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Biochimica et Biophysica Acta 1190 (1994) 199–212

BBA Biochimica et Biophysica Acta

Modulation of GTP-dependent fusion by linoleic and arachidonic acid in derivatives of rough endoplasmic reticulum from rat liver

Jacques Paiement [a,*], Christine Lavoie [a], Grace R. Gavino [b], Victor C. Gavino [b]

[a] *Département d'anatomie; Faculté de médecine, Université de Montréal, C.P. 6128, Succursale A, Montréal, Que., Canada H3C 3J7*
[b] *Département de nutrition, Faculté de médecine, Université de Montréal, C.P. 6128, Succursale A, Montréal, Que., Canada H3C 3J7*

(Received 10 September 1993)

Abstract

The effect of modulation of the content of unsaturated free fatty acids on GTP-dependent fusion of stripped rough microsomes from rat liver was determined. Cytidine monophosphate, CDP and CTP were all observed to be able to stimulate free fatty acid accumulation and coincident membrane fusion. GTP was required for membrane fusion in the presence of cytidine nucleotide but was not required for free fatty acid accumulation. In the presence of GTP and cytidine nucleotide, the addition of ATP and CoA led to the synthesis of triacylglycerol and marked inhibition of both free fatty acid accumulation and membrane fusion. Delipidated bovine serum albumin also inhibited both free fatty acid accumulation and membrane fusion. Analysis by gas chromatography indicated that linoleic acid and arachidonic acid were the most actively fluctuating of the accumulated free fatty acids. Comparison by quantitation indicated a high correlation between GTP-dependent membrane fusion and changes in amount of unesterified linoleic acid and arachidonic acid. The results suggest that polyunsaturated free fatty acids may be required for GTP-dependent membrane fusion.

Key words: Rough endoplasmic reticulum; Membrane fusion; Gas chromatography; GTP; CMP; Polyunsaturated free fatty acid

1. Introduction

The endoplasmic reticulum (ER) is a dynamic structure capable of undergoing extensive morphological alteration within eukaryotic cells. Sometimes it exists as a continuum, and sometimes it may be observed as a number of isolated components that can fuse to form a continuum [1]. Structural changes implicating fusion among membranes of this system in interphase cells have been shown to correlate with changes in cellular metabolism [2–8], as well as with changes in the organization of the cytoskeleton [9–12]. The exact significance of these changes is not known, but since the endoplasmic reticulum is a major store for intracellular calcium, fluctuations in metabolic activities dependent on this cation may be related to both its location and its form [13]. The form of the ER has also been observed to change during the cell cycle [14–16], and in such cases ER reconstitution was shown to result from membrane fusion following vesiculation during cell division. Vesiculation during division is thought to be important for the efficient redistribution of ER in post mitotic cells [17].

The molecular mechanism responsable for either vesiculation or fusion of ER membranes is not well understood. However, cell-free incubation systems have recently become available to permit a molecular dissection of the sequence of events involved in ER formation. Endoplasmic reticulum formation involves both membrane transport and membrane–membrane interaction. Membrane-membrane interaction involves a specific recognition step (for the ER this implies self recognition, i.e., homotypic interaction) and sequencial coalescence to form a continuous network. Dabora and Sheetz [18] were able to reconstitute a dynamic tubulovesicular membrane network (endoplasmic reticulum) using extracts of cultured chick embryo fibroblasts. Both ATP and microtubules were shown to be essential for network formation and although the ATP-dependent step was not identified the microtubules were implicated in membrane transport by a process

* Corresponding author. Fax: +1 (514) 3432459.

SSDI 0005-2736(93)E0357-G

defined as membrane tethering [18]. The nucleotide ATP was also shown to be required for the movement of rough ER membranes along stationary actin cables [11]. Vale and Hotani [19] were also able to reconstitute a network of ER tubulovesicles and this was done in the presence of kinesin and ATP.

Whereas the well-defined kinesin-microtubule (actin) motility assays described above have been extremely useful for the examination of the process of ER membrane movements in vitro, the small quantities of membrane obtained have precluded detailed biochemical characterization of the interacting membranes. Paiement et al. [20], using stripped rough microsomes from rat liver and cell free incubation conditions, identified GTP as a key factor involved in the interaction (fusion) of ER membranes. Since this initial report several other laboratories have confirmed GTP-dependent effects on ER membranes [21,22]. The availability of such systems should facilitate the identification of the components involved in membrane fusion. GTP has also been shown to be involved in membrane traffic between various organelles from interphase cells [23–25]. In these systems GTP-binding proteins have been defined but their exact role in membrane–membrane interaction remains enigmatic.

