosphate, bisphosphate.

ammoniacal solvent (see Methods), and in chloroform–methanol–acetic acid–water 50:32:11:3 (by vol). Furthermore, when brain lipids were labeled with myo-[^{3}H]inositol, radioactivity was found in phosphatidylinositol and the two fractions identified as polyphosphoinositides. **Fig. 2** shows the fractionation of such a labeled extract. The sample in *A* contains the phosphoinositides and a fourth, unidentified component near the origin of the chromatographic plate. The fraction eluted from the neomycin column with 620 mM ammonium acetate (*B*) contains virtually only phosphatidylinositol phosphate, while the ammoniacal eluate (*C*) contains phosphatidylinositol bisphosphate and the fourth component. The tritiated lipids comigrate with previously prepared ^{32}P-labeled polyphosphoinositides (hatched areas).

Purity of the fractions. Purity of the fractions was determined from thin-layer chromatography as above. Phosphate reagent (18) gave only one spot while a contamination of less than 5% would have been detected. Ninhydrin-positive material was absent. Over 90% of the radioactivity was consistently associated with the appropriate lipid. Some ^{32}P was always detectable at the origin of the chromatographic plate and, occasionally, in a distinct band below the lipids (Fig. 1, fractions 5, 6, 9, 10).

Capacity of the material. With several batches of immobilized neomycin, it was observed that 1 ml of beads was sufficient for the separation of lipids from a guinea pig brain (3–3.5 g wet weight). Obviously, the capacity of the material is determined by the number of functional groups on the native beads and the efficiency of the coupling with neomycin. Neomycin density on the beads can be estimated by titrating the amino groups ($pK_{neomycin} = 8.6$). Such analysis of one preparation yielded a concentration of approximately 7 μmol of neomycin per ml of beads. The binding capacity was about 2 μmol polyphosphoinositide-phosphate.

Recycling of the material. The neomycin–glass adduct appears to be relatively stable in organic solvents and to the brief exposures to ammonia or acid. After the last elution, the column can be returned to the starting buffer and used again. Because of the rigidity of the supporting matrix, the material can remain packed in the column for several cycles.

Discussion

The method presented here offers several advantages over previous isolation procedures for polyphosphoinositides. *1*) It is quantitative for both phosphatidylinositol phosphate and bisphosphate. There are no steps such as prior neutral extractions or fractionations in which polyphosphoinositides may be lost. *2*) It is rapid. Only one tissue extract is prepared and directly applied to the column. Elution of polyphosphoinositides is accomplished in two steps. No gradient elution is necessary. *3*) The purity of the fractions is high. *4*) The presence of other lipids in the lipid extract does not interfere with the binding of polyphosphoinositides. The procedure should therefore be suitable for the isolation of these lipids from tissues with low levels of polyphosphoinositides. *5*) It can be modified to a batch-wise procedure if speed of isolation is favored over purity of the fractions. *6*) It saves solvents over

previous procedures and considerably reduces the use of toxic chloroform.

The high affinity of neomycin for the polyphosphoinositides becomes apparent when one compares this method to phospholipid chromatography on DEAE-cellulose (10). As a primary amine, neomycin acts as a weaker ion exchanger than DEAE. Most phospholipids are eluted from the neomycin column at a salt concentration of 100 mM or less while for their elution from DEAE approximately 200–250 mM salt is required. In contrast, 400–500 mM ammonium acetate is needed for the elution of phosphatidylinositol phosphate from both exchangers. The bisphosphate is removed from DEAE at 600 mM salt while an efficient elution from neomycin is not even accomplished with 1 M ammonium acetate.

The selective binding of neomycin to the polyphosphoinositides strongly supports our previous suggestions (11, 14, 16) that these lipids may be membrane receptors for aminoglycosidic antibiotics in vivo. This interaction may play a crucial role in the nephro- and ototoxicity of these drugs.

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