Role for phosphatidylinositol in nuclear envelope formation

Banafshé LARIJANI^{*†}, Teresa M. BARONA[†] and Dominic L. POCCIA^{*}

^{*}Department of Biology, Amherst College, Amherst, MA 01002, U.S.A., and [†]Faculdade de Medicina de Lisboa, Nucleo de Endocrinologia Diabetes e Metabolism, Hospital de Santa Maria, 1750 Lisbon, Portugal

PtdIns is a minor membrane phospholipid that is important in signal transduction. Recently, derivatives of PtdIns phosphorylated at the 3-position of the inositol ring have been implicated in the regulation of constitutive membrane traffic and in membrane fusion events. Assembly of the nuclear envelope (NE), a crucial step in the progress of mitosis, is also likely to involve membrane fusion reactions. We therefore investigated the role of PtdIns and phosphoinositide 3-kinase (PI-3K) activity in NE formation *in vitro*. GTP-induced NE formation was blocked by wortmannin and LY294002, two specific inhibitors of PI-3K, suggesting a role for PtdIns phosphorylated at the 3-position. PtdIns-specific phospholipase C mimicked GTP hydrolysis as an inducer of NE

formation. This induction was dependent on a membrane vesicle subfraction (MV1) that was highly enriched in PtdIns, as determined by heteronuclear two-dimensional NMR spectroscopy. On the basis of these results, we suggest that the MV1 population serves as a source of membranes rich in PtdIns that might facilitate fusion, possibly through the production of the membrane-destabilizing lipid diacylglycerol.

Key words: membrane vesicle fusion, phosphatidylinositol-specific phospholipase C, phosphoinositide 3-kinase, two-dimensional NMR.

INTRODUCTION

The role of phosphatidylinositol polyphosphates in signalling membrane traffic and fusion has attracted the attention of many researchers [1–6]. Several studies have provided evidence for a mediating role of phospholipase C (PLC) and inositol phospholipid pathways in both the formation of vesicles and their fusion to acceptor membranes [2,3,7–11].

Phosphoinositide kinases have a direct role in the fusion of endosomal vesicles [7,9]. For example, endosome fusion is inhibited *in vitro* by blocking phosphoinositide 3-kinase (PI-3K) with the specific inhibitors wortmannin and LY294002 [10,11]. Moreover, the addition of a constitutively activated form of the catalytic subunit of PI-3K to cell extracts resulted in a 3-fold increase in endosome fusion [10]. Because the binding and fusion of vesicles are involved in the formation of the nuclear envelope (NE) *in vitro*, we were interested to see whether PtdIns metabolism also has a role in NE dynamics.

We have shown previously that sea urchin cell-free extracts initiate NE assembly by binding cytoplasmic membrane vesicles (MVs), or NE precursors, to chromatin. The binding of MVs to chromatin requires ATP; the fusion of chromatin-bound MVs is induced by GTP hydrolysis (and is blocked by guanosine 5'-[γ-thio]triphosphate), resulting in a NE [12,13]. Additional ATP results in the import and assembly of lamin B and swelling of the nucleus accompanied by further membrane fusion.

Total MVs (MV0), derived from 10000 g supernatants of egg homogenates, are composed of four fractions differing in buoyant density. Two of these, MV1 and MV2, are involved in NE assembly. MV1 binds exclusively to the tips of the sperm nuclei and contributes a minor fraction to the envelope. It has unusual properties, suggesting a crucial role in nuclear membrane fusion [12]. Most of the NE is contributed by MV2, which binds around

the entire nuclear periphery. Two subfractions of MV2, MV2α and MV2β, contain enzymic markers of Golgi and endoplasmic reticulum (ER) respectively. MV2β also contains lamin B and lamin B receptor.

With this system we demonstrate that two specific inhibitors of PI-3K block GTP-induced NE formation, suggesting that a form of PtdIns phosphorylated at the 3-position is involved in NE formation. We also show that the addition to the system of a bacterial PtdIns-specific PLC (PI-PLC) promotes NE formation in the absence of GTP, an effect dependent on the PtdIns-rich fraction MV1. We discuss models incorporating a role for PtdIns-rich membranes in NE formation.