As well as stimulating rough ER membrane fusion GTP causes membrane permeability changes [26,27], Ca^{2+} release [21,28], protein translocation [29], core glycosylation [30,31] and CDP-diacylglycerol formation [32,33] in ER membranes. Although numerous GTP-binding proteins have been identified by high resolution 2-D gel analysis in enriched fractions of rough microsomes [34] it is not yet known whether the numerous GTP-dependent ER functions mentioned above are subserved by different GTP-binding proteins or by one or several such proteins working through a common metabolic pathway. Recently GTP-dependent rough ER fusion was observed to occur coincident with the accumulation of unsaturated free fatty acids (UFAs) [35]. Since GTP itself did not stimulate accumulation of UFAs it was suggested that this nucleotide stimulates metabolic events upstream from those occuring during actual membrane fusion [35]. Consistent with this suggestion is the recent report that exogenous UFAs can stimulate fusion between rough microsomes independent of the presence of GTP [36]. Although fatty acid accumulation was not stimulated by the presence of GTP both GTP-dependent membrane fusion [36] and fatty acid accumulation were potentiated by CTP [35]. CTP by itself at sufficiently high concentration (3 mM), and 2 mM $MnCl_2$ resulted in both the accumulation of fatty acids and diacylglycerol [35] and membrane fusion [36].

In this paper we identify the UFAs which accumulate during GTP-dependent membrane fusion as linoleic and arachidonic acid and demonstrate modulation of their content during both stimulated and inhibited fusion conditions. Data is also presented suggesting that polyunsaturated fatty acids are released consequent to the back reaction of both CDP-diacylglycerol inositol phosphatidyltransferase and cholinephosphotransferase, and subsequent diacylglycerol lipase activity.

2. Materials and methods

Stripped rough microsomes (SRM) were prepared from rat liver homogenates as previously described [31]. Unless otherwise indicated, incubations were carried out for 120 min at 37°C in medium containing 100 mM Tris-HCl pH 7.4, and 5 mM $MgCl_2$. The cell-free membrane fusion assay involved morphometric measurement of embedded and sectioned membranes using electron microscopy [37]. Samples destined for analysis by electron microscopy were prepared in duplicate and repeated in at least three separate experiments.

Endogenous lipids from SRM were extracted by a modification of the Schact procedure [38] as outlined by Smith and Wells [39]. The organic extract was used fresh or was stored no longer than 12 h at 4°C under nitrogen gas before use. In some cases incubated samples were kept frozen at −75°C before extraction. Storage conditions had no adverse effects on results.

For identification of lipids in densitometric studies, aliquots (10 μl) of lipid extracts from triplicate or quadruplicate samples were spotted using a Drummond Microdispenser (Drummond Scientific, Broomall, PA, USA) on thin-layer plates of silica gel 60 which had been pre-activated for 1 h at 110°C. Application of lipid extracts was done in a room with controlled humidity (60 ± 3%) and temperature (21 ± 1°C). Chromatograms were developed in a rectangular glass tank (N-tank) with n-hexane/diethyl ether/acetic acid (70:30:1, v/v) [40]. The chambers were lined with Whatman 3 MM filter paper wetted with developing solvent. The lipids were detected by staining with 10% ethanolic phosphomolybdic acid using the dipping technique and a dipping chamber of 125 ml capacity (Chromatographic Specialties Inc. Boucherville, Canada). The lipids were identified by co-chromatographed phospholipid and fatty acid standards detected by the same procedure. Unsaturated free fatty acids were quantitated by densitometric scanning using the LKB Ultra Scan XL laser densitometer with Gel Scan XG software (LKB Pharmacia, Montréal, Canada) after exposure of the plate to ammonia vapors to decolorize the background. A linear relationship for the range of absorbance units (area of absorbance curves, 0–4.8), and the amount of phosphomolybdic acid staining was observed for the different concentra-

tions of the lipid standards employed (arachidonic acid, from Sigma, St Louis, MO, USA at 0.5–8 μg, triacylglycerols, from pig liver, Serdary Research, London, Canada, at 0.5–20 μg) (data not shown). Under our conditions saturated fatty acids gave negligible staining.

