POLYPHOSPHOINOSITIDE METABOLISM IN RAT LIVER NUCLEAR ENVELOPES AND ITS ROLE IN REGULATING NUCLEOSIDE TRIPHOSPHATASE ACTIVITY

By

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ABSTACT

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Studies were initiated to investigate the role of the phosphorylation of nuclear envelope components in the regulation of the function of this membrane system. Nuclear envelopes isolated from rat liver were incubated with [γ-^{32}P]ATP and the labeled products were characterized. In addition to a number of protein phosphorylation products, a low molecular weight material was observed and identified as a mixture of labeled phosphatidic acid, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). The enzymes responsible for the synthesis of these phospholipids were identified as nuclear envelope components and were further characterized. Degradation of labeled PIP was observed after depletion of the radioactive ATP. The enzyme responsible for the hydrolysis of this lipid was identified as a PIP-specific phosphomonoesterase. Therefore, nuclear envelopes are able to interconvert phosphatidylinositol (PI) and PIP through a simple phosphorylation / dephosphorylation cycle; a process well suited to be a regulatory mechanism in nuclear envelope function.

The intranuclear distribution of PI kinase and PIP phosphomonoesterase were examined using two techniques: 1) examination of their relative sensitivities to inactivation by free and immobilized trypsin, and 2) physical separation of the inner and outer nuclear membranes by derivatization with citraconic anhydride. Both techniques indicated that PI kinase and PIP phosphatase are located on the inner nuclear membrane. Studies of the distribution of newly synthesized PIP indicated that it accumulates in both the inner and outer nuclear membranes. Therefore, phospholipids but not proteins can exchange between the two membranes.

The role of polyphosphoinositides in regulating the nucleoside triphosphatase of the nuclear envelope was investigated by solubilizing the enzyme and delipidating it by ion exchange chromatography. Removal of the solubilized phospholipids and RNA by these procedures resulted in suppression of the ATPase activity. This activity could be partially restored through the addition of a polynucleotide. The quantitatively major phospholipids were ineffective in stimulating the ATPase activity; however, PIP in the presence of RNA reconstituted the ATPase to nearly full activity. We therefore believe that polyphosphoinositide are actively metabolized in the nuclear envelope and that they may be important in regulating nuclear envelope function.

To Staci and Scott

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ABBREVIATIONS

Abbreviations not listed below are standard usage according to the Journal of Biological Chemistry, Instructions to Authors, January, 1984.

ATPase	adenosine triphosphatase
CDTA	cyclohexanediaminetetraacatic acid
DEAE	diethylaminoethane
DG	diacylglycerol
DNase I	deoxyribonuclease I
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high pressure liquid chromatography
IP_3	myo-inositol-1,4,5-trisphosphate
MES	2-(N-morpholino)ethane sulfonic acid
NTPase	nucleoside triphosphatase
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIPase	phosphatidylinositol 4-phosphate phosphomonoesterase
PS	phosphatidylserine

SDS	sodium dodecylsulfate
Tricine	N-tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminomethane
2-ME	2-mercaptoethanol

INTRODUCTION

The phosphorylation and dephosphorylation of membrane components has often been proposed as a mechanism for the regulation of membrane activity. In 1981, Dr. Chrisitine Collins was studying the possibility that the phosphorylation of lysosomal membrane proteins might be involved in the hormonal regulation of lysosome activity. She came upon a phosphorylation product that was unusual in that it migrated with very high mobility during SDS-PAGE and could be extracted from gels with organic solvents. At this time, I began to study the phosphorylation of nuclear envelopes to see if a similar component might be present there. Extensive phosphorylation of nuclear envelope-associated proteins by endogenous protein kinase activity was observed, characterized, and is described in the Journal of Biological Chemistry, 258, 9360-9367 (1983). This manuscript is reproduced in part in Chapter I. In addition to the protein phosphorylation, the low molecular weight-organic soluble material was observed. This material was identified as a mixture of PA, PIP, and PIP_2, and the enzymes responsible for their synthesis were identified and characterized. These studies were published in the Journal of Biological Chemistry, 258, 9368-9373 (1983), which is paraphrased in Chapters I and

II. This led to further investigations of phosphoinositide metabolism in nuclear envelopes as described in Chapters III and IV. Chapter III is currently in press at Archives of Biochemistry and Biophysics. Chapter IV has been submitted to the Journal of Cell Biology. Demonstration of the active metabolism of PIP in nuclear envelopes prompted studies of its possible roles in nuclear envelope function as described in Chapter V. This manuscript is currently in press at the Journal of Biological Chemistry to be published in October or November of 1984.

LITERATURE REVIEW

By definition, a eucaryotic cell contains a nucleus which is enclosed by a bimembrane system called the nuclear envelope. The nuclear envelope therefore serves as a biological interface between the cytoplasm and the nucleoplasm of the cell. As such, it stands in the unique position of being able to regulate the exchange of information between these two cellular compartments. Although the nuclear envelope has been extensively studied at the morphological level, biochemical characterizations of its components and studies of the mechanisms by which its functions are regulated remain incomplete (reviewed in References 1-5).

Architecture of the eucaryotic nucleus. Delineating the nucleus is the nuclear envelope which is composed of two unit membranes; the outer nuclear membrane in contact with the cytoplasm and the inner nuclear membrane in contact with the nucleoplasm. This double membrane system is a universal feature of eucaryotes. The reason that the nuclear envelope has evolved as a bimembrane system remains unknown; although it may imply that the inner and outer nuclear membranes are involved in different functions (discussed below).

The outer nuclear membrane is continuous with the endoplasmic reticulum (6,7) providing a pathway for membrane flow between these organelles as proposed by Morre, et al. (8). The outer nuclear membrane is also thought to be continuous with the inner nuclear membrane at the sites of nuclear pore complexes (1-4). In work described in Chapter IV of this thesis (9), the pathway of membrane flow has been expanded to include the inner nuclear membrane. Ribosomes are often found to be attached to the outer nuclear membrane (1-5). These nuclear ribosomes have been proposed to be a functionally distinct subgroup of polyribosomes involved in the translation of nuclear proteins (10) and therefore may act as a mechanism for coupling specific protein synthesis with nuclear events (11,12).

Between the inner and outer nuclear membranes is the perinuclear space which is continuous with the lumen of the endoplasmic reticulum (1-5). This space is therefore thought to be filled with fluid similar to that of the cisternae of the endoplasmic recticulum. Antibodies have been observed in the perinuclear space of lymphoid cells (13,14), suggesting that nuclear ribosomes may be the sites of immunoglobulin synthesis in these cells. Franke, et al. (15), have described filaments in the perinuclear space which seem to link the outer and inner nuclear membranes. These structures may account for the

maintainance of the intermembrane distance (usually approximately 20 nm) and the resistance of the nuclear membranes to separation under mechanical stress.

Immediately underlying the inner nuclear membrane is an area called the internal dense lamellar region or the lamina. Ultrastructurally, this region appears as an area of densely packed proteins (1-3). However, using whole mount electron microscopy with detergent-extracted cells, a meshwork of fibers associated with the inner membrane can be distinguished (16,17). It is believed that this lamina serves as a scaffold for the nuclear envelope thus providing the structural rigidity of the nucleus. The lamina can be isolated from nuclear envelopes by extracting other components with detergent and high salt concentrations (18,19). Typically, three predominate proteins with apparent molecular weights of 60,000 to 80,000 daltons are observed. These proteins have been named lamins A, B, and C in order of decreasing molecular weight (20). Lamins A and C demonstrate considerable immunological crossreactivity (20,21), similarity in ionic properties (22-25), and sequence homology (24,26,27). In fact, there is evidence suggesting that lamins A and C are derived from a common precursor (28-30). In contrast, lamin B appears to be quite different from lamins A and C (22-27), and is composed of a family of several isoelectric point variants (24,31,32). The lamins appear

to be highly conserved across species lines (24). The lamins occur exclusively at the nuclear periphery in interphase cells (28,33), and are excluded from the areas of nuclear pore complexes. The lamins are DNA-binding proteins (34). In addition, lamin B has a hydrophobic domain which inserts into the inner nuclear membrane (21,25) and so this protein is thought to provide the link between the nuclear envelope and the heterochromatin condensed at the nuclear periphery (1-5).

The lamina is an active structure, in that it is reversibly depolymerized during cell division. During mitosis, the lamins become dispersed throughout the cytoplasm and are quantitatively recovered during the reassambly of the nuclear envelopes in the daughter cells (21). This depolymerization and reassembly correlates with changes in the level of phosphorylation of lamin B (21), suggesting that lamin B phosphorylation disrupts the interactions among lamins and leads to lamina breakdown. A different scenario occurs during a meiotic cell division (35). The lamina disappears during prophase leaving the nuclear membranes and pores structurally intact. The lamina then reassembles after the pachytene stage. This correlates with the degree of chromatin condensation in these cells, suggesting that the lamina may play an important role in organizing nuclear DNA (35). This is supported by studies of lamina structure during

spermiogenesis in which the lamina is completely absent in the nuclei of spermatocytes and spermatids (36) which exhibit a high degree of chromatin condensation.

The interior of the nucleus is filled with an array of filamentous structures collectively termed the internal nuclear matrix (16,17,37). This nuclear matrix is thought to provide the anchorage sites required to unwind DNA during its replication and the transcription of RNA (38-41). Evidence supporting these proposals come from experiments demonstrating that newly synthesized DNA is found to be selectively associated with the nuclear matrix (40,42-45). In addition, newly synthesized bulk RNA (46-51) and transcribed gene sequences (52-55) are associated with the nuclear matrix. This association appears to be involved in gene activation since, for example, the ovalbumin gene is associated with the matrix in the chicken oviduct where it is actively undergoing transcription, but it is not associated with the matrix in the liver where the gene is inactive (52). Similarily, steroid hormones bind to the nuclear matrix in responsive tissues but not in unresponsive cells (56). Specific proteins such as DNA polymerase (57), "prompt" heat shock proteins (58), polyoma T antigen (59), and adenovirus proteins (60,61) are also found to be associated with the nuclear matrix.

Like the lamina, the internal nuclear matrix appears

to be capable of undergoing reversible depolymerization. Chick erythrocyte nuclei, which are inactive in RNA and DNA synthesis, lack an internal nuclear matrix (62,63). However, a nuclear matrix is generated in these nuclei when they are fused with mouse L-cell cytoplasts (64). This fusion also results in the reactivation of chromatin condensation and recovery of DNA and RNA synthesis in the chick nuclei (65-69). Proliferation of the nuclear matrix is also seen during concanavalin A-induced transformation and proliferation of human peripheral blood lymphocytes (70). Modulation of nuclear matrix function may be mediated through the phosphorylation of matrix proteins by nuclear protein kinases (71,72).

The nuclear envelope is punctuated by octagonal nuclear pore complexes which penetrate both nuclear membranes and project annular material into both the nucleoplasm and the cytoplasm. The nuclear pores are thought to be the sites of the energy-dependent nucleocytoplasmic exchange of macromolecules (1-5). The energy required for these processes is provided through the hydrolysis of ATP by the nuclear envelope-associated NTPase, as will be discussed below. Nuclear pore density (1 to 3 pores/um^2 to 50 to 60 pores/um^2), pore number (10^2 to 5 x 10^7/nuclei), and the pattern of pore distribution vary widely between different types of nuclei (1-5). These variations seem to correlate with the degree of

nuclear activity in these cells (3,4,73-77). During the breakdown and reassembly of the nuclear envelope in mitosis, most nuclear pores dissociate, although some pores remain apparently intact in association with envelope fragments (5).

Although nuclear pores are retained in preparations of lamina, it has not yet been possible to isolate the pore complexes away from the lamina or other nuclear envelope components, and therefore the annular subunit protein(s) has not been identified. It has been proposed that the pore subunits may in fact represent a complex mixture of minor nuclear envelope proteins (78) because of two observations. First, there is no single polypeptide in nuclear envelope preparations which is present at a high enough concentration to produce the pore density observed in these membranes. Second, there are no major differences in the polypeptide patterns of nuclear envelopes isolated from cells containing or devoid of nuclear pores. However, a 190,000 dalton nuclear membrane glycoprotein has been identified that appears to be physically associated with the pore complex (79). It was proposed that this protein may be involved in anchoring the pore complexes to nuclear membranes (79).

Advances in techniques in electron microscopy, monoclonal antibody production, and nuclear subfractionation have led to the rapid expansion of

knowledge of nuclear ultrastructure. However, the molecular interactions among the nuclear components described above, and the biochemical mechanisms by which nuclear structure and function are regulated, remain to be elucidated. The remainder of this review will focus on nuclear envelope compostition, function, and possible mechanisms for regulating its activity.

Properties of the nuclear envelope. Four major methods for isolating nuclear envelopes from purified nuclei have been reported. These include sonication of the nuclei followed by extraction of chromatin at high (80-83) or low (84-86) ionic strength; rupture of nuclei with hypertonic buffer at high ionic strength (87); digestion of nuclei with DNase I either at high (88-91) or low (1,92-96) ionic strength; and lysis of nuclei with polyanions such as heparin (97,98). These different isolation methods result in highly varied nuclear envelope preparations, ranging from small single membrane vesicles to intact nuclear ghosts exhibiting double membranes and intact nuclear pore complexes. In general, it is beleived that the mild isolation methods using low ionic strength are preferable since the resulting intact nuclear ghosts are likely to more closely represent the in vivo structure of the nuclear envelope. The mild isolation conditions

would also be preferable for studies of the biochemical makeup of the nuclear enveloope, since it has been demonstrated that nuclear envelope associated enzymes can be dissociated during the harsh isolation procedures (99,100).

The gross chemical compostion of representative nuclear envelope preparations isolated by the various methods are summarized in Table I. Approximately 70% of the nuclear envelope weight is contributed by protein, while phospholipids make up another 25%. The amount of DNA recovered in the nuclear envelopes depends on the ionic strength used in the isolation procedure, such that gentle methods using low ionic strength result in preparations with higher levels of residual chromatin. Small, but reproducible, amounts of RNA can be detected in most nuclear envelope preparations. This RNA may be associated with nuclear pore complexes (101) or with ribosomes attached to the outer nuclear membrane.

Nuclear envelope lipids have been analyzed in preparations from several sources (87,102-107). Phospholipids account for 86% of the total lipid in nuclear envelopes (108). A summary of the phospholipid composition of rat liver nuclear envelopes is described by Table II. These analyses indicate that the

Table I

Chemical composition of rat liver nuclear envelope preparations

Isolation Method	Ionic Strength	% composition by weight				
		Protein	Phospholipid	DNA	RNA	Reference
Sonication	low	64.0	23.0	8.0	5.0	84
	high	67.4	26.1	0.0	6.6	83
DNase digestion	low	65.7	26.7	3.9	3.6	93
	high[a]	70.4	22.7	1.1	5.8	88
Hypertonic lysis	high	73.0	23.0	0.6	3.0	87

[a] Bovine liver

TABLE II

Phospholipid composition of rat liver nuclear envelopes

Phospholipid	Reference 103	105	108
Sphingomyelin	5.8	3.2	2.7
Phosphatidylcholine	57.7	61.4	59.7
Phosphatidylethanolamine	24.2	22.7	18.8
Phosphatidylserine	1.8	3.6	>14.6
Phosphatidylinositol	6.4	8.6	
Phosphatidic acid		<1.0	
Lysophosphatidylcholine	2.6	1.5	2.4
Lysophosphatidylethanolamine	2.0		

phospholipid profile of the nuclear envelope closely parallels that of the endoplasmic reticulum. However, analysis of the non-polar lipids has indicated that the ratio of cholesterol to cholesterol ester is four times lower in the nuclear envelope than in microosomes (104).

It is generally assumed that under normal conditions, all of the nuclear lipid is present in the nuclear envelope. This is supported by studies indicating that the phospholipid profiles of nuclei and nuclear envelopes are essentially identical (81,104,107, C.D. Smith, unpublished observations). However, intranuclear membranes have been observed in anterior pituitary cells after the stimulation of protein synthesis by oestrogen (109), and in human salivary-gland nuclei (110). Invaginations of the nuclear envelope which appear to protrude into the nucleus are often observed in transformed cells (111,112) and hypertrophied human cardiac muscle (113). Intranuclear lipid also occurs after infection of cells with herpes or similar viruses during the formation of the nucleocapsid envelope (114). The capsule of these viruses is apparently formed by budding of the inner nuclear membrane (115-117).

The fate of nuclear envelope lipids during mitosis has been studied in several systems (118-120). In <u>amoeba proteus</u> (118) and chinese hamster ovary (CHO) cells (119), the synthesis of nuclear envelope phospholipid occurs in

G_2, with little or no synthesis during mitosis or S phase. An additional burst of nuclear envelope-specific phospholipid biosynthesis was detected during early G_1 in the CHO cells (119). By labeling nuclear envelope phosphatidylcholine with [^{3}H]choline, it was shown that the phospholipids become dispersed throughout the cell during mitosis, but are quantitatively recovered in the nuclear envelopes of the daughter cells during telophase (118). These results indicate that the dispersed nuclear envelope-derived vesicles retain a marker which enables them to be targeted back to the nucleus during reassembly. A possible candidate for this role might be lamin B since it is a membrane protein which is exclusively nuclear during interphase and contains a DNA binding site that could target it toward the reforming nucleus. A similar reutilization of nuclear envelope protein and lipid has been demonstrated during mitosis in CHO cells (119) and during nuclear envelope reformation after drug-induced breakdown in Pachymedusa dacnicolor melanophores (121).

Studies of lectin binding to isolated nuclei indicate that exposed carbohydrate exists on nuclear membranes (122-125). Concanavalin A binding sites in thymocyte nuclei appear to be distributed between both the inner and outer nuclear membranes, and occur at a similar density to those found on the plasma membrane (124). A similar number of Con A binding sites are found in rat ascites

hepatoma nuclei and normal rat liver nuclei; however, the tumor cell nuclei bind approximately 10 times as many molecules of Ricinus communis agglutinin than do the normal nuclei (122). Chemical analysis of the carbohydrate found in rat liver nuclear envelopes indicated significant amounts of bound mannose, glucose, and glucosamine (29.4, 34.0, and 10.6 umoles/g of protein, respectively) and small amounts of galactose, galactosamine, and sialic acid (126). This carbohydrate is not extracted from nuclear envelopes with chloroform : methanol, indicating that it is present in glycoproteins and not glycolipids (104). The major glycoprotein in rat liver nuclear envelopes exhibits an apparant molecular weight of between 174,000 (127) and 190,000 (79) daltons and is associated with the nuclear pore complex. [^{14}C]Glucosamine can be incorporated into protein in isolated nuclear membranes, indicating that the carbohydrate moieties of glycoproteins in the nuclear envelope may be synthesized in situ (126).

The protein composition of various nuclear envelope preparations have been extensively studied (1-5). The polypeptide patterns revealed by SDS-PAGE are somewhat similar to those for microsomes; however, many polypeptides with equivalent mobilities differ significantly in their amino acid composition (128) indicating that the nuclear envelope and microsome

proteins are not necessarily analogous. In addition to these differences, several nuclear envelope-specific proteins can be distinguished. The quantitatively major polypeptides in nuclear envelope preparations correspond to the lamina proteins which are also isolated with the membranes. Nuclear envelopes isolated using mild conditions also contain significant amounts of histones due to the residual chromatin.

Attempts have been made to identify proteins specifically associated with the inner and outer nuclear membranes by physically separating the two membranes. Two major techniques have been described for this purpose. In the first, low concentrations of Triton X-100 were proposed to selectively remove the outer membrane (18,129); and in the second method, citric acid was thought to chemically disrupt the membranes resulting in their separation (130,131). Both of these methods have been critisized (1,132). Chemical modification of nuclear proteins by derivatization with citraconic anhydride, however, seems to provide a method for selectively removing outer nuclear membranes (132,Chapter IV). The integrity of the inner nuclear membrane after this modification procedure has been assessed by electron microscopy, phospholipid analysis, use of lamin B as an inner membrane marker, and lateral mobility measurements on phospholipids in the residual nuclei (132). An

additional method for investigating the topology of nuclear envelope-associated enzymes by measuring their relative sensitivities to inactivation by free and immobilized trypsin has also been described (9,Chapter IV). This method is similar to a less specific method for analyzing the topology of nuclear envelope proteins by iodination catalyzed by free or immobilized lactoperoxidase (1). Studies using these new methods will help refine our knowledge of the chemical composition and interactions between the two nuclear membranes.

Nuclear envelope proteins turnover with heterogeneous half-lives (133-136) averaging 2.5 days (134). Mechanisms for replacing proteins in intact nuclear envelopes remain undetermined, although a continuous pathway of membrane from endoplasmic reticulum to the outer nuclear membrane to the inner nuclear membrane does exist. In preparation for mitosis, synthesis of proteins destined for daughter cell nuclear envelopes occurs during G_1 phase (119). Proteins from the mother cell are also extensively reutilized (119). Nuclear envelope proteins appear to be synthesized and assembled in a concerted manner during the process of nuclear swelling induced by carcinogens (137).

Several reviews (1,2,138-142) summarize the many studies examining the relationship between the enzymology of the nuclear envelope and that of the endoplasmic reticulum. The nuclear envelope possesses a complement of

enzymes very similar to that of the endoplasmic reticulum; although the specific activities in the nuclear envelope are generally lower. This may be due, in part, to the presence of lamins in nuclear envelope preparations, which contribute protein and thus lower the specific activities of membrane-associated enzymes. It has been postulated that the enzyme profile of the outer nuclear membrane would be particularily similar to that of the endoplasmic reticulum because of their close physical and functional association (1,2). Cytochemical and immunohistological techniques have also been used to investigate the topology of nuclear envelope associated enzymes (1-4).

A particularily interesting activity in the nuclear envelope is the nucleoside triphosphatase (NTPase; EC 3.6.1.15) (80,83,143). This enzyme has received attention because of its proposed role in the nucleocytoplasmic transport of RNA (discussed below). The enzyme has been termed an NTPase to indicate its relative non-specificity in hydrolyzing nucleoside triphosphates (144). In addition, Mg^{2+}, Ca^{2+}, Mn^{2+}, or Co^{2+} will serve as the counterion for the nucleotide (144). The physiological substrate would therefore most likely be Mg-ATP. The ATPase is not stimulated by Na^{+} or K^{+} and is not inhibited by ouabain (80,145,146). The ATPase is however stimulated by RNA, poly[A], or poly[G] if the endogenous RNA is first degraded (147). Cytochemical staining procedures have

been used to try to determine the localization of the ATPase in nuclear envelopes. Although certain groups have reported that the ATPase is associated with nuclear pore complexes (138,148-151), this has not been universally accepted (152-1544). Photoaffinity labeling studies using S-dinitrophenyl-6-mercaptopurine riboside triphosphate have suggested that the ATPase is located on the inner nuclear membrane (155). A 174,000 dalton polypeptide in Drosophila nuclear matrix-pore complex-lamina fraction was identified as an ATPase by photoaffinity labeling studies (127,156). This protein is distinct from the major glycoprotein in these preparations which migrates with an identical apparent molecular weight (127). Attempts to purify the nuclear envelope ATPase have not yet been sucessful; although the initial steps in a possible procedure and the requirements for reconstituting activity have been described (157, Chapter V). Antibodies against this protein would be extremely useful in conclusively demonstrating its localization in nuclear envelopes, as well as for studies of its role in RNA transport.

Roles of the nuclear envelope in cellular function. Several roles for the nuclear envelope have been proposed and are summarized in Table III. In its most obvious

Table III

Roles of the nuclear envelope in cellular function

1. Serves as a boundary between the cytoplasm and nucleoplasm
2. Involved in the nucleocytoplasmic transport of RNA and protein
3. Serves as a stuctural component of the cell
4. Involved in the mediation of hormonal stimulation
5. Contributes to the general metabolism of the cell

role, the nuclear envelope serves as a boundary between the cell's cytoplasm and the nucleoplasm thereby segregating nuclear events from cytoplasmic events. This segregation is not complete since, for example, the transcription of RNA occurs in the nucleus while its translation occurs in the cytoplasm. Similarily, nuclear proteins are synthesized in the cytosol but must then migrate into the nucleus to function. This transport of RNA and protein provides for the exchange of information between the nucleoplasm and the cytoplasm and is required for proper cell function (158).

Studies of the permeability of the nuclear envelope have indicated that water, Na^+, K^+, and small molecular

weight organic compounds diffuse freely between the nucleoplasm and the cytoplasm (1,2,159,160). Therefore, it is not believed that the nuclear envelope is involved in ion transport. Proposals of oxidative phosphorylation by nuclei (161,162) do not seem plausible.

The functional diameter of nuclear pores has been assessed by microinjecting colloidal gold particles into intact cells. Particles of diameter up to 13.5 nm are able to diffuse into Amoeba proteus nuclei (163), while nuclei of Chaos chaos exclude particles of 16 nm (164). Similar studies using proteins of known size indicate Periplaneta oocyte nuclei are freely permeable to proteins with diameters less than 4.5 nm, and a molecular seiving effect is observed on proteins up to 12 nm in diameter (165). Studies using iodinated proteins (166) or tritiated dextrans (167) and amphibian oocytes, have indicated a functional pore diameter of approximately 4.5 nm. Solute charge also affects the rates of nucleocytoplasmic exchange since nuclear pore complexes exhibit a net positive charge at physiological pH (168,169).

The role of the nuclear envelope in regulating the nucleocytoplasmic exchange of proteins is receiving increasing attention. The nuclear envelope does not seem to represent a significant barrier to nuclear proteins. Comparison of the protein content of manually isolated

Xenopus oocyte nuclei and those isolated by the common technique of centrifugation through heavy sucrose (170), demonstrated that proteins diffuse out of the nucleus with a half-time of migration of approximately 250 seconds (171). Therefore, nuclei isolated by centrifugation would contain less than 5% of the in vivo protein content (171). Correcting for the smaller size of somatic cell nuclei, the half-time of loss in these nuclei would be 2-3 seconds or less.

Microinjection of cellular proteins into Xenopus oocytes has demonstrated that these proteins possess determinants that direct their distribution between the two major cellular compartments (172). For example, injection of labeled cytosolic proteins followed by autoradiographic analysis of their distribution, demonstrates that some of these proteins seem to be specifically excluded from the nucleoplasm (173). Examples of proteins that are excluded from the nucleus include tubulin (172,173), proteins that bind to U snRNA (174), and transcription factor III_A (172). The mechanisms by which small cytosolic proteins are excluded from nuclei are unknown. In contrast, injected labeled nuclear proteins rapidly accumulate in the nucleus (173,175).

Two mechanisms for accumulating nuclear proteins have been discussed (172). These are 1) specific transport of

nuclear proteins across the nuclear envelope, and 2) binding of nuclear proteins to substuctures in the nucleoplasm. Feldherr's group has proposed that the nuclear envelope is not important in the concentration of nuclear proteins since the accumulation of labeled nuclear proteins microinjected into Xenopus oocytes is not interupted when the nuclei are mechanically disrupted (176-179). A possible problem with these studies is indicated by the above described study of protein loss during nuclear isolation (171). The nuclear proteins that were isolated, labeled, and used in microinjection studies (176-179) would have belonged to the subset of tightly bound nuclear proteins. It seems likely that these proteins would be mainly structural, and so it is not surprizing that they would tightly bind to nuclear substructures even in the presence of a compromised nuclear envelope. Repitition of the microinjection studies using total nuclear proteins would be more useful in determining the role of binding in nuclear protein concentration.

Specific intranuclear transport of certain proteins has been demonstrated. Study of the rate of nuclear accumulation of a 148,000 dalton Rana oocyte polypeptide indicated that simple diffusion into the nucleus would proceed at only 5% of the observed rate of uptake of this protein (180). Therefore, a transport process must exist

to facilitate the movement of this protein across the nuclear envelope. Nucleoplasmin, the quantitatively major nuclear protein in Xenopus oocytes, also rapidly accumulates in the nucleus after microinjection into oocytes (181). Enzymatic dissection of this protein results in the formation of "core" and "tail" polypeptides (182). Core fragments are not able to enter the nucleus, but are retained if microinjected into nuclei. In contrast, the tail fragments rapidly enter and accumulate in the oocyte nuclei (182). These results indicate that a structural domain on nuclear proteins is involved in targeting them for transport across the nuclear envelope. Similar indications were obtained using a simian virus 40-adenovirus 7 hybrid virus mutant [PARA(cT)] that produces a mutant T antigen that fails to accumulate in the nucleus as the wild type T antigen does (183,184). To characterize the alteration in this mutant, an SV40(cT) mutant containing the SV40 sequences found in the PARA(cT) hybrid virus was constructed (185). Mapping and sequence analysis of the SV40(cT) mutant revealed a single point mutation that correlated with the lack of nuclear transport of the T antigen (185). These results indicate that very subtle changes in the transport domain of nuclear proteins can eliminate their ability to be transported across the nuclear envelope. This SV40(cT) mutant offers the exciting possibility of using molecular

biology techniques to alter protein structure for the investigation of an important biological process. A similar system which might prove even more powerful would be the study of the targeting of viral oncogene products to the nucleus. Both the myc (186,187) and myb (188) oncogenes code for proteins which accumulate in the nucleus. Use of site-directed mutagensis of oncogenes and structural analysis of the resulting proteins, might provide a method for analyzing the target domain and the mechanisms by which nuclear envelopes transport these proteins.

The role of the nuclear envelope in regulating the nucleocytoplasmic transport of RNA has been studied quite extensively. Nuclear pores are generally presumed to be the sites of RNA efflux (1-5) and RNA can be demonstrated to be associated with nuclear pores in intact nuclei(1-4). Certain groups have proposed that the membrane is not important in the retention of nuclear RNA since mechanical disruption of nuclear envelopes does not result in the release of significant quantities of RNA from the nuclei (189). This indicates that the ribonucleoprotein particles are bound to part of the nuclear substructure. These binding sites might be present in either the internal nuclear matrix, the lamina, or the pore complexes. Bulk RNA may be associated with the internal matrix during its synthesis (46-51) and then transported

along the matrix to the pore complex (190). Matrix fibers have been seen to extend from the interior of the nucleus to pore complexes (16,17,37). This tranport mechanism would seem to be most efficient in that diffusion of ribonucleoprotein particles would be eliminated. This mechanism would require energy and might be analogous to the actomyosin system in muscle. Intranuclear ATPases have been demonstrated (190) and two that are apparently associated with ribonucleoprotein particles have been isolated (191). A fundamental problem in cell biology is to determine the mechanisms by which specific RNAs are transported out of the nucleus. Transport along the matrix might allow sites for the selection of these specific RNAs to be introduced.

Although the membrane may not be required for the retention of RNA in the nucleus, it appears likely that the nuclear envelope-associated NTPase is involved in the transport of ribonucleoprotein particles across the nuclear envelope. The NTPase is thought to be localized in the nuclear pore complexes (127,138,148-151), in position for translocating ribonucleoprotein particles. RNA efflux from isolated nuclei is energy-dependent, requiring the hydrolysis of one high-energy phosphate per nucleotide transported out of the nucleus (192). This energy is thought to be provided by the hydrolysis of ATP by the nuclear envelope-associated NTPase. Two lines of

evidence support the proposal for a role for the ATPase in this process. First, correlations have been drawn between the activity of the NTPase and the rate of RNA efflux from isolated nuclei. For example, the release of prelabeled RNA from isolated nuclei demonstrates a nucleotide and cation requirement that correlates well with the substrate specificity of the NTPase (193-195). Similarily, inhibitors of the NTPase also inhibit the energy depend efflux of RNA (193-197). Both the NTPase (198) and RNA transport (199) are stimulated by treatment of nuclei with insulin. A parallel increase in NTPase activity and enhanced transport of RNA are observed in nuclei of rats treated with carcinogens (200,201).

In the second type of experiment, removal of RNA from nuclear envelopes leads to a reduction in NTPase activity. For example, digestion of nuclear envelopes with RNase A produced a 30% lowering of ATPase activity (202). This activity could be restored by the addition of exogenous RNA, poly[A], or poly[G] (202,203). In studies described in Chapter V (157), the ATPase has been solubilized and separated from certain other nuclear envelope components. Removal of RNA from the ATPase resulted in almost complete inactivation of the enzyme. Reconstitution of the activity was dependent on the addition of polynucleotides. Therefore, the ATPase appears to be almost completely dependent on RNA for activity, suggesting that

interactions of these nuclear envelope components are important in ATPase function.

