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Freeze-fracture analysis of the effects of intermediates of the phosphatidylinositol cycle on fusion of rough endoplasmic reticulum membranes

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While searching for the identity of the effector of the putative GTP-binding protein involved in fusion of rough endoplasmic reticulum (RER) cell-free incubation conditions were found permitting fusion in a GTP-independent manner. Membrane fusion was obtained using medium required to study synthesis of phosphatidylinositol (PI). We now report on the effects of various co-factors and intermediates of the PI cycle on the interaction of rough microsomes. By freeze-fracture, fusion of rough microsomes was defined as the appearance of fracture-planes of membrane larger than those of unincubated membrane. Cytosine triphosphate (CTP, 3 mM) in the presence of 2 mM $MnCl_2$ was most effective in stimulating fusion. Guanosine triphosphate (GTP) at the same concentration, could substitute for CTP to stimulate fusion, ATP, ITP, UTP and guanosine 5'-[γ-thio]triphosphate (GTPγS) could not. When combined together in the same medium CTP potentiated the effect of GTP. Arachidonic acid (20 μg/ml) also stimulated fusion in the presence of $MnCl_2$. This led to the appearance of large fracture-planes of membrane with a heterogeneous distribution of intramembranous particles. Other saturated fatty acids at the same concentration did not stimulate fusion. Phosphatidylinositol (PI, 50 μg) and 2 mM $MnCl_2$ had a similar effect as arachidonic acid and $MnCl_2$ in stimulating fusion. The PI effect was largely augmented in the presence of CTP. Our results are consistent with the concept that metabolism of phospholipids may modulate GTP-dependent fusion of RER membranes.

Introduction

Several GTP-binding proteins have been described in association with the secretion apparatus of the cell. GTP has been implicated in membrane fusion and is thought to play a role in membrane traffic between divers organelles [1–10]. GTP has also been implicated in membrane fusion events believed to be important for organelle assembly [11,12]. Thus, as suggested by Warren [13], the fusion mechanism which permits organelle assembly during mitosis may be similar to the one that operates during interphase allowing intracellular transport. The mechanism of action of GTP in membrane fusion has yet to be better defined. The present concensus is that GTP-binding proteins play a key role in the establishment of specificity during membrane–membrane interaction [14]. How specificity of interaction may be elaborated has yet to be determined both for membrane traffic in interphase cells and for organelle reconstitution in post-mitotic cells.

Recently, in a search for the identity of the effector of the GTP-binding protein involved in fusion of RER membranes, cell-free incubation conditions were found permitting fusion in a GTP-independent manner. RER membranes were observed to fuse in the absence of GTP if allowed to incubate in the presence of co-factors required for the synthesis of phosphatidylinositol [15]. Thus, phospholipid metabolism may be an intervening step between GTP stimulation and membrane fusion. In this paper we report the effects of addition of exogenous intermediates of the PI cycle on the fusion of membrane derivatives prepared from rat liver RER. Our results suggest that intermediates of the PI cycle may be important for RER membrane fusion.

Materials and Methods

Preparation and treatment of rough microsomes

Rough microsomes were prepared from rat liver [16] and stripped of their associated RNA as previously described [11]. These microsomes will be referred to as

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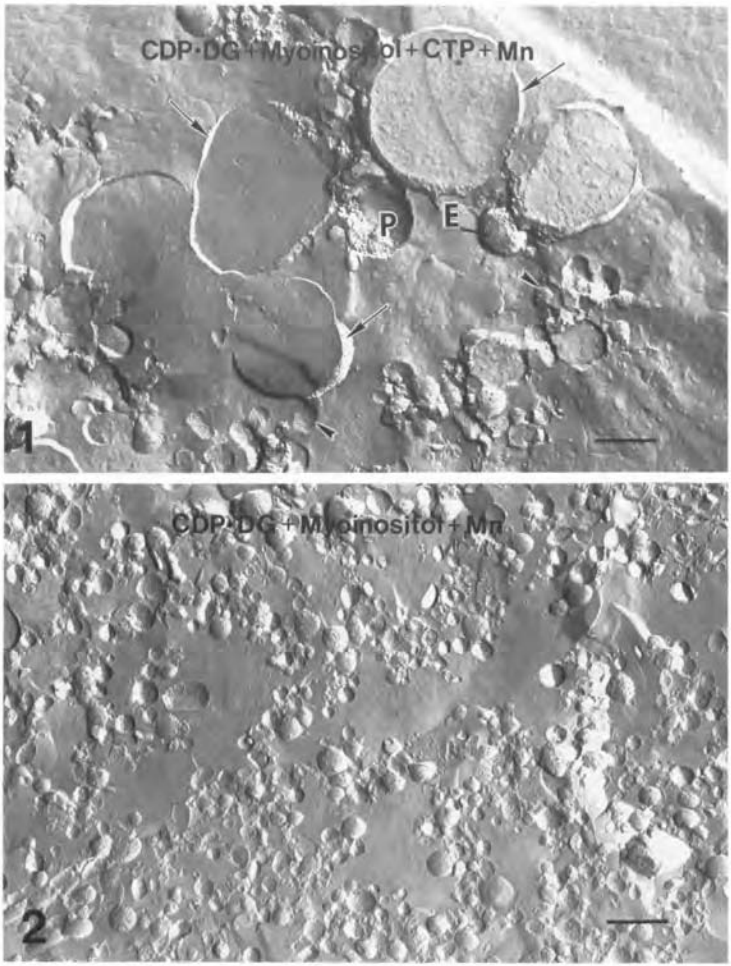