For identification of radioactive lipids, aliquots of lipid extracts obtained from SRM after incubation with ^{14}C-labelled precursors were spotted on thin-layer plates of silica gel 60 which had been preactivated for 1 h at 110°C. Chromatograms were developed and labelled lipids visualized by radioautography on Kodak X Omat AR photographic film and identified by co-chromatographed phospholipid standards detected by phosphomolybdic acid staining.

For gas chromatography membrane lipids were extracted as stated above. Heptadecanoic acid, (1,2) diheptadecanoin and triheptadecanoin (Nu-Check-Prep, MN, USA) were used as recovery standards. Lipids were separated by thin-layer chromatography using n-hexane/diethyl ether/acetic acid (70:30:1, w/v) as solvent [40]. Butylhydroxytoluene (0.004%) was present in the diethyl ether. Free fatty acids were methyl esterified [41]. Diacylglycerol and triacylglycerol spots were transesterified to fatty acid methyl esters by treatment with sodium methoxide [42]. The fatty acid methyl esters were analyzed using a 30-m bonded Carbowax-30N fused silica capillary column (Supelco, Oakville, Canada) installed in a Hewlett-Packard gas chromatograph model 5890, equipped with flame ionization detector and a Hewlett-Packard model 3396 electronic integrator [43]. For the gas chromatographic studies assays were performed in duplicate and repeated in at least three separate experiments.

3. Results

Unsaturated free fatty acids were shown to accumulate during GTP-dependent fusion of stripped rough microsomes and both membrane fusion and UFA accumulation were potentiated in the presence of CTP [35]. We have now examined the effects of different cytidine nucleotides and analogues on rough microsomal lipid composition. Microsomes were incubated in the presence of 500 μM GTP plus one of the additional nucleotides, 5′-CTP, 5′-CDP, 5′-CMP, 3′-CMP, 2′-CMP, 5′-GMP or 5′-AMP, also at 500 μM. Quantitation of lipids by densitometry of stained lipids after separation by thin-layer chromatography revealed a stimulation of basal accumulation of UFAs in the presence of 5′-cytidine nucleotides (Fig. 1). 5′-CMP was more effective than 5′-CDP, which was more effective than 5′-CTP (Fig. 1). The analogues 3′-CMP, 2′-CMP, 5′-GMP and 5′-AMP were ineffective (Fig. 1). The presence of guanosine triphosphate led to a small

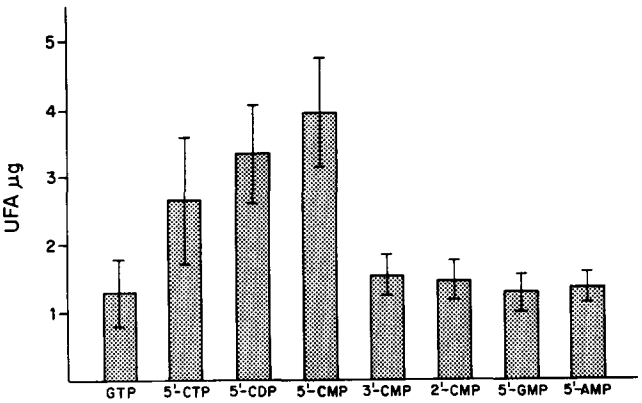


Fig. 1. Effect of nucleotides on the accumulation of unsaturated free fatty acids. Stripped rough microsomes (150 μg membrane protein) were incubated in the presence of 5 mM $MgCl_2$, 0.5 mM GTP and in the absence or in the presence of one of the following nucleotides at 0.5 mM; 5′-CTP, 5′-CDP, 5′-CMP, 3′-CMP, 2′-CMP, 5′-GMP, or 5′-AMP. Incubations were carried out for 120 min at 37°C. Lipids were then extracted and separated by thin-layer chromatography using n-hexane/diethyl ether/acetic acid (70:30:1, v/v). Quantitation of free fatty acids was carried out by densitometry as indicated in Materials and methods. Three concentrations of purified arachidonic acid were used to generate standard curves for the experiments and these were used to convert absorbance values to μg units. Bars represent mean values ±S.D. from three independent experiments carried out in triplicates.

amount of membrane fusion as evidenced by the presence of large membrane-bound vesicles (asterisks, Fig. 2A). Cytidine triphosphate had a synergistic effect and stimulated GTP-dependent membrane fusion as indicated by the presence of much larger membrane-bound vesicles (asterisk, Fig. 2B). The synergistic effect was observed with either 5′-CDP or 5′-CMP but not with the other analogues including 3′-CMP, 2′-CMP, 5′-GMP or 5′-AMP (data not shown). The cytidine nucleotides also led to the accumulation of an additional lipid which co-migrated with 1,2-diacylglycerol on TLC plates (data not shown).