The nuclear envelope is thought to be involved in the organization of both nucleoplasmic and cytoplasmic structures. As discussed above, the lamina associated with the inner nuclear membrane serves in the organization of chromatin (3,4). An example of this interaction is the demonstration that chromatin condensation during prophase initiates at the nuclear periphery (1-5,204,205). Direct interaction of chromatin with the inner nuclear membrane can also be observed in some nuclei (5).

Nuclear envelope involvement in the nucleation and organization of microtubules has also been discussed at length (206-211). Anchorage to the nuclear envelope also appears to protect microtubules from disassembly (212). Microtubules connect the nuclear envelope to the preprophase band of microtubules (213), which is involved in defining the plane of cytoplasmic division during mitosis (214,215). This association may provide an anchor against which force may be generated during cell division (212,216,217). Bundles of microtubules lie parallel to the nuclear envelope in spermatids and form a manchette around the caudal region of the nucleus (218). Microfilaments are also associated with the nuclear envelope (219), and may be involved in positioning cytoplasmic organelles relative to the nucleus (220).

Recently, roles for the nuclear envelope in the transduction of hormonal stimulation have been proposed. For example, binding of dihydrotestosterone to the nuclear envelope exhibits a K_D of 8.4 nm (221). The nuclear envelope also binds activated cytoplasmic steroid hormone receptor with a specific activity 10 times higher than the plasma membrane (222). These studies suggest that the nuclear envelope may be involved in the translocation of steroid hormones into the nucleus (221,222).

High affinity binding sites for peptide hormones have also been identified in nuclear envelopes. These include receptors for gonadotropins (223), epidermal growth factor (224,225), nerve growth factor (226-228), and insulin (229-231). Of these, the insulin sytem has been best characterized. Nuclear receptors for insulin have been demonstrated by several groups (232-235), and further localized to the nuclear envelope (236,237). Comparison of insulin receptors in the plasma membrane and the nuclear envelope have demonstrated a similar specificity; however, several differences in the physical properties of binding occur (229-231). In addition, antibodies isolated from sera of patients with severe insulin resistance block the binding of insulin to the plasma membrane receptor (238-241), but not to the nuclear envelope receptor (241). Therefore, the molecular structure of these two classes of receptor must differ significantly.

Analyses of the cellular uptake of fluorecent derivatives of insulin (242-244) or radiolabeled insulin (245-247) have demonstrated that insulin can enter target cells and bind to intracellular membranes including the nuclear envelope. Therefore, the nuclear receptors may indeed be physiologically relevent. In intact cells, insulin is known to regulate the synthesis of both DNA (248-251) and RNA (252,253), and in vitro, insulin stimulates the nuclear envelope-associated NTPase (198) and augments RNA efflux from isolated nuclei (199). Continued study of hormone receptors in the nuclear envelope are necessary to elucidate their functions and the mechanisms for the transduction of hormonal stimulation across this membrane system.

A final role of the nuclear envelope is its contribution to the general metabolism of the cell. As previously discussed, the enzymatic profile of the outer nuclear membrane is thought to be similar to that of the endoplasmic reticulum (1,2,138-142). These enzymes include at least 4 species of cytochrome P-450 (254) that respond to induction differently than cytochrome P-450s of the endoplasmic reticulum (141,255-257). Metabolism of polycyclic aromatic hydrocarbons by the nuclear envelope-associated mixed function oxidase system may be an especially significant factor in DNA modification because of its close proximity with chromatin (254).

Mechanisms for regulating nuclear envelope function. From the preceeding discussion, it should be apparent that a significant amount of work remains to be done to even define the roles of the nuclear envelope in cellular function. As would be expected, delineation of the mechanisms involved in regulating these functions remain even more obscure. However, in recent years, some mechanisms involved in regulating other membrane systems have come under study in the nuclear envelope. These include post-tranlational modifications of proteins, of which phosphorylation / dephosphorylation is the most common example; and modulation of the lipid environment in the membranes. These possible mechanisms for regulating the functions of the nuclear envelope will be discussed below.

Protein phosphorylation has proven to be an important mechanism for regulating the activity of several soluble enzymes (258,259). Phosphorylation of cytoskeletal components may also be involved in modulating cell structure (260,261). It therefore seems reasonable to predict that the phosphorylation and dephosphorylation of membrane proteins may serve as a mechanism for regulating membrane transport systems and other membrane-associated enzyme activities (258,262). Protein kinase activity and specific phosphorylation products have been demostrated in several biological membrane systems including plasma

membranes (263-269), microsomal membranes (270-273), chloroplast membranes (274), pancreatic zymogen granules (275,276), mitochondrial membranes (277), sarcoplasmic reticular membranes (278), and synaptic membranes (279). In all of these systems, endogenous protein phosphorylation products can be demonstrated; however, since the identitiy of the proteins are generally unknown, it is difficult to ascertain the effects of protein phosphorylation on membrane function.

Protein kinase activity was first described in rat liver nuclear envelopes by Steer, et al. (280). Incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous as well as into the exogenous protein substrates dephosphophosvitin and lysine-rich histone was observed. Lam and Kasper (281) demonstrated the selective phosphorylation of a 68,000 dalton protein in isolated nuclear envelopes. They suggested that this might be a protein of the nuclear pore complex and postulated that protein phosphorylation may be involved in the nucleocytoplasmic transport of macromolecules. Isolated pore complex-lamina fractions demonstrate a protein kinase specific activity of approximately twice that of the whole nuclear envelope (282). This activity was reported to be stimulated by Co^{2+}. Kletzien studied the phosphorylation of nuclear envelopes isolated from cultures of baby hamster kidney cells (283). He was able to show that the

growth state of the culture has a significant effect on nuclear envleope protein phosphorylation since envelopes isolated from proliferating cells exhibited a 3- to 5-fold greater incorporation of ^{32}P *in vitro* than did envelopes isolated from quiesent cells (283). Protein kinase activity was also observed in nuclear envelopes prepared from hamster seminal-vesicle epithelium (284). Nuclear envelope-associated phosphoprotein phosphatase activity that hydrolyzes endogenous and exogenous phosphoproteins has also been described (285,286).

Analyses of the number of endogenous protein substrates in rat liver nuclear envelopes have produced contradictory results (281,283,286). This problem has been resolved by analyzing the endogenous substrates in nuclear envelopes isolated by different methods (99). The use of relatively mild procedures such as the lysis of nuclei with heparin or digestion with DNase I produces envelopes with high kinase activity toward endogenous proteins, resulting in the labeling of at least 12 proteins; while envelopes isolated by the harsher sonication methods possess lower kinase activity and phosphorylate only 2 endogenous proteins (99).

Nuclear envelope-associated protein kinase activity is not affected by cyclic nucleotides (99,280-284), or Ca^{2+} in the presence of calmodulin or phosphatidylserine (99). Multiple protein kinases can not be distinguished

on the basis of treatment with various inhibitors and SDS-PAGE analysis of the phosphorylation products (99). However, differences were detected in the phosphorylation products produced with different nucleotides and cations (99).

The role of protein phosphorylation and dephosphorylation in regulating nuclear envelope functions remains under investigation. Studies have suggested that this process may be involved in regulating nucleocytoplasmic transport (281), or cell cycle events (283). The relationship between nuclear envelope phosphorylation and polyribonucleotide binding has been investigated (287). It was suggested that RNA binds preferentially to phosphorylated nuclear envelopes and stimulates protein dephosphorylation (287). Purrello, et al., have demonstrated that insulin (288), anti-insulin receptor antibodies (289), and Concanavalin A (289) stimulate nuclear envelope phosphoprotein phosphatase activity. Since insulin stimulates RNA efflux from isolated nuclei (199), it was proposed that nuclear envelope dephosphorylation is involved in the nucleocytoplasmic transport of RNA.

From these studies, it appears that phosphorylation of nuclear envelope proteins may represent an attractive mechanism for modulating nuclear envelope function; however, several problems remain to be solved.

Determination of the multiplicity of nuclear envelope-associated protein kinases and their substrate specificities remain to be investigated. The topology of the nuclear envelope phosphorylation systems also requires further studies to determine the interactions among nuclear envelope-associated kinases, pore-lamina proteins, and other nuclear components, such as chromatin, which may serve as substrates for the kinases. Further investigations of the regulation of nuclear envelope protein phosphorylation, and the affects of the phosphorylation on nuclear envelope function should provide a better understanding of nuclear envelope structure and function interactions.

The second major mechanism for regulating membrane activity is through modulation of protein-phospholipid interactions. Phospholipids are the numerically major component of biological membranes. Integral proteins remain in membranes because of hydrophobic interactions between the proteins and membrane lipids (290). It is therefore not surprising that the activities of many membrane-associated enzymes are affected by the phospholipids that they contact (reviewed in 291-294), and that phospholipid alterations may be involved in biological signal transduction (295). Many of these effects may be mediated by alterations in membrane fluidity which can be regulated by phospholipids

(296-298).

The possible influence of phospholipids on nuclear envelope function has received exceedingly little attention. Most studies of nuclear envelope phospholipid metabolism have focused on determining the fate of phospholipids during mitosis (118-120). Enzymes involved in the de novo biosynthesis of the major phospholipids are not found in isolated nuclear envelopes (299); although acyl-CoA : 1-acyl-sn-glycero-3-phosphorylcholine acyltransferase has been observed in neuronal nuclear membranes (300). Since phospholipids can be mobilized from the endoplasmic reticulum (8), nuclear envelope phospholipids have a continual source of resupply.

Possible phospholipid effects on regulating nuclear envelope NTPase activity (194) and RNA transport (301-304) have been described. Agutter, et al. (194), described a discontinuous Arrhenius plot for nuclear envelope-associated ATPase activity. Discontinuities in such plots for membrane-associated enzymes have often been proposed to be due to differences in protein-phospholipid interactions below the temperature at which thermotropic lipid clustering occurs. Therefore, it seems possible that phospholipid interactions may be involved in regulating nuclear envelope ATPase activity. Similar temperature effects have been noted in the rate of RNA efflux from isolated Tetrahymena nuclei, in which a

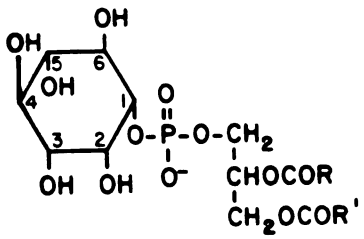
transition occurs at approximately 17 C such that RNA efflux at lower temperatures is inhibitied (303). An essentially identical transition temperature was measured by electron spin resonance using 5-doxylstearic acid as a spin label for lipid fluidity in these nuclei (303). A linear decrease in RNA release upon temperature lowering is observed in nuclei treated with Triton X-100, indicating that membrane phospholipids probably mediate this effect (303). A different approach to determining the effects of lipids on RNA transport was used by Yannarell and Awad (304), in which rats were fed diets enriched in either saturated or polyunsaturated fatty acids. RNA transport was reduced from nuclei isolated from animals fed the saturated fatty acid diet as compared with those maintained on the diet containing polyunsaturated fatty acids (304). Such a treatment has been shown to alter the fatty acyl composition of nuclear envelope phospholipids (305).

Obviously, many additional studies are required to determine the effects of phospholipids on nuclear envelope function, and to elucidate the mechanisms involved in modulating nuclear envelope protein-phospholipid interactions. Perhaps the most intense area of current lipid research centers on the inositol phospholipids. As described in the body of this thesis, the phosphorylated derivatives of phosphatidylinositol have been identified

as nuclear envelope components (100,Chapter I). In the remainder of this review, the metabolism and cellular functions of phosphoinositides will be discussed.

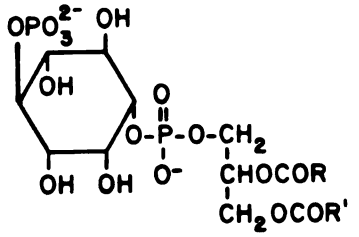
Metabolism of inostiol phospholipids. The existance of an inostiol-containing phospholipid, phosphatidylinositol (PI), was first described by Folch and Woolley in 1942 (306). Several years later, polyphosphoinositides, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), were isolated (307); but it was not until 1961 that their chemical structures (Figure 1) were established (308). Since then, extensive investigation of the structures, subcellular distributions, metabolism, and possible physiological functions of these phospholipids has occured. This intense interest in phosphoinositides has resulted in several recent reviews (309-314) and an international conference (315) devoted to glorifying these phospholipids. Therefore in the interests of brievity, only a cursory review of this field will be attempted here.

PI serves as the parent compound for the phosphoinositides and typically constitutes 2 - 12 % of the membrane phospholipids (316-318) (approximately 7 % for nuclear envelopes). The amounts of



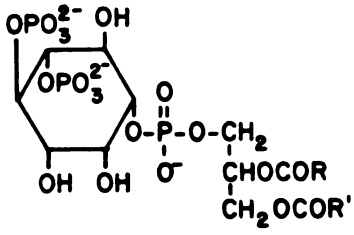
phosphatidylinositol

(PI)



phosphatidylinositol 4-phosphate

(PIP)



phosphatidylinositol 4,5-bisphosphate

(PIP_2)

Figure 1. Structures of inositol phospholipids

polyphosphoinositides in cellular membranes have been difficult to estimate because of their rapid hydrolysis upon the depletion of cellular energy (319), and their requirement for acidic conditions for quantitative extraction (320). Neural tissue demonstrates the highest levels of polyphosphoinositides, especially in the myelin sheath (321,322). In other tissues, polyphosphoinositides probably account for approximately 0.1% of the total phospholipids (318,323). Inositol phospholipids in animals are highly enriched with stearoyl and arachidoyl residues at positions 1 and 2, respectively (324).

The biosynthesis of PI through the action of CDP-diglyceride : inositol transferase of the endoplasmic reticulum has been well characterized (200). Enrichment in the arachidonyl moiety appears to be largely due to deacylation and reacylation occurring at the 2 postion of PI involving an acyltransferase that is highly selective for arachidonyl CoA (325-327). PI can be transported from the endoplasmic reticulum to other cellular membranes by cytoplasmic carrier exchange proteins (reviewed in 328).

Catabolism of PI occurs via two mechanisms: 1) deacylation by phospholipase A_2 (329) producing lyso-PI and a fatty acid (mostly arachidonate), and 2) hydrolysis by a PI-specific phosphodiesterase (PI-specific phospholipase C) producing 1,2-diacylglycerol and a mixture of <u>myo</u>-inositol-1-phosphate and <u>myo</u>-inositol 1:2

cyclic phosphate (330,331). Stimulation of phospholipase A_2 action on PI has been proposed as a mechanism for mobilizing arachidonate for prostaglandin synthesis (332-334); although this mechanism has been challenged (335). The PI-specific phospholipase C has been subjected to an enormous amount of study because of its postulated role in stimulated PI turnover (309-314,331,336).

Agonist-stimulated PI metabolism was first demonstrated by Hokin and Hokin (337) and is usually measured as an increased incorporation of label from [γ-^{32}P]ATP into PA and PI in response to cellular stimulation. PI turnover is enhanced in many tissues in response to numerous stimuli (309-314) including calcium mobilizing agents such as vasopressin, angiotension II, and α-adrenergic agonists, muscarinic agents, histamine, and secretagogues in leukocytes. In this model of cell stimulation, it is assumed that receptor occupancy stimulates PI hydrolysis by the PI-specific phospholipase C. This enzyme has been characterized in a variety of tissues (331), and in all cases requires Ca^{2+} concentrations much higher than <u>in vivo</u> values. Therefore, controversy raged as to whether calcium mobilization led to PI breakdown, or vice versa. This has calmed somewhat due to the recognition of the role of the breakdown products of PIP and PIP_2 in the mobilization of intracellular calcium (discussed below).

PIP is synthesized by the transfer of the γ-phosphate from ATP to the 4 position of PI, catalyzed by the action of PI kinase. This enzyme has been characterized in a variety of tissues including brain (338-341), kidney (342,343), adrenal glands (344-347), parotids (348), erythrocytes (349-352), heart (353), skeletal muscle (353), leukocytes (353), and liver (353,354). In most cases, PI kinase has been described as being associated with the plasma membrane. However; recently, the subcellular distribution of PI kinase in the liver has been reevaluated (355). PI kinase activity has been identified in highly purified prepartions of plasma membranes (100,355), microsomes (100,273,355), lysosomal membranes (355,356), Golgi (357), and nuclear envelopes (100,355). In addition, studies of the subcellular sites of incorporation of ^{32}P into PIP in isolated hepatocytes, indicate that although the highest specific activity of PIP is found in the plasma membrane, PIP synthesis also occurs throughout the cell (355,358). Therefore, it appears that PIP is a normal component, widely distributed in biological membranes. A notable exception is the observation that mitochondria contain virtually no PI kinase activity and PIP synthesis does not occur in the membranes of this organelle (355).

Mechanisms for the regulation of PI kinase activity remain very poorly understood. The enzyme exhibits a

neutral pH optimum (100,353) and is inhibited slightly by Ca^{2+} (100,353,356). PI kinase requires high levels of Mg^{2+} to express maximal activity (100,353,354,356). At physiological concentrations of Mg^{2+}, PI kinase would be essentially inactive. Recently, the effects of various cellular polyamines (putrescine, spermidine, and spermine) have been examined as possible regulators of PI kinase activity (359). Physiological concentrations of spermine stimulate PI kinase activity approximately 3-fold when Mg^{2+} is present at low (physiological) concentrations. This stimulatory effect may be due to binding of spermine to the substrate PI since it was also found that spermine has an affinity for binding to PI that is 2 - 3 orders of magnitude higher than that of Mg^{2+} (360). Therefore, it seems possible that modulation of intracellular polyamine levels could affect polyphosphoinositide metabolism, and therefore alter membrane processes regulated by these phospholipids (discussed below).

PIP_2 is synthesized by the transfer of the γ-phosphate from ATP to the 5 position of PIP, catalyzed by the action of PIP kinase. This enzyme has been less well characterized than PI kinase, but has been described as being associated with erythrocyte membranes (339,352), plasma membranes (361) and Golgi (343) in rat kidney cortex, and cytosol in rat brain (339). The subcellular distribution of PIP kinase in liver has only recently been

described (355). In this tissue, plasma membranes exhibit a specific activity 5-, 6.5-, and 9-fold higher than that of the cytosol, nuclear envelope, and microsomes, respectively; the only other sites in which it is located (355). Studies on the synthesis of PIP_2 in isolated hepatocytes indicate that the specific activity of PIP_2 is at least 20-fold higher in the plasma membrane than in any other organelle (355,358). Therefore, PIP_2 may be involved in plasma membrane specific functions, such as the transduction of receptor-mediated events (further discussed below).

Two enzyme systems exist for the degradation of polyphosphoinositides: 1) phosphomonoesterases which hydrolyze PIP_2 to PIP to PI with the release of inorganic phosphate; and 2) phosphodiesterases which hydrolyze PIP_2 and PIP into diacylglycerol and inositol phosphates. The inositol phosphates are further degraded to myo-inositol and inorganic phosphate.

Phosphomonoesterase activity toward PIP_2 has been described in brain (362,363), kidney (364-366), erythrocytes (367,368), and iris smooth muscle (369). In all of these cases, PIP_2 hydrolysis requires the presence of Mg^{2+}. Studies on the subcellular distribution of PIP_2 phosphatase suggest a cytosolic localization in brain (363), while the kidney (366) and iris smooth muscle (369) enzymes are particulate. Analysis of Mg^{2+}-stimulated PIP_2

hydrolysis in rat liver subcellular fractions revealed high activity in the plasma membrane and mitochondria (355).

Hydrolysis of PIP by a specific metal-independent phosphomonoesterase has been reported in kidney (365), liver (370,Chapter III), and erythrocyte membranes (371). Due to the scarcity of studies on PIP phosphatase, the subcellular distribution of this enzyme remains to be characterized. Preliminary experiments with rat liver have however suggested that it is widely distributed throughout intracellular membranes (355).

It appears that the subcellular distributions of PI kinase and PIP kinase are very similar to that of their antagonistic phosphatases. This sets up the possibility of regulating the membrane levels of polyphosphoinositides through alterations of this phosphorylation / dephosphorylation cycle. This mechanism would allow for the rapid and precise regulation of membrane processes which involve these phospholipids (discussed below). It is interesting to speculate on the possible role of polyamines in this model. As previously discussed, spermine stimulates the synthesis of PIP and, therefore, PIP_2. In addition, spermine inhibits the liver PIP phosphatase (370,Chapter III). Therefore, increased spermine levels would tend to promote the accumulation of polyphosphoinositides in biological membranes. An example

of support for this proposal comes from studies demonstrating increased cellular levels of polyamines (372,373) and polyphosphoinositides (374-376) in transformed cells.

Phosphodiesterases which hydrolyze polyphosphoinositides have been demonstrated in brain (377-380), erythrocyte membranes (381-383), platelets (384,385), hepatocytes (386), and iris smooth muscle (369). Despite their importance in recently described models for intracellular calcium mobilization, these enzymes have not been well characterized. In all cases, these phosphodiesterases require Ca^{2+} for activity; although the form of the substrate appears to tremendously effect the level of Ca^{2+} required (386). Therefore, the primary mechanism for regulating the activity of these enzymes may involve modulation of the structure of the substrate phospholipids.

Roles of polyphosphoinositides in cellular function. Two major classes of polyphosphoinositide effects have been described. These include: 1) direct effects of these phospholipids on membrane structure and functions, and 2) effects of the products released by the receptor-mediated stimulation of polyphosphoinositide

hydrolysis.

Polyphsophoinositides are a special subset of phospholipids because of their highly anionic character. This property is thought to mediate most of the membrane effects of polyphophoinositides. For example, phosphoinositides have been shown to tightly bind divalent metal cations, especially Ca^{2+} (387-391). Because of this, polyphosphoinositides were proposed to provide the binding sites for membrane-associated pools of Ca^{2+} (309,391-394). This has since been disputed since the affinity for Mg^{2+} is only slightly lower than that for Ca^{2+}, while the cellular Mg^{2+} concentration would be at least 3-orders of magnitude higher. Therefore, Mg^{2+} would essentially completely displace Ca^{2+} under physiological conditions (396). Recent studies have demonstrated that spermidine and spermine bind to phosphoinositides with affinities 2-3 orders of magnitude higher than those for Ca^{2+} and Mg^{2+} (359,360). Therefore, polyamines may represent the biological counterions for phosphoinositides. Effects of this association on the interconversion of PI and PIP have been described above (359).

Metal binding to polyphosphoinositides tremendously increases the hydrophobicity of these phospholipids (388). This effect on lyso-PIP_2 has been reported to enable the lipid to penetrate into the membrane and provide a channel

for univalent cations (389). Such effects on membrane permeability have been proposed to play a role in the generation or propagation of action potentials in neurons (391,397).

The ionic properties of polyphosphoinositides may be responsible for the effects these lipids on membrane fluidity. Interactions between divalent cations and acidic phospholipids have been proposed to be important in the process of membrane fusion (398,399). Thus, phosphodiesteric breakdown of polyphosphoinositides would promote membrane fusion (400). Such a mechanism has been suggested by studies on the fusion of chick embryo myoblasts (401) and fusion of vesicles with the plasma membrane during exocytosis (402). PIP_2 has been shown to activate glycoprotein lateral diffusion in erythrocyte membranes, possibly by disrupting interactions with the erythrocyte membrane skeleton (403).

Phosphoinositides also interact strongly with membrane proteins. An insoluble ternary complex of allergic encephalitogenic protein-PIP_2-Ca was formed with physiological concentrations of the three components (404). Extraction of glycophorin from erythrocyte membranes yields a preparation with polyphosphoinositides tightly bound (405,406). Similarily, isolated nicotinic cholinergic receptors from cerebral cortex contain associated PIP_2 (407,408). Studies described in Chapter V

of this thesis (157) demonstrate that procedures commonly used for delipidating solubilized membrane proteins, do not necessarily remove polyphosphoinositides. Therefore, studies on phospholipid effects in the reconstitution of membrane-associated enzymes may contain polyphosphoinositides in addition to the exogenously added phospholipid.

These interactions between membrane proteins and phosphoinositides may be involved in the regulation of the activities of certain membrane-associated enzymes. For example, the (Na^+,K^+)-ATPase of kidney microsomes (409) and Torpedo marmorata (410) require phosphoinositides for activity. The Ca^{2+}-pump ATPase of erythrocyte plasma membranes has been particularily well characterized (411) and is strongly activated by PIP and PIP_2 (412). The sarcoplasmic reticular Ca^{2+}-transport ATPase is activated by the phosphorylation of an associated PI, producing a very tightly bound PIP (413). It has also been suggested that the canine renal (Na^+,K^+)-ATPase is regulated by its interactions with phosphoinositides (414). Delipidated nuclear envelopes require PIP or PIP_2 for the reconstitution of ATPase activity (157). Polyphosphoinositides inactivate the low K_m cAMP phosphodiesterase from rat adipocyte microsomes (415), and stimulate the cleavage of the side chain of cholesterol by cytochrome P-450 (416). Coupling of β-adrenergic

receptors to the adenylate cyclase complex also appears to be affected by phosphoinositides (417). Effects of polyphosphoinositides are generally overlooked in studies of the regulation of membrane-associated enzymes by phospholipids; therefore, increased awareness of the polyphosphoinositides will probably result in demonstrations of their effects on many enzymes.

Phosphoinositides may also be involved in anchoring certain enzymes in membranes. This was suggested by studies in which a phosphoinositide-specific phospholipase C was found to selectively release alkaline phosphatase from cell membranes while leaving other membrane-bound enzymes such as 5'-nucleotidase unaffected (418,419). Similar experiments using erythrocyte membranes, resulted in the solubilization of acetylcholinesterase (420).

Presently, a very active area of investigation is the study of the mechanisms by which phosphoinositide hydrolysis leads to the elevation of intracellular calcium (421). The receptor-mediated stimulation of PI metabolism is now being reaxamined in many systems. In several cases, it is now apparent that the initial event in this response is the hydrolysis of PIP_2, and in some cases also PIP, rather than the hydrolysis of PI (386,422-433). The products released by the phosphodiesteric cleavage of PIP_2 have both been found to be important mediators in cell stimulation. The diacylglycerol produced is thought to

stimulate the phospholipid-dependent protein kinase, protein kinase C (434,435). The role of protein phosphorylation by the stimulation of this kinase remains to be established. The other product released from PIP_2 is myo-inositol-1,4,5-trisphosphate (IP_3). IP_3 has been demonstrated to cause the release of Ca^{2+} from the endoplasmic reticulum in hepatocytes (436,437) and pancreatic acinar cells (438). The mechanism for this process is unknown, but through this sytem, intracellular calcium levels can be increased without the entry of extracellular Ca^{2+}. This increased level of Ca^{2+} may then further increase the activity of phosphodiesterases active against PI as well as PIP and PIP_2.

Two recent reports promise to stimulate even more interest in polyphosphoinositide metabolism. These are the demonstrations that the products from the oncogenes src (375) and ros (376) act as PI kinases as well as tryosine protein kinases. Therefore, oncogenes may stimulate cell division by increasing the levels of polyphosphoinositides and their hydrolysis products, diacylglycerol and IP_3. Discerning the molecular mechanisms involved in these events will require a significant amount of further work.

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CHAPTER I

IDENTIFICATION OF PHOSPHATIDYLINOSITOL 4-PHOSPHATE AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE IN NUCLEAR ENVELOPES

ABSTRACT

Incubation of nuclear envelopes isolated from normal rat liver with [γ-^{32}P]ATP and $MgCl_2$ resulted in the rapid phosphorylation of a number of endogenous proteins by nuclear envelope-associated protein kinase activity. In addition, a low molecular weight, protease-insensitive, ^{32}P-labeled material which could be extracted from the membranes by treatment with acidified chloroform : methanol was observed. The ^{32}P-labeled components of this chloroform-soluble material were identified as phosphatidic acid, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate based on their chromatographic mobilities on Silica Gel H and cellulose thin layer plates, and by analysis of their glycerylphosphorylester deacylation products by high pressure liquid chromatography.

INTRODUCTION

The eucaryotic nuclear envelope serves as a biological interface between the cytoplasm and the nucleoplasm of a cell. It is thought to be involved in a number of cellular processes, such as the regulation of transport of ribonucleoprotein complexes (1,2), organization of chromatin (2-4), and transduction of hormonal stimuli (5-8). The disassembly and reformation of the nuclear envelope during mitosis demonstrate the stringent control the cell maintains over this structure. In spite of the importance of these processes in normal and aberrant cell function, the biochemical basis for their regulation is not understood.

In recent years, a few studies have explored the possibility that nuclear envelope function may be regulated by the phosphorylation and dephosphorylation of certain membrane proteins (9-13). Phosphorylation of the supporting lamina may also be important in regulating nuclear structure (14). These studies have begun to characterize the nuclear envelope phosphorylation / dephosphorylation systems and to indicate that nuclear envelope phosphorylation may be important in some cellular processes such as nucleocytoplasmic transport of macromolecules and passage through the cell cycle.

In a study of protein phosphorylation in isolated rat

liver nuclear envleopes (13), we described a number of phosphoproteins and characterized nuclear envelope-associated protein kinase activity. In addition to the protein phosphorylation products, a low molecular weight, chloroform : methanol : HCl-extractable, ^{32}P-labeled material is formed. In this Chapter, this organic soluble material is identified as a mixture of phosphatidic acid (PA), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2).

EXPERIMENTAL PROCEDURES

Materials. Adult male Sprague-Dawley rats (180-200 g) were used for all studies. (^{32}P)Orthophosphoric acid, carrier-free, was purchased from ICN Pharmaceuticals, Inc. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (15) as modified by Walsh, et al. (16). Phospholipids were obtained from Sigma and Serdary Research Laboratories. Thin layer cellulose (Avicel) plates were purchased from Analtech. Glass-distilled organic solvents were from MCB. Materials for gel electrophoresis and an Aminex A-27 anion exchange HPLC column (250 x 4mm) was obtained from Bio-Rad. Heparin (sodium salt), Escherichia coli alkaline phosphatase, Streptomyces griseus protease, bovine pancreas ribonuclease A, Bacillus cereus phospholipase C, and bovine pancreas deoxyribonuclease I were from Sigma.

Nuclei isolation. Nuclei were isolated by the method of Blobel and Potter (17), except that the livers were homogenized with a Tekmar Tissuemizer in a hypotonic buffer, HB (10mM HEPES, pH 7.5, 25mM KCl, 5mM $MgCl_2$), to eliminate contamination by erythrocytes. Sucrose (2.4M) in HB was then added to give a final sucrose concentration of 0.25M. Phenylmethylsulfonyl fluoride (75 ug/ml) was included throughout the purification procedure to inhibit

serine protease activity. The isolated nuclei were washed twice with 0.25M sucrose, 2mM $MgCl_2$, 10mM HEPES, pH 7.5, by sedimentation at 600 x g for 10 min and were then resuspended in the same buffer. Approximately 75% of the total homogenate DNA, determined by the method of Karsten and Wollenberger (18) as modified by Kurtz and Wells (19), was recovered in these purified nuclei.

Nuclear envelope isolation. Nuclear envelopes were isolated by the heparin method according to Hildebrand and Okinaka (20). Purified nuclei were washed with HB, and heparin dissolved in HB was added to a final concentration of 1mg of heparin/$2x10^8$ nuclei. The suspension was gently homogenized and incubated at 30 C for 20 min with occassional gentle rehomogenization. The suspension was then diluted with 6 volumes of HB and centrifuged at 18,000 x g for 30 min. The pelleted crude membranes were resuspended in 0.25M sucrose, 10mM HEPES, pH 7.5, and then centrifuged at 98,000 x g for 1 h on a discontinuous gradient of 2.0, 1.8, 1.4, and 0.25M sucrose in 10mM HEPES, pH 7.5. Membranes banding at the 0.25/1.4M and 1.4/1.8M interfaces were pooled, diluted to 0.25M sucrose, and pelleted by centrifugation at 40,000 x g for 30 min. The purified nuclear envelopes were then resuspended in 0.25M sucrose containing 10mM HEPES, pH 7.5.

Assessment of nuclear envelope purity. The purity of isolated nuclear envelope preparations was assessed by marker enzyme analyses as described by Leighton, et al. (21). Marker enzymes included fumarase to measure mitochondrial contamination (22), β-N-acetyl-glucosaminidase for lysosomal contamination (23), urate oxidase for peroxisomal contamination (24), alkaline phosphodiesterase I for plasma membrane contamination (25) and NADH:cytochrome c reductase for microsomal contamination (26).