CDP-DG + Myoinositol + CTP + Mn
P
E
1
CDP-DG + Myoinositol + Mn
2

TABLE I

Morphometric comparison of the effect of co-factors required for the synthesis of PI on fusion of SRM

Stripped rough microsomes were incubated in 40 mM Tris-HCl (pH 7.4) containing 100 μg CDP-DG, 2 mM $MnCl_2$ and 10 mM *myo*-inositol in the absence or presence of 3 mM CTP for 120 min at 37°C. Following incubation the microsomes were frozen without fixation or cyroprotection and processed for freeze-fracture analysis and morphometric measurement as described in Materials and Methods.

Experiment	Incubation condition	Total membrane (μm^2)	Mean membrane area (μm^2)	Minimum membrane area (μm^2)	Maximum membrane area (μm^2)	Standard deviation	Fusion index (%)
I	SRM − CTP	18.246	0.018	0.001	0.116	0.013	0
	SRM + CTP	93.068	0.093 *	0.004	5.549	0.263	80.4
II	SRM − CTP	20.744	0.021	0.002	0.109	0.015	0
	SRM + CTP	90.143	0.090 *	0.003	10.104	0.465	76.9

Morphometry and calculation of the fusion index are described in Material and Methods.
* P vs. −CTP, $P < 0.001$.

stripped rough microsomes (SRM). Protein concentrations were determined using the procedure of Lowry et al. [17] with bovine serum albumin as standard.

Thin-section electron microscopy

Microsomes were fixed with or without previous incubation using 1.5% ice-cold glutaraldehyde in 50 mM phosphate buffer (pH 7.4). These fractions were recovered by filtration on Millipore membranes (0.45 μm pores; Millipore, Bedford, MA) and processed for electron microscopy (EM) as previously described [11].

Morphometric quantitation of membrane fusion was carried out as previously outlined [12]. Briefly, after incubation microsomes were fixed and embedded in Epon. Electron micrographs of sectioned vesicles were prepared. The lengths of the membranes of 737 sectioned vesicles were measured and summated. Since small vesicles fuse to form large vesicles under fusion conditions the sum of the membrane lengths for 737 vesicles is always higher than that for an equivalent amount of vesicles incubated using non-fusion conditions. Therefore, the total membrane length for vesicles incubated using non-fusion condition was subtracted from the total membrane length for vesicles incubated using fusion conditions and the difference expressed as a percentage of the amount of total membrane length for vesicles incubated using fusion conditions. This value gives the fusion index.

Freeze-fracture electron microscopy

For freeze-fracture electron microscopy, the microsomes were first placed on ice for 15 min. With a pipette with a tapered end, several microliters of incubation medium containing the microsomes were placed between two gold discs (Balzers-type specimen support plates) and rapidly frozen in partially solidified Freon 22 cooled at the temperature of liquid nitrogen without prior fixation and cryoprotection. For freeze-fracture frozen samples were stored in liquid nitrogen until fracturing. The samples were fractured at −130°C under a vacuum of $2 \cdot 10^{-6}$ torr in a Balzers freeze-etch apparatus (BA 400 T; Balzers AG, Balzers, Liechtenstein). Replicas were obtained after sequential shadowing, first with platinum at a fixed angle of 45° followed by carbon at 90°, using electron beam gun evaporators and an oscillating quartz for monitoring thickness of the replica (2 nm for platinum and 20 nm for carbon). Replicas were cleaned by floating on sodium hypochlorite solution (1–3 h), washed three times with distilled water, and mounted on parlodion-coated grids.

The freeze-fracture electron micrographs were mounted with the shadow direction from bottom to top and the terminology used to identify the fracture-faces and membrane surfaces was that proposed by Branton et al. [18].