The 5′-cytidine nucleotides in the presence of GTP also provoked dramatic structural changes amongst the microsomal membranes. As well as stimulating GTP-dependent membrane fusion, the cytidine nucleotides provoked the formation of tight membrane aggregates termed multilamellar membrane structures (arrow, Fig. 2 B). The analogues 3′-CMP, 2′-CMP, 5′-GMP or 5′-AMP did not lead to such changes (data not shown). The morphological and biochemical results thus demonstrated that CMP-stimulated phenomena is specific for 5′-CMP and is not reproduced by other nucleoside monophosphates or isomers of CMP. Quantitation of UFA accumulation (Fig. 1) and membrane fusion (data not shown) revealed that CMP was most active followed by CDP and then CTP. When GTP was omitted from the incubation medium, CMP (at 500 μM) or CTP (at 3 mM) stimulated accumulation of diacylglycerol and UFAs, provoked a small amount of

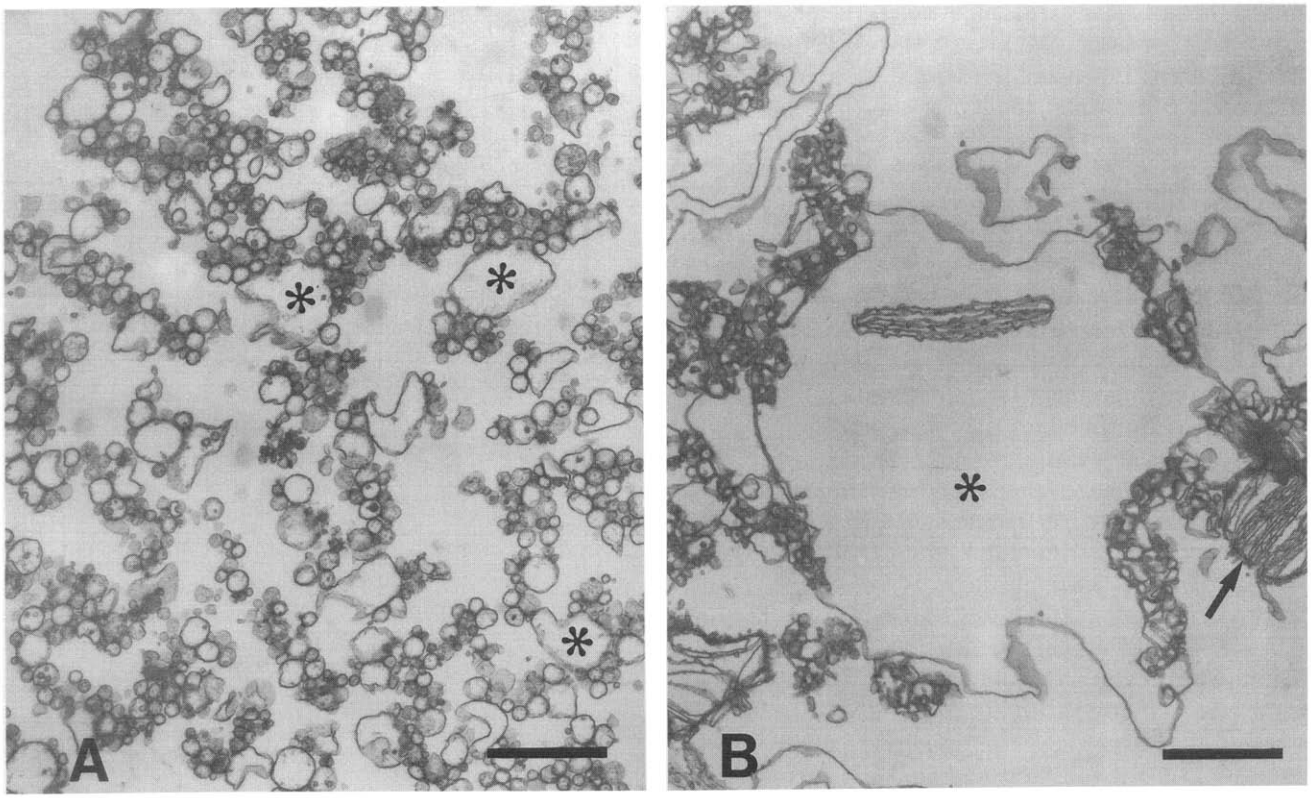


Fig. 2. Effect of nucleotides on the structure of stripped rough microsomes. Stripped rough microsomes were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 0.5 mM GTP in the absence or presence of 0.5 mM CTP. Incubation of SRM in the presence of GTP alone led to a small amount of membrane fusion and the production of fusion products (asterisks) (A). Incubation in the presence of GTP plus 5′-CTP (B) greatly stimulated membrane fusion as evidenced by the presence of very large membrane-bound fusion products (asterisk). The cytidine nucleotide also provoked the formation of multilamellar membrane structures (arrow in B). Bars, 1 μm.

fusion but did not induce the formation of multilamellar membrane structures (data not shown).

We next examined the effects of different concentrations of 5′-CMP on diacylglycerol and free fatty acid accumulation as well as on GTP-dependent membrane fusion. Lipid analysis was carried out by gas chromatography and membrane fusion was quantitated by morphometry. Incubation of membranes in the presence of GTP alone led to the accumulation of