Nuclear envelope phosphorylation and SDS-PAGE. Nuclear envelopes (50 ug of protein (27) in 25 ul) were preincubated at 30 C for 5 min before phosphorylation was initiated by the addition of an equal volume of mixture containing 100mM HEPES, pH 7.5, 200mM NaCl, 10mM $MgCl_2$, and 200uM [γ-^{32}P]ATP (500-2000 cpm/pmol). Incubations were carried out at 30 C and were terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid containing 10mM sodium pyrophosphate. After 5 min on ice, the precipitates were pelleted by centrifugation at 15,000 x g for 5 min. The pellets were washed once with 10% trichloroacetic acid and twice with distilled water. Phosphorylated membrane samples were then resuspended in 50 ul of 50mM sodium borate, pH 9.0, containing 2% SDS, boiled for 1 min and allowed to cool. Fluorescamine (5ul

of 2mg/ml in acetonitrile) was added with immediate mixing. An equal volume of sample buffer (20% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 100mM Tris-Cl, pH 7.5) was then added to each sample. Samples were electrophoresed on 12.5% polyacrylamide gels according to the procedure of Laemmli (28). Following electrophoresis, a photograph of the gel was taken under ultraviolet light to reveal the protein banding patterns. The gel was then dried and exposed to Kodak XAR-5 x-ray film for autoradiography.

Phospholipid extraction. Nuclear envelopes were phosphorylated for 2 min at 30 C as described above, except that, the reactions were terminated by the addition of 1.5ml of chloroform : methanol (1:2,v/v) followed by the addition of 0.5ml of chloroform and 0.5ml of 2.4N HCl (29). Phases were separated by centrifugation, the lower phase was removed, and the upper phase was reextracted with 1ml of chloroform. The combined lower phases were then extracted twice with 2ml of methanol : 1N HCl (1:1, v/v) and evaporated under a stream of nitrogen at 40 C. The lipid residue was dissolved in 50 ul of chloroform : methanol : H_2O (75:25:2, v/v/v) and analyzed chromatographically as described below.

Thin layer chromatography of lipids. The lipid phosphorylation products were analyzed in three thin layer chromatography systems. System A- Aliquots of the lipid extract were applied to thin layer cellulose (Avicel) plates along with the appropriate phospholipid standards. Chromatography was performed in n-butanol : acetic acid : water (85:10:25, v/v/v) similar to the method of Marche et al. (30). After development, chromatograms were exposed to x-ray film for autoradiography. The positions of the standards were determine by iodine staining. System B- Aliquots of the lipid extract were applied to Silica Gel H thin layer plates which had been previously activated at 110 C for 1 h and chromatographed with appropriate phospholipid standards in n-propanol : 4.3N NH_4OH containing 10mM CDTA (65:35, v/v) as described by Hauser, et al. (31). Radioactive products and phospholipid standards were located as described for System A. System C- Aliquots of the phospholipid extract were applied to Silica Gel H thin layer plates previously activated at 110 C for 1 h and chromatographed with appropriate phospholipid standards in chloroform : acetone : methanol : acetic acid : water (10:4:2:2:1, by volume). Radioactive products and phospholipid standards were located as described for System A.

HPLC of glycerylphosphorylester deacylation products. Aliquots of the lipid extract and phospholipid standards were deacylated in weak alkali by the method of Kates (32). The deacylation products were analyzed by high pressure liquid chromatography as described by Seyfred and Wells (33). A 250 x 4mm column of Aminex A-27 in the formate form was equilibrated with 20mM ammonium borate containing 0.1M ammonium formate at pH 9.5. The glycerylphosphoryl derivatives of the phospholipids were separated by increasing the ammonium formate concentration to 0.75M in 20 mM ammonium borate, pH 9.5, as indicated in Figure 3. The flow rate was 0.6ml/min and 1.5 ml fractions were collected. The positions of the deacylation products from standard phospholipids were determined by total phosphate analyses (34) of the fractions. Radioactive products were detected by scintillation counting.

RESULTS

Assessment of nuclear envelope purity. The lack of a suitable marker enzyme for nuclear envelopes necessitated an indirect assessment of purity through the demonstration of the absence of marker enzymes for other cellular organelles. As demonstrated in Table I, the purified nuclear envelopes were virtually free of contamination by mitochondria, lysosomes, peroxisomes, and plasma membranes. The calculation of microsomal contamination ignores the fact that NADH:cytochrome c reductase activity is also located in the nuclear envelope (1,3) and so is an overestimation of the actual amount of contamination by endoplasmic reticulum remnants. For each of the enzymes studied, at least 80% of the total activity in the whole homogenate could be accounted for in the various washes in the nuclear envelope isolation procedure. A minimum purity of 84% was calculated for the isolated nuclear envelopes by this enzymatic analysis.

Characterization of nuclear envelope-associated phosphorylation products. Analysis of nuclear envelope phosphorylation products by SDS-PAGE and autoradiography (Figure 1) indicated that nuclear envelopes isolated by the heparin method contain at least 13 ^{32}P-labeled

TABLE I

Assessment of nuclear envelope purity

Marker (organelle)	Amount of organelle in liver[a]	RSA[b]	Contamination[c]
	%		%
Fumarase (mitochondria)	20.2	0.00 ± 0.02	0
β-N-Acetylglucoaminidase (lysosomes)	2.0	0.33 ± 0.10	0.7
Urate oxidase (peroxisomes)	2.5	0.18 ± 0.05	0.5
Alkaline phosphodiesterase I (plasma membranes)	2.6	0.57 ± 0.09	1.5
NADH:cytochrome c reductase (endoplasmic reticulum)	21.5	0.60 ± 0.12	13

Whole homogenates and purified nuclear envelopes were assayed for marker enzymes as described under "Experimental Procedures". Relative specific activity (RSA) values represent the mean ± SEM for six complete analyses.

[a] Values represent the percentage of total liver protein contributed by the organelle.

[b] Specific activity in purified nuclear envelopes/specific activty in whole homogenate.

[c] Percentage of organelle in purified nuclear envelopes = percentage of organelle in liver X RSA.

Figure 1. Characterization of nuclear envelope phosphorylation products

Nuclear envelopes were phosphorylated for 2 min as described under "Experimental Procedures", resuspended in 100 ul of 250mM HEPES, pH 7.5, and treated as follows. Lane 1 was incubated on ice for 30 min; lane 2 was treated with 1 N NaOH at 90 C for 10 min; lane 3 was treated with 100mM NH_2OH at 30 C for 30 min; lane 4 was extracted with chloroform : methanol : HCl as described under "Experimental Procedures". Lanes 5-10 were incubated at 37 C for 30 min with the following additions: lane 5, no further addition; lane 6, 50 ug of alkaline phosphatase; lane 7, 50 ug of protease (S. griseus); lane 8, 50 ug of phospholipase C; lane 9, 50 ug of ribonuclease A; lane 10, 50 ug of deoxyribonuclease I. Following incubation, samples were precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE. The molecular weight standards were phosphorylase a (92,500 daltons), bovine serum albumin (68,000), ovalbumin (43,000), ribonuclease A (13,700), and bovine trypsin inhibitor (6,100).

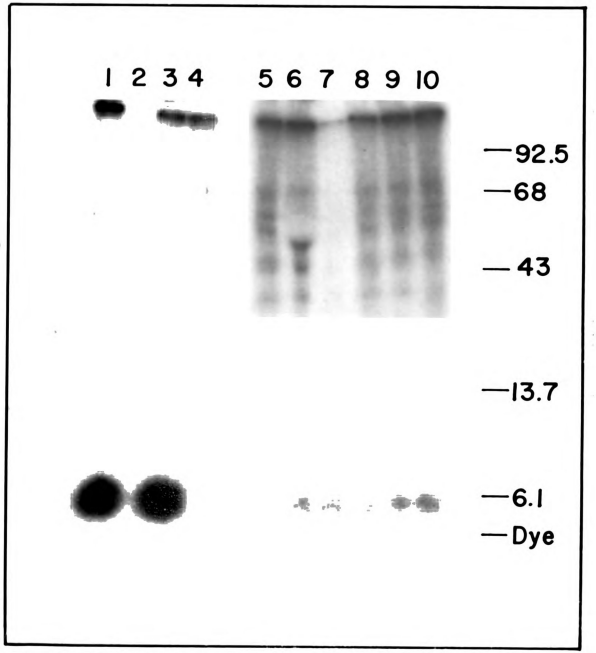

Figure 1. Characterization of nuclear envelope phosphorylation products

products which range in apparant molecular weight from 3000 to 120,000. ATP and P_i migrated well ahead of the dye front on these 12.5% polyacrylamide gels and so did not interfere with the autoradiographic analyses. Nuclear envelopes were phosphorylated, resuspended in 100mM HEPES, pH 7.5, and subjected to various chemical and enzymatic treatments to assess the nature of the phosphate linkages and the chemical class of the phosphorylation products. Incubation of the resuspended phosphorylated envelopes in 10% trichloroacetic acid for 30 min at 4 C resulted in the loss of 5% of the radioactivity, measured as Cerenkov radiation, when the membranes were reprecipitated. Treatment with 1N NaOH at 90 C for 10 min resulted in the loss of 96% of the radioactivity and the disappearence of all of the phosphorylation products seen in SDS-PAGE (Figure 1). Treatment with 100mM hydroxylamine led to the loss of 10-38% of the total radioactivity; however, SDS-PAGE analysis of the products did not indicate that ^{32}P was lost from any of the products resolved by this technique (see Reference 13 for a discussion of this finding). Extraction of the phosphorylation products with chloroform : methanol:HCl resulted in the partioning of approximately 30% of the radioactivity into the chloroform phase. This extraction removed material which migrated in the 3000 dalton region of the SDS gels. Further analyses of this material are described below and indicate that it

is a mixture of phospholipids. Choloform : methanol : HCl extraction also eliminated an 11,000 dalton product. These same products can be removed from the gels by washing with acidified organic solvents, such as 25% isopropanol + 10% acetic acid as is used in the Coomassie blue staining procedure (data not shown). Incubation of the resuspended phosphoryaltion products in 100mM HEPES, pH 7.5, at 37 C for 30 min resulted in the loss of 20-47% of the radioactivity in the reprecipitated membranes. The radioactivity was lost predominantly from the phospholipid products. Incubation of the resuspended membranes with alkaline phosphatase, phospholipase C, RNase A, or DNase I caused only minor losses of radioactivity. Incubation with protease eliminated all of the phosphorylation products except for the labeled phospholipids.

Identification of ^{32}P-labeled lipids in nuclear envelopes. The chloroform-soluble fraction of phosphorylated nuclear envelopes was prepared and analyzed by thin layer chromatography in three different analytical systems (Figure 2). Unlabeled carrier PA, PIP, and PIP_2 were added to the extracts before chromatography. The positions of the radioactive products corresponded to the positions of PA, PIP, and PIP_2 standards in all three chromatography systems. The calcium chelator, CDTA, was included in solvent system B since the calcium salts of

Figure 2. Thin layer chromatography of lipid phosphorylation products

Nuclear envelopes were phosphorylated for 2 min and extracted as described under "Experimental Procedures". Unlabeled carrier PA, PIP, and PIP_2 were added to the extracts and thin layer chromatography was performed using the following systems:

Lane 1- Thin layer cellulose plates developed with n-butanol : acetic acid : water (85:10:25, v/v/v)

Lane 2- Silica Gel H plates developed with n-propanol : 4.3N NH_4OH containing 10mM CDTA (65:35, v/v)

Lane 3- Silica Gel H plates developed with chloroform : acetone : methanol : acetic acid : water (10:4:2:2:1, by volume)

An autoradiogram is shown for each of these systems. The positions of phospholipid standards as detected by iodine staining are indicated.

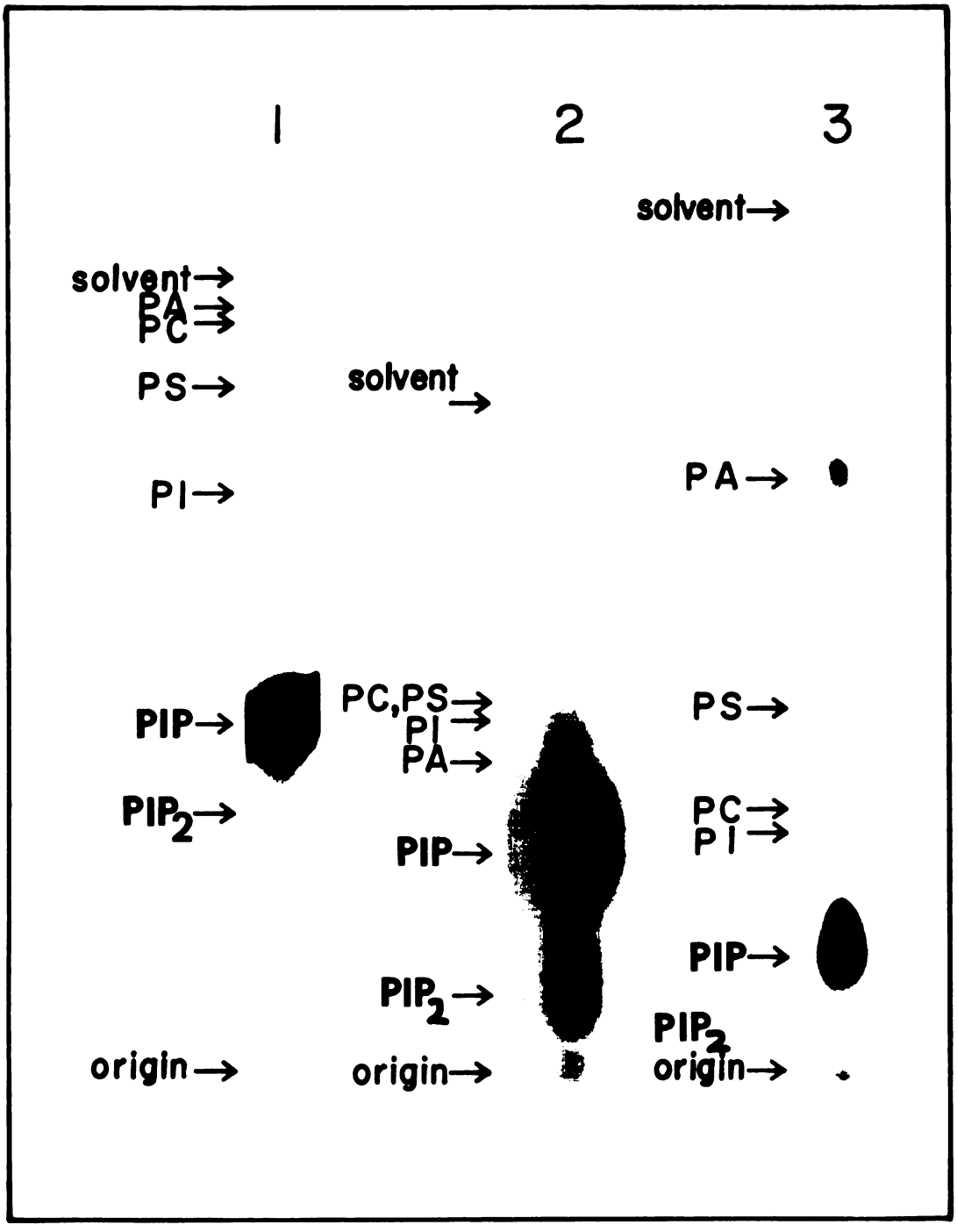

Figure 2. Thin layer chromatography of lipid phosphorylation products

the polyphosphoinositides are quite insoluble and remain near the origin on Silica Gel plates as in solvent system C. PIP accounted for approximately 79% of ^{32}P-labeled lipid, while PA and PIP_2 comprised 9% and 12%, respectively. In agreement with the thin layer chromatographic analyses, anion exchange high pressure liquid chromatography of deacylated lipid extracts produced a major peak of radioactivity eluting with the deacylation product of PIP, and minor peaks eluting with the deacylation products of PA and PIP_2 standards (Figure 3).

Figure 3. High pressure liquid chromatography of deacylated lipid phosphorylation products

An extract of phosphorylated nuclear envelopes was prepared as described for Figure 2. The extract was then subjected to deacylation in mild alkali and HPLC as described under "Experimental Procedures". Radioactive products were detected by scintillation counting (▲). The concentration of ammonium formate in the elution gradient is shown (--). The positions of elution of deacylated phospholipid standards are indicated.

Abbreviations are as follows:

GPC, glycerylphosphorylcholine
GPE, glycerylphosphorylethanolamine
GPI, glycerylphosphorylinositol
GP, glycerylphosphate
GPS, glycerylphosphorylserine
GPIP, glycerylphosphorylinositol 4-phosphate
$GPIP_2$, glycerylphosphorylinositol 4,5-bisphosphate.

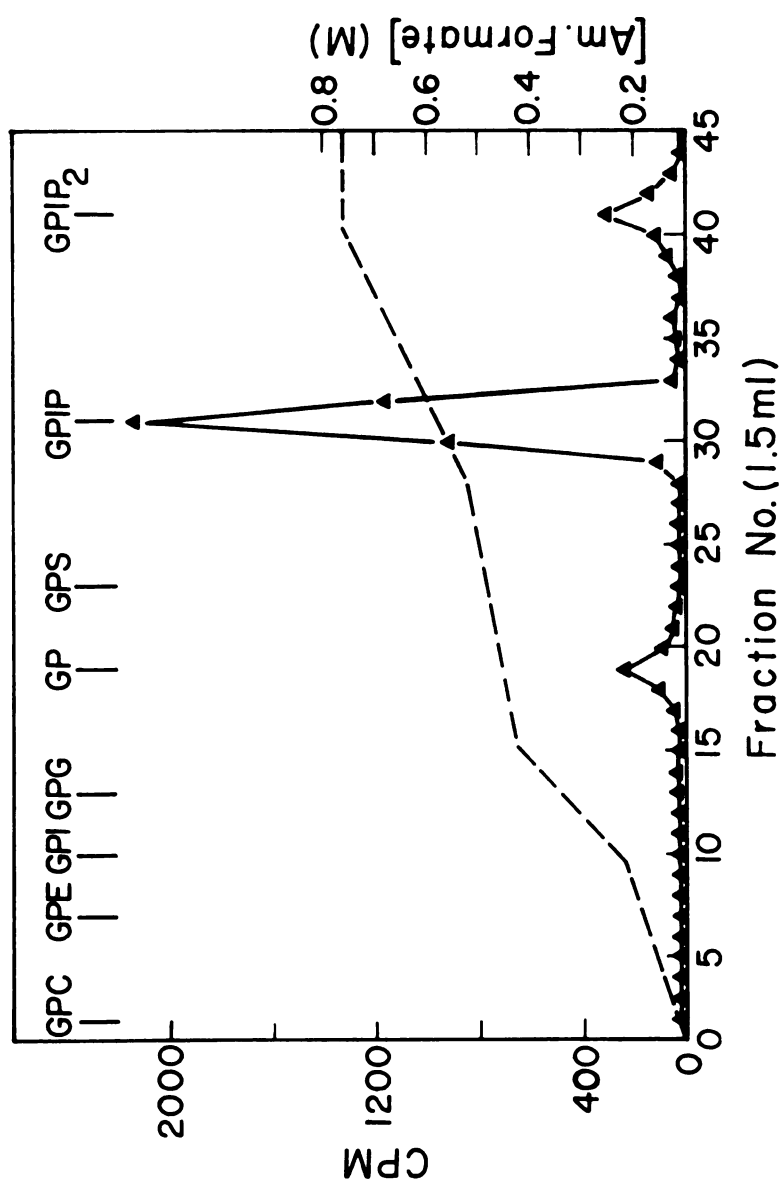

Figure 3. High pressure liquid chromatography of deacylated lipid phosphorylation products

DISCUSSION

Although protein kinase activity has been previously identified in isolated rat liver nuclear envelopes, the presence of low molecular weight, chloroform:methanol: HCl-extractable phosphorylation products has not been previously demonstrated. This is mainly due to the use of trichloroacetic acid precipitation-filter assays when studying protein kinase activity (9,35,36), which does not resolve phosphorylation products. The risk in using this assay for studying membrane phosphorylation, pointed out by Behar-Bannelier and Murray (37) and supported here, is that labeled phospholipids can also be precipitated and lead to an overestimation of protein kinase activity. In previous studies where nuclear envelope phosphorylation products have been subjected to polyacrylamide gel electrophoresis (10,11,38), the gels have been stained with organic dyes before autoradiography was performed to identify phophorylation products. As previously discussed, the labeled phospholipids can be removed by the organic solvents used in these procedures and so were not detected.

The chloroform-soluble ^{32}P-labeled material has been identified as a mixture of PA, PIP, and PIP_2 by four chromatographic procedures. PIP and PIP_2 have not been

previously identified as nuclear envelope components, possibly for the following reasons: 1) analyses of nuclear envelope phospholipids (39-43) have not used methods sensitive enough to detect the low levels of polyphophoinositides likely to be present in isolated nuclei; 2) the methods used in these analyses will not resolve the polyphosphoinositides; and 3) the polyphosphoinositides are probably degraded during the isolation of nuclei.

Characterization of the mechanisms for the metabolism of PIP and PIP_2 are described in Chapters II, III, and IV. A possible role for these phospholipids in regulating nuclear envelope-associated ATPase activity is described in Chapter V.

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CHAPTER II

CHARACTERIZATION OF NUCLEAR ENVELOPE-ASSOCIATED PHOSPHATIDYLINOSITOL KINASE AND PHOSPHATIDYLINOSITOL 4-PHOSPHATE KINASE

ABSTRACT

The synthesis of phosphatidylinositol [4-^{32}P]phosphate (PIP) and phosphatidylinositol [4,5-^{32}P]bisphosphate (PIP_2) in isolated rat liver nuclear envelopes was further characterized. Phosphatidylinositol kinase and PIP kinase were identified as authenic nuclear envelope-associated enzymes by comparison with the same activities in plasma membranes and microsomes. The amount of ^{32}P associated with PIP peaked at 2 to 5 min of incubation and rapidly declined thereafter. In comparison, [^{32}P]PIP_2 levels remained relatively constant after 2 min of incubation. The greatest amount of ^{32}P-incorporation into PIP occurred at pH 7.5 in the presence of 25mM $MgCl_2$, while PIP_2 synthesis was optimal at pH 8.0 and 25mM $MgCl_2$. Synthesis of PIP required ATP and $MgCl_2$; however, some PIP_2 was synthesized in the presence of ATP and $MgCl_2$, $CaCl_2$, or $CoCl_2$, or with GTP and $MgCl_2$. PIP synthesis was inhibited by $CaCl_2$, $CoCl_2$, $MnCl_2$, and was strongly inhibited by $CuCl_2$ and $LaCl_3$. PIP sythesis was also inhibited by disulfides and N-ethylmaleimide. PIP_2 synthesis was also inhibited by metals, disulfides, and N-ethylmaleimide. PIP synthesis was inhibited by exogenously added phospholipids, especially phosphatidylglycerol, phosphatidylserine, and PIP_2. PIP_2 synthesis was stimulated by exogenous PIP but inhibited by phosphatidylglycerol and PIP_2.

INTRODUCTION

The phosphphorylation of nuclear envelope components has been postulated to be involved in the regulation of nucleocytoplasmic transport of macromolecules (1,2) and cell cycle events (3). In chapter I, the organic-soluble material that becomes labeled with ^{32}P during the phosphorylation of rat liver nuclear envelopes was identified as a mixture of phosphatidic acid, PIP, and PIP_2. In recent years the importance of polyphosphoinositides (PIP and PIP_2) in modulating biological membrane structure and function has been discussed (4,5). Studies of polyphosphoinositide effects on maintainance of membrane-bound calcium pools (4), membrane fluidity (6,7), ATPase activity (8,9), and mediation of hormonal stimulation (10-13) have been reported. It had been assumed that polyphosphoinositide metabolism is confined primarily to the plasma membrane, but it has now been shown that PI kinase activity is also associated with highly purified preparations of microsomes (14), lysosomal membranes (15), and Golgi (16).

Phospholipids of isolated rat liver nuclei have been characterized (17-19); however, polyphosphoinositides have not previously been identified in these preparations. Studies of nuclear envelope phospholipid metabolism have focused primarily on determining the fate of the

phospholipids during mitosis (20-22). Phospholipid metabolism in isolated nuclear envelopes has received little attention, although acyl-CoA:1-acyl-sn-glycero-3-phosphorylcholine acyltransferase activity has been observed in neuronal nuclear envelopes (23). In the present study, PI kinase (EC 2.7.1.67) and PIP kinase (EC 2.7.1.68) activities in nuclear envelopes isolated from rat liver have been further characterized. An enzymatic activity which degrades PIP has also been identified and is further characterized in Chapter III. These results suggest that nuclear envelopes actively metabolize phosphoinositides. This metabolism may be important in the regulation of nuclear envelope function since it has been suggested that nucleoside triphosphatase activity (24) and RNA transport (25) are affected by the lipid environment in the envelope.

EXPERIMENTAL PROCEDURES

Materials. Adult male Sprague-Dawley rats (180 - 200 g) were used for all studies. (^{32}P)Orthophosphoric acid, carrier free, was purchased from ICN Pharmaceuticals, Inc. [γ-^{32}P]ATP and [γ-^{32}P]GTP were synthesized according to the method of Glynn and Chappell (26), and isolated as described by Walsh, et al. (27). Phospholipids were obtained from Sigma and Serdary Research Laboratories. All other chemicals were of reagent grade.

Assay of lipid phosphorylation. In routine assays of lipid phosphorylation, the various effectors tested were preincubated with nuclear envelopes (isolated as described in Chapter I) for 10 min at 30 C before the reactions were initiated by the addition of an equal volume of phosphorylation mixture (100mM HEPES, pH 7.5, 200mM NaCl, 10mM $MgCl_2$, and 200uM [γ-^{32}P]ATP, 500 - 1500 cpm/pmol). Incubations were carried out at 30 C for 1 or 2 min. The reactions were terminated by the addition of 1.5 ml of chloroform : methanol (1:2, v/v) followed by the addition of 0.5 ml of chloroform and 0.5 ml of 2.4N HCl (28). The lower phase was removed and the upper phase and interface were extracted with 1.0 ml of chloroform. The combined lower phases were the extracted twice with 2 ml of methanol : 1N HCl (1:1, v/v) and evaporated under a stream

of nitrogen at 30 C. The lipid residues were dissolved in 50 ul of chloroform : methanol : H_2O (75:25:2, v/v/v) and applied to thin layer cellulose (Avicel) plates along with the appropriated phospholipid standards. Chromatography was performed in n-butanol : acetic acid : water (85:10:25, v/v/v) and the plates were subjected to autoradiography as previously described (Chapter I). Areas of the thin layer plates corresponding to the positions of PA, PIP, and PIP_2 were scraped into vials and the incorporated radioactivity was determined by scintillation counting. The coefficients of variation for each of the kinase assays were less than 5% among duplicate samples and less than 9% among different nuclear envelope preparations.

Other methods. Rat hepatic plasma membranes were prepared as described by Emmelot, et al. (29). Microsomes were pelleted from a post-15,000 x g supernatant by centrifugation at 100,000 x g for 60 min. The purities of the subcellular fractions were assessed by marker enzyme analyses (30) as previously described (Chapter I). Protein concentrations were determined by the method of Lowry, et al. (31), using bovine serum albumin (Sigma) as the standard.

RESULTS

Comparison of lipid phosphorylation in nuclear envelopes, plasma membranes, and microsomes. An analysis of lipid phosphorylation in subcellular fractions isolated from rat liver is described in Table I. The marker enzymes NADH:cytochrome c reductase and alkaline phosphodiesterase I were assayed to determine the extent of cross-contamination by microsomes and plasma membranes, respectively (30). Analysis of alkaline phosphodiesterase I indicated that microsomes contained less than 5% contamination by plasma membranes. Analysis of NADH:cytochrome c reductase showed that plasma membranes contained up to 23% contamination by microsomes. Approximately 13% and 1.5% of the nuclear envelope protein could be contributed by contaminating microsomes and plasma membranes, respectively. However, as previously discussed (Chapter I), the estimate of contamination by microsomes is an overestimate since nuclear envelopes contain endogenous NADH:cytochrome c reductase activity (32).

^{32}P incorporation into PA, PIP, and PIP_2 was observed for all three subcellular fractions. The activity of diacylglycerol kinase was greatest in the microsomes, with nuclear envelopes and plasma membranes exhibiting 82% and 46% of the microsomal specific activity, respectively.

Table I

Comparison of lipid phosphorylation in nuclear envelopes, microsomes, and plasma membranes

Preparation	Relative specific activity[a]		^{32}P-incorporation		
	NADH:cytochrome c reductase	Alkaline phosphodiesterase I	PA	PIP	PIP_2
			pmol/mg protein		
nuclear envelopes	0.60	0.57	8.7	122.8	25.0
microsomes	5.15	1.69	10.6	186.0	20.6
plasma membranes	1.12	30.52	4.9	114.4	186.4

Nuclear envelopes, microsomes, and plasma membranes were assayed for cross-contamination and ^{32}P incorporation into PA, PIP, and PIP_2 as described under "Experimental Procedures". Values represent the mean of three determinations.

[a] Relative specific activity = specific activity in preparation/specific activity in whole homogenate.

Significant PI kinase activity was present in all three fractions but the highest specific activity was in the microsomes. The highest specific activity for PIP kinase was expressed by the plasma membranes with nuclear envelopes and microsomes exhibiting only 13.4% and 11.1% as much incorporation of ^{32}P into PIP_2, respectively. However, these values are greater than the amount of plasma membrane contamination in these fractions. These results indicate that nuclear envelope lipid phosphorylation cannot be attributed to contamination by microsomes and plasma membranes. Therefore, diacylglycerol kinase, PI kinase and PIP kinase represent enzymatic activities endogenous to the nuclear envelope. The remainder of this Chapter will deal only with PI kinase and PIP kinase. For further discussion of the characterization of diacylglycerol kinase, see Reference 33.

Time course of lipid phosphorylation. The time course of ^{32}P incorporation into PIP and PIP_2 is shown in Figure 1. Labeling of PIP peaked by 2 min of incubation with 70% of the labeled PIP being degraded in the next 18 min. PIP_2 labeling also peaked at 2 min with approximately 30% of the labeled PIP_2 being degraded in the next 18 min of incubation.

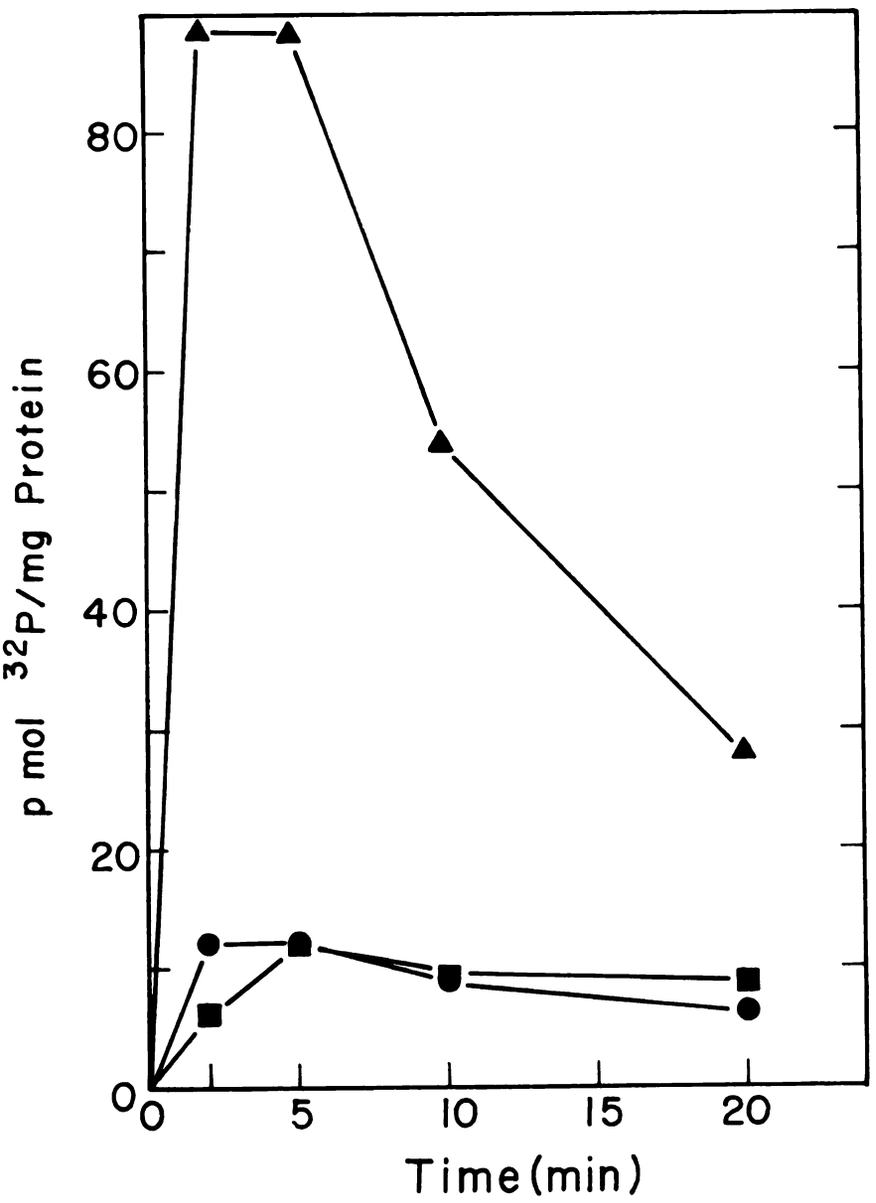

Figure 1. Time course of labeling of PIP and PIP_2

Nuclear envelopes were phosphorylated for the times indicated, extracted, and products were analyzed as described under "Experimental Procedures". Incorporation of ^{32}P into PA (■), PIP (▲), and PIP_2 (●) were determined. Values represent the mean of duplicate experiments.