Morphometry of freeze-fracture electron micrographs was carried out as follows. Membrane fractures

Fig. 1. Freeze-fracture EM micrograph showing evidence for fusion of SRM in the presence of co-factors required for the synthesis of PI. Stripped rough microsomes were incubated in 40 mM Tris-HCl (pH 7.4) containing 100 μg CDP-DG, 3 mM CTP, 2 mM $MnCl_2$ and 10 mM *myo*-inositol for 120 min at 37°C. Following incubation the microsomes were frozen without fixation or cryoprotection and processed for freeze-fracture analysis as described in Materials and Methods. Fusion products appear as large freeze-fractured vesicles (arrows) (see Fig. 2 for comparison). Unfused microsomes are seen as small fracture-planes of vesicles aggregated at the periphery of the large fracture-planes (arrowheads). P, protoplasmic fracture-face, E, exoplasmic fracture-face. Bar, 0.5 μm; magnification: ×28800.

Fig. 2. Freeze-fracture EM micrograph showing SRM incubated in a medium identical to the one used in Fig. 1 but in absence of CTP. Most of the SRM appear as individual, unfused vesicles in different planes of fracture. The absence of large fracture-planes of membrane indicates the absence of fusion of SRM membranes. Bar, 0.5 μm; magnification: ×28800.

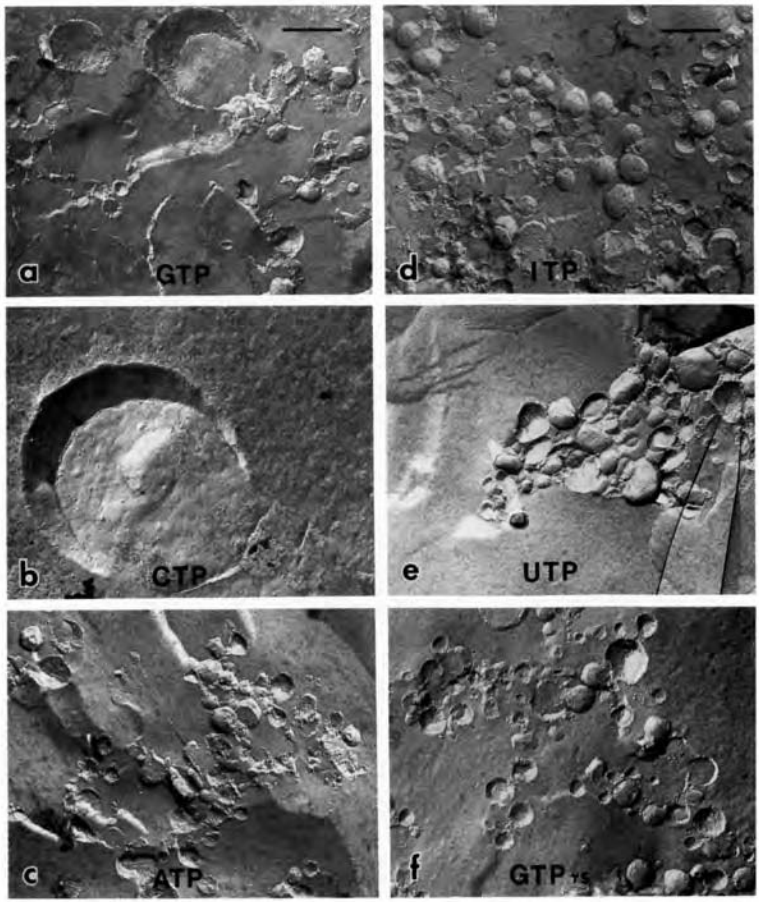


Fig. 3. Effect of nucleotides on the formation of large fracture-planes of membrane. Stripped rough microsomes were incubated in buffer containing 40 mM Tris-HCl (pH 7.4) and 7.5 mM $MgCl_2$ plus 3 mM of one of the nucleotides indicated. Large fracture-planes of membrane indicating the presence of fusion products are observed in microsomal preparations incubated in the presence of GTP (a), and CTP (b), however, no fusion products are observed amongst the microsomes incubated in the presence of ATP (c), ITP (d), UTP (e), or GTPγS (f). Bars, 0.5 μm; magnification: ×27000.

were located in replicas and photographed. Negatives were printed at a final magnification of ×28 800 and used in all stereological analysis. The prints were fastened to the measuring tablet (Graphic Master, Numonics, Montgomeryville, PA, USA) of a Sigma-Scan measurement system (Jandel Scientific, Corte Madera, CA, USA), and the contours of fracture-planes of membranes (both convex and concave as well as cross-fracture planes) were traced manually with the Sigma-Scan stylus, yielding the surface areas for the structures identified. 1000 fracture-planes were analysed for each incubation condition. The units of surface areas were obtained in square micrometers after calibration of the measurement system using a grating replica of 54 800 lines/inch (J.B. EM Services, Montreal, Canada). The surface areas were automatically stored in computer memory and digitizing morphometric software was used to compare measurements obtained after different experimental treatments. The fusion index was calculated as described for thin-section electron microscopy, except that surface area measurements were used instead of perimeter measurements.