EXPERIMENTAL

Buffers and reagents

Nuclear preparation buffer (SXN), egg lysis buffer (LB) and membrane wash buffer (MWB) were prepared as described previously [14]. *Lytechinus pictus* (sea urchins) were purchased from Marinus (Long Beach, CA, U.S.A.). PI-PLC from *Bacillus thuringiensis* and 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH₃) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Wortmannin, LY294002 and the Ca²⁺ ionophore A23187 were purchased from Sigma (St Louis, MO, U.S.A.). The lipophilic membrane probe 3,3'-dihexyloxacarbocyanine iodide (DiOC₆) was obtained from Molecular Probes. The ATP-generating system was composed of 1 mM ATP (SigmaUltra grade; Sigma), 20 mM creatine phosphate (Sigma) and 1 mg/ml creatine kinase (type I; Sigma) in LB. The GTP-generating system was made of 1 mM ATP, 20 mM creatine phosphate, 1 mg/ml creatine kinase and 1 mM GTP (type II; Sigma) in LB.

Abbreviations used: DAG, diacylglycerol; DiOC₆, 3,3'-dihexyloxacarbocyanine iodide; ER, endoplasmic reticulum; ET-18-OCH₃, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; HMQC-NMR, heteronuclear multiple-quantum coherence NMR; LB, lysis buffer; MV, membrane vesicle; MWB, membrane wash buffer; NE, nuclear envelope; PI-3K, phosphoinositide 3-kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; PLC, phospholipase C; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

[†] To whom correspondence should be addressed. Present address: Cell Biophysics Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K. (e-mail b.larijani@icrf.icnet.uk).

Figure 1 Wortmannin and LY294002 inhibit the induction of NE formation by GTP

NEs were assembled *in vitro* on permeabilized sperm nuclei in cytoplasmic extracts of sea urchin eggs and their sensitivity to PI-3K inhibitors was assessed. (A) DNA stained with Hoechst 33342; (B–G) membranes stained with DiOC₆. Nuclei were observed with a BHS-RFC Olympus fluorescence microscope with a Zeiss Neofluar 100 $\times$ oil-immersion objective with appropriate filter sets (as specified in the Experimental section). (A) Decondensed nucleus (4 μ m in diameter). (B) MV binding with 1 mM ATP. (C) Membrane fusion with 1 mM GTP. (D) Nuclear swelling (to 7 μ m in diameter) with the addition of 2 mM ATP. (E) Inhibition of GTP-induced NE formation by 25 nM wortmannin. (F) Inhibition of GTP-induced NE formation by 10 μ M LY294002. (G) DMSO (1.5%) does not inhibit GTP-induced NE formation. (H, I) Dose-dependent inhibition of NE formation by LY294002 (H) and wortmannin (I). The experiments were performed three times; error bars indicate S.D.

Nuclei and egg extracts

Sperm nuclei were demembrated with 0.1% (v/v) Triton X-100 as described previously [14]. Demembrated nuclei were washed and resuspended to 10⁸ nuclei/ml. Nuclei were diluted 1:25 and added to egg extracts to a final concentration of approximately one sperm nucleus per egg equivalent.

Eggs of *L. pictus* were collected and fertilized as described previously [14]. Fertilized eggs were washed in Millipore-filtered seawater and LB, resuspended in an equal volume of LB and homogenized on ice with two vigorous passes of a 22-gauge needle and a glass syringe at 10 min after fertilization. The lysate was centrifuged at 10000 *g* for 10 min at 4 °C in a microcentrifuge and the clear cytoplasmic layer (S₁₀) was withdrawn. Cytosol was prepared from S₁₀ by centrifugation for 2 h at 100000 *g* at 4 °C in a Ti50 rotor (Beckman Instruments, Carlsbad, CA, U.S.A.). The clear cytosolic fraction S₁₀₀ was recentrifuged for 1 h at 100000 *g* to collect the remaining pelleted membrane fractions. Cytosol (S₁₀₀) was frozen in liquid nitrogen and stored at –80 °C.