Characterization of nuclear envelope-associated PI kinase and PIP kinase. Nuclear envelope-associated PI kinase expressed maximal activity at pH 7.0 and with 25mM $MgCl_2$ (Figures 2A and 3A). PIP kinase expressed maximal activity at pH 8.0 and with 25mM $MgCl_2$ (Figures 2B and 3B). Citrate and glycine were rejected as buffers since citrate strongly inhibited both kinases, and glycine slightly stimulated PIP_2 kinase (data not shown). PI kinase and PIP kinase were both inhibited by 50mM $MgCl_2$.

Substrate specificities of PI kinase and PIP kinase. The cation and nucleotide requirements for nuclear envelope-associated PI kinase and PIP kinase were examined (Table II). No lipid phosphorylation occurred in the absence of added divalent metal cation. With [γ-^{32}P]ATP, PI kinase activity was significantly expressed only in the presence of $MgCl_2$. [γ-^{32}P]GTP was not used as a substrate by PI kinase in the presence of either $MgCl_2$ or $CaCl_2$. PIP kinase activity was observed with [γ-^{32}P]ATP in the presence of $MgCl_2$, $CaCl_2$ and $CoCl_2$, and with [γ-^{32}P]GTP in the presence of $MgCl_2$.

Figure 2. pH profiles of nuclear envelope-associated PI kinase and PIP kinase

Nuclear envelopes were phosphorylated as described under "Experimental Procedures" except that the following buffers were substituted at a final concentration of 50mM: sodium acetate (▲), MES (■), HEPES (●), and Tris-Cl (△). Phosphorylation products were extracted and analyzed as previously described. Values represent the mean of duplicate experiments.

A. PI kinase activity

B. PIP kinase activity

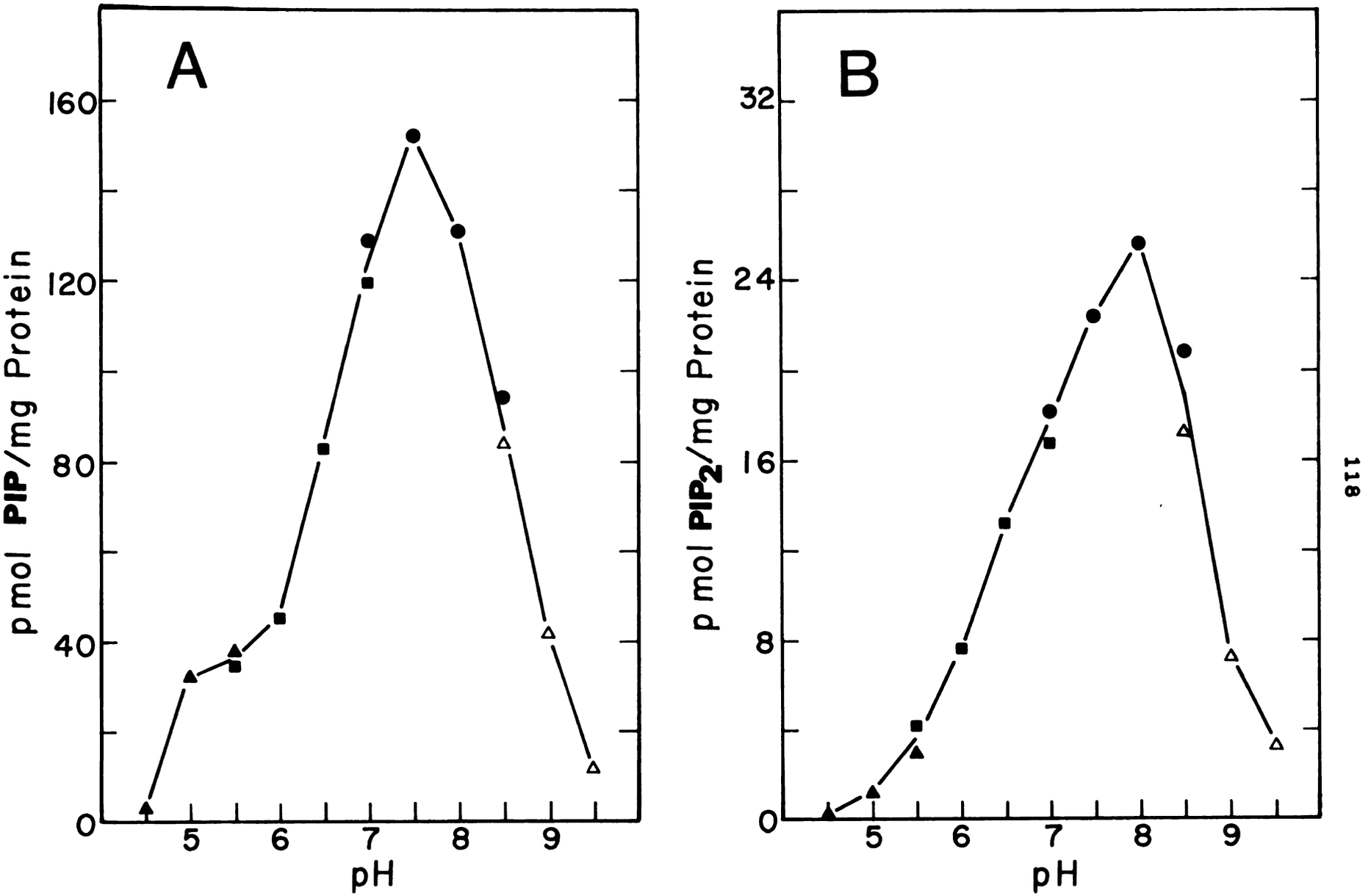
A
160
120
80
40
0
p mol PIP/mg Protein
5
6
7
8
9
pH
B
32
24
16
8
0
p mol PIP2/mg Protein
5
6
7
8
9
pH

Figure 3. Effect of $MgCl_2$ concentration on nuclear envelope -associated PI kinase and PIP kinase

Nuclear envelopes were phosphorylated as described under "Experimental Procedures" except that $MgCl_2$ was varied to give the indicated final concentration. Phosphorylation products were extracted and analyzed as previously described. Values represent the mean of duplicate experiments.

A. PI kinase activity

B. PIP kinase activity

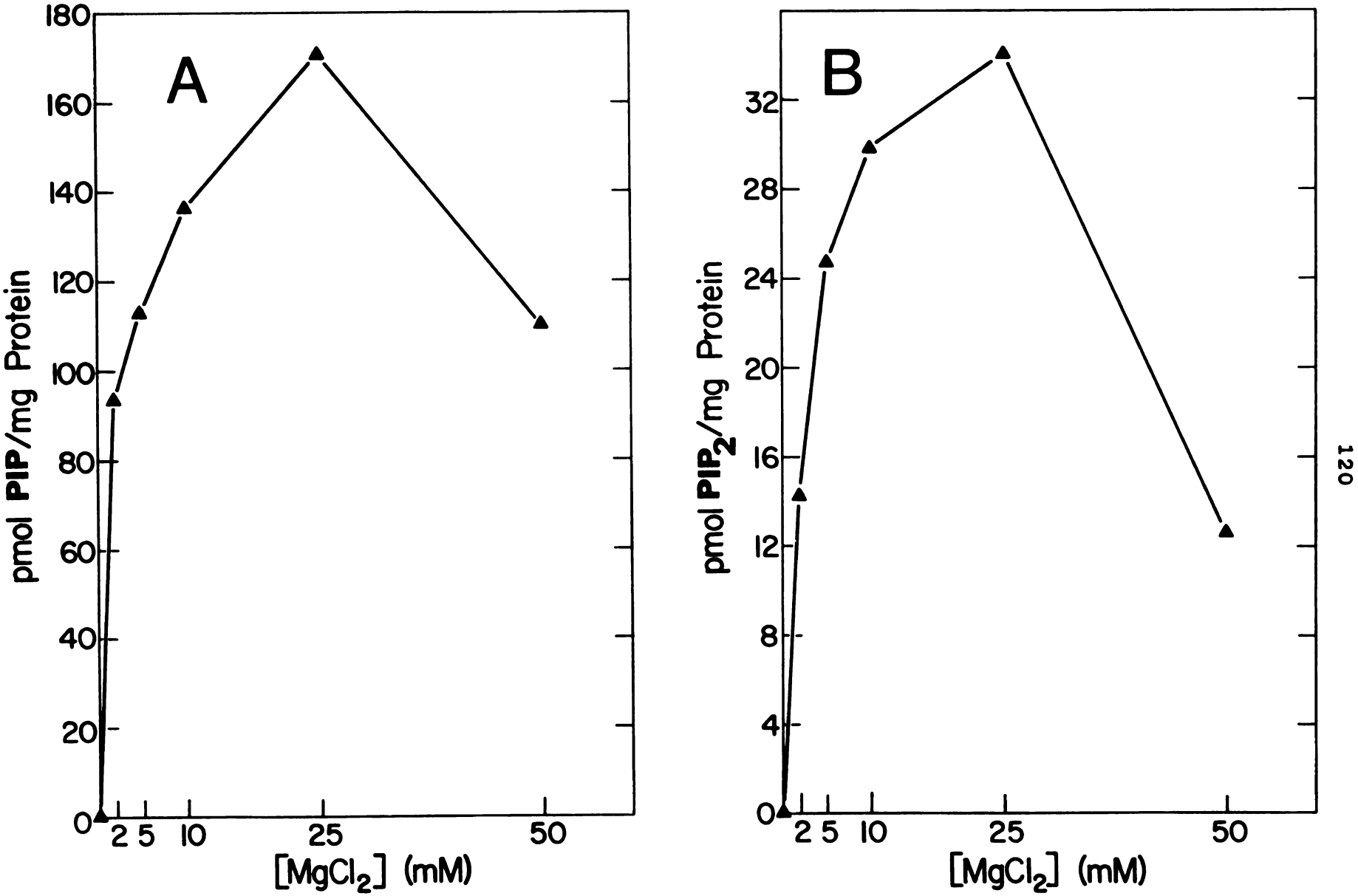
A
B
pmol PIP/mg Protein
pmol PIP2/mg Protein
[MgCl2] (mM)
[MgCl2] (mM)
0
20
40
60
80
100
120
140
160
180
0
4
8
12
16
20
24
28
32
2 5 10 25 50
2 5 10 25 50

Table II

Substrate specificities of PI kinase and PIP kinase

Nucleotide	Metal	PIP	PIP_2
		% of control[a]	
ATP	none[b]	0	0
	none	0	0
	$MgCl_2$	100	100
	$CaCl_2$	9	38
	$MnCl_2$	15	17
	$CoCl_2$	9	41
	$ZnCl_2$	0	5
	$CuCl_2$	1	27
	$NiCl_2$	2	7
GTP	$MgCl_2$	1	47
	$CaCl_2$	2	2

Nuclear envelopes were phosphorylated for 2 min in the presence of 50mM HEPES, pH 7.5; 5mM metal; and 100uM [γ-^{32}P]ATP or [γ-^{32}P]GTP as indicated below. Phosphorylation products were extracted and analyzed as described under "Experimental Procedures". Values represent the mean of three experiments.

[a] Control values were those expressed with ATP + $MgCl_2$.

[b] Contained 5mM EDTA.

Effects of metals on lipid phosphorylation. Inclusion of $CaCl_2$, $MnCl_2$, or $CuCl_2$ in the phosphorylation assay, which contained 5mM $MgCl_2$, led to a dose-dependent inhibition of both kinases (Table III). $CoCl_2$ (0.1mM) slightly stimulated PI kinase, while 1mM $CoCl_2$ inhibited both kinases. $LaCl_3$ (0.1mM) also strongly inhibited both activities.

Effects of sulfhydryl reagents on lipid phosphorylation. Table IV summarizes the effects of sulhydryl reagents on PI kinase and PIP kinase. PI kinase was slightly inhibited by 5mM DTT, 2-ME, and cysteine, but was strongly inhibited by 5mM GSH, and 0.5mM GSSG, cystine, DTNB, and NEM. PIP kinase was stimulated by DTT and 2-ME, and was strongly inhibited by GSH, GSSG, cystine, DTNB, and NEM.

Effects of phospholipids on lipid phosphorylation. The effects of lipids and the nonionic detergent, Triton X-100, on PI kinase and PIP kinase activities are summarized in Table V. Lipids were added as multilamellar vesicles dispersed in 25mM HEPES, pH 7.5, and in the case of PI, as mixed micelles with 0.1% Triton X-100. PI kinase activity was unaffected by PC and PE, but was inhibited by PG, PA, and PS. PIP kinase activity was stimulated by PA and PS, but was inhibited by PG.

Table III

Effects of metals on lipid phosphorylation

Addition	Concentration[a]	PIP	PIP_2
	mM	% of control[b]	
none		100	100
$CaCl_2$	0.1	89	78
	1.0	56	41
$CoCl_2$	0.1	111	79
	1.0	28	23
$MnCl_2$	0.1	76	58
	1.0	30	26
$CuCl_2$	0.1	8	25
	1.0	2	15
$LaCl_3$	0.1	14	41

Nuclear envelopes were preincubated with the indicated compounds for 10 min at 30 C. Phosphorylation, extraction and analysis of products were carried out as described under "Experimental Procedures". Values represent the mean of three experiments.

[a] Values represent the final concentration in the phosphorylation assay. The preincubation concentration was twice that indicated.

[b] Control values were those expressed with no addition to the standard phosphorylation assay.

Table IV

Effects of sulfhydryl reagents on lipid phosphorylation

Addition[a]	PIP	PIP_2
	% of control[b]	
none	100	100
5mM dithiothreitol	81	146
5mM 2-mercaptoethanol	90	121
5mM cysteine	85	101
5mM glutathione (reduced)	9	14
0.5mM glutathione (oxidized)	23	27
0.5mM cystine	12	13
0.5mM DTNB	9	14
0.5mM iodoacetamide	91	83
0.5mM N-ethylmaleimide	6	16

Nuclear envelopes were preincubated with the indicated chemicals for 10 min at 30 C. Phosphorylation, extraction, and analysis of products were carried out as described under "Experimental Procedures". Values represent the mean of three experiments.

[a] Values represent the final concentration in the phosphorylation assay. The preincubation concentration was twice that indicated.

[b] Control values were those expressed with no addition to the standard phosphorylation assay.

Table V

Effects of phospholipids on lipid phosphorylation

Addition	Concentration[a]	PIP	PIP_2
	mM	% of control[b]	
none		100	100
PG	0.2	16	32
	1.0	14	45
PA	0.2	71	351
	1.0	62	202
PS	0.2	57	216
	1.0	9	360
PI	1.0	48	89
	5.0	12	57
Triton X-100	16.0	51	75
	90.0	10	24
PI + 16mM Triton X-100	1.0	113	86
	5.0	77	66
PIP	0.1	81	116
	0.5	67	152
PIP_2	0.1	53	96
	0.5	10	46

Nuclear envelopes were preincubated with the indicated lipids for 10 min at 30 C. Phosphorylation, extraction, and analyses of products were carried out as described under "Experimental Procedures". Values represent the mean of 2 to 4 experiments.

[a] Values represent the final concentration in the phosphorylation assay. The preincubation concentration was twice that indicated.

[b] Control values were those expressed with no addition to the standard phosphorylation assay.

Addition of either PI or Triton X-100 alone inhibited both kinases. Addition of PI in the presence of Triton X-100, however, produced greater incorporation of ^{32}P into PIP than with either agent alone. This suggests that exogenously added PI is not used as a substrate by PI kinase unless detergent is also present. Addition of PIP led to increased labeling of PIP_2 but inhibited PI kinase. Exogenously added PIP_2 was strongly inhibitory to PI kinase, and was moderately inhibitory to PIP kinase. These results suggest that phospholipids can influence the activity of nuclear envelope-associated lipid kinases; however, a more refined understanding of these effects will require studies with purified enzymes reconstituted into vesicles containing substrate and test phospholipids.

DISCUSSION

In Chapter I, PA, PIP and PIP_2 were identified as phosphorylation products in isolated rat liver nuclear envelopes. The enzymes responsible for their synthesis have now been characterized in greater detail. Previous studies of nuclear envelope phospholipid metabolism have focused primarily on determining the fate of the phospholipids during mitosis (20-22). Phospholipid metabolism in isolated nuclear envelopes has received very little attention. This may be due in part to the lack of any previous demonstration of the association of phospholipid biosynthetic enzymes with the nuclear envelope. However, when studying the subcellular distribution of enzymes, the nucleus is often prematurely disregarded. Since the crude nuclear fractions commonly used in such studies are heavily contaminated with mitochondria, plasma membranes, and other organelles, the use of these crude fractions does not accurately reflect the nuclei-associated activity. The use of highly purified nuclei is necessary to assess the nuclear activity. In studying membrane-associated enzymes, the specific activity should be calculated using isolated nuclear envelopes. Studies with whole nuclei underestimate the specific activities of membrane-associated enzymes by a factor of at least 10,

since only 6-9% of the nuclear protein is in the membrane (34). Care must also be used in selecting the method of isolating the envelopes from purified nuclei. In studies of the phosphorylation of nuclear envelope components, it was found that using the relatively mild heparin and DNase I methods for isolating nuclear envelopes, produced membranes with higher kinase activity than did using the harsher sonication isolation method (33,35).

PI kinase activity has been reported to be primarily associated with plasma membranes in the rat liver (36,37); however, highly purified preparations of microsomes (14), lysosomal membranes (15), and Golgi (16) have subsequently been identified as sites of PI kinase in the hepatocyte. PI kinase activity has been reported in crude nuclear fractions of homogenates of rat liver (36-39) and kidney (40), but this activity was attributed to plasma membrane contamination. Highly purified nuclei and nuclear envelopes have not previously been examined for PI kinase activity. In this study, it was established that purified nuclear envelopes have this activity. The specific activity of PI kinase in the nuclear envelope is similar to that in the plasma membrane and slighlty lower than the microsomal activity. Nuclear envelope-associated PI kinase activity exhibits a pH profile and response to $CaCl_2$ similar to that observed for the plasma membrane activity (37). The enzyme described here is strongly

inhibited by sulfhydryl reagents, suggesting that a reduced sulfhydryl is required for activity. PI kinase is also inhibited by exogenously added phospholipids, except for PC which produced a slight stimulation. PIP_2, PG, and PS were particularly strong inhibitors. Exogenously added PI did not serve as substrate unless it was added with detergent; however, since the detergent inhibited the activity, no substantial increase in PIP labeling could be attained by adding PI to the assay.

PIP kinase has been described as being associated with plasma membranes (41) and Golgi (40) in the rat kidney cortex and cytosol in the rat brain (42). The subcellular distribution of PIP kinase in the liver has not previously been described. In this study, PIP kinase has been identified in purified nuclear envelopes, microsomes, and plasma membranes. Additional studies have indicated that PIP kinase also occurs in the cytosol of rat liver (43). Microsomes and nuclear envelopes have similar specific activities for PIP kinase, while the specific activity in the plasma membrane is approximately 8-fold higher. Nuclear envelope-associated PIP kinase is inhibited by $CaCl_2$, sulfhydryl reagents, detergent, and PIP_2, similar to the kidney enzyme (40,41) but exhibits a higher pH optimum than those PIP kinases previously described.

The amount of ^{32}P associated with the phospholipids

was reduced after 2 min of incubation. By this time, 40-50% of the [γ-^{32}P]ATP had been hydrolyzed by the nuclear envelope nucleoside triphosphatase (data not shown). This probably allowed PIP and PIP_2 degradative activities to surpass the kinase activities, resulting in decreased PIP and PIP_2 labeleing in the later part of the time course. The nature of the activity which degrades PIP is further described in Chapter III.

Polyphosphoinositide metabolism was originally studied in the brain where it has been proposed to be important in the generation and propagation of action potentials in neurons (44). More recently, polyphosphoinositides in nonexcitable membranes have been shown to affect the lateral mobility of glycoproteins, presumably by affecting membrane fluidity (8). This effect on membrane fluidity has been proposed as a mechanism for regulating (Na^+,K^+)-ATPase activity in canine kidney (9). The anchorage of specific proteins in membranes may also involve polyphosphoinositides. This has been suggested by experiments in which phosphoinositide-specific phospholipase C released alkaline phosphodiesterase and 5'-nucleotidase from liver cell membranes while leaving other membrane-associated enzymes unaffected (45). A theory on the role of polyphosphoinositides in membrane fusion events has also been presented (46).

Polyphosphoinositides may be involved in similar functions in the nuclear envelope. It has been suggested that both nucleoside triphosphatase activity (24) and RNA transport (25,47) in isolated nuclei are affected by the fluidity of the nuclear envelope. The nuclear envelope is also known to form tight associations with both structural proteins and chromatin-associated proteins, although the biochemical nature of this association has not been described (32,48). The reformation of the nuclear envelope following mitosis requires the fusion of many small vesicles which coalesce on the chromosome surfaces (48). Further investigation of the metabolism of polyphosphoinositides and thier involvement in these processes may lead to a more complete understanding of the regulation of nuclear envelope structure and function.

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CHAPTER III

CHARACTERIZATION OF A PHOSPHATIDYLINOSITOL 4-PHOSPHATE SPECIFIC PHOSPHOMONOESTERASE IN RAT LIVER NUCLEAR ENVELOPES

ABSTRACT

Incubation of rat liver nuclear envelopes with [γ-^{32}P]ATP results in the synthesis of phosphatidylinositol [4-^{32}P]phosphate (PIP). Degradation of endogenously labeled PIP is observed upon the dilution of the labeled ATP with an excess of unlabeled ATP. This degradation is most rapid in the presence of EDTA, and is inhibited by $MgCl_2$ and $CaCl_2$. To further characterize the degradative activity, phosphatidylinositol [4-^{32}P]phosphate and phosphatidylinositol [4,5-^{32}P]bisphosphate (PIP_2) were synthesized and isolated from erythrocyte plasma membranes. The ^{32}P-labeled phospholipids were then resuspended in 0.4% Tween 80, a detergent that did not inhibit degradation of endogenously labeled PIP, and mixed with nuclear envelopes. [^{32}P]PIP and [^{32}P]PIP_2 were degraded at rates of 2.25 and 0.04 nmol/min/mg of nuclear envelope protein, respectively. Only ^{32}P was released from phosphatidyl[2-^{3}H]inositol [4-^{32}P]phosphate, indicating that hydrolysis of PIP is due to a phosphomonoesterase activity (EC 3.1.3.36) in nuclear envelopes. Similarly, anion exchange chromatographic analysis of the water-soluble products released from [^{32}P]PIP indicated that inorganic phosphate was the sole ^{32}P-labeled product. Hydrolysis of PIP was most rapid at neutral pH and was not affected by inhibitors of acid

phosphatase or alkaline phosphatase. Hydrolysis of PIP was also not inhibited by non-specific phosphatase substrates, such as, glycerophosphate, p-nitrophenyl phosphate, AMP, or glucose-6-phosphate. Hydrolysis was stimulated by putrescine and was inhibited by inositol-2-phosphate, spermidine, spermine, and neomycin.

INTRODUCTION

Although the pollyphosphoinositides (PIP and PIP_2) constitute only a small fraction of the total amount of phospholipids in biological membranes, increasing attention is being paid to their roles in membrane function (1-3). Most of these studies have focused on the receptor-mediated breakdown of phosphoinositides in response to hormones (4-6) and other factors (7). Other studies have proposed roles for polyphosphoinositides in Ca^{2+} mobilization (1,8,9), regulation of membrane-bound enzyme activity (10), alteration of membrane fluidity (11,12), and mediation of hormonal stimulation (13-15). While PIP_2 metabolism appears to be primarily associated with the plasma membrane (16), phosphatidylinositol (PI) kinase, the enzyme responsible for PIP biosynthesis, has been reported in highly purified preparations of microsomes (17), Golgi (18), lysosomal membranes (19), and nuclear envelopes (20, also Chapter II).

The nuclear envelope is a particularily interesting, although understudied, organelle since it is thought to be involved in a number of important cellular processes, such as the regulation of the nucleocytoplasmic transport of RNA (21,22), organization of chromatin (22-24), and transduction of hormonal stimuli (25-28). In spite of the importance of these processes in cell function, the

biochemical basis for their regulation is not understood. While characterizing PI kinase in nuclear envelopes isolated from rat liver, we described the degradation of ^{32}P-labeled PIP after the depletion of [γ-^{32}P]ATP. These results suggested that the nuclear envelope contains enzymes involved in both the synthesis and degradation of PIP, and led us to postulate roles for PIP in nuclear envelope function (20). In the present study, we have identified this degradative activity as a PIP-specific phosphomonoesterase. This indicates that the interconversion of PI and PIP in the nuclear envelope occurs through a simple phosphorylation and dephosphorylation pathway, a process well suited to be a regulatory mechanism in nuclear envelope function.

EXPERIMENTAL PROCEDURES

Materials. Adult male Sprague-Dawley rats (180-200 g) were used for all studies. (^{32}P)orthophosphate, carrier-free, was purchased from ICN Pharmaceuticals, Inc. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (29) as modified by Walsh, et al. (30). myo-[2-^{3}H]inositol was a product of New England Nuclear. PIP, PIP_2, phospholipase C (Clostridium welchii), glycerophosphate, p-nitrophenylphosphate, AMP, glucose-6-phosphate, inositol-2-phosphate, neomycin sulfate, putrescine, spemidine, and spermine were obtained from the Sigma Chemical Co. Glass distilled organic solvents were from MCB (Cincinnati, OH). Tween 80 was obtained from the Nutritional Biochemicals Corp (Cleveland, OH). Neomycin was immobilized on Glycophase-CPG beads (Pierce Chemical Co., Rockford, IL) as described by Schacht (31).

Assay of [^{32}P]PIP in nuclear envelopes. Nuclei were isolated according to a modification of the method of Blobel and Potter (32) as previously described (33). Nuclear envelopes were prepared as described by Kay, et al. (34), and resuspended in 0.25M sucrose containing 10mM HEPES, pH 7.5. As previously described (33), these preparations of nuclear envelopes are virtually free of

contamination by mitochondria, lysosomes, peroxisomes, plasma membranes, and microsomes, exhibiting relative specific activities of 0.0, 0.3, 0.2, 0.6, and 0.6 of their respective marker enzymes. Nuclear envelopes (50 ug of protein in 15 ul) were preincubated at 30 C for 5 min. and phosphorylation was initiated by the addition of an equal volume of phosphorylation mixture (100mM HEPES, pH 7.5; 10mM $MgCl_2$; 2mM [γ-^{32}P]ATP, 2000-3000 cpm / pmol). Further additions, as described in the results, were made after 5 min, and the suspensions were incubated for an additional 5 or 10 min. The reactions were terminated by the addition of 1.5 ml of chloroform : methanol (1 : 2, v/v), and the phospholipids were extracted as described by Schacht (31). The lipid residues were redissolved in 50 ul of chloroform : methanol : H_2O (75 : 25 : 2, v/v/v) and analyzed by thin layer chromatography on Silica Gel H plates developed with chloroform : methanol : 20% methylamine (60 : 36 : 10, v/v/v) as described by Volpi, et al. (35). The plates were exposed to Kodak XAR-5 x-ray film for autoradiography. Spots corresponding to PIP were scraped into scintillation vials and counted with Safety Solve (Research Products Int.) scintillation fluid.

Preparation of ^{32}P-labeled phospholipids.

Erythrocyte ghosts, prepared by the method of Schneider and Kirschner (36), were incubated with 2mM [γ-^{32}P]ATP,

10mM $MgCl_2$, and 50mM HEPES, pH 7.5, for 60 min at room temperature. Phospholipids were then extracted and phosphatidylinoitol [4-^{32}P]phosphate and phosphatidylinositol [4,5-^{32}P]bisphosphate were purified by affinity chromatography on a column of immobilized neomycin as described by Schacht (31). [^{32}P]phosphatidic acid was recovered in the unbound fraction and was purified by thin layer chromatography (35). Analysis of the phospholipid products by thin layer chromatography, as described above, indicated radiochemical purities of greater than 90%, 95% and 93% for [^{32}P]PA, [^{32}P]PIP, and [^{32}P]PIP_2, respectively. The labeled phospholipds were stored in chloroform : methanol : H_2O (75 : 25 : 2) at -20 C and were reextracted before use.

Assay of [^{32}P]PIP hydrolysis. Nuclear envelopes (typically 50 ug of protein in 15 ul) were preincubated with various compounds, e.g. phosphatase inhibitors, for 5 to 10 min at 30 C. The assay was initiated by the addition of 15 ul of 0.6mM [^{32}P]PIP in 0.8% Tween 80 containing 25 mM HEPES, pH 7.5. The reactions were terminated by the addition of 1.5 ml of chloroform : methanol (1 : 2, v/v), 0.5 ml of chloroform, and 0.5 ml of 2.4N HCl. The mixtures were vortexed and then centrifuged to separate the phases. Radioactivity in the aqueous phase was determined as Cerenkov radiation and the amount

of PIP hydrolyzed was calculated. Blanks consisted of [^{32}P]PIP incubated in the absence of enzyme or stopped immediately after the addition of the nuclear envelopes.

Preparation of phosphaatidyl[2-^{3}H]inositol [4-^{32}P]phosphate. Rat liver microsomes were pelleted from a post-15,000 x g supernatant by centrifugation at 100,000 x g for 60 min. The microsomes (10mg of protein) were then incubated at 30 C with 100uCi of myo-[2-^{3}H]inositol in the presence of 1mM $MnCl_2$ and 25mM HEPES, pH 7.5, to incorporate the [^{3}H]inositol into PI (37). After 4 hr, 25mM $MgCl_2$ and 2mM ATP were added and the suspension was incubated an additional 15 min. Phospholipids were extracted and [^{3}H]PIP was isolated by chromatography on a column of immobilized neomycin as previously described. [^{3}H]PIP and [^{32}P]PIP were mixed to give an equal number of counts per min of ^{3}H and ^{32}P.

Analysis of water soluble degradation products from [^{32}P]PIP and [^{3}H,^{32}P]PIP. Nuclear envelopes were incubated with [^{32}P]PIP or [^{3}H,^{32}P]PIP in the presence of 10mM EDTA, 1mM $MgCl_2$, or 1mM $CaCl_2$ as described above. Phospholipids were extracted and the resulting phases were dried under N_2 at 37 C. The products from [^{3}H,^{32}P]PIP were dissolved in scintillation fluid and the amounts of ^{3}H and ^{32}P were analyzed by dual channel counting on a

Beckman LS 7000 liquid scintillation counter programmed with automatic quench compensation. The water-soluble products from [^{32}P]PIP were dissolved in 5ml of 0.1N formic acid and applied to a 0.5ml column of Dowex-1 (formate) and washed with an additional 5ml of 0.1N formic acid. Inorganic phosphate was eluted with 5ml of 0.1N formic acid + 0.2M ammonium formate (Buffer A), and inositol-bisphosphate was eluted with 5 ml of 0.1N formic acid + 0.4M ammonium formate (Buffer B) as described by Downes, et al. (38). The eluant from each wash was collected and assayed for radioactivity by scintillation counting.

Other methods.The concentration of PIP in the assay mixtures was determined by total phosphate analysis by the method of Ames (39). Protein concentrations were determined by the method of Bohlen, et al. (40), except that the samples were incubated with 1% sodium dodecylsulfate before derivatization with fluorescamine. Bovine serum albumin (Sigma) was used as a standard.