Lipid treatments

Exogenous lipids were diluted from stock solutions containing 10 mg lipid/ml and added to incubation tubes to give amounts as indicated in the results. The lipids were dried under a stream of nitrogen gas and resuspended by sonication in 40 mM Tris-HCl buffer (pH 7.4) just prior to incubation.

Incubation conditions

Unless otherwise indicated, SRM (300 μg of microsomal protein) were incubated at 37°C for 120 min in 200 μl of buffer containing 40 mM Tris-HCl (pH 7.4) and 2 mM $MnCl_2$. Unless otherwise indicated all experiments described were repeated three times or more with similar results obtained each time.

Miscellaneous reagents

Bovine liver L-phosphatidylinositol (L-PI) was from Calbiochem-Behring (La Jolla, CA). All fatty acids, CDP-diacylglycerol (CDP-DG) and diacylglycerol (DG) were from Serdary Research (London, Ontario, Canada). The highest grade available CTP was obtained from three commercial sources: Sigma Chemical (St. Louis, USA), Calbiochem-Behring (La Jolla, CA, USA), Boehringer (Mannheim, Germany).

Results

Stripped rough microsomes were incubated in the presence of 100 μg CDP-DG, 3 mM CTP, 2 mM $MnCl_2$ and 10 mM *myo*-inositol. These constituents have previously been shown to be co-factors required for the synthesis of PI [19]. Freeze-fracture preparations of stripped rough microsomes incubated in the defined medium indicated the production of large membrane fusion products (Fig. 1). In this study, fusion is defined by the presence of fusion products characterized as fracture-planes of membrane which reveal intramembranous particles and which are larger than those of unincubated membranes. In these preparations, a quantitation was carried out to estimate the average surface area of the fractured vesicle membranes. This was done by measuring the area of the vesicles in various planes of fracture. These measurements indicated the formation of large fracture-planes with increased surface areas (Table I). Withdrawal of CTP (Fig. 2) or $MnCl_2$ (not shown) from the medium led to complete inhibition of membrane fusion as shown by the lack of production of large fracture-planes of membrane (Fig. 2). Surface-area measurements of fractured membranes which were incubated in the absence of CTP indicated fracture planes which were significantly smaller than those of membranes incubated in the presence of this nucleotide (Table I). Withdrawal of CDP-DG or *myo*-inositol from the medium, however, did not inhibit membrane fusion (not shown). This data confirms our previous report indicating GTP-independent fusion of stripped rough microsomes after incubation in the medium required for PI synthesis [15].

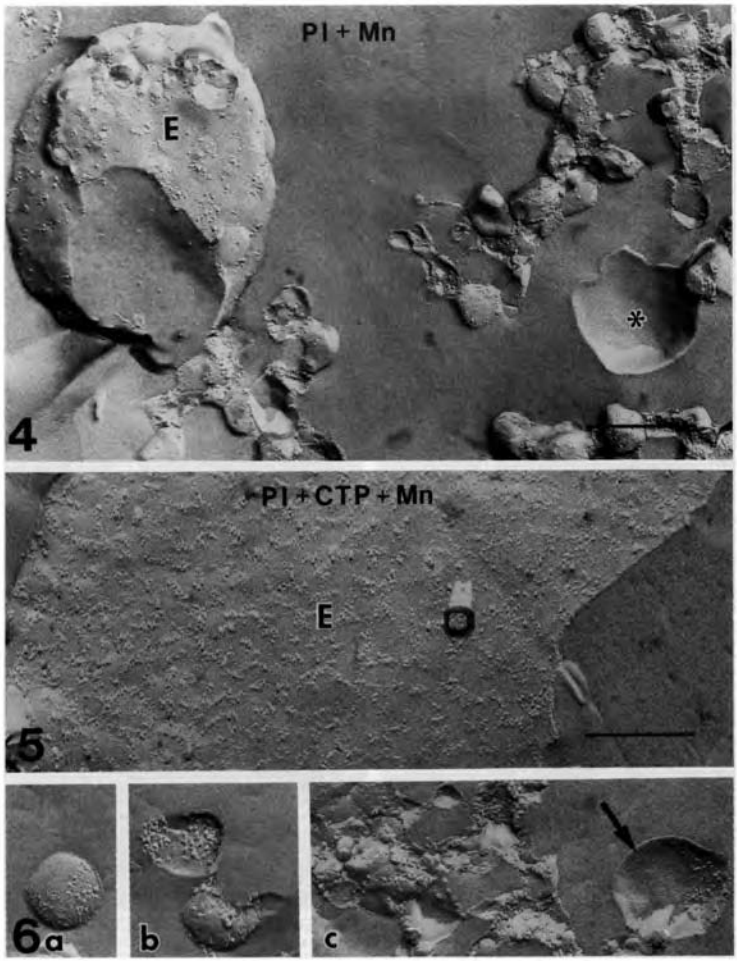
Since it was previously reported that only GTP could stimulate RER membrane fusion [11] we re-examined the nucleotide specificity of the reaction under the new incubation conditions employed. Membranes were incubated in the presence of 7.5 mM $MgCl_2$ plus one of the following nucleotides at 3 mM concentrations; ATP, CTP, ITP, GTP, UTP or GTPγS (guanosine 5'-[γ-thio]triphosphate). Guanosine triphosphate and CTP were both found to be effective in stimulating