Binding assays

For S₁₀ MV binding assays, to a 1.5 ml Eppendorf tube were added 20 μ l of S₁₀, 1.2 μ l of ATP-generating system and 2 μ l

of demembrated sperm nuclei (final concentration 8 $\times$ 10⁵ nuclei/20 μ l). The mixture was incubated at room temperature for 1 h. Then 0.2 μ l of 0.1 mg/ml DiOC₆ (dissolved in methanol) was added to the suspension to label membranes; 5 μ l of this suspension was observed with a BHS-RFC Olympus fluorescence microscope with a Zeiss Neofluar 100 $\times$ oil-immersion objective under an FITC filter set (λ_{ex} 460 $\pm$ 20 nm; λ_{em} > 500 nm). Binding was assessed by observing the attachment of fluorescent green membranes to the nuclei (confirmed by centrifugation through 0.5 M sucrose to remove unbound vesicles). Images were captured in grey scale with a Hamamatsu Photonics C2400 SIT video camera (objective 40 $\times$) by using a frame grabber (Digital Vision, Dedham, MA, U.S.A.) and were processed after image averaging and background subtraction with a Hamamatsu Argus-10 image processor. Raw images were cropped and printed with Aldus Photostyler and Corel Draw 8 software.

Nuclear dimensions were calculated from an internal control of condensed conical sperm nuclei (4 μ m height by 1.5 μ m base). With the ATP-generating system, more than 85% of the nuclei decondensed and there was binding of MVs to nuclei [14]. For obtaining the number of decondensed or membrane-bound nuclei, 100 nuclei per experiment were counted; each experiment was repeated three times.

Table 1 Swelling assays for PI-3K inhibitor experiments

Expt 1 shows that 25 nM wortmannin-inhibited nuclei did not swell; Expt 2 shows that nuclei inhibited with 10 μ M LY249002 did not swell. Swelling was promoted by the addition of extra ATP. Results are means $\pm$ S.D.

Addition	Average diameter of decondensed and swollen nuclei (μ m)	
	Expt 1	Expt 2
GTP (fusion)	5.2 $\pm$ 0.4	5.5 $\pm$ 0.4
ATP (swelling)	9.6 $\pm$ 0.9	13.1 $\pm$ 0.4
Wortmannin + GTP + ATP	5.5 $\pm$ 0.4	—
LY249002 + GTP + ATP	—	5.6 $\pm$ 0.5

Fusion assay

Fusion of MVs occurs with exogenously added GTP [12,14]. To 1.5 ml Eppendorf tubes were added 20 μ l of S_{10} , 8×10^5 nuclei and 1.6 μ l of the GTP-generating system. The mixture was incubated at room temperature for 1 h, and 0.2 μ l of 0.1 mg/ml DiOC₆ was added to the mixture before observation under the microscope. The criterion for fusion was a continuous NE, in contrast with patches of bound membrane. If an enclosing smooth envelope was seen in more than 80% of the nuclei, a swelling assay was performed to confirm fusion.

Swelling assay

Swelling of decondensed sea urchin male pronuclei formed *in vitro* depends on the addition of extra ATP to the egg extract [14]. To the mixture of the fusion assay was added 2.4 μ l of the

ATP-generating system. Nuclei swell to approx. 7 μ m or more in diameter within 30 min. A ratio of swollen to unswollen nuclear diameters of at least 1.75 (7 μ m/4 μ m) was taken to confirm successful membrane fusion.

Inhibitor assays

A stock solution of 9.6 mM ET-18-OCH₃ was made in DMSO. The inhibitor was first dissolved in chloroform. A thin film of the dry compound was deposited on the walls of the container by evaporating the chloroform with a stream of argon. DMSO was added and the solution was vortex-mixed and heated to 50 °C to dissolve. Stocks were stored at –20 °C for up to 3 months.

A stock solution of 2.3 μ M wortmannin in DMSO was made and stored at –20 °C for no more than 1 month. The stock of 3.2 mM LY294002 was made in ethanol and stored at –20 °C for no more than 1 week.