RESULTS

Degradation of endogenously synthesized PIP

Effect of metals on PIP hydrolysis. Incubation of rat liver nuclear envelopes with [γ-^{32}P]ATP results in the synthesis of [32P]phosphatidic acid, phosphatidylinositol [4-^{32}P]phosphate and phosphatidylinositol [4,5-^{32}P]bisphosphate (20). Dilution of the [γ-^{32}P]ATP with unlabeld ATP enables the degradation of [^{32}P]PIP to be observed (Figure 1). Degradation was most rapid when 10mM EDTA was included, resulting in the hydrolysis of 51% of the labeled PIP in 10 min. Inclusion of 5mM $MgCl_2$ or 5mM each of $MgCl_2$ and $CaCl_2$ inhibited this degradation by 35% and 49%, respectively. The radioactivity associated with phosphatidic acid and PIP_2 remained constant for at least 10 min after the addition of the unlabeled ATP (data not shown).

Effect of detergent on PIP hydrolysis. The effects of certain detergents on the degradation of endogenously synthesized PIP are described by Figure 2. Sodium deoxycholate, Triton X-100, and octyl-glucoside inhibited degradation at both concentrations of detergent tested. Brij 35 stimulated degradation at a concentration of 0.1% and Tween 80 stimulated degradation at concentrations of 0.1% and 0.4%

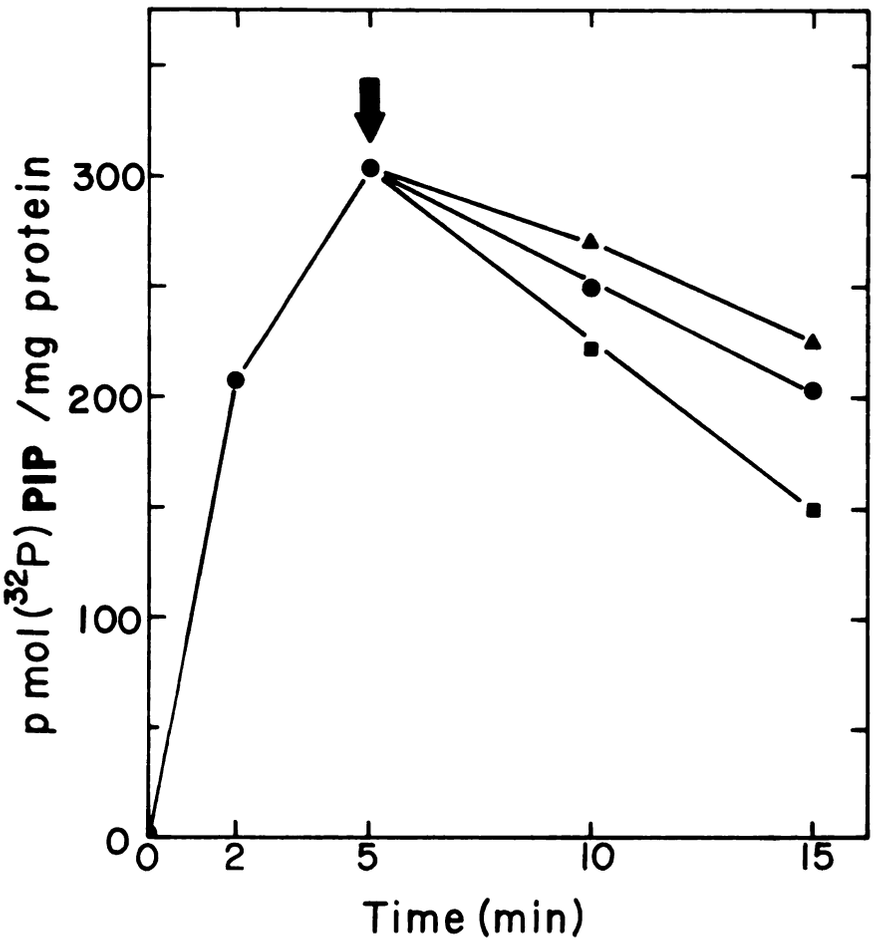

Figure 1. Effect of metals on hydrolysis of endogenous [^{32}P]PIP

Nuclear envelopes were phosphorylated as described under "Experimental Procedures". After 5 min of incubation, indicated by the arrow, additions were made to give final concentrations of 50mM ATP + 10mM EDTA (■), 50mM ATP + 5mM $MgCl_2$ (●), or 50mM ATP + 5mM $MgCl_2$ + 5mM $CaCl_2$ (▲). The suspensions were incubated an additional 5 or 10 min before [^{32}P]PIP levels were assayed as described under "Experimental Procedures".

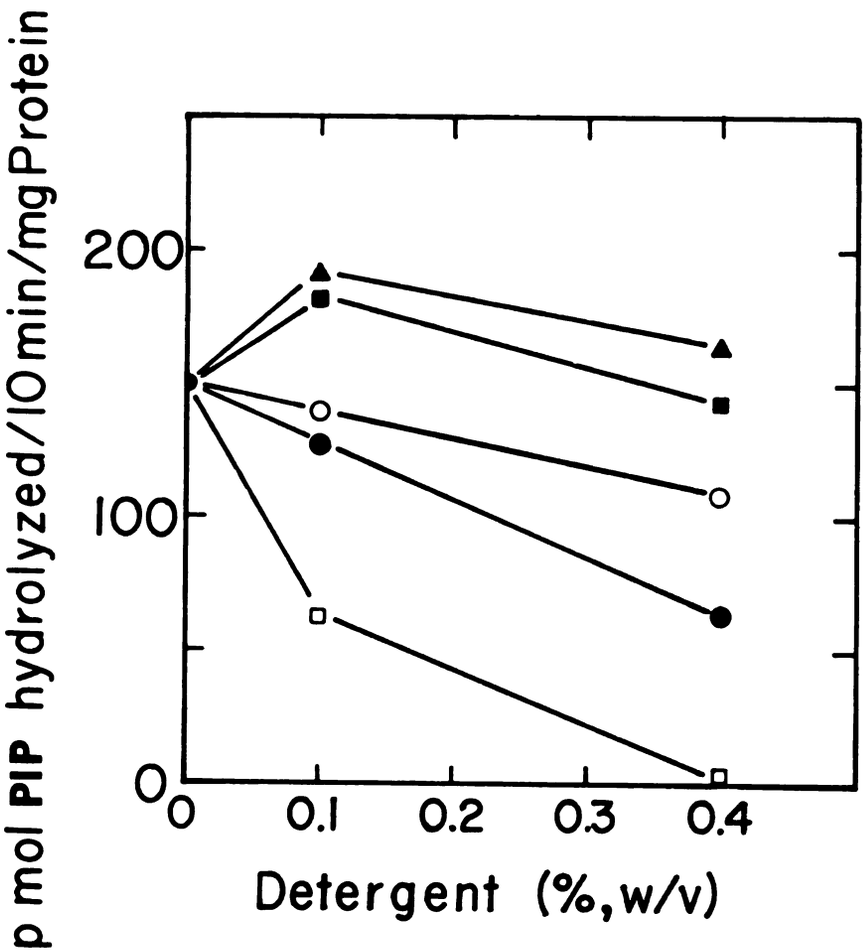

Figure 2. Effect of detergents on hydrolysis of endogenous [^{32}P]PIP

Nuclear envelopes were phosphorylated for 5 min, as described above. Additions were made to give 50mM ATP, 10mM EDTA, and the detergent concentrations indicated, and the suspensions were incubated for an additional 10 min before [^{32}P]PIP levels were assayed. Detergents used were Tween 80 (▲), Brij 35 (■), octyl-glucoside (○), Triton X-100 (●), and sodium deoxycholate (□).

Degradation of exogenous [^{32}P]PIP and [^{32}P]PIP$_2$

Time course of PIP hydrolysis. The hydrolysis of exogenously added PIP was assayed by incubating nuclear envelopes with phosphatidylinositol [4-^{32}P]phosphate in the presence of 0.4% Tween 80, a concentration that did not inhibit the degradation of endogenous [^{32}P]PIP. The amount of radioactivity released to the aqueous phase upon phospholipid extraction was linear for at least 10 min in the presence of 10mM EDTA, indicating a rate of 2.25 nmol PIP hydrolyzed/min/mg of nuclear envelope protein (Figure 3). $MgCl_2$ and $CaCl_2$, at 1mM, inhibited degradation to the same extent, producing rates of hydrolysis 61% of that observed with 10mM EDTA.

Degradation of exogenous [^{32}P]PIP$_2$ and [^{32}P]PA. Assay of the degradation of [^{32}P]PIP$_2$ indicated a rate of hydrolysis of 0.04 nmol/min/mg of nuclear envelope protein in the presence of 10mM EDTA. Hydrolysis was not stimulated by 5mM $MgCl_2$ or $CaCl_2$ (data not shown). No hydrolysis of [^{32}P]PA could be detected under any of the above conditions, indicating a rate of less than 0.01 nmol/min/ mg of nuclear envelope protein.

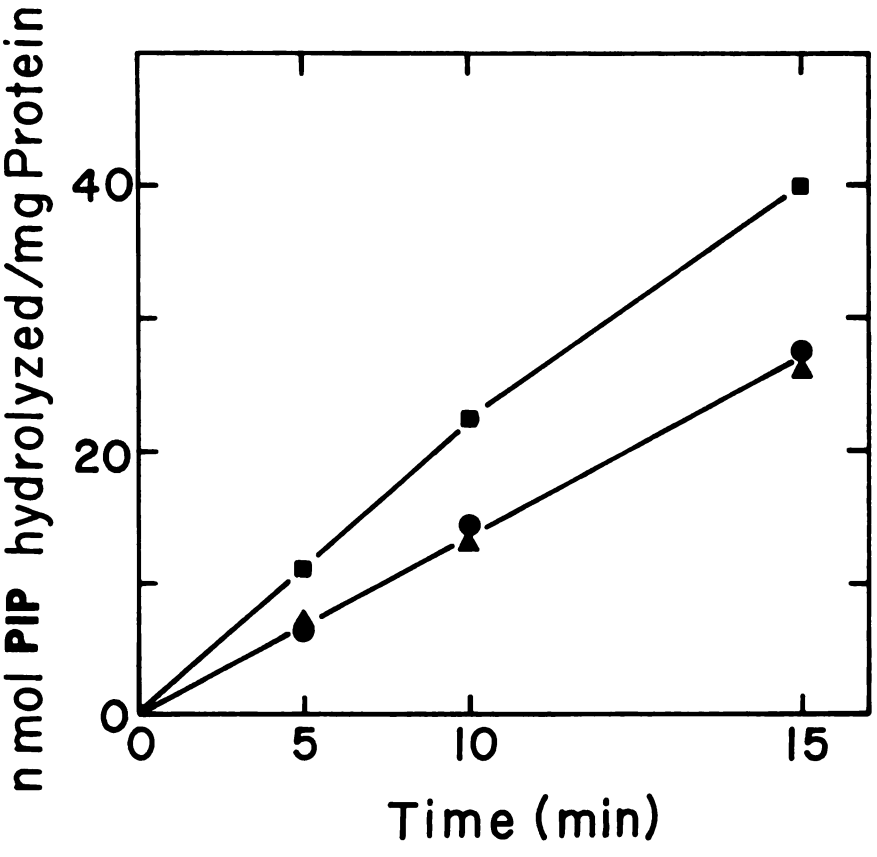

Figure 3. Time course of PIP hydrolysis

Nuclear envelopes were incubated with [^{32}P]PIP for the times indicated in the presence of 10mM EDTA (■), 1mM $MgCl_2$ (●), or 1mM $CaCl_2$ (▲) and PIP hydrolysis was assayed as described under "Experimental Procedures".

Identification of water-soluble deggradation products from PIP. Incubation of liver microsomes with myo-[2-^{3}H]inositol in the presence of 1mM $MnCl_2$, followed by 2mM ATP + 25mM $MgCl_2$ resulted in the incorporation of 46% of the [^{3}H]inositol into PI, 0.5% into PIP, and less than 0.1% into PIP_2. [^{3}H]PIP was combined with [^{32}P]PIP to give an equal number of counts per min for ^{3}H and ^{32}P and incubated with nuclear envelopes. Only ^{32}P was released to the aqueous phase in the presence of 10mM EDTA, 1mM $MgCl_2$, or 1mM $CaCl_2$ (Table I), indicating that hydrolysis is due to a phosphomonoesterase activity. Analysis of the water-soluble products released by the hydrolysis of [^{32}P]PIP is described by Table II. Elution of the Dowex-1 (formate) columns with 0.1N formic acid + 0.2M ammonium formate eluted at least 88% of the radioactivity of the sample containing [^{32}P]orthophosphate and the samples containing the hydrolysis products of [^{32}P]PIP incubated with nuclear envelopes in the presence of either 10mM EDTA, 1mM $MgCl_2$, or 1mM $CaCl_2$. In contrast, incubation of [^{32}P]PIP with commercial phospholipase C (C. welchii) produced a product that eluted from the Dowex column with 0.1N formic acid + 0.4M ammonium formate, in the place of inositol-bisphosphate (38). The activity of the phospholipase C was extremely low toward PIP, approximately 0.1% that of the activity toward phosphatidylcholine (data not shown).

Table I

Analysis of water-soluble products from [^{3}H,^{32}P]PIP

Sample	Addition	Chloroform phase		Aqueous phase	
		^{3}H	^{32}P	^{3}H	^{32}P
Blank		8556 ± 372	8148 ± 294	223 ± 39	571 ± 47
Nuclear Envelope	10mM EDTA	8537 ± 198	7316 ± 233	209 ± 47	1889 ± 76
Nuclear Envelope	1mM $MgCl_2$	8478 ± 225	7671 ± 313	210 ± 22	1125 ± 43
Nuclear Envelope	1mM $CaCl_2$	8673 ± 197	7709 ± 256	178 ± 31	1085 ± 55

Nuclear envelopes (NE) were incubated for 20 min with [^{3}H,^{32}P]PIP in the presence of 10mM EDTA, 1mM $MgCl_2$, or 1mM $CaCl_2$, as indicated above, and extracted as described under "Experimental Procedures". The phases were separated, dried, and analyzed for ^{3}H and ^{32}P. Values represent the mean ± SEM cpm for 3 experiments.

Table II

Chromatographic analysis of water-soluble degradation products from [^{32}P]PIP

Radioactive compound	Sample	Addition	Elution Buffer A	Elution Buffer B
			% of total cpm	
[^{32}P]P_i	nuclear envelope	none	87	5
[^{32}P]PIP	phospholipase C (C. welchii)	none	7	90
	nuclear envelope	10mM EDTA	91	8
	nuclear envelope	1mM $MgCl_2$	86	6
	nuclear envelope	1mM $CaCl_2$	89	7

Nuclear envelopes were incubated for 20 min with [^{32}P]PIP in the presence of 10mM EDTA, 1mM $MgCl_2$, or 1mM $CaCl_2$, extracted and analyzed as described under "Experimental Procedures". Inorganic phosphate was eluted with Buffer A and inositol bisphosphate was eluted with Buffer B. Values represent the percentage of the total counts which were eluted with the indicated buffer and are the mean of at least 4 experiments.

Effect of $MgCl_2$ and $CaCl_2$ on PIP hydrolysis. Hydrolysis of PIP was inhibited by all concentrations tested of both $MgCl_2$ and $CaCl_2$ (Figure 4). $MgCl_2$ and $CaCl_2$ inhibited to virtually identical extents at concentrations lower than 1mM, while $CaCl_2$ was a slightly more potent inhibitor than $MgCl_2$ at higher concentrations.

pH profile of PIP hydrolysis. The effect of pH on the hydrolysis of PIP is described by Figure 5. The activity was highest at neutral pH and was eliminated by decreasing the pH to 4.5.

Effect of phosphatase inhibitors on PIP hydrolysis. Hydrolysis of PIP was not decreased by acid phosphatase inhibitors (41) such as 5mM sodium fluoride, sodium potassium tartrate, or sodium pyrophosphate (Table III). Hydrolysis was also not affected by inhibitors of alkaline phosphatase (42) such as 5mM inorganic phosphate or cysteine. In addition, 0.5mM cystine and 0.5 - 5mM GSH and GSSG had no significant effect on PIP hydrolysis (data not shown).

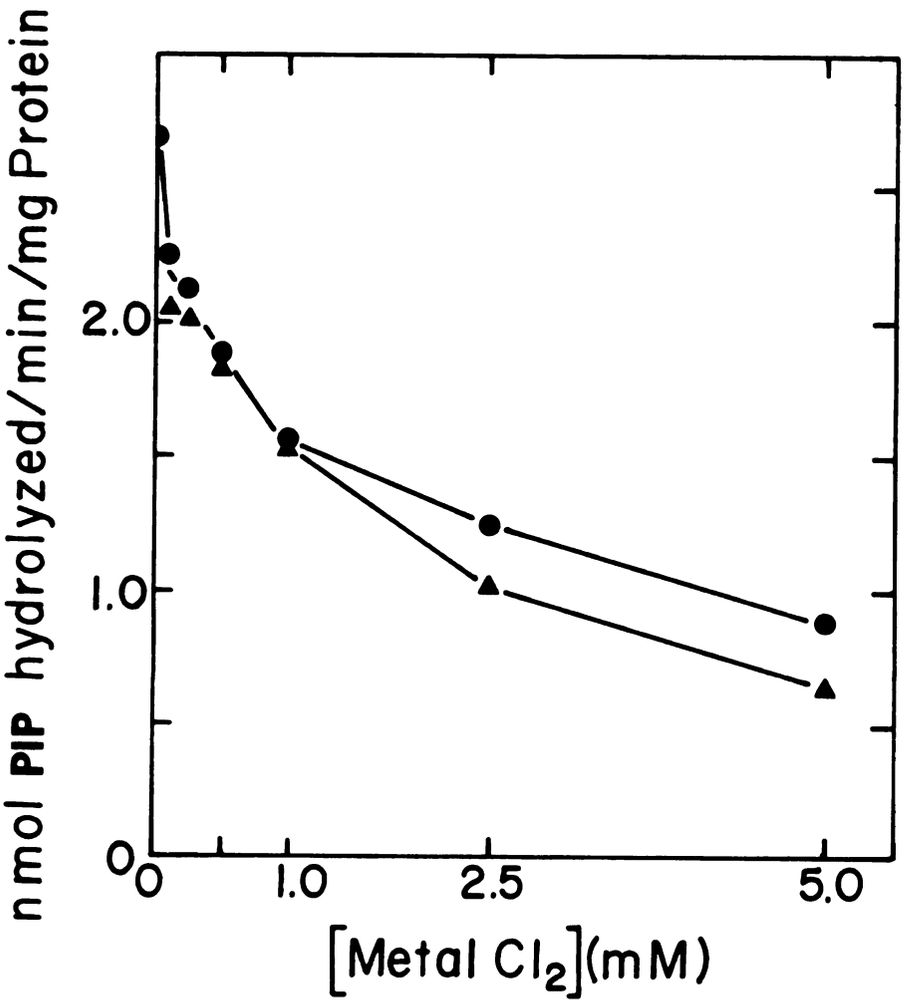

Figure 4. Effect of [Metal,Cl_2] on PIP hydrolysis

Nuclear envelopes were incubated with [^{32}P]PIP for 10 min in the presence of the indicated concentrations of $MgCl_2$ (●) or $CaCl_2$ (▲) and PIP hydrolysis was assayed as described under "Experimental Procedures".

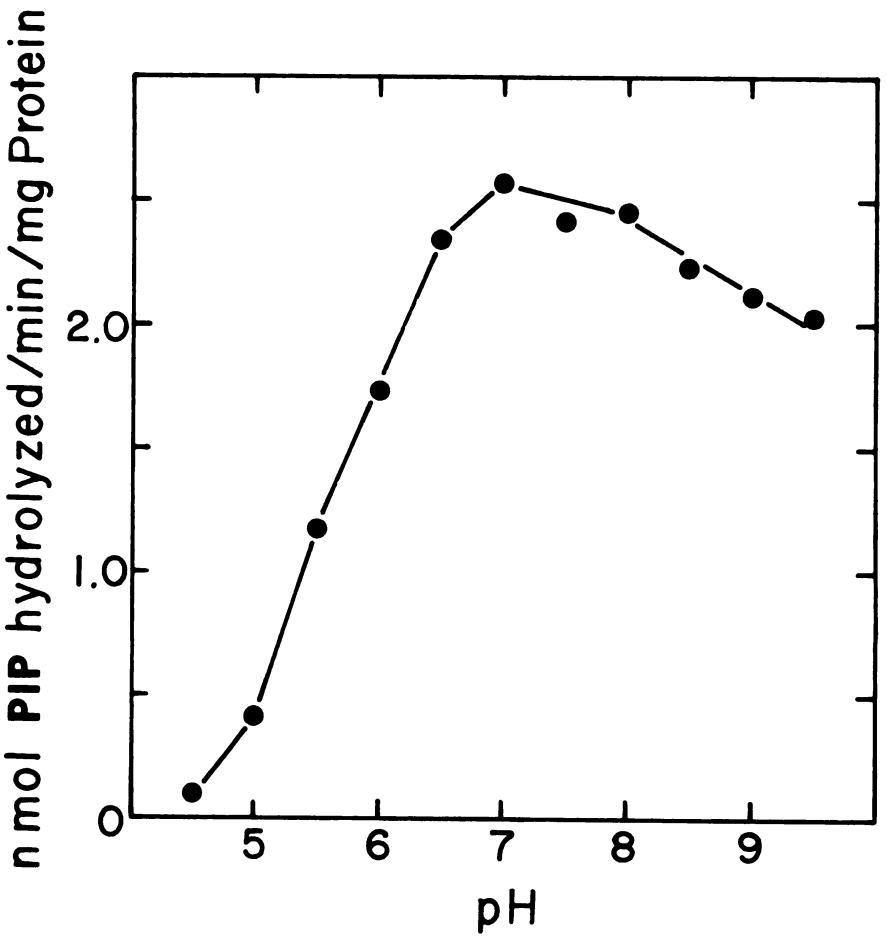

Figure 5. pH profile of PIP hydrolysis

Nuclear envelopes were incubated with [^{32}P]PIP for 10 min in the presence of 50mM sodium acetate (pH 4.5, 5.0), 50mM MES (pH 5.5, 6.0, 6.5), 50mM HEPES (pH 7.0, 7.5, 8.0), or 50mM Tricine (pH 8.5, 9.0, 9.5), and PIP hydrolysis was assayed as described under "Experimental Procedures".

Table III

Effect of phosphatase inhibitors on PIP hydrolysis

Addition	Concentration mM	PIP hydrolysis nmol/min mg protein	% of control
none		2.07 ± 0.10	100
NaF	0.5	1.96 ± 0.06	95
	5.0	2.10 ± 0.08	101
Na,K,tartrate	0.5	2.10 ± 0.12	101
	5.0	2.04 ± 0.09	99
Na pyrophosphate	0.5	1.80 ± 0.04	87
	5.0	1.95 ± 0.10	94
Na phosphate	0.5	1.94 ± 0.07	94
	5.0	2.00 ± 0.11	97
cysteine	0.5	2.13 ± 0.12	103
	5.0	2.56 ± 0.17	124

Nuclear envelopes were preincubated with the indicated compounds for 5-10 min at 30 C before PIP hydrolysis was assayed as described under "Experimental Procedures". Values represent the mean ± SEM of 3 experiments.

Substrate specificity of PIP phosphomonoesterase. To assess the specificity of the phosphomonoesterase, common phosphatase substrates were tested for their ability to compete with PIP for hydrolysis (Table IV). No inhibition of PIP degradation was observed in the presence of 0.5 or 5.0mM alpha- or beta-glycerophosphate, p-nitrophenyl phosphate, AMP, or glucose-6-phosphate. Inositol 2-phosphate, however, caused a dose-dependent decrease in the rate of PIP hydrolysis, suggesting that this compound may be competing with PIP.

Effect of neomycin on PIP hydrolysis. The effect of neomycin sulfate on the degradation of PIP is described by Figure 6. At concentrations of 0.1mM or less, neomycin caused a slight stimulation of PIP hydrolysis; however at higher concentrations, neomycin acted as a strong inhibitor of PIP degradation.

Effect of polyamines on PIP hydrolysis. The effects of putrescine, spermidine, and spermine on the degradation of PIP are indicated in Figure 7. The addition of putrescine resulted in stimulation of PIP hydrolysis, while spermidine caused a slight inhibition of degradation and spermine stongly inhibited degradation.

Table IV

Substrate specificity of PIP phosphomonoesterase

Addition	Concentration mM	PIP hydrolysis nmol/min mg protein	% of control
none		1.87 ± 0.08	100
α-glycerophosphate	0.5	1.88 ± 0.09	101
	5.0	1.81 ± 0.13	97
β-glycerophosphate	0.5	1.79 ± 0.07	96
	5.0	1.97 ± 0.09	105
p-nitrophenyl-phosphate	0.5	2.05 ± 0.08	110
	5.0	2.09 ± 0.14	112
AMP	0.5	1.90 ± 0.05	102
	5.0	2.06 ± 0.14	110
glucose-6-phosphate	0.5	1.91 ± 0.09	102
	5.0	2.02 ± 0.14	108
inositol-2-phosphate	0.5	1.20 ± 0.08	64
	5.0	0.53 ± 0.15	28

Nuclear envelopes were preincubated with the indicated compounds for 5-10 min at 30 C before PIP hydrolysis was assayed as described under "Experimental Procedures". Values represent the mean ± SEM of 3 experiments.

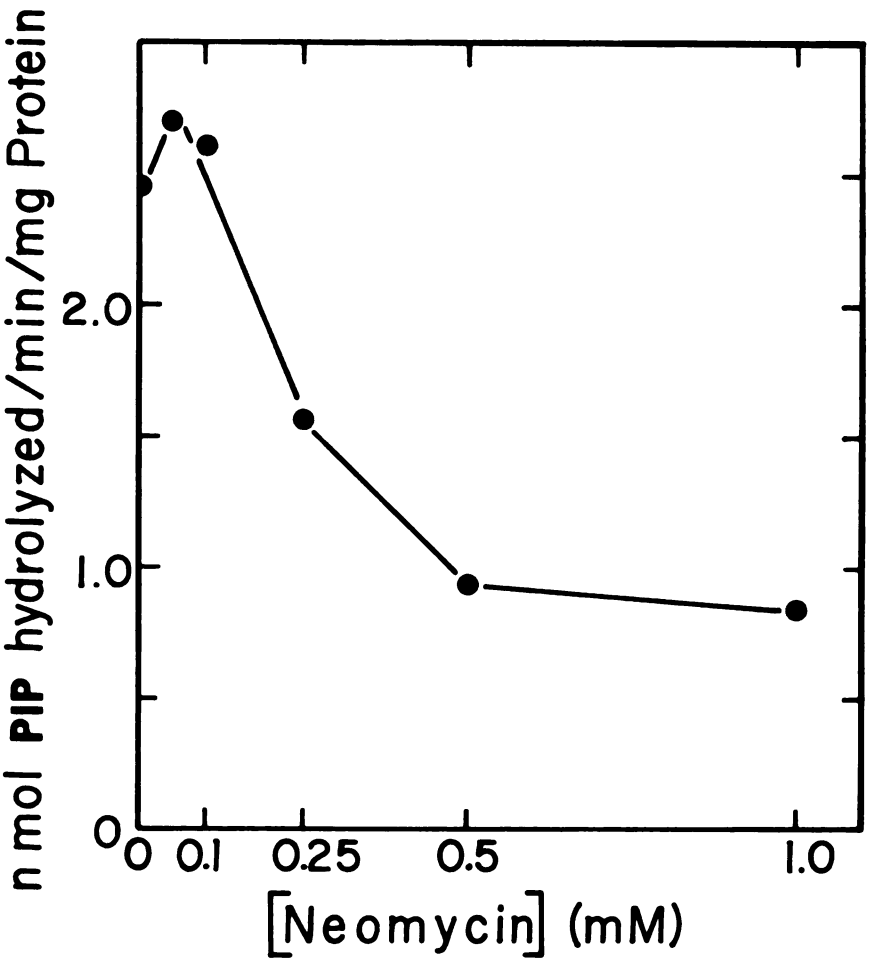

Figure 6. Effect of neomycin on PIP hydrolysis

Nuclear envelopes were incubated with [^{32}P]PIP for 10 min in the presence of the indicated concentrations of neomycin sulfate, and PIP hydrolysis was assayed as described under "Experimental Procedures".

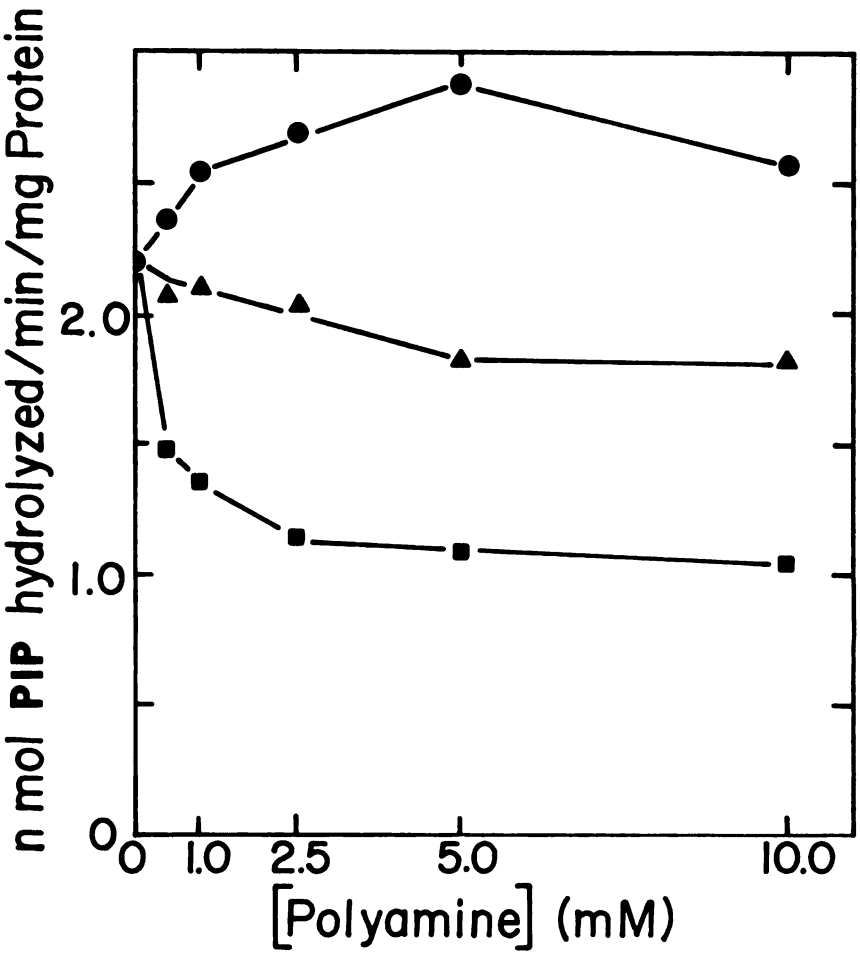

Figure 7. Effect of polyamines on PIP hydrolysis

Nuclear envelopes were incubated with [^{32}P]PIP for 10 min in the presence of the indicated concentrations of putrescine (●), spermidine (▲), or spermine (■), and PIP hydrolysis was assayed as described under "Experimental Procedures".

Discussion

In a previous report, we described the synthesis of PIP and PIP_2 in isolated rat liver nuclear envelopes (20). A nuclear envelope-associated activity which degraded the endogenously labeled PIP was observed, and has now been characterized more fully. Exogenously prepared [^{32}P]PIP provided a substrate for a simple assay of this activity. Inorganic phosphate was the only water-soluble product released from the [^{32}P]PIP in the presence of nuclear envelopes and EDTA, $MgCl_2$, or $CaCl_2$, indicating that hydrolysis of PIP is due to a phosphomonoesterase activity. Phosphomonoesterase activity was also indicated by the release of only ^{32}P from [^{3}H,^{32}P]PIP.

The hydrolysis of both endogenously labeled PIP and exogenous PIP was most rapid in the presence of 10mM EDTA, and was inhibited by both $MgCl_2$ and $CaCl_2$. This is similar to the results presented for a PIP-phosphomonoesterase from rat kidney which was not activated by metals (43), and a cation-independent PIP phosphatase in erythrocyte membranes (44), but contrasts with descriptions of other polyphosphoinositide phosphomonoesterases which are stimulated by Mg^{2+} (45-49) or Ca^{2+} (50). Alternately, the hydrolysis of PIP and PIP_2 by phosphodiesterases appears to be Ca^{2+}-dependent in all cases reported (51).