TABLE II

Morphometric comparison of the effect of GTP and/or CTP on the fusion of stripped rough microsomes from rat liver

Stripped rough microsomes were incubated in the presence of nucleotide as indicated plus 5 mM $MgCl_2$ for 120 min at 37°C. Samples were then fixed and processed for morphometric analysis of membrane fusion using thin-section electron microscopy as described in Materials and Methods. Values indicated in parentheses represent data obtained from a separate experiment using membranes from a different fractionation experiment. n.d., not determined.

Nucleotide	Fusion index (%)
GTP (0.5 mM)	26.6 (21.6)
CTP (0.5 mM)	0 (0)
CTP (3 mM)	22.1 (n.d.)
GTP (0.5 mM) + CTP (0.5 mM)	39.6 (27.5)


PI + Mn
E
*
4
PI + CTP + Mn
E
5
6a
b
c

membrane fusion as indicated by the formation of large fracture-planes of membrane (Fig. 3a and b). A semi-quantitative analysis based on diameter measurements of more than 50 fracture-planes per incubation condition indicated the production of fracture-planes of membrane greater than 2 μm after incubation in either GTP or CTP. Other nucleotides including ATP, ITP, UTP as well as the non-hydrolyzable analogue of GTP, GTPγS did not promote the formation of large fracture-planes of membrane (Figs. 3c, d, e and f). This was confirmed by the lack of evidence of an increase in size of the microsomal vesicles. An estimation of the average diameter of the freeze-fractured microsomal vesicles incubated in presence of the nucleotides as shown in Fig. 3 are as follows: ATP (0.23 ± 0.02 μm); ITP (0.24 ± 0.01 μm); UTP (0.24 ± 0.02 μm) and GTPγS (0.22 ± 0.01 μm). Among these preparations, none of the vesicles had a diameter exceeding 0.37 μm. Similar results were obtained using 2 mM $MnCl_2$ in place of 7.5 mM $MgCl_2$ (not shown).

In two separate experiments GTP- and CTP-dependent membrane fusion were compared by quantitative assay using thin-section electron microscopy (Table II). Fusion in the presence of 0.5 mM GTP was consistantly higher than that in the presence of 0.5 mM CTP (Table II). In fact when highly purified preparations of CTP from different suppliers were used, the amount of fusion was very low or undetectable at concentrations of 0.5 mM (Table II) but evident at 3 mM (Fig. 3b and Table II). When the two nucleotides were combined in the same incubation medium at equal concentrations (0.5 mM) this led to a significant increase in amount of fusion compared to that obtained with either nucleotide alone (Table II). Thus CTP potentiates the effect of GTP.