MV subfractionation

The combined pellets of MVs (MV0) were washed twice by resuspension in MWB and centrifuged at 40000 *g* for 10 min. All solutions contained fresh protease inhibitor (1 mM PMSF). MV0 was subfractionated by sedimentation to density equilibrium in a linear sucrose gradient (0.2–2 M). Suspensions of 200 μ l of MV0s in MWB were diluted with 0.5–1.0 ml of ice-cold 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM PMSF.

Two-dimensional heteronuclear (³¹P–¹H) multiple-quantum coherence NMR (HMQC-NMR) spectroscopy

Details of this method have been described previously [15]. A modified Folch procedure adapted by Glonek and Merchant [16] was used for the extraction of MV subfractions. Each subfraction was resuspended in 200 μ l of MWB and added to 4 ml of

Figure 2 Two-dimensional phospholipid profile of MV1 and MV2

Cytoplasmic MVs were fractionated by buoyant density in a sucrose gradient. Extracted phospholipids were analysed by two-dimensional NMR on a 500 MHz Bruker machine. (A) PtdIns (PI) is the main component of MV1. The peak at –0.2, 3.67 p.p.m. (³¹P, ¹H) represents the identity peak of PtdIns-H⁶ from the inositol head group. PtdCho (PC) is present as a minor fraction. (B) Phospholipid profile of MV2, with a different profile from MV1. MV2 is composed of PtdCho, PtdIns, PtdSer (PS) and PtdEtn (PE) in proportions typical of ER and Golgi membranes.

ice-cold chloroform/methanol (2:1, v/v). It was then filtered and K_4 -EDTA was added. The lower chloroform phase was collected and evaporated under a stream of argon. The dried lipid samples were either used immediately or stored at -80°C .

All spectra were obtained on a Bruker AMX-2 500 MHz NMR instrument with a Bruker 5 mm broadband indirect probe tuned to 202 and 500.13 MHz. The probe temperature was stabilized at 298 K and samples were left to stabilize for 20 min at 298 K (the magnetic field homogeneity and hence the line width are sensitive to temperature variation). The parameters for the HMQC two-dimensional spectra shown in this work are as follows: ^1H and ^{31}P 90° pulses, respectively 8.0 and 9.5 μs , recycle delay 2 s, acquisition time 0.68 s, sweep width F_2 2008 Hz in 2750 real points, sweep width F_1 607 Hz in 64 slices, MLEV17 mixing at 7.1 kHz power for 101 ms (45 cycles), GARP ^{31}P decoupling during acquisition at 2.4 kHz power, refocus delay 0.071 s ($J_{\text{P-H}} = 7$ Hz). Data sets were processed with zero-shifted sine-bell window functions in both directions and displayed in magnitude mode for two-dimensional integration. The number of scans was varied to give sufficient signal. Runs for 2 h with 48 scans were performed for concentrated extracts; runs for 20 h with 480 scans were performed for submicromolar lipid extracts.

PI-PLC assays with MV0 and MV2

PI-PLC (0.8 mg = 5 units) was dissolved in 1 ml of 144 mM NaCl/10 mM Tris/HCl (pH 7.4)/0.02% (w/v) BSA. The enzyme was stored for up to 1 month at -8°C with 60% (v/v) glycerol added to the buffer. It was used in reactions at a final concentration of 0.12–0.16 unit/ml [17]. A 2 μl sample of either MV0 or MV2 (MV0 from which MV1 had been removed) was added to 20 μl of S_{100} . Then 2 μl of nuclei and 1.2 μl of ATP-generating system were added and the mixture was brought to 0.12 unit/ml PI-PLC. These assays were performed without the GTP-generating system. Binding and fusion were evaluated after 1 h. The experiments with PI-PLC and S_{10} were repeated three times and the number of nuclei with bound or fused MVs were quantified by counting 70–100 nuclei.

Ca^{2+} ionophore A23187 and Ca^{2+} ionophore assay

A stock solution of A23187 (1 mg/ml) was made in DMSO. The concentration of A23187 used in the fusion assays was 100 μM . These assays were performed without the GTP-generating system.