The nuclear envelope-associated PIP monoesterase activity is highly specific for PIP since PIP_2 was hydrolyzed at a rate 55-fold lower than that of PIP hydrolysis even though the 4-phosphate was radiolabeled in both substrates. Furthermore, no hydrolysis of exogenously added PA could be detected. Hydrolysis of PIP occurred most rapidly at neutral pH, and was not due to either acid phosphatase or alkaline phosphatase. The hydrolysis of PIP was not inhibited by non-specific phosphatase substrates, again suggesting that the activity is specific for PIP. The enzyme also appears to be insensitive to sulfhydryl oxidation by either cystine or oxidized glutathione.

Hydrolysis of PIP was inhibited by neomycin, an amino-glycosidic antibiotic which is known to interact strongly with polyphosphoinositides (52). Hydrolysis of prelabeled polyphosphoinositides in preparations of guinea-pig cerebral cortex (53) and inner ear tissue (54) is also inhibited by neomycin, presumably due to an ionic interaction between the antibiotic and the substrate phospholipids. Neomycin also inhibits PI-specific phospholipase C from rat kidney cortex (55) and human amnion (56). The effect of neomycin on the amnion activity was similar to that reported here, producing stimulation at low concentrations of neomycin and inhibition at higher levels.

PIP hydrolysis by nuclear envelopes was stimulated by all concentrations of putrescine tested, while spermidine was slightly inhibitory and spermine acted as a strong inhibitor. Similar effects for these polyamines have been reported on PI-specific phospholipase C from rat brain (57). As with neomycin, these effects may be due to interactions between the polyamines and PIP. This is supported by preliminary experiments that suggest that the higher order polyamines, particularily spermine, form precipitates when mixed with polyphosphoinositides (unpublished observation). Cellular and nuclear levels of polyamines undergo profound changes during mitogenic stimulation and cellular transformation (58), and so could affect polyphosphoinositide metabolism at the nuclear envelope and other membrane sites. Therefore, interactions between the metabolism of polyphosphoinositides and polyamines may be important in regulating membrane events.

The role of polyphosphoinositide metabolism in nuclear envelope function remains undefined. As previously described (20), we believe that these lipids may be important in regulating nuclear envelope-associated enzyme activity. The present results support this proposal since we have now demonstrated that nuclear envelopes have the capacity to both synthesize and degrade PIP. Since the intercoversion of PI and PIP in the

nuclear envelope occurs through a simple phosphorylation / dephosphorylation pathway, the levels of PIP in the nuclear envelope could be easily regulated. An alternative mechanism for PIP hydrolysis, involving a phosphodiesterase activity, would require PI to be resynthesized in the nuclear envelope or to be transported there from the endoplasmic reticulum, and so probably would not be as favorable for the rapid and precise regulation of PIP levels. A particularily interesting activity in the nuclear envelope is the nucleoside triphosphatase which is thought to provide the energy required for the nucleocytoplasmic transport of RNA (59). We have conducted studies which suggest that polyphosphoinositides are involved in the regulation of the activity of this enzyme (60). Therefore, the metabolism of PIP in the nuclear envelope may be involved in the expression of genetic information. Further investigations of the functions of polyphosphoinositide metabolism and its possible interactions with polyamine metabolism should help elucidate the mechanisms of events that occur at the nuclear envelope and other cellular membranes.

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CHAPTER IV

TOPOLOGY OF PHOSPHATIDYLINOSITOL 4-PHOSPHATE METABOLISM IN RAT LIVER NUCLEI. EVIDENCE FOR PHOSPHOLIPID EXCHANGE BETWEEN THE INNER AND OUTER NUCLEAR MEMBRANES

ABSTRACT

The intranuclear distribution of phosphatidylinositol (PI) kinase and phosphatidylinositol 4-phosphate (PIP) phosphomonoesterase (PIPase) were examined using two techniques. In the first method, isolated rat liver nuclei were treated with free trypsin or trypsin immobilized on Agarose beads. Both PI kinase and PIPase were inactivated by treatment with free trypsin but not with the immobilized enzyme. Similar results were obtained for the internal marker enzyme, RNA polymerase; however, NADH:cytochrome c reductase was equally sensitive to the two forms of trypsin. In the second method, nuclei were treated with citraconic anhydride to selectively remove the outer nuclear membrane. At least 85% of the PI kinase and PIPase activitites were found to be associated with the pelleting inner nuclear membranes. Therefore, both of these methods provided results suggesting that PI kinase and PIPase are located on the inner nuclear membrane.

To examine the distribution of PIP, nuclei were incubated with [γ-^{32}P]ATP to synthesize phosphatidylinositol [4-^{32}P]phosphate in the nuclear envelope. The nuclei were then treated with the anhydride to separate the membranes. Phosphorylation of nuclei at 30 C for 5 min resulted in [^{32}P]PIP being equally

distributed between the inner and outer nuclear membranes. However, phosphorylation at 4 C resulted in a more rapid accumulation of [^{32}P]PIP in the inner membrane than in the outer membrane. These results indicate that PIP synthesis occurrs on the inner nuclear membrane, and that the phospholipid can be rapidly transported to the outer membrane at physiological temperature.

INTRODUCTION

By definition, a eucayotic cell contains a nucleus which is surrounded by a bimembrane system termed the nuclear envelope (1-4). The nuclear envelope therefore serves as a biological interface between the cytoplasm and the nucleoplasm of the cell. As such, it is in the unique position of being able to regulate the exchange of information between these two cellular compartments. Nuclear envelopes are composed of distinct inner and outer nuclear membranes which may be involved in different nuclear functions (1-3). Roles for the nuclear envelope have been proposed in the regulation of the nucleocytoplasmic transport of RNA (1-3), organization of chromatin (2-5), and transduction of hormonal stimuli (6-9). Although the nuclear envelope has been extensively studied at the morphological level (10), biochemical characterizations of its components and studies of the mechanisms by which its functions are regulated remain incomplete.

A potentially important mechanism for regulating membrane function is through modification of the phospholipids in the membrane. We have previously demonstrated that phosphatidylinositol 4-phosphate (PIP) is synthesized in isolate rat liver nuclear envelopes through the action of phosphatidylinositol (PI) kinase

(11,Chapter II). This phospholipid can be reconverted into PI by a PIP-specific phosphomonoesterase that is also present in nuclear envelopes (12,Chapter III). Polyphosphoinositide metabolism has been proposed to play important roles in the regulation of biological membrane structure and function (13-16). In the nuclear envelope, PIP appears to be involved in the regulation of nucleoside triphosphatase activity (17,Chapter V), and so may be involved in regulating the nucleocytoplasmic transport of RNA (18-22). To further define the role of PIP in nuclear function, we have attempted to determine the topology of PIP metabolism in the nucleus. Two techniques have been used to investigate the distribution of PI kinase and PIPase in the nuclear envelope. In addition, the fate of newly synthesized PIP was examined.

EXPERIMENTAL PROCEDURES

Materials. Adult male Sprague-Dawley rats (180-200 g) were used for all studies. (^{32}P)Orthophosphoric acid, carrier free, was obtained from ICN. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (23) and purified as described by Walsh, et al. (24). [5-^{3}H]UTP was obtained from New England Nuclear. Phosphatidylinositol [4-^{32}P]phosphate was synthesized and purified as previously described (12). [^{3}H]Hemoglobin was prepared by incubating bovine hemoglobin (Sigma, Type I) with [^{3}H]acetic anhydride, followed by purification of the hemoglobin by gel filtration (25). Trypsin and Agarose-bound trypsin (bovine pancreas, Type III), soybean trypsin inhibitor (Type I-S), PIP, cytochrome c (horse heart, Type III), citraconic anhydride, and nucleotides were obtained from Sigma. All other chemicals were of analytical grade.

Assay of trypsin activity. The Agarose-bound trypsin was washed throughly and resuspended in 50mM HEPES, pH 7.5. The activity of the suspensions of trypsin and Agarose-bound trypsin were determined by incubating aliquots with [^{3}H]hemoglobin for 30 min at 30 C in the presence of 50mM HEPES, pH 7.5. The reactions were terminated by the addition of 1ml of cold 500mM

perchloric acid and the samples were incubated on ice for 10 min. The increase in acid-soluble radioactivity was determined after centrifugation of the samples at 5000 x g for 10 min. The solutions were then adjusted so that the proteolytic activity of the stock solutions of free trypsin and Agarose-bound trypsin were equal.

Isolation of nuclei and trypsinization. Nuclei were isolated from rat liver as previously described (26), washed twice with 0.25M sucrose containing 25mM HEPES, pH 7.5, and resuspended to 10 mg protein (27)/ml of buffer. Various concentrations of trypsin or Agarose-bound trypsin were added to 0.25 ml aliquots of the purified nuclei and incubated at 30 C for 30 min. The trypsinization was terminated by the addition of 50 ul of 2mg trypsin inhibitor/ml buffer and the samples were placed on ice.

Enzyme assays. Aliquots of the trypsinized nuclei were assayed for NADH:cytochrome c reductase activity (28) and RNA polymerase activity (29). PI kinase activity in whole nuclei was measured by incubating samples with 1mM [γ-^{32}P]ATP (1000 cpm/pmol), 20mM $MgCl_2$, and 50mM HEPES, pH 7.5, for 1 min at 30 C. The reactions were terminated by the addition of acidified chloroform : methanol (30) and the labeled phospholipids were extracted. The radioactivity associated with the lipid extracts was

determined as Cerenkov radiation. Greater than 90% of the lipid-associated radioactivity was associated with PIP (data not shown). PIPase activity was measured as previously described (12) using purified phosphatidylinositol [4-^{32}P]phosphate.

Citraconylation of nuclei. Purified nuclei were washed with modification buffer (0.25M sucrose, 1mM $MgCl_2$, and 50mM HEPES, pH 8.5) and resuspended in the same buffer. Citraconylation was performed as described by Schindler (31) using up to 15 ul of 1.1M citraconic anhydride in EtOH per 100 ul of nuclei. The inner and outer nuclear membranes were then separated by centrifugation at 15,000 x g for 5 min (32). Pellets (inner nuclear membranes) were resuspended to the original volume in 0.25M sucrose containing 200mM HEPES, pH 7.0, and supernatants (outer nuclear membranes) were adjusted to pH 7.0 with HEPES. The samples were then incubated overnight at 4 C to hydrolyze the citraconyl-protein derivatives. Aliquots were then extracted with acidified chloroform : methanol as described above and assayed for phospholipid (33). Aliqouts were also assayed for PIP phosphomonoesterase and PI kinase as described above, except that the PI kinase assays also contained 0.5mM PI and 0.2% Triton X-100.

In some experiments, nuclei suspended in modification

buffer were incubated at either 30 C or 4 C with 1mM [γ-^{32}P]ATP and 20mM $MgCl_2$ for up to 10 min. The phosphorylations were terminated by the addition of the anhydride and the membranes were separated. The resulting pellets and supernatants were extracted and analyzed for phospholipid and [^{32}P]PIP as previously described.

RESULTS

Trypsinization of nuclear enzymes. The basis for the expected differential sensitivity to free trypsin and Agarose-bound trypsin is diagramed in Figure 1. Proteins on the outer nuclear membrane should serve as substrates for both forms of trypsin, while the proteins on the inner membrane should be sensitive to free trypsin, which is small enough to pass through nuclear pores, but not to Agarose-bound trypsin which is too large to penetrate the nucleoplasm. NADH:cytochrome c reductase, which is present on the outer nuclear membrane (34), showed equal sensitivity to proteolysis by both forms of trypsin (Figure 2A). RNA polymerase, present in the nucleoplasm, was inactivated by free trypsin but not by the immobilized trypsin (Figure 2B). A slight loss of activity occurred at high concentrations of Agarose-bound trypsin, but this may be due to dissociation of some bound trypsin or to a generalized breakdown of the nuclear envelope. Both PI kinase (Figure 2C) and PIPase (Figure 2D) were inactivated by free trypsin but not by the Agarose-bound trypsin, indicating that these enzymes are located in the nuclear interior. Since previous studies have indicated that PI kinase and PIPase are located in the nuclear envelope (11,12), these enzymes are most likely present in the inner nuclear membrane.

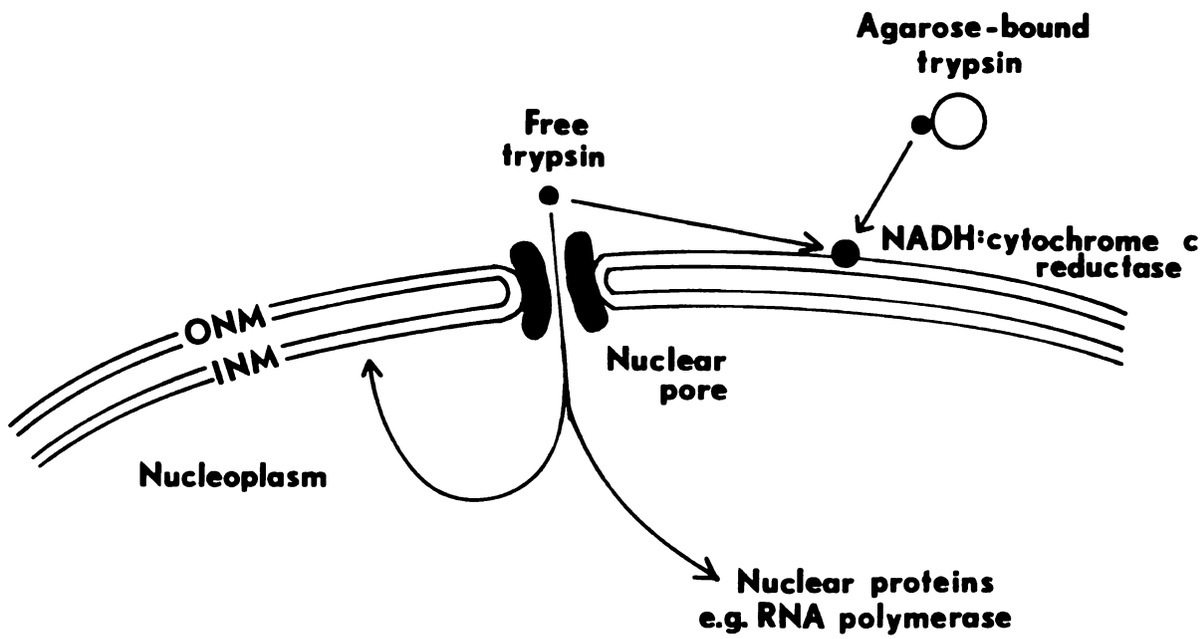

Figure 1. Determination of the topology of nuclear envelope-associated enzymes

Intact nuclei are incubated in isotonic buffer with either free trypsin or trypsin immobilized on Agarose beads. Enzymes associated with the outer nuclear membrane (ONM) should be sensitive to inactivation by both forms of trypsin, while enzymes associated with the inner nuclear membrane (INM) should be sensitive to free trypsin but not to the immobilized trypsin.

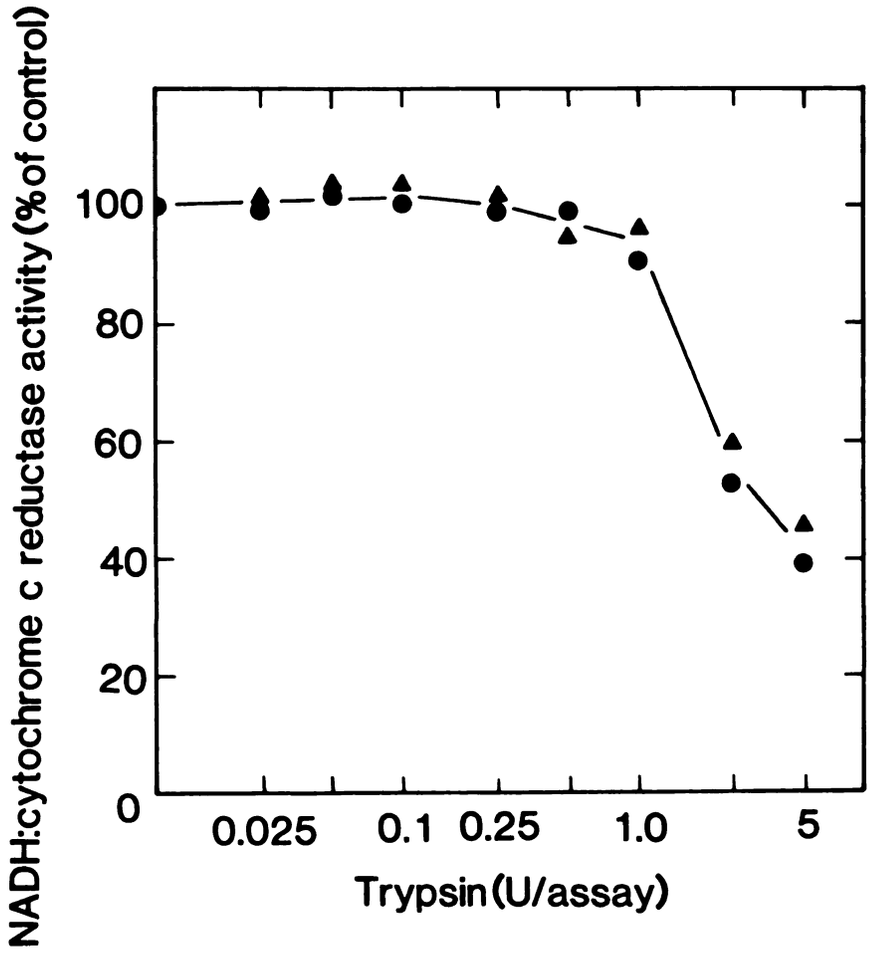

Figure 2. Trypsinization of nuclear enzymes

A. NADH:cytochrome c reductase

Nuclei were incubated with the indicated amounts of trypsin (●) or Agarose-bound trypsin (▲) for 30 min at 37 C. Aliquots were then assayed for NADH:cytochrome c reductase activity as described under "Experimental Procedures".

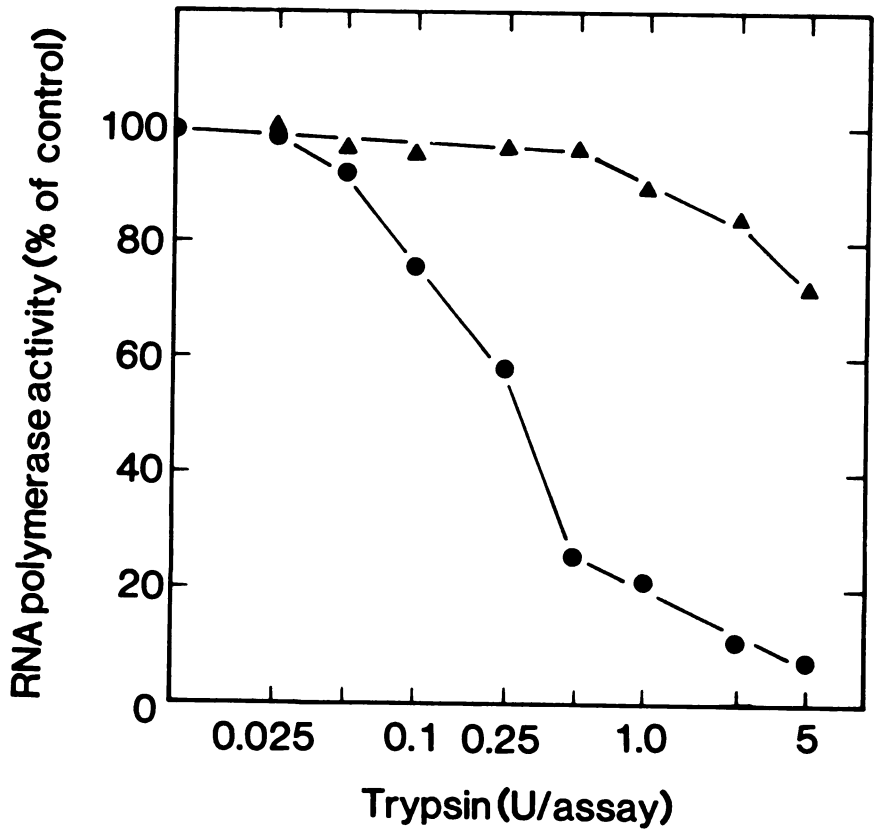

Figure 2. Trypsinization of nuclear enzymes

B. RNA polymerase

Nuclei were incubated with the indicated amounts of trypsin (●) or Agarose-bound trypsin (▲) for 30 min at 37 C. Aliquots were then assayed for RNA polymerase activity as described under "Experimental Procedures".

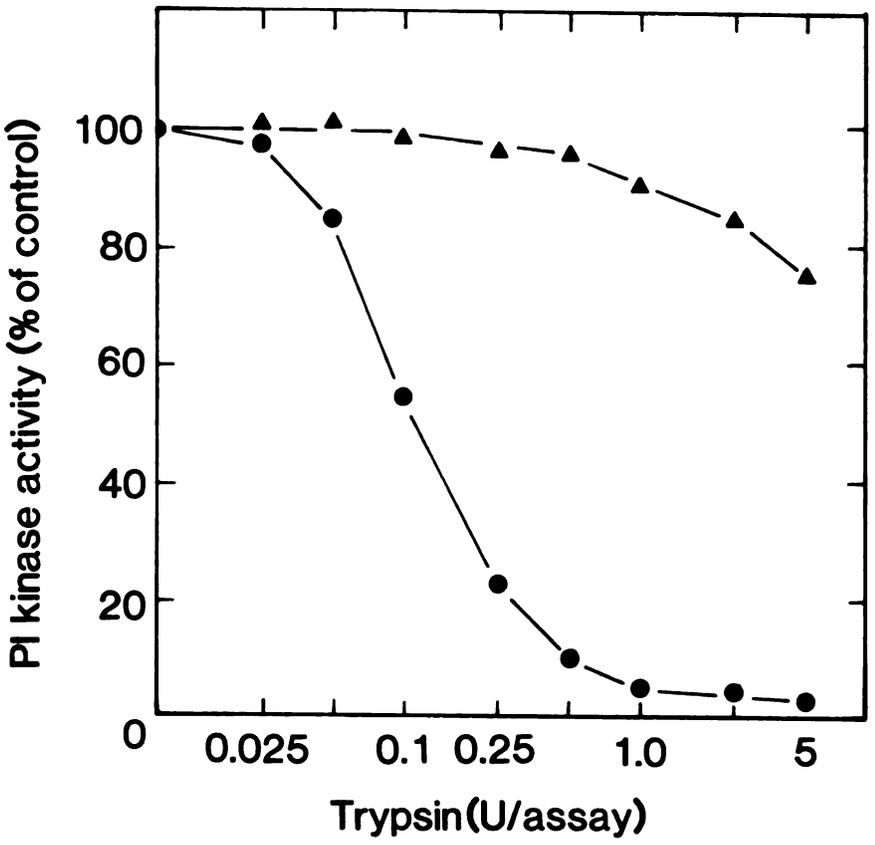

Figure 2. Trypsinization of nuclear enzymes

C. PI kinase

Nuclei were incubated with the indicated amounts of trypsin (●) or Agarose-bound trypsin (▲) for 30 min at 37 C. Aliquots were then assayed for PI kinase activity as described under "Experimental Procedures".

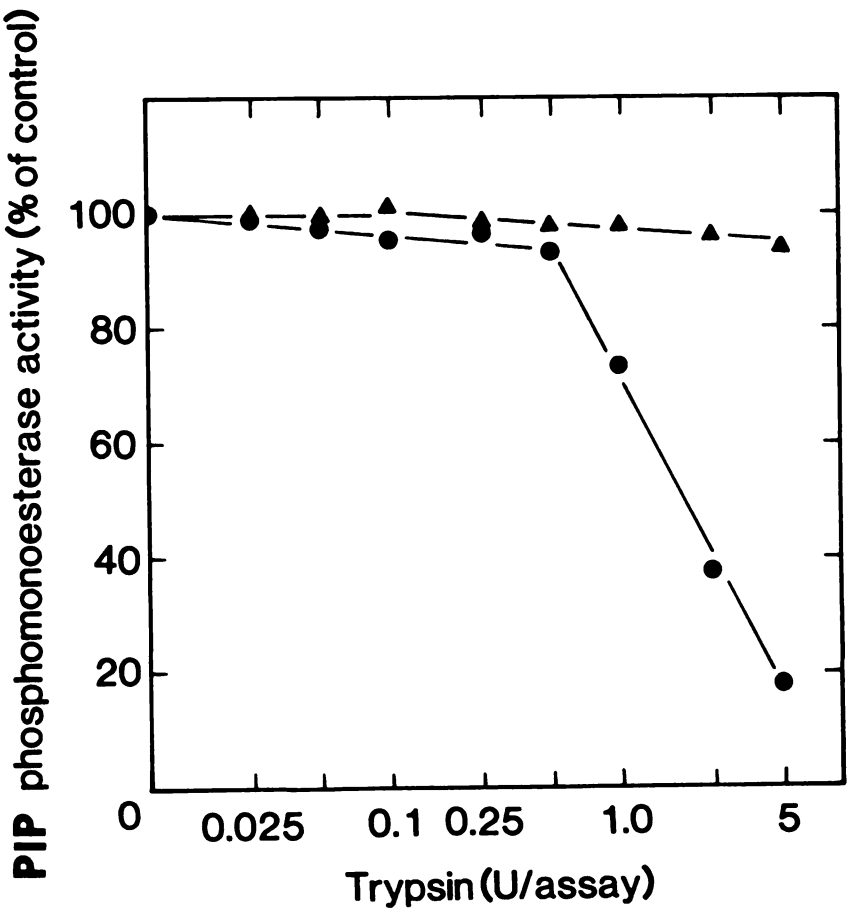

Figure 2. Trypsinization of nuclear enzymes

D. PIP phosphomonoesterase

Nuclei were incubated with the indicated amounts of trypsin (●) or Agarose-bound trypsin (▲) for 30 min at 37 C. Aliquots were then assayed for PIP phosphomonoesterase activity as described under "Experimental Procedures".

Effect of citraconylation on PI kinase and PIPase. Treatment of nuclei with citraconic anhydride has been shown to provide a method for separating inner and outer nuclear membranes (32). Therefore, this procedure was also used to investigate the distribution of PI kinase and PIPase in the nuclear envelope. Although citraconyl-protein derivatives are hydrolyzed by incubation at neutral pH, treatment of nuclei with citraconic anhydride led to an irreversible loss of PI kinase and PIPase activity (Figure 3). Presumably, this indicates that the derivatization of these enzymes with the anhydride causes a conformational change which cannot be completely reversed upon removal of the citraconyl groups. In spite of this inactivation, PI kinase and PIPase expressed enough activity to be accurately measured even after treatment with 44mM citraconic anhydride.

Distribution of PI kinase and PIPase in nuclear membranes separated by citraconylation. Nuclei were treated with up to 44mM citraconic anhydride and separated into fractions containing inner nuclear membranes (pellets) and outer nuclear membranes (supernatants). After neutralization and incubation overnight at 4 C, the samples were assayed for phospholipid, PI kinase, and PIPase. As indicated in Figure 4, approximately 55% of the nuclear phospholipid was removed by treatment with

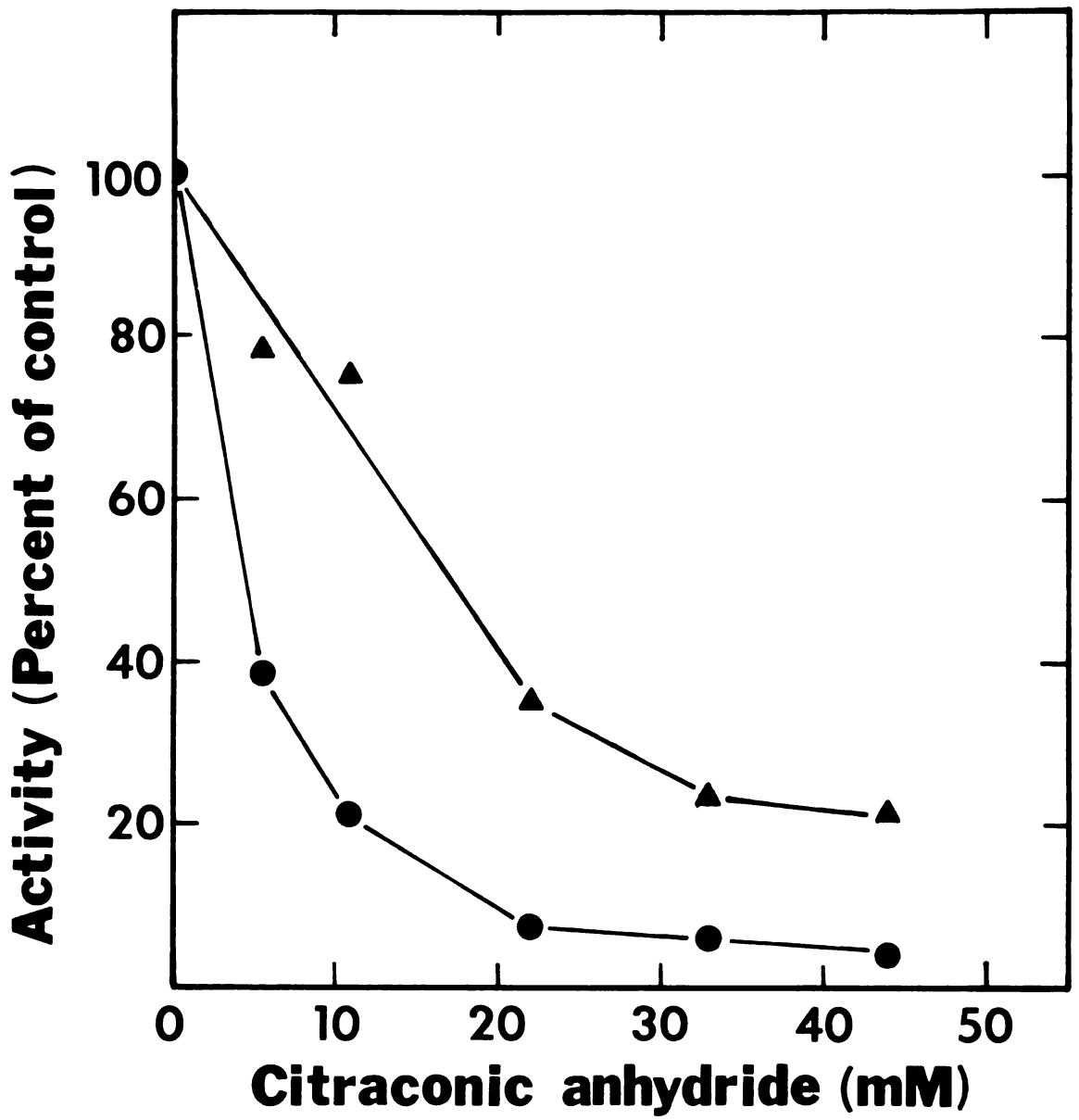

Figure 3. Effect of citraconylation on PI kinase and PIPase

Nuclei were incubated with the indicated concentrations of citraconic anhydride at pH 8.5 for 5 min at 4 C. The samples were then neutralized with HEPES and incubated overnight at 4 C before being assayed for PI kinase (●) and PIPase (▲) as described under "Experimental Procedures".