five major free fatty acid constituents, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and arachidonic acid (20:4) (Fig. 3A). Addition of CMP at increasing concentrations stimulated significantly the accumulation of both linoleic and arachidonic acid (2–3 fold), stimulated to a lesser degree the accumulation of palmitic and stearic acid ($\approx$ 1.5 fold) and stimulated only slightly the accumulation of oleic acid (Fig. 3 A). The fatty acid profile of the diacylglycerol was the same as that for the free fatty acids generated in the presence of CMP with the exception that stearic acid was the major saturated fatty acid (Fig. 3B). Increasing concentrations of CMP resulted in the selective accumulation of polyunsaturated fatty acid-enriched diacylglycerol (Fig. 3B). Cytidine monophosphate stimulated GTP-dependent membrane fusion $\approx$ 2 fold (Fig. 3C). Stimulation of accumulation of free fatty acids, diacylglycerol and membrane fusion were maximal at concentrations of CMP of about 500 μM (Fig. 3). A dose dependent increase in the accumulation of free fatty acids and membrane fusion was observed. Of all the fatty acids detected the accumulation of linoleic and arachidonic acid gave the highest correlations with membrane fusion ($r = 0.998$ and $r = 0.997$, respectively). The correlation coefficients were significant with P values less than 0.01. There was also a significant positive correlation between the accumulation of diacylglycerol and free fatty acid, consistent with a precursor-product relationship for these two lipid classes in our system.

We next determined the effects of reducing the amount of free fatty acids on GTP-dependent membrane fusion. To do this we employed incubation conditions which caused the inhibition in accumulation of free fatty acids. Inhibition was achieved by two separate methods. Firstly, delipidated bovine serum albumin (BSA) was added to sequester accumulated free fatty acids. Secondly, ATP and coenzyme A (CoA) were added to promote acyl-CoA formation and trans-