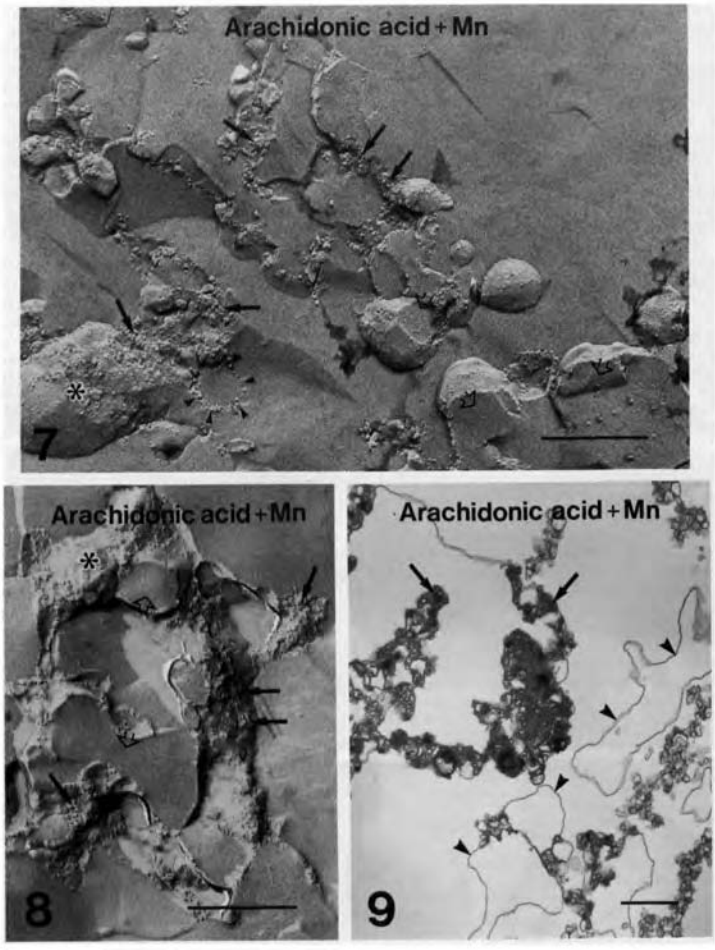
The effect of PI on membrane fusion was next examined. When SRM were incubated in the presence of PI (50 μg) and $MnCl_2$ (2 mM), this led to the formation of two types of large fracture-planes of membrane (Fig. 4). The first type was characterized by the total absence of intramembranous particles and was believed to represent fracture-planes of reconstituted PI liposomes (Fig. 4 asterisk). The second type was characterized by fracture-planes of membrane having heterogeneously distributed intramembranous particles (Fig. 4, large endoplasmic fracture-plane E). When membranes were incubated in the presence of PI and $MnCl_2$ as well as 3 mM CTP the response was potentiated leading to the appearance of vast fracture-planes of membrane (Fig. 5). Many of the fracture-planes of membrane displayed a heterogeneous distribution of intramembranous particles (Figs. 5 and 6). When PI (50 μg) was incubated in the presence of $MnCl_2$ (2 mM) without SRM this led to the formation of unilamellar membranes many of which had free ends (not shown). Since our fusion assay requires the analysis of intact vesicles we did not study these structures any further.

Since arachidonic acid is a product of PI metabolism and since this fatty acid was previously shown to be fusogenic [20,21], the effect of this polyunsaturated free fatty acid on membrane fusion was determined. Incubation of SRM in the presence of 20 μg arachidonic acid and 2 mM $MnCl_2$ led to the appearance of large fracture-planes of membrane. The fracture-planes of the large fused vesicles were often characterized by the presence of aggregates of globular structures (Figs. 7 and 8). When thin-sections of similar fusion products were prepared and examined in the electron microscope, the large membranes produced in the presence of arachidonic acid were characterized by having numerous small vesicles densely aggregated at their periphery (Fig. 9). Fatty acid specificity of membrane fusion was then analyzed. Stripped rough microsomes were incubated in the presence of 2 mM $MnCl_2$ and 20 μg of one of the following fatty acids: palmitic, stearic, erucic, oleic, linoleic, linolenic or arachidonic acid (Fig. 10). For comparison incubation of SRM in the presence of 3 mM CTP and 2 mM $MnCl_2$ led to the formation of large fusion products (Fig. 10a). None of the saturated fatty acids (i.e., palmitic or stearic) was able to promote the formation of large fusion products (Figs. 10b and c). Erucic acid a long chain carbon molecule (22 carbons) with one double bond also did

Fig. 4. Effect of the presence of phosphatidylinositol and $MnCl_2$ on the formation of large fracture-planes of membrane. Stripped rough microsomes were incubated in the presence of 50 μg PI and 2 mM $MnCl_2$. The microsomes were then processed for freeze-fracture analysis as described in Materials and Methods. The convex exoplasmic fracture-plane of a large fusion product is shown (E) as well as the fracture-planes of many unfused vesicles. In this preparation, the protoplasmic face of a microsome (liposome?) is shown devoid of intramembranous particles (asterisk). Bar, 0.5 μm; magnification: $\times$51,300.

Fig. 5. CTP potentiates the formation of large fracture-planes of membrane in the presence of PI. SRM were incubated as in Fig. 4 but with additional 3 mM CTP. The micrograph shows the vast expanse of the exoplasmic membrane half of a portion of a membrane fusion product. Bar, 0.5 μm; magnification: $\times$51,300.

Fig. 6. Freeze-fracture micrographs showing the fracture-planes of SRM incubated as in Fig. 5. The vesicles display highly variable surface distributions of intramembranous particles as shown by both exoplasmic faces (a) as well as protoplasmic faces (b). Figure (c) shows the fracture-plane of a membrane with discreet particle-free (arrow) and particle-rich areas. Bar, 0.5 μm; magnification: $\times$51,300.