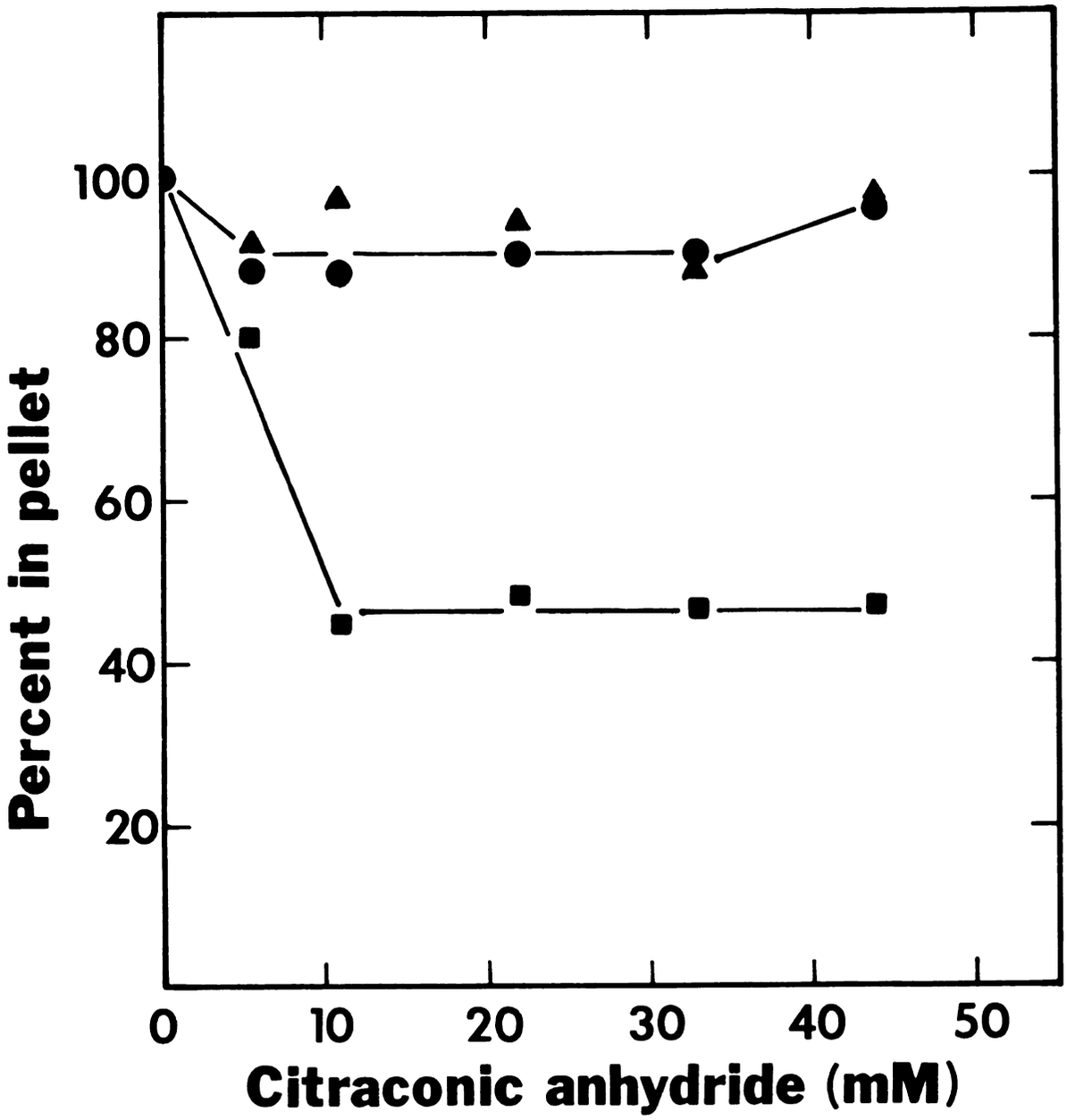

Figure 4. Distribution of PI kinase and PIPase in nucear envelopes

Nuclei were treated with the indicated concentrations of citraconic anhydride, centrifuged, neutralized and incubated overnight at 4 C as described under "Experimental Procedures". Aliquots were then assayed for phospholipid (■), PI kinase (●), and PIPase (▲). Values represent the percentage of the total amount remaining that was recovered in the pellet.

11mM anhydride. No additional loss of phospholipid occurred at citraconic anhydride concentrations up to 44mM. This indicates that the outer nuclear membrane had been removed from the pelleting inner nuclear membrane. Greater than 85% of the total amount of PI kinase and PIPase activities were recovered with the inner nuclear membranes at all anhydride concentrations. Therefore, physical separation of the membranes by citraconylation supports the trypsinization data in indicating that PI kinase and PIPase are associated with the inner nuclear membrane.

Distribution of PIP in nuclear envelopes. Previous results have suggested that the enzymes involved in PIP metabolism are confined to the inner nuclear membrane. To determine if the phospholipid itself is similarily localized, nuclei were phosphorylated with [γ-^{32}P]ATP at 30 C for 5 min to produce [^{32}P]PIP in the nuclear envelope (11). Separation of the nuclear membranes and analysis of the resulting fractions indicated that the distribution of [^{32}P]PIP was identical to that of the total nuclear phospholipid (Figure 5). These results indicate that although PIP is synthesized in the inner nuclear membrane, at 30 C, it rapidly equilibrates with the outer nuclear membrane.

The time course of [^{32}P]PIP distribution in inner and

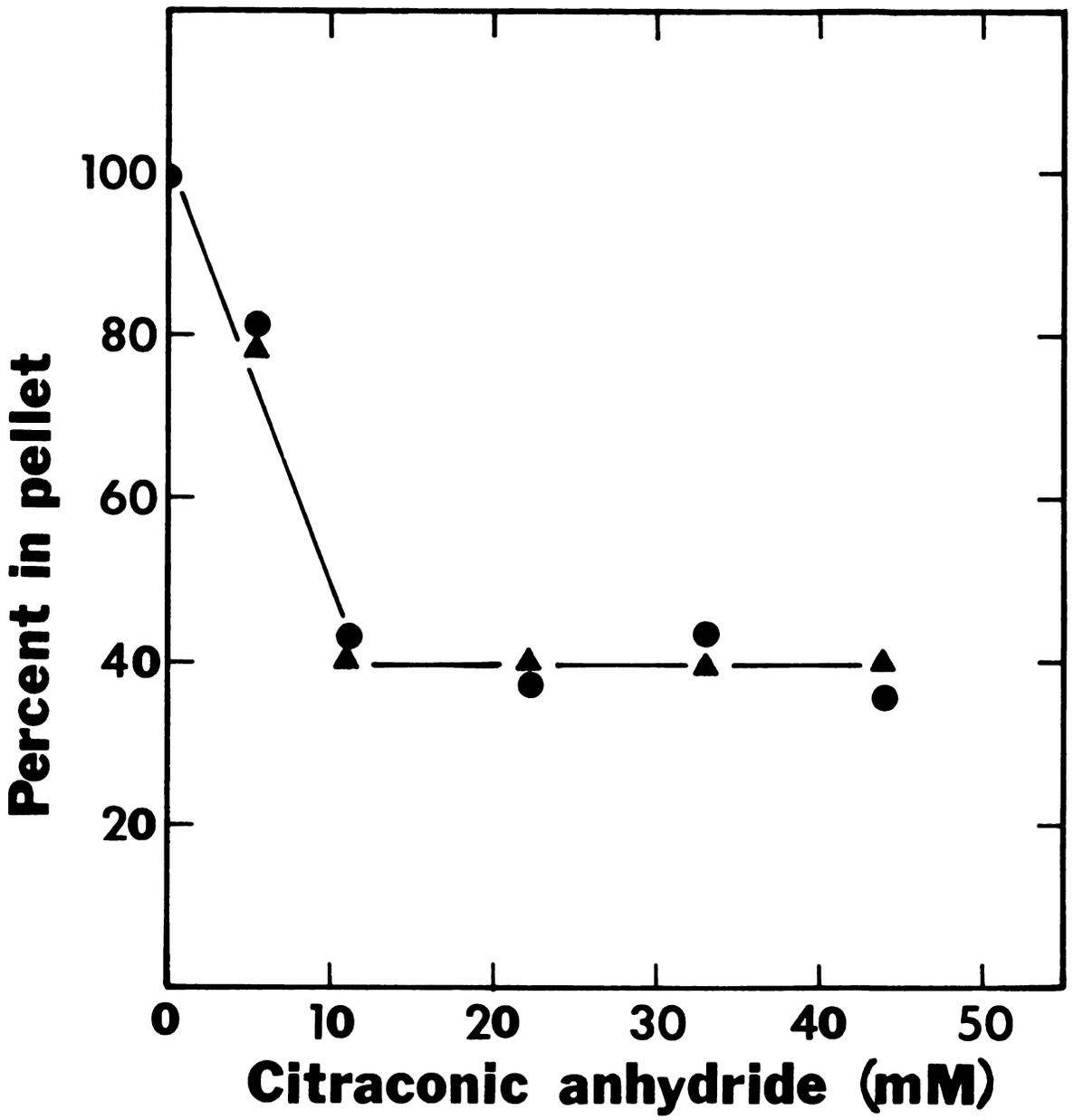

Figure 5. Distribution of PIP in nuclear envelopes

Nuclei were phosphorylated at 30 C for 5 min as described under "Experimental Procedures", before being treated with the indicated concentrations of citraconic anhydride. Inner and outer nuclear membranes were then separated and analyzed for phospholipid (●) and [^{32}P]PIP (▲) as previously described.

outer nuclear membranes phosphorylated at either 4 C or 30 C is described by Figure 6. The membranes were separated by treating the labeled nuclei with 25mM citraconic anhydride which resulted in 51 $\pm$ 3 % of the phospholipid remaining in the pellet (inner nuclear membrane). At 30 C, the amount of [^{32}P]PIP in the inner membrane increased rapidly, peaking at 1 min of phosphorylation. Subsequent to that, [^{32}P]PIP decreased reflecting PIPase activity after the [γ-^{32}P]ATP had been depleted (11). [^{32}P]PIP in the outer nuclear membrane increased less rapidly and peaked at 2 min of phosphorylation. This slower rate and lag in peak time may reflect the time required for the intermembrane transport of the phospholipid. At 4 C, the difference in the rate of labeling of the two membranes was slightly more pronounced; however, no period was apparant in which [^{32}P]PIP was exclusively confined to the inner nuclear membrane.

Figure 6. Time course of PIP distribution in nuclear envelopes

Nuclei were phosphorylated as described under "Experimental Procedures" for the indicated times either at 4 C (open symbols) or 30 C (closed symbols) before being treated with 25mM citraconic anhydride. Inner nuclear membranes (circles) and outer nuclear membranes (triangles) were then separated and analyzed for [^{32}P]PIP as previously described.

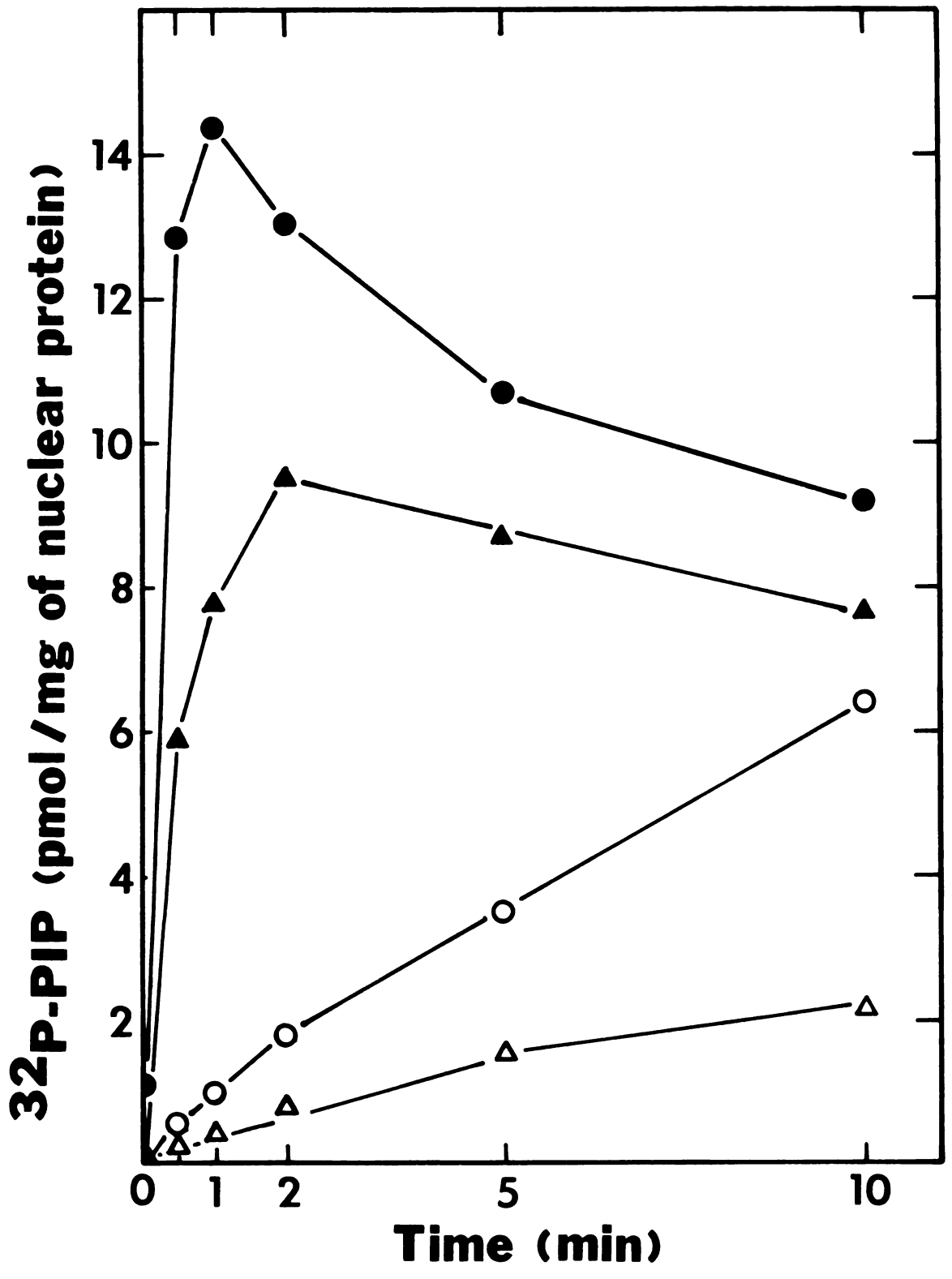

Figure 6. Time course of PIP distribution in nuclear envelopes

DISCUSSION

Determination of the topology of enzymes in the nuclear envelope has previously relied on the use of cytochemical or immunohistochemical methods. Unfortunately, such procedures have yielded contradictory information on the localization of some enymes (1-3). In the present investigation, we have used two new methods for determining the topology of nuclear envelope-associated enzymes. In the first method, the sensitivities of nuclear envelope-associated enzymes to inactivation by free trypsin and immobilized trypsin were measured. Differential sensitivity to the two forms of protease would imply that the enzyme is located in the inner nuclear membrane. Although similar results could be obtained if the enzyme is protected from proteolysis due to steric hinderence by the Agarose beads, this did not occur for NADH:cytochrome c reductase which was equally sensitiive to both forms of trypsin. Since both PI kinase and PIPase were inactivated in a manner analogous to that of RNA polymerase, it is most likely that these enzymes are associated with the inner nuclear membrane. In the second localization method, inner and outer nuclear membranes were physically separated by chemical modication of nuclear proteins with citraconic anhydride (32). The proposal that this derivatization procedure results in the

separation of the two membranes is based on evidence from electron microscopy, phospholipid analyses, use of lamin B as an inner nuclear membrane marker, and measurement of the lateral mobilities of phospholipids in the inner and outer nuclear membranes (32). PI kinase and PIPase remained preferentially associated with the residual membrane after treatment of nuclei with citraconic anhydride. These results therefore support the trypsinization data in indicating that these activities are located in the inner nuclear membrane. These results also provide further evidence that citraconylation of nuclei can be used to separate inner and outer nuclear membranes.

Citraconylation of nuclei also provided a method for studying the distribution of PIP in the nuclear envelope. Although the enzymes involved in the metabolism of this phospholipid are present in the inner nuclear membrane, [^{32}P]PIP could be demonstrated in both membranes. This provides evidence that phospholipid exchange between the inner and outer nuclear membranes can occur. The phospholipid profiles of the two nuclear membranes are essentially identical (32), again suggesting that phospholipids are free to distribute throughout both membranes. The mechanism by which phospholipids can be transfered between the nuclear membranes remains unexplored; however, since the two membranes are thought

to be continuous at the sites of nuclear pore complexes (35), flow through these junctions may mediate the equilibration of phospholipids between the two membranes. The lateral mobilities of phospholipids in the inner and outer nuclear membranes are similar to those reported for other membrane systems (32), indicating that intermembrane movement of phospholipids could be rapid. Lowering the temperature of the incubations to 4 C resulted in a slower movement of [^{32}P]PIP from the inner to the outer nuclear membrane, as would be expected for a phospholipid flow mechanism. The rates of inter-nuclear membrane transport of other phospholipids have not been measured; however, it might be anticipated that they would be somewhat faster than that for PIP which could be retarded through interactions with other membrane components due to its high charge density.

The roles of PIP in nuclear envelope function remain under investigation. In other membranes, polyphosphoinositides have been shown to be involved in agonist-dependent calcium mobilization from the endoplasmic reticulum (36,37) and the regulation of membrane-associated enzyme activity (38-41). We have shown that delipidated nuclear envelopes express ATPase activity only when reconstituted with a polynucleotide and PIP or phosphatidylinositol 4,5-bisphosphate (17,Chapter V). Therefore PIP may be involved in regulating the

nuclear envelope-associated nucleoside triphosphatase which has been implicated as an important component of the RNA transport system (18-22). Other functions of PIP in the nuclear membrane may include an effect on the organization of chromatin, possibly through interactions with lamin B, an integral inner nuclear membrane protein (42). Further studies on the roles of PIP in nuclear function may provide insight into the mechanisms for regulating these events.

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CHAPTER V

SOLUBILIZATION AND RECONSTITUTION OF A NUCLEAR ENVELOPE ASSOCIATED ATPase. SYNERGISTIC ACTIVATION BY RNA AND POLYPHOSPHOINOSITIDES

ABSTRACT

Treatment of isolated rat liver nuclear envelopes with 1% Triton X-100 solubilized 20 - 30 % of the nuclear envelope protein and 85% of the ATPase activity. Chromatography on DEAE-Sepharose in 1% Triton X-100 at pH 7.5 removed all of the chemically measurable phospholipid from the bound ATPase activity; however, endogenously synthesized phosphatidylinositol [4-^{32}P]phosphate (PIP) co-chromatographed with the ATPase activity on this column. Further purification by chromatography on heparin-Agarose, removed all of the [^{32}P]PIP and RNA from the ATPase; however, the recovery of ATPase activity from this column was very low. RNA, polyadenylic acid and polyguanylic acid stimulated the delipidated ATPase activity 4- to 6-fold. This activity was not further stimulated by the addition of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or phosphatidic acid; but was stimulated 2-fold by the addition of phosphatidylinositol. Addition of 40 uM PIP and 50 ug of RNA/ml resulted in a 25-fold stimulation of basal ATPase activity. Phosphatidylinositol 4,5-bisphosphate, in the presence of RNA, was also able to stimulate ATPase activity but to a lesser extent than that of PIP. Therefore, the nuclear envelope-associated ATPase appears to require interaction with a polynucleotide and

PIP to express full activity. PIP, which is actively metabolized in the nuclear envelope, may therefore be involved in the regulation of the activity of this enzyme.

INTRODUCTION

The nucleoside triphosphatase (NTPase; EC 3.6.1.15) activity of mammalian nuclear envelopes (1-3) exhibits broad substrate specificities toward nucleotides and divalent metal cations (4). Hydrolysis of ATP by this enzyme is thought to provide the energy required for the nucleocytoplasmic transport of RNA (5), since inhibitors of the NTPase also inhibit the efflux of prelabeled RNA from isolated nuclei (6-9). NTPase activity is stimulated by RNA containing poly[A] or poly[G] sequences (10-11) and by treatment of the nuclear envelopes with insulin (12). Previous characterizations of the NTPase have been performed predominantly on whole nuclear envelopes due to the lack of a method for solubilizing the enzyme in an active form (4). Therefore, characterization of the physical properties of the enzyme and studies of its interactions with other nuclear membrane components remain incomplete.

Phospholipid-protein interactions are known to markedly affect the activities of many membrane-associated enzymes (13,14), including several cation-transport ATPases. For example, the Ca^{2+}-pump ATPase of erythrocyte plasma membranes has been particularily well characterized (15) and is strongly activiated by acidic phospholipids, especially the polyphosphoinositides, phosphatidylinositol

4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (16). The sarcoplasmic reticular Ca^{2+}-transport ATPase is activated by the phosphorylation of an associated phosphatidylinositol, producing a very tightly bound PIP (17). It has also been suggested that the canine renal (Na^+,K^+)-ATPase is regulated by its interactions with phosphoinositides (18). These effects of polyphosphoinositides may be mediated through localized changes in membrane fluidity caused by the highly polar head groups of these phospholipids (19,20).

PIP is widely distributed throughout intracellular membranes (21), and is activiely metabolized in nuclear envelopes (22, also Chapters II and III). The fluidity of nuclear membrane lipid has been shown to influence RNA transport (23-26); however, it is not known if this is due to modulation of the NTPase activity or to an effect on the nuclear pore complex, the presumed site of RNA efflux (27). Therefore, in the present study, we have investigated the role of phospholipids, particularly the phosphoinositides, in the regulation of rat liver nuclear envelope-associated ATPase activity.

EXPERIMENTAL PROCEDURES

Materials. Adult male Sprague-Dawley rats (180-200 g) were used in all studies. (^{32}P)Orthophosphoric acid, carrier free, was purchased from ICN Pharmaceuticals, INC. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (28) as modified by Walsh, et al. (29). Bovine pancreas deoxyribonuclease I, yeast RNA, polyadenylic acid (5'), polyguanylic acid (5'), phospholipids, bovine serum albumin and chromatography materials were obtained from the Sigma Chemical Co. Triton X-100 was from Research Products International Corp. All other chemicals were of reagent grade.

Nuclear envelope isolation. Nuclei were isolated from rat livers by a modification of the method of Blobel and Potter (30) as previously described (31). Nuclear envelopes were isolated from purified nuclei as described by Kay, et al. (32). The nuclear envelopes were then resuspended in 0.25M sucrose containing 10mM HEPES, pH 7.5, and frozen at -80 C. No loss in ATPase activity occurred in nuclear envelopes frozen for up to 4 weeks.

ATPase assay. Nuclear envelope samples were preincubated at 30 C for 5-10 min before the assay was

initiated by the addition of an equal volume of 100mM HEPES, pH 7.5, containing 4mM $MgCl_2$ and 4mM [γ-^{32}P]ATP (2000-4000 cpm/nmol). Incubations were carried out for 10 min at 30 C, and were terminated by the addition of 1ml of 300mM perchloric acid containing 5mg of activated charcoal. The samples were then incubated at room temperature for 10 min to allow adsorption of the nucleotide to the charcoal, followed by centrifugation at 3000 x g for 5 min. An aliquot (200ul) of the supernatant was removed and the amount of $^{32}P_i$ in the supernatant was determined as Cerenkov radiation (counting efficiency of 40%). Samples without enzyme, or stopped immediately after the addition of the [γ-^{32}P]ATP, were used as blanks. The coefficients of variation for ATPase assays were less than 1% among duplicate samples and less than 5% among different nuclear envelope preparations.

Protein assay. Protein concentrations were determined by the method of Bohlen, et al. (33), except that the samples were incubated with 1% sodium dodecylsulfate before derivatization with fluorescamine. Bovine serum albumin (Sigma) was used as the standard.

Assay of protein and ATPase solubilization. Nuclear envelopes were treated with Triton X-100 for 10 min at room temperature. The samples were then centrifuged at

22,000 x g for 20 min. The supernatants were removed and assayed for protein and ATPase activity. The pellets were resuspended to the original volume with detergent solutions of the same concentration as their corresponding supernatants and then assayed. The percentage of ATPase and protein solubilized was calculated as the percentage of the total amount which was recovered in the supernatant. The total amount recovered after centrifugation was not significantly different from that of uncentrifuged samples.

Delipidation of nuclear envelope protein. Nuclear envelopes were thawed, centrifuged at 22,000 x g for 20 min and resuspended in 0.25M sucrose containing 10mM HEPES, pH 7.5, to a concentration of 5 mg of protein per ml. Triton X-100 was then added to give a final concentration of 1%. The suspensions were incubated at room temperature for 10 min and centrifuged at 22,000 x g for 20 min. The resulting supernatant was removed and Triton X-100 was added to a concentration of 5%. The solubilized ATPase was then subjected to chromatography on DEAE-Sepharose, heparin-Agarose, and Sephacryl S-200 as described under "Results". Fractions were analyzed for protein and ATPase activity as previously described. Aliquots were also extracted with acidified chloroform : methanol as described by Schacht (34) and analyzed for

phospholipid-associated phosphate according to the method of Ames (35).

In some experiments, nuclear envelopes were phosphorylated by incubation with 200uM [γ-^{32}P]ATP (2000 cpm/pmol) in the presence of 5mM $MgCl_2$ and 50mM HEPES, pH 7.5, at 30 C for 5 min to synthesize phosphatidylinositol [4-^{32}P]phosphate in the membrane (22). The envelopes were then pelleted by centrifugation at 22,000 x g for 20 min, treated with Triton X-100, and chromatography was performed as described above. The amount of [^{32}P]PIP was determined as the amount of Cerenkov radiation associated with the phospholipid extracts.

Reconstitution of ATPase activity. Delipidated nuclear envelope protein, prepared as described above, was incubated with polynucleotides and/or phospholipids for 5 min at 30 C before the standard ATPase assay was performed. Phospholipids were added as mixed micelles with 0.5% Triton X-100.

RESULTS

Effects of detergents of ATPase activity. The effects of various detergents on nuclear envelope-associated ATPase activity are indicated in Figure 1. Sodium deoxycholate completely inhibited ATPase acivity at concentrations of 0.25% or higher. Octyl-glucoside was also completely inhibitory at concentrations of 1% or higher. Tween 80 slightly stimulated ATPase activity at concentrations of 0.1% or lower and inhibited at higher concentrations. Triton X-100 also stimulated at very low detergent concentrations and slightly inhibited at higher concentrations. Triton X-100 was used in further studies since this detergent had the smallest effect on the ATPase activity in the range of concentrations that would be used.

Solubilization of ATPase activity with Triton X-100. The ability of Triton X-100 to solubilize nuclear envelope-associated protein and ATPase activity is described by Figure 2. Freezing and thawing nuclear envelopes solubilizes 20-30% of the nuclear envelope-associated protein, but none of the ATPase activity. Increasing the Triton concentration to 0.5% resulted in the solubilizattion of at least 90% of the ATPase activity and approximately 50% of the nuclear

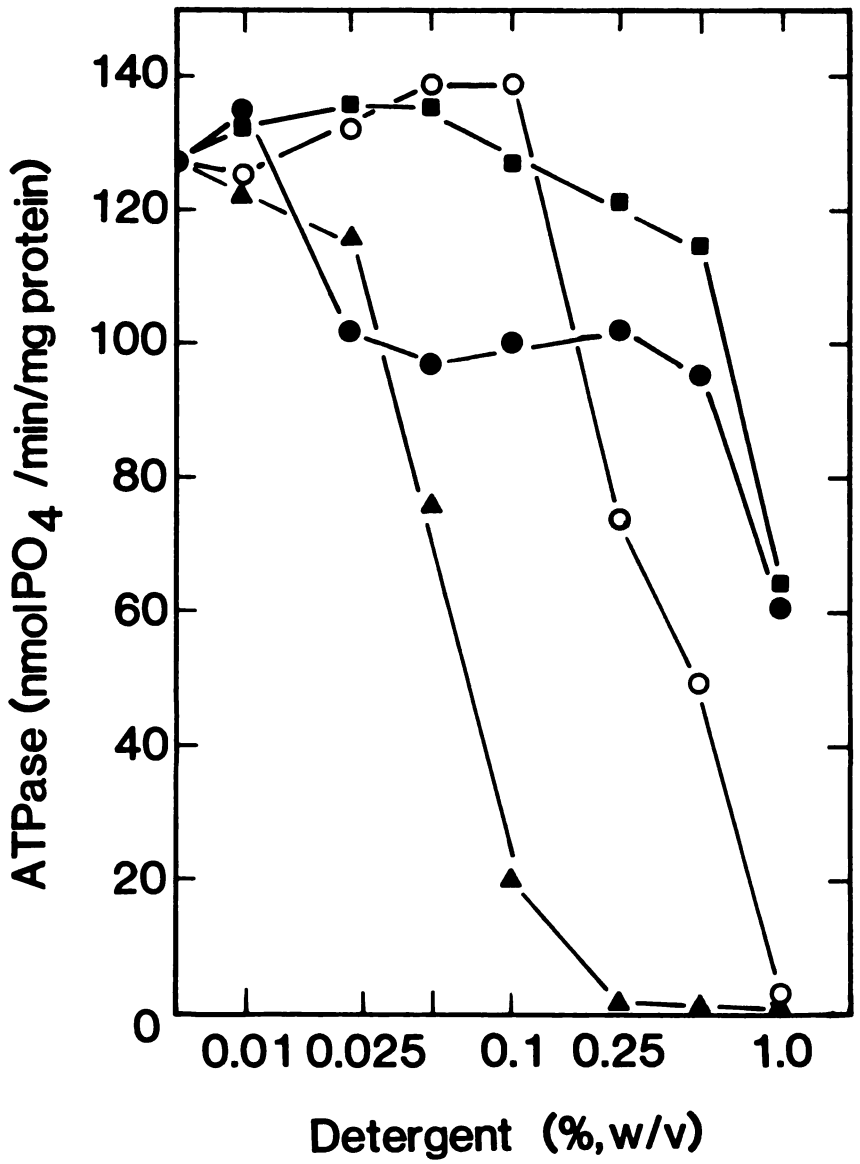

Figure 1. Effects of detergents on nuclear envelope-associated ATPase activity

Nuclear envelopes (5mg of protein/ml) were incubated with the indicated concentration of sodium deoxycholate (▲), octyl-glucoside (○), Tween 80 (■), or Triton X-100 (●) for 5 min at 30 C. ATPase activity was then assayed as described under "Experimental Procedures". Values represent the means of 4 to 6 experiments.

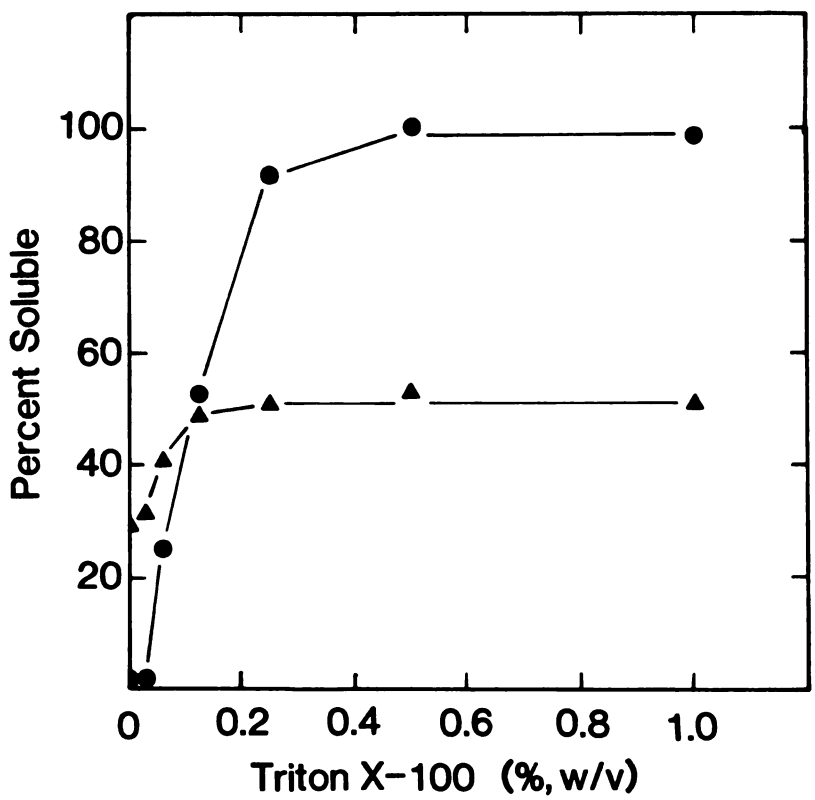

Figure 2. Solubilization of nuclear envelope protein and ATPase activity

Nuclear envelopes (5mg of protein/ml) were incubated with the indicated concentrations of Triton X-100 for 10 min at room temperature. The percentage of protein (▲) and ATPase activity (●) that was solubilized was determined as described under "Experimental Procedures". Values represent the means of 3 experiments.

envelope protein. The remaining 50% of the nuclear envlope protein remained insoluble in Triton X-100 concentrations up to 5% and represents the "pore-lamina" fraction (36) of nuclear envelopes (data not shown).