RESULTS

GTP-induced NE formation is blocked by PI-3K inhibitors *in vitro*

We tested whether the PI-3K pathway was involved in nuclear membrane formation by using the inhibitors wortmannin and LY294002 under conditions of GTP-promoted fusion. Figure 1(A) shows a 4 μm decondensed sperm nucleus and Figure 1(B) the binding of DiOC₆-labelled membranes to this nucleus. After the addition of GTP, the patchy appearance of membranes was converted to a smooth enclosure, indicating that a NE had formed (Figure 1C). Swelling to approx. 7 μm occurred with additional ATP (Figure 1D). Smooth enclosure and swelling were both used as criteria of envelope formation (Table 1).

Figure 1(E) and Table 1 show the inhibition of membrane fusion by 25 nM wortmannin (IC_{50} 5–50 nM). To verify the specificity, experiments were repeated with another PI-3K in-

Figure 3 MV fusion induced by a bacterial PI-PLC

NEs were assembled in the cytoplasmic extract as in Figure 1 with GTP or with PI-PLC in the absence of GTP. (A) ATP-dependent MV binding. (B) GTP-dependent fusion. (C) PI-PLC (0.12 unit/ml) without GTP induces membrane fusion. (D) Swelling of nuclei from (C) with excess ATP. (E) Inhibition of PI-PLC-induced membrane fusion by ET-18-OCH₃, an inhibitor of PI-PLC. (F) GTP-induced NE formation is not inhibited by ET-18-OCH₃. The bar chart (bottom panel) quantifies PI-PLC induction and ET-18-OCH₃ inhibition of NE formation. The experiments were performed three times; error bars indicate S.D. The scale on the y-axis corresponds to the percentage of nuclei enclosed.

Table 2 Swelling assays for PI-PLC induction experiments

Expt 1 shows that 0.12 unit/ml bacterial PI-PLC induced NE formation and swelling with MV0 and that the effect was blocked by the PI-PLC inhibitor ET-18-OCH₃. Expt 2 shows that GTP-induced NE formation with the MV2 fraction resulted in NE formation and swelling. Expt 3 shows that PI-PLC did not induce NE formation and swelling with MV2. Swelling was promoted by the addition of extra ATP. Results are means $\pm$ S.D.

Addition	Membranes used ...	Average diameter of decondensed and swollen nuclei (μ m)		
		Expt 1 MV0	Expt 2 MV2	Expt 3 MV2
GTP	—	—	5.3 $\pm$ 0.6	—
PI-PLC	—	5.2 $\pm$ 0.2	—	5.3 $\pm$ 0.6
ATP	—	9.5 $\pm$ 0.1	9.2 $\pm$ 0.6	5.4 $\pm$ 0.3
ET-18-OCH ₃	—	5.5 $\pm$ 0.3	—	—

hibitor, LY294002 at 10 μ M (IC₅₀ 1.4–50 μ M). Again only membrane binding was observed (Figure 1F). DMSO alone (the solvent for wortmannin) did not inhibit MV fusion (Figure 1G).

Figures 1(H) and 1(I) quantify these effects. Inhibitions of 50 % occurred at approx. 15 nM wortmannin and at 7 μ M LY294002, close to their IC₅₀ values. These results suggest that the PI-3K pathway is involved in the regulation of NE formation in the system *in vitro*.

Phospholipid composition of MV subfractions by HMQC-NMR spectroscopy

To determine the amounts of PtdIns in the membrane fractions contributing to the NE, we developed a sensitive quantitative NMR-based assay and analysed three fractions: MV1 and MV2, which are precursors of the envelope, and MV3, which is not.

The lipids were determined by HMQC-NMR spectroscopy. The peak assignments and the relative quantification of the phospholipids have been described previously [15]. Figure 2(A) shows the phospholipid profile of MV1. The proton peaks and their unique correlation with phosphorus define a fingerprint profile. The peak at -0.2 , 3.67 p.p.m. (³¹P, ¹H) represents the identity peak of PtdIns-H⁶. The identity peak for phosphatidylcholine (PtdCho) is at $(-0.5, 3.6$ p.p.m.). PtdIns and PtdCho were the only two phospholipids in MV1 (89 $\pm$ 5 mol % PtdIns and 11 $\pm$ 5 % PtdCho) [15]. MV2 (Figure 2B) was composed of PtdCho, PtdIns, phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) [62 % PtdCho ($-0.5, 3.5$ p.p.m.), 6 % PtdIns ($-0.2, 3.67$ p.p.m.), 5 % PtdSer (0.2, 3.7–3.8 p.p.m.) and 28 % PtdEtn (0.44, 3.04 p.p.m.)]. MV3 was composed of 73 % PtdCho, 10 % PtdIns, 4 % PtdSer and 13 % PtdEtn (results not shown).