Arachidonic acid + Mn
7
Arachidonic acid + Mn
8
Arachidonic acid + Mn
9

not promote membrane fusion (Fig. 10d). In contrast, large membrane fusion products were observed after incubation in the presence of one of the unsaturated fatty acids (i.e., oleic, linoleic, linolenic and arachidonic acid) (Figs. 10e–h). As judged by the size of the fusion products (here defined as large membrane-bounded vesicles) the capacity to promote fusion was least with oleic and linoleic acid (smaller and fewer membrane fusion products, Figs. 10e and f) and greatest with linolenic and arachidonic acid (larger and more membrane fusion products, Figs. 10g and h).

Discussion

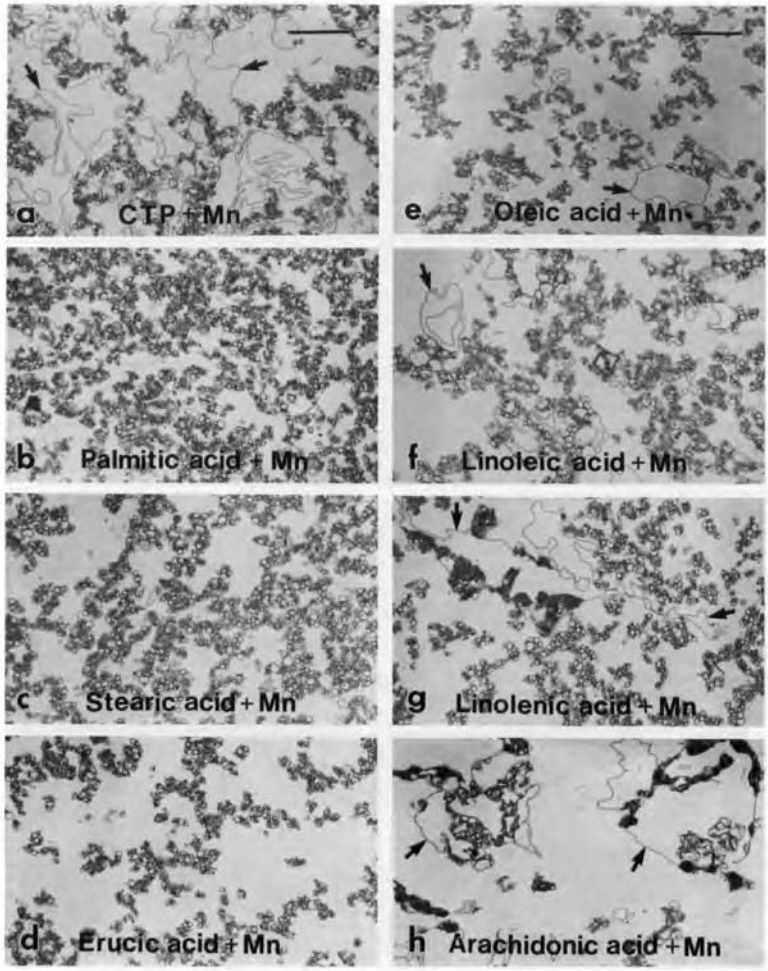
In the present study, we have taken advantage of the membrane splitting property of the freeze-fracture technique to examine the effects of intermediates of the PI cycle on fusion of RER membranes. Since freeze-fracture exposes large surfaces of individual membrane leaflets and permits distinction between natural and synthetic membranes (based on the presence or absence of intramembranous particles), we therefore defined fusion in the present study as the presence of fracture-planes of membrane which show intramembranous particles and which are larger than those of unincubated membranes. Fusion of membrane derivatives of the rough endoplasmic reticulum was observed in this study to occur after incubation in the presence of the co-factors known to be required for the synthesis of PI [19]. This data confirms our previous report [15] of this phenomenon. We further demonstrate in this paper that other intermediates of the PI cycle including PI and arachidonic acid can promote membrane fusion in a GTP-independent manner. The results are consistent with the suggestion that phospholipid metabolism implicating intermediates of the PI cycle may be an intervening step between GTP stimulation and membrane fusion [15], and are corroborated by the previous finding that polyunsaturated free fatty acids are generated coincident with GTP-dependent fusion [22].

Systematic removal of the enzyme co-factors employed for PI synthesis revealed a requirement for the presence of CTP and $MnCl_2$ in RER membrane fusion. Since fusion was observed in the absence of *myo*-inositol, CTP is thought to induce its effect via an intermediate of the PI cycle other than PI itself. This intermediate could be either arachidonic acid or diacylglycerol since the formation of these products has been observed in the presence of CTP [22]. We cannot however completely exclude the possibility that PI could be involved since endogenous *myo*-inositol could be transfered on to CDP-DG by a *myo*-inositol exchange enzyme known to exist in the ER [19]. It should be pointed out that the association between inositol phospholipid metabolism and membrane fusion has previously been made for myoblast fusion [23]. This relationship may have broader implications than was previously suspected.