Delipidation of nuclear envelope protein. Nuclear envelopes were thawed, centrifuged at 22,000 x g for 20 min and ATPase activity was extracted with 1% Triton X-100. This treatment yielded 85-95% of the ATPase activity and only 20-30% of the total protein. Triton X-100 was then added to give a final concentration of 5% to insure dissociation of protein and phospholipid. The extract was then applied to a 20 x 7 mm column of DEAE-Sepharose equilibrated with 1% Triton X-100 in 25mM HEPES, pH 7.5. Approximately 45% of the protein and all of the ATPase activity bound to the column and could be eluted with 0.25M NaCl (Figure 3A). All of the measurable phospholipid phosphate passed through the column without binding (Figure 3B); however, when the distribution of endogenously synthesized [^{32}P]PIP was analyzed, it was found that this highly polar phospholipid bound to the DEAE-column and was also eluted with 0.25M NaCl. Purified [^{32}P]PIP and [^{32}P]PIP_2 can also bind to DEAE-columns under identical conditions but in the absence of protein (data not shown). These results suggest that the ionic interactions between these phospholipids and the cationic

Figure 3. DEAE-Sepharose chromatography of solubilized nuclear envelopes

Nuclear envelopes were phosphorylated by incubation with [..-^{32}P]ATP for 5 min, centrifuged, and extracted with 1% Triton X-100 as described under "Experimental Procedures". The solubilized material was then chromatographed on DEAE-Sepharose in 1% Triton X-100 containing 20mM HEPES, pH 7.5. Bound material was eluted by washing with 0.25M NaCl in the above buffer, added as indicated by the arrow.

A- Aliquots were assayed for protein (●) and ATPase activity (▲).

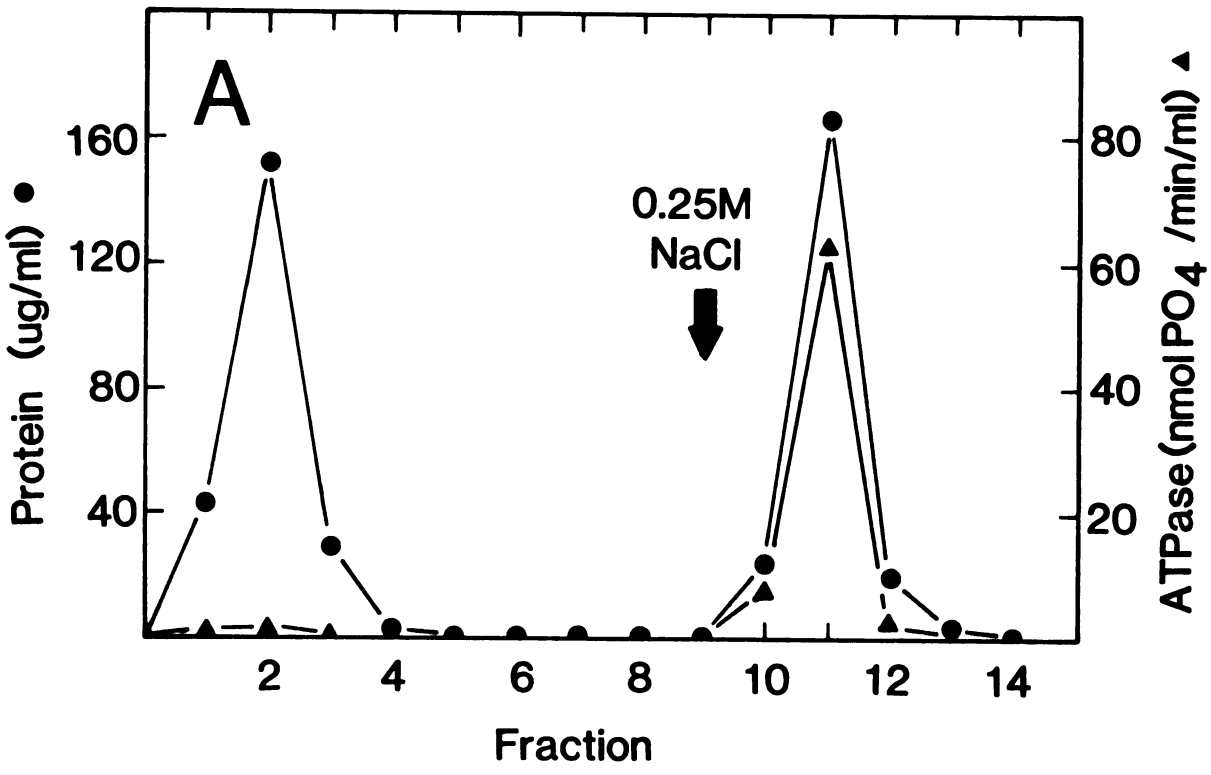

Figure 3A. DEAE-Sepharose chromatography of solubilized nuclear envelopes

Figure 3. DEAE-Sepharose chromatography of solubilized nuclear envelopes

B- Aliquots were extracted with acidified chloroform : methanol and assayed for phospholipid-associated phosphate (■) and [^{32}P]PIP (○) as described under "Experimental Procedures".

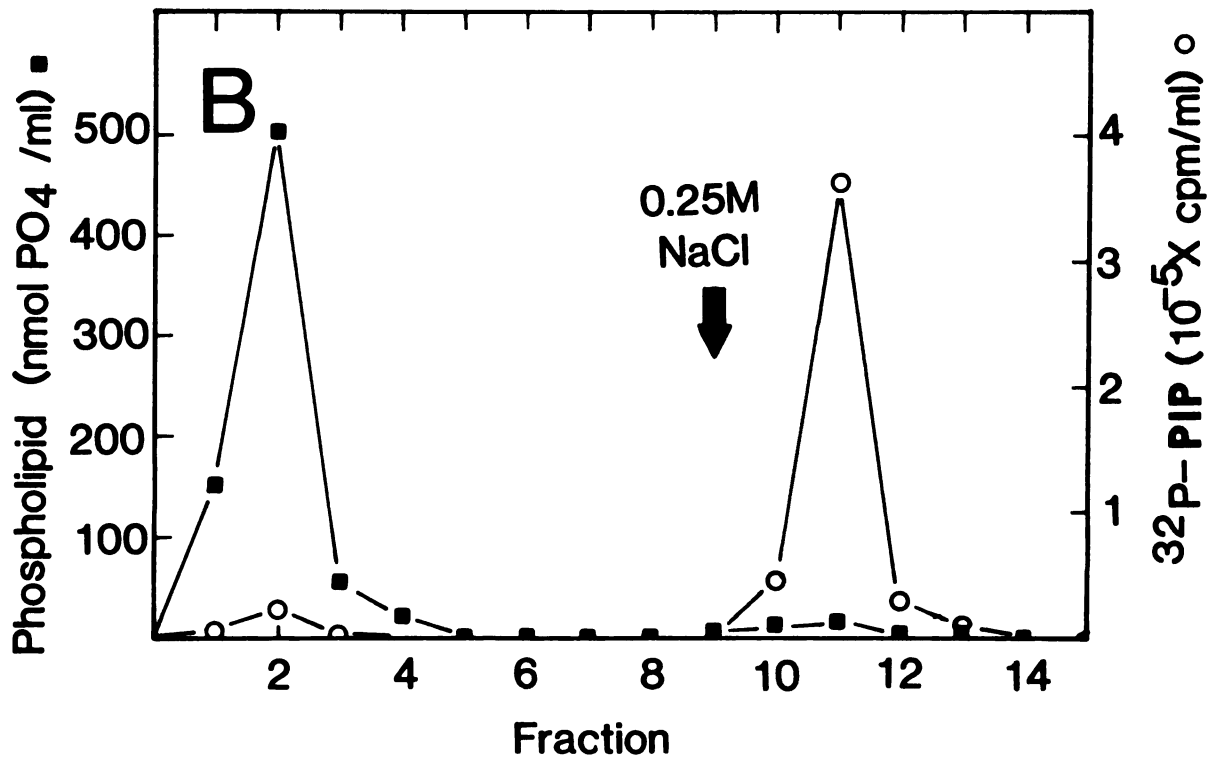

Figure 3B. DEAE-Sepharose chromatography of solubilized nuclear envelopes

column support outweigh the tendency for the lipid to be removed through hydrophobic interactions with the Triton X-100 in the eluant.

Fractions containing ATPase activity were diluted with 4 volumes of 1% Triton X-100 in 10mM HEPES, pH 7.5, and applied to a 20 x 7 mm column of heparin-Agarose equilibrated with the same buffer. As indicated in Figure 4, approximately 80% of the protein and all of the ATPase activity bound to the column and could be eluted with 0.5M NaCl. [^{32}P]PIP did not bind to this anionic column support, and so was removed from the ATPase activity. The recovery of ATPase activity from this column appears to be only approximately 10%; however, this activity could be stimulated by polynucleotides and phospholipids as described below. The fractions containing ATPase activity were chromatographed on a 50 x 1.5 cm column of Sephacryl S-200 equilibrated with 0.5% Triton X-100 in 10mM HEPES, pH 7.5. The ATPase activity eluted with an apparant molecular weight of 200,000 daltons (data not shown). This delipidated ATPase preparation was used in the reconstitution studies described below.

Figure 4. Heparin- Agarose chromatography of partially purified ATPase

Nuclear envelopes were phosphorylated, extracted with 1% Triton X-100, and subjected to chromatography on DEAE-Sepharose as described under "Experimental Procedures". The peak of ATPase activity was diluted with 4 volumes of 20mM HEPES, pH 7.5, containing 1% Triton X-100 and applied to a 20 x 7 mm column of heparin-Agarose equilibrated with the same buffer. Bound material was eluted with 0.5M NaCl as indicated by the arrow. Fractions were assayed for protein (●), ATPase activity (▲), and [^{32}P]PIP (■) as previously described.

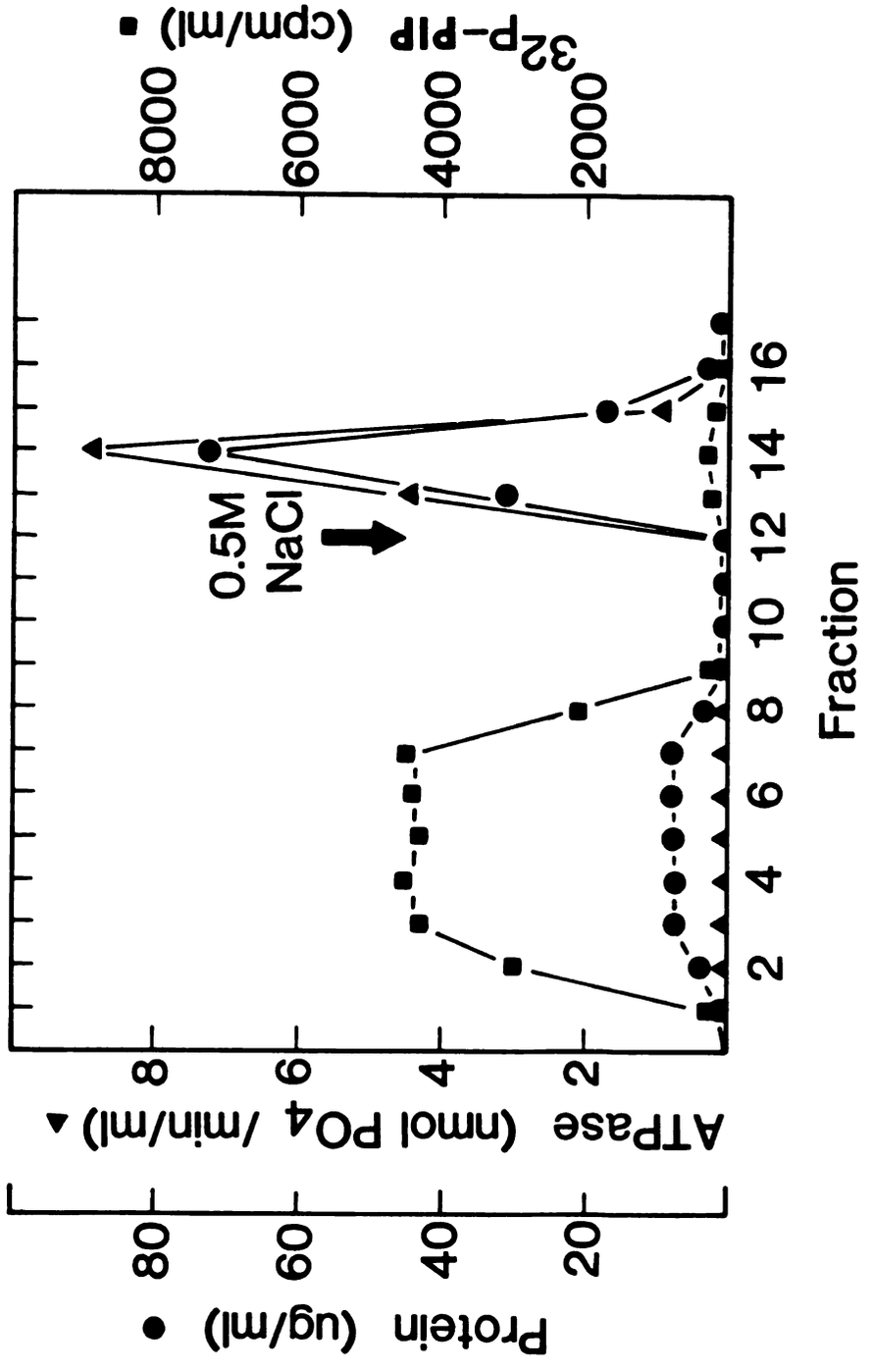

Figure 4. Heparin-Agarose chromatography of partially purified ATPase

Reconstitution of ATPase activity with polynucleotides. The effects of RNA, polyadenylic acid, and polyguanylic acid on the ATPase activity expressed by the delipidated nuclear envelope protein, prepared as described above, are shown in Figure 5. Addition of 50 ug RNA per ml resulted in a 6-fold stimulation of ATPase activity. Higher levels of RNA produced slightly less stimulation. Poly[A] and poly[G] were also effective in stimulating ATPase activity, although their maximal stimulation amounted to only 4- and 3-fold, respectively.

Reconstitution of ATPase activity with phospholipids. Table I describes the effects of certain phospholipids on the delipidated ATPase in the absence and presence of 50 ug of RNA/ml. Up to 1mM phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid had essentially no effect on ATPase activity, while 1mM phosphatidylglycerol inhibited both the basal and RNA stimulated activity by approximately 50%. Phosphatidylinositol (1mM) stimulated ATPase activity by about 2-fold.

The effects of PIP and PIP_2 on the activity of the delipidated ATPase are shown in Figure 6. In the absence of added RNA, PIP slightly stimulated the ATPase activity while a 4-fold stimulation was obtained with 20 uM PIP_2. Higher concentrations of PIP_2 inhibited the ATPase

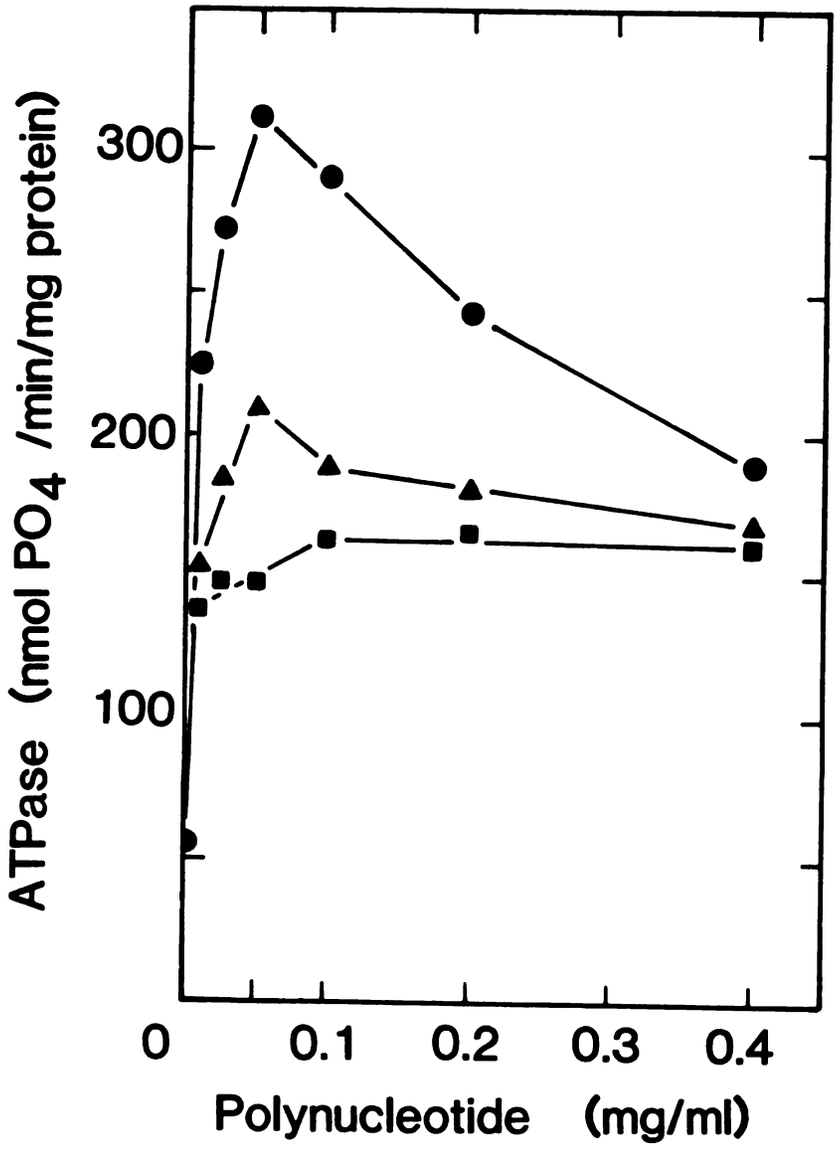

Figure 5. Reconstitution of delipidated ATPase with polynucleotides

Nuclear envelope ATPase activity was solubilized and delipidated by chromatography on DEAE-Sepharose and heparin-Agarrose as described under "Experimental Procedures". The fractions containing ATPase activity were then pooled. Samples were preincubated at 30 C for 5 min with the indicated concentrations of RNA (●), polyadenylic acid, (▲), or polyguanylic acid (■), and assayed for ATPase activity. Values represent the means of 3 experiments.

Table I

Effects of phospholipids on delipidated ATPase activity

Phospholipid	Concentration	ATPase activity	
		no addition	+ RNA
	mM	nmol PO_4/min/mg protein	
none		47	282
phosphatidylcholine	0.1	53	274
	1.0	49	292
phosphatidylethanolamine	0.1	38	271
	1.0	36	265
phosphatidylserine	0.1	57	302
	1.0	49	297
phosphatidylglycerol	0.1	36	223
	1.0	17	141
phosphatidic acid	0.1	42	259
	1.0	53	281
phosphatidylinositol	0.1	63	321
	1.0	98	425

Delipidated nuclear envelope protein was preincubated with the indicated phospholipids for 5 min at 30 C either alone or in the presence of 50 ug of RNA/ml of buffer before the standard ATPase assay was performed. Values represent the means of three or four experiments.

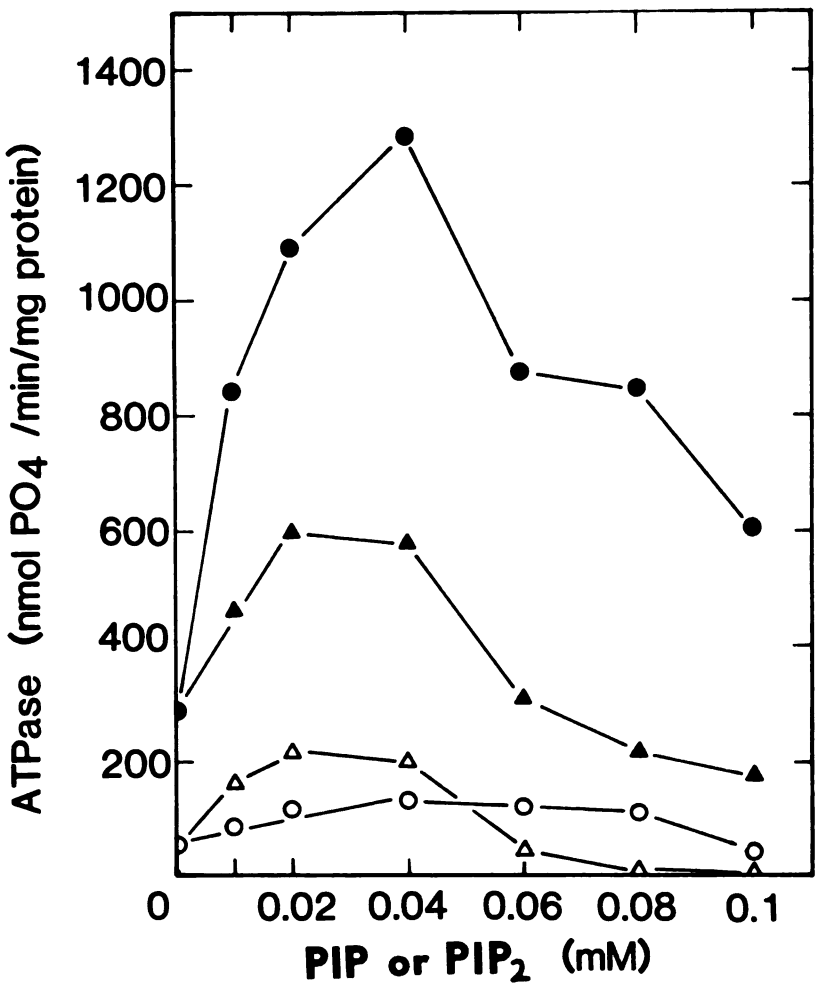

Figure 6. Reconstitution of ATPase activity with polyphosphoinositides and RNA

Delipidated nuclear envelope protein, prepared as described under "Experimental Procedures", was preincubated with the indicated concentrations of PIP (●, ○) or PIP_2 (▲, △) in the absence (○, △) or presence (●, ▲) of 50 ug RNA/ml before ATPase activity was assayed. Values represent the means of 2 to 4 experiments.

activity. When 50 ug of RNA/ml was included in the reconstitution, 40uM PIP stimulated the basal ATPase activity 25-fold to approximately 1250 nmol of PO_4 released/min/mg of protein. Higher levels of PIP inhibited this stimulation. PIP_2 maximally stimulated at 20uM producing an ATPase activity of approximately 600 nmol of PO_4 released/min/mg of protein. This stimulation was also eliminated by incubation with higher levels of PIP_2. Approximately 80% of the original ATPase activity could be reconstituted at the optimal levels of RNA (50 ug/ml) and PIP (40uM).

DISCUSSION

The nuclear envelope stands in the unique position of being able to regulate the exchange of information between the cytoplasm and the nucleoplasm of a eucaryotic cell. This transfer of information occurs through the nucleocytoplasmic exchange of RNA and protein (37). RNA transport is energy-dependent, requiring the hydrolysis of one high-energy phosphate per nucleotide transfered out of the nucleus (5). This energy is thought to be provided through the hydrolysis of ATP by the nuclear envelope-associated NTPase. Two lines of evidence support the proposal for a role of this enzyme in RNA transport. First, agents which inhibit nuclear envelope NTPase activity also inhibit the energy-dependent efflux of prelabeled RNA from isolated nuclei (6-9). Similarly, insulin which stimulates the efflux of RNA (38) also stimulates nuclear envelope NTPase activity (12). Second, nuclear envelope NTPase activity can be reduced 30% by treating the membranes with RNase A (10). This activity can be restored by the addition of exogenous RNA. Both of these approaches suggest that the ATPase is involved in the transport of RNA; however, since these studies were conducted with whole nuclear envelopes, the molecular details of the interactions between the NTPase, RNA, and other membrane components could not be characterized.

Characterization of the nuclear envelope-associated NTPase itself has also remained incomplete due to the lack of a purification procedure for this enzyme. Putative ATPases have been identified by photoaffinity labeling nuclear envelopes with ATP analogues and analyzing the products by sodium dodecylsulfate-polyacrylamide gel electrophoresis. These studies have produced contradictory results proposing a 174-kilodalton protein (39) and a 47-kilodalton protein (40) as the ATPase. The initial problem in purifying a membrane-associated enzyme is finding a method to solubilize the activity without permanently inactivating it. It was reported by Agutter, et al. (4) that all attempts to solubilize the nuclear envelope ATPase, including treatment with detergents, salts, and phospholipases, resulted in the inactivation of the enzyme. However, in a report by Kondor-Koch, et al. (38), Triton X-100 solubilized ATPase activity from rat liver nuclear envelopes. In the present study, we have used a similar method to solubilize this enzyme. The components of this Triton X-100 extract can be further resolved through chromatographic procedures. Although not necessary for the present studies, further purification of the ATPase is being pursued to more fully characterize the molecular properties of this enzyme.

To study the effects of phospholipids on the ATPase, it was first necessary to remove the lipids which were

solubilized from the membrane with the ATPase. A common procedure for removing phospholipids from solubilized proteins involves chromatography of the extract on a DEAE-column at a pH where the protein will bind (41). The phospholipid is eluted from the column because of its hydrophobic interaction with the detergent in the buffer. As demonstrated in the present study, this procedure is adequate for removing the major phospholipids; however, the highly polar polyphosphoinositides remain associated with the bound protein under these conditions. These quantitatively minor phospholipids may have pronounced effects on the activity of membrane-bound enzymes (15) and therefore should be considered in studies of phospholipid effects on enzyme activity. The polyphosphoinositides can be removed by chromatography of the detergent extracts on an anionic support such as heparin-Agarose.

Triton X-100 extracts of nuclear envelopes displayed approximately 85% of the original ATPase activity. At least 90% of this activity was recovered after chromatography on DEAE-Sepharose, indicating that the bulk phospholipid removed by this procedure is not required for ATPase activity. However, ATPase activity is lost if the Triton X-100 is removed by chromatography on Bio-Beads SM-2 (data not shown), indicating that the enzyme does require a hydrophobic environment for activity. Chromatography on heparin-Agarose, a polyanionic support,

removes the PIP and RNA from the ATPase, resulting in a marked loss of activity. This activity could be partially restored by the addition of a polynucleotide, supporting the proposal that this enzyme interacts with RNA. The mechanistic basis for this stimulation, as well as possible requirements for specific nucleotide sequences, are currently under study. Almost full activity could be reconstituted by the addition of both RNA and PIP.

We have shown that PIP is a component of rat liver nuclear envelopes through the demonstration of enzymes responsible for its synthesis, phosphatidylinositol kinase (22, Chapter II), and its degradation, PIP phosphomonoesterase (42, Chapter III), in isolated nuclear envelopes. Therefore, phosphatidylinositol and PIP can be interconverted in the nuclear envelope through a simple phosphorylation - dephosphorylation cycle. This metabolism of PIP may be linked to the regulation of the nucleocytoplasmic transport of RNA through the effects of this phospholipid on the nuclear envelope-associated ATPase.

Recent work has demonstrated that the products from the oncogenes src (43) and ros (44) possess phosphatidylinositol kinase activity as well as tyrosine kinase activity. It is also interesting to note that the src gene product associates predominantly with the nuclear envelope when rat cells (RR1022) are transformed with Rous

sarcoma virus (45). It therefore seems possible that increased amounts of PIP may accumulate in the nuclear envelope during transformation. In view of the present work, this could be expected to stimulate the nuclear envelope-associated NTPase. This may provide the basis for the increased amounts of RNA transport observed in transformed cells (46). Increased polyphosphoinositide metabolism in the nuclear envelope may also be involved in the mediation of mitogenic stimulii or the regulation of nuclear structure.

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SUMMARY

The phosphorylation and dephosphorylation of biological membrane components has often been proposed as a mechanism for regulating membrane function. Therefore, it seemed plausible that protein phosphorylation in the nuclear envelope may be involved in modulating the structure and function of this membrane system. We therefore initiated studies to investigate this possibilty. A number of phosphoproteins in the nuclear envelope were identified by SDS-PAGE and autoradiography. In addition to these protein phosphorylation products, a ^{32}P-labeled material which migrated with high mobility during SDS-PAGE, was insensitive to protease digestion, and which could be extracted from the labeled membranes (or the polyacrylamide gels) with acidified organic solvents was observed. This material was identified by thin layer and high pressure liquid chromatography as a mixture of ^{32}P-labeled PA, PIP, and PIP_2. Although the phosphoproteins may represent important components involved in the regulation of nuclear envelope function, their roles in these processes would be difficult to determine since their identities remain unknown. In contrast, the lipid phosphorylation products could be exactly identified. Therefore, it was judged that investigation of the metabolism and possible roles of

polyphosphoinositides might be more easily approached.

PI kinase and PIP kinase were identified as authenic nuclear envelope-associated enzymes, proving that the lipid phosphorylation observed in the nuclear envelope preparations was not due to contaminating organelles. Both kinases expressed maximal activity at pH 7.5-8.0, and in the presence of high concentrations of $MgCl_2$. In studies not described in this thesis, polyamines, particularily spermine, have been found to stimulate PI kinase at low (physiological) Mg^{2+} concentrations. Rapid degradation of PIP in nuclear envelopes was observed upon the depletion of the labeled ATP. This degradative activity was identified as a PIP-specific phosphomonoesterase. Ulike previously described phosphatases which act on polyphosphoinositides, the nuclear envelope-associated enzyme did not require divalent metal for activity. The PIPase was not inhibited by common phosphatase inhibitors; however, spermine was found to be a reasonably strong inhibitor. Therefore, it appears that cellular polyamine levels may be important in regulating the levels of polyphosphoinositides in biological membranes by stimulating the synthesis and inhibiting the degradation of PIP. These effects appear to be mediated through the binding of the polyamines to the phospholipids, thus neutralizing the charge on the substrate lipids. It is interesting to note that many

conditions, e.g. cellular transformation, result in increased levels of both polyamines and polyphophoinositides. Further studies on the interactions between the metabolism of these two important families of cell components should provide insight into the mechanisms of the regulation of membrane function.

Since the nuclear envelope is a bimembrane system, it seemed of interest to try to determine the topology of polyphosphoinositide metabolism in the nuclear envelope. Two new techniques were used to investigate the distribution of PI kinase and PIPase between the inner and outer nuclear membranes. Both techniques provided data suggesting that these enzymes were located on the inner nuclear membrane. However, when the distribution of newly synthesized PIP was determined, the phospholipid was found in both membranes. These results provide the first evidence that phospholipids are free to move between the inner and outer nuclear membranes, while proteins can be restricted to a single membrane.

Having exhaustively studied the metabolism of PIP in nuclear envelopes, it became necessary to initiate studies on possible roles for this phospholipid in nuclear envelope function. The best characterized enzyme in the nuclear envelope is the NTPase which is thought to be involved in the nucleocytoplasmic transport of RNA. Therefore, the effects of polyphosphoinositides on this

enzymes were investigated. In contrast to previously published reports, the NTPase was easily solubilized with Triton X-100 at low ionic strength. The ATPase was completely delipidated using two chromatographic steps. The second step was shown to be necessary for removing the highly polar polyphosphoinositides. This observation indicates that studies of phospholipid effects on other enzymes may have to be reevaluated since polyphosphoinositides might still have been present in most other reconstitution studies. The delipidated nuclear envelope ATPase was almost completely inactive; however, the activity could be partially reconstituted with polynucleotides. Nearly full reconstitution of the activity could be obtained by incubating with PIP and RNA. Therefore, it appears that polyphosphoinositides may be involved in the regulation of the ATPase, and thus RNA transport.

Additional studies on the roles of polyphosphoinositide metabolism in the nuclear envelope could address several questions. For example, possible roles of PIP in regulating the activity or specificity of nuclear envelope-associated protein kinase(s) could be explored. PIP might also interact with lamin B and therefore might alter the structure of the associated chromatin, perhaps resulting in gene activation. Cell cycle changes in the levels of PIP might be involved in

the disassembly or reassembly of the nuclear envelope. Studies described herein also suggest other lines of investigation. For example, in the studies of the phosphorylation of nuclear envelopes, the possibilty that a component becomes modified as an acyl-phosphorylated product was discussed. Addition studies not described in this thesis have identified this acyl-phosphate in a 90,000 dalton protein. Since this product can be formed with either ATP or GTP in the presence of $MgCl_2$ or $CaCl_2$, it seems likely that it represents a catalytic intermediate of the NTPase. Therefore, purification of the NTPase and reconstitution as described in Chapter V, could provide useful information about the molecular details of the RNA transport system and its regulation by PIP. As previously mentioned, there appear to be interations between polyamine metabolism and polyphosphoinositide metabolism. Further charaacterization of these effects may provide information on this potentially important means for regulating cell function.