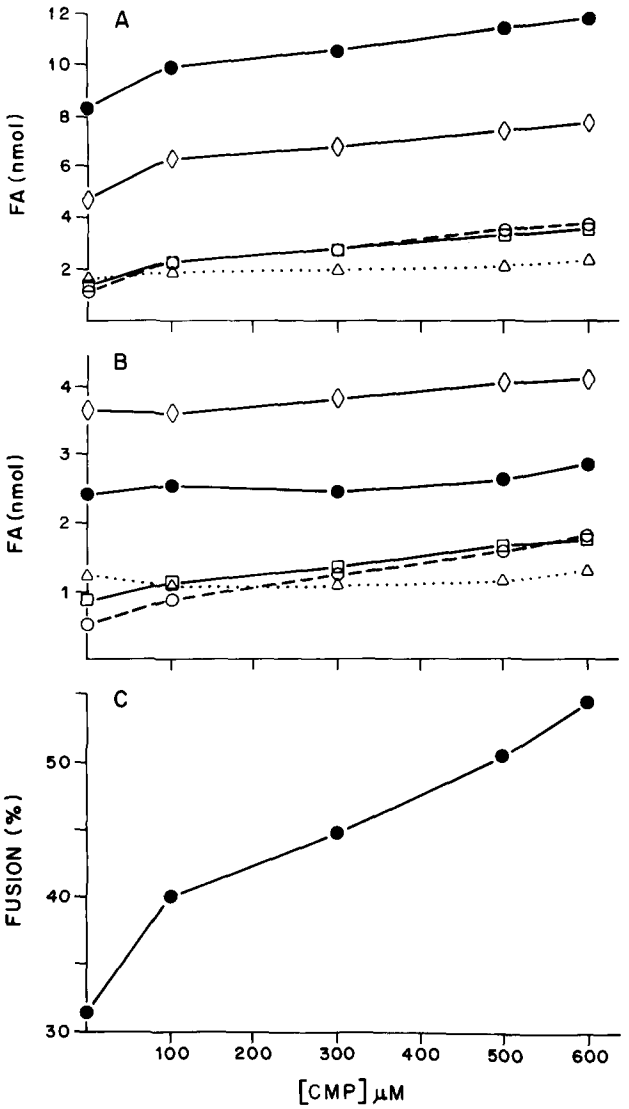


Fig. 3. Comparison of free fatty acid composition, diacylglycerol content and GTP-dependent membrane fusion as a function of CMP concentration. Stripped rough microsomes were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM GTP and increasing concentrations of CMP at 37°C for 120 min. Lipids were extracted and analyzed by gas chromatography as indicated in the Materials and methods. Duplicate samples were fixed and processed for morphometric analysis of membrane fusion as previously described [37]. Amounts of free fatty acids (A) are expressed as nanomoles, amounts of fatty acids in diacylglycerol (B) are expressed as nanoequivalents and amounts of fusion (C) as percent values. ●——●, palmitic acid; ◇——◇, stearic acid; △ ··· △, oleic acid; □ —— □, linoleic acid; ○——○, arachidonic acid. The biochemical results shown are representative of three independent experiments. The morphometric results are representative of six separate experiments.

fer of fatty acids to triacylglycerol lipid. In the first case stripped rough microsomes were incubated in the presence of 500 μM GTP, 500 μM CMP and in the absence or presence of 10 mg/ml delipidated BSA. Following incubation for different time periods the microsomes were centrifuged to produce a pellet containing membrane and a supernatant with or without BSA. The free fatty acids accumulated in the two fractions during incubation in the presence of BSA were compared to those accumulated in the membrane pellet of microsomes incubated in the absence of BSA. In the absence of BSA, accumulation of total free fatty acids in the microsomes occured in a time dependent manner (Fig. 4A). Arachidonic acid was the most rapidly accumulated free fatty acid (data not shown). Incubations in the presence of BSA dramatically reduced accumulation of free fatty acids in the microso-

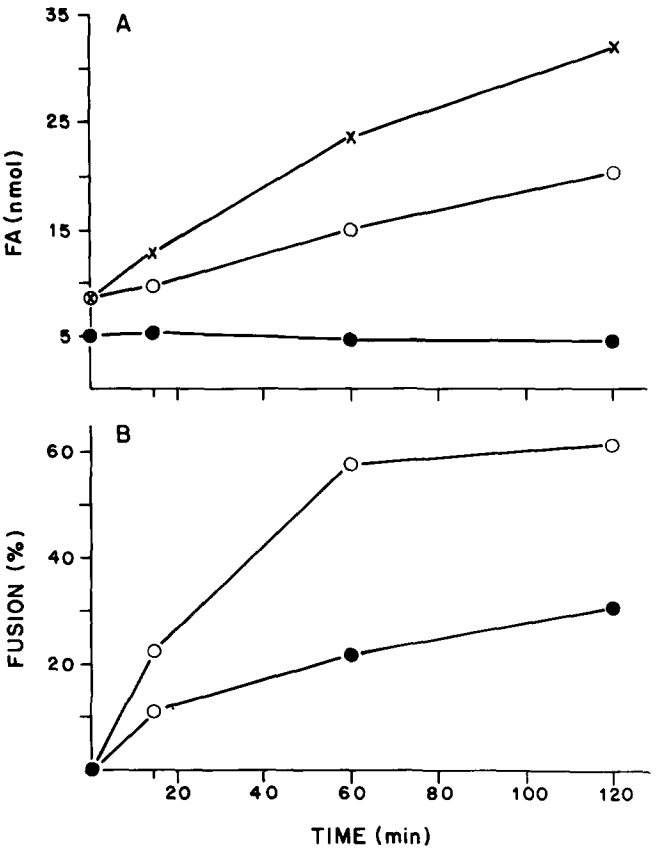