Previous studies have suggested that arachidonic acid and/or diacylglycerol may be important for membrane fusion [20,21,24–27]. Our results with arachidonic acid are consistent with this suggestion. Although we have not yet studied the effects of diacylglycerol, we do know that it's production as well as that of arachidonic acid is stimulated by CTP [22]. The question therefore arises, how does the GTP effect relate to the CTP effect? Our present working hypothesis is that GTP stimulates metabolic events which occur at the level of the initiation of membrane–membrane interaction before actual membrane coalescence occurs. This is in accord with the model proposed by Bourne [14] implicating GTP in membrane recognition and targeting during secretion. With regards to CTP we suggest that this nucleotide can either potentiate or bypass initial GTP-dependent events by virtue of its capacity to stimulate formation of both arachidonic acid and diacylglycerol. The data presented in this paper are consistent with a role for arachidonic acid in membrane fusion and indicate that this occurs in a GTP-independent manner. We also observed membrane fusion in the presence of exogenous PI. Exogenous PI may promote the appearance of diacylglycerol and/or arachidonic acid especially in the presence of CTP. Indeed the formation of DG in the presence of CTP has previously been demonstrated using microsomal preparations and shown to occur as a consequence of the back reaction of CDP-diacylglycerol–inositol phosphatidyltransferase [28]. Such lipids would then cause bilayer lipid disorganization (possibly by induc-

Figs. 7 and 8. Effect of arachidonic acid and $MnCl_2$ on the formation of large fracture-planes of membrane. Stripped rough microsomes were incubated in the presence of 20 μg arachidonic acid and 2 mM $MnCl_2$. Fusion products are shown here as large fracture-planes of membrane (asterisk) some of which are cross-fractured exposing their sizable luminal space (open arrows). Aggregates of globular structures are indicated along the surfaces of fractured membranes (arrows). These globular structures are also visible along the membranes of cross-fractured microsomes (arrowheads). Bar, 0.5 μm; magnification: ×51300.

Fig. 9. Thin-section EM micrograph showing evidence for fusion of SRM after incubation as in Figs. 7 and 8. The microsomes were fixed and processed for EM as described in Materials and Methods. Membranes of large fusion products are indicated by arrowheads. Dense membrane aggregates are observed at the periphery of the fusion products (arrows) and are thought to correspond to the globular structures seen in freeze-fracture replicas shown in Figs. 7 and 8. Bar, 1 μm; magnification: ×13000.


a CTP + Mn
e Oleic acid + Mn
b Palmitic acid + Mn
f Linoleic acid + Mn
c Stearic acid + Mn
g Linolenic acid + Mn
d Erucic acid + Mn
h Arachidonic acid + Mn

tion of type II non-bilayer lipid structures [29]) in regions of membrane apposition and provoke membrane fusion.

Exogenous arachidonic acid was previously shown to stimulate fusion of chromaffin granules [20] and fusion of neutrophil granules with liposomes [21]. As in chromaffin granule fusion [20] RER membrane fusion was found to occur in the presence of similar concentrations of arachidonic acid (10–20 μg). This concentration corresponds closely to the amount of endogenous polyunsaturated free fatty acid previously detected in rough microsomes [22]. Furthermore, as in the study of chromaffin granule fusion, we found that saturated fatty acids were incapable of stimulating fusion and polyunsaturated fatty acids were more effective at stimulating fusion in correspondence with the degree of unsaturation. Other membrane properties shown to be affected by polyunsaturated free fatty acids include membrane fluidity [30], chloride transport [31] and liberation of Ca^{2+} [32].

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Fig. 10. Thin-section EM micrographs showing the effect of various fatty acids on fusion of SRM. Stripped rough microsomes were incubated in the presence of 20 μg of fatty acid and 2 mM $MnCl_2$. For comparison incubation of SRM in the presence of 3 mM CTP and 2 mM $MnCl_2$ led to the formation of large fusion products (a). Incubation in the presence of palmitic acid, stearic acid or erucic acid did not provoke formation of fused vesicles (b–d). Few membrane fusion products were observed after incubation in the presence of oleic and linoleic acid (e and f). Larger and more frequent fusion products were obtained after incubation with linolenic (g) and arachidonic acid (h). Dense membrane aggregates are evident at the periphery of the fused vesicles after incubation with either linolenic or arachidonic acid (g and h). Fusion products are indicated by arrows. Bars, 2 μm; magnification: ×7000.