MV2 and MV3 had compositions typical of ER or Golgi membranes; MV1 was highly enriched in PtdIns.

Bacterial PI-PLC induces NE formation

It has been suggested that the PtdIns-rich MV1 fraction is essential in NE formation [12]. To see whether the hydrolysis of PtdIns was involved, we added bacterial PI-PLC to our assay system in the absence of GTP. Bacterial PI-PLC prefers unphosphorylated PtdIns and cannot act on PtdIns phosphorylated at the 4- or 5-positions [18–20].

Figure 3(A) shows membrane-bound nuclei and Figure 3(B) shows GTP-induced NE formation. Figure 3(C) illustrates that, in the absence of GTP, PI-PLC produces the same smooth NE as those seen in Figure 3(B). When excess ATP was added, nuclei swelled (Figure 3D and Table 2).

Figure 4 Ca²⁺ ionophore A23187 does not induce NE formation

NE formation was attempted with A23187 to release membrane-bound stores of Ca²⁺ in the extract. (A) Hoechst staining of a decondensed nucleus. (B) ATP-dependent MV binding. (C) GTP-dependent membrane fusion. (D) Binding but no fusion of membranes with 100 μ M A23187 in the absence of added GTP.

To verify that the PI-PLC enzyme activity was promoting fusion, we added 19.6 μ M ET-18-OCH₃, a specific inhibitor of PI-PLC with an IC₅₀ of 0.4–9.6 μ M [21]. We also added ET-18-OCH₃ to the GTP-induced fusion assay mixture. As seen in Figure 3(E) and Table 2, ET-18-OCH₃ inhibited MV fusion induced by PI-PLC, but it did not affect GTP-induced fusion (Figure 3F). Figure 3 (bottom panel) quantifies these effects and includes DMSO and buffer controls. These results suggest that the fusion of MVs might be linked to PtdIns hydrolysis, but that GTP triggers fusion in the absence of an activity equivalent to the exogenous activity.

Basal levels of Ca²⁺ are required for membrane fusion [22,23]. NEs formed in 12 mM EGTA, in which Ca²⁺ levels were measured at less than 100 nM, but not in 5 mM BAPTA

Figure 5 PI-PLC induces NE formation in the presence of wortmannin or LY294002

NEs were assembled in the cytoplasmic extract as in Figure 1 with GTP or with PI-PLC in the absence of GTP and in the presence of PI-3K inhibitors. (A) ATP-dependent binding of MV. (B) Membrane fusion induced by PI-PLC (no GTP). (C) Inhibition of GTP-induced NE formation by 25 nM wortmannin. (D) NE formation induced by adding PI-PLC to the experiment in (C). (E) NE inhibition by 10 μ M LY294002. (F) NE formation induced by adding PI-PLC to the experiment in (E).

[bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid], indicating that extremely low levels of Ca^{2+} are required. Although bacterial PI-PLC is not expected to result in $\text{Ins}(1,4,5)P_3$ -mediated pathways of Ca^{2+} release, we tested whether fusion might be caused by an indirect release of Ca^{2+} from internal stores. Extracts were treated with excess Ca^{2+} ionophore A23187 (100 μ M) in the absence of GTP (Figure 4). No envelope formation was detected. This supports the notion that fusion was not due to a general increase in Ca^{2+} .

To determine whether PI-3K activity is necessary for the PI-PLC-induced formation of NEs, we performed the reaction in the presence of 50 nM wortmannin or 10 μ M LY294002 (Figure 5). Neither affected exogenous PI-PLC-induced fusion (Figures 5D and 5F). These results show that, although GTP-induced NE formation was sensitive to the two PI-3K inhibitors, exogenous PI-PLC induction was insensitive and could restore NE formation.

PI-PLC-induced membrane fusion requires MV1

To determine whether the PtdIns-rich MV1 fraction was required for NE formation induced by PI-PLC, we compared reactions with the MV1-depleted fraction MV2 with reactions containing both MV1 and MV2. (MV1 alone is insufficient to yield a full NE [12].)

In the absence of MV1, PI-PLC did not result in NE formation (Figure 6E). However, under the same conditions, GTP induced NEs, but at a lower frequency (Figure 6C and bottom panel). Therefore the PtdIns-rich MV1 fraction mediated the induction by PI-PLC.

Figure 6 PI-PLC does not induce NE formation in the absence of MV1

NE assembly was attempted with total and MV1-depleted cytoplasmic membrane vesicles. (A) NE formation with MV0, induced by 0.12 unit/ml PI-PLC. (B) ATP-dependent binding of MV2 to nuclei in S_{100} . (C) Fusion of MV2 in S_{100} with the addition of 1 mM GTP. (D) Nuclear swelling in the reaction shown in (C) with additional ATP (two nuclei shown). (E) No NE formation from MV2 with PI-PLC. The bar chart (bottom panel) quantifies these results. PI-PLC induces NE formation in 90% of the nuclei with MV0 and none with MV2. GTP results in 60% of nuclei forming NEs with MV2 only. The experiments were performed three times; error bars indicate S.D.

DISCUSSION

As in systems derived from other organisms [24], NE formation in cell-free sea urchin egg homogenates can be triggered by GTP hydrolysis. Here we show for the first time that GTP-induced NE formation is blocked by two relatively specific inhibitors of PI-

3K, establishing a parallel with the regulation of endosomal membrane fusion, in which it is believed that PtdIns phosphorylated at the 3-position helps to recruit machinery required for fusion. GTP-induced NE formation does not seem to require MV1, but we cannot yet rule out a role for hydrolysis of PtdIns phosphorylated in the 3-position in this pathway in our system.

The most unusual aspect of NE formation in our system *in vitro* is MV1, which has a remarkably high content of PtdIns and facilitates fusion [12,15]. These observations led us to investigate the effect of hydrolysis of PtdIns on NE formation. Our results show that the NE formation triggered by GTP hydrolysis can be mimicked by the addition of a bacterial isoform of PI-PLC to extracts of sea urchin egg cytoplasm. Furthermore, this effect requires the MV1 fraction.

These results lead to the question of what role, if any, MV1 might have in NE formation *in vivo*. The derivation of NE precursors is still uncertain. Several studies suggest that the NE is derived from a redistribution of parts of the ER [25–27]. *In vivo*, concentrated PtdIns domains might exist in natural membranes [28], or distinct vesicles enriched in PtdIns might be present. It seems to us unlikely that our MV1 fraction rich in PtdIns would be created by homogenization [29].

We can suggest a mechanism by which MV1 would lead to membrane fusion after PtdIns hydrolysis. The bacterial PI-PLC we used prefers unphosphorylated PtdIns and cannot act on PtdIns(4,5) P_2 [18]. It therefore forms diacylglycerol (DAG) and D-myo-inositol 1,2-cyclic phosphate [18]. Basanez et al. [30] have shown that the formation of DAG from the hydrolysis of phospholipids by a PLC in unilamellar vesicles *in vitro* stimulates fusion. DAG destabilizes the lamellar phase and promotes non-lamellar phases that favour fusion [31,32].

The observation that MV1 is not necessary for GTP-induced NE formation does not mean that it has no role. Most NE membrane precursors bind initially at the tips of the nucleus and continue towards the middle [12]. The peculiar restriction of MV1 binding to only the tips of the sperm nucleus indicates that it might be involved in the spatial regulation of initiation of fusion.

In conclusion, our experiments have led to us to propose that PI-3K is involved in NE formation. In addition, a fraction with a large amount of hydrolysable PtdIns, a novel feature of our system, is consistent with a structural role for DAG in destabilization of the lipid bilayer, which might facilitate fusion or regulate its site of initiation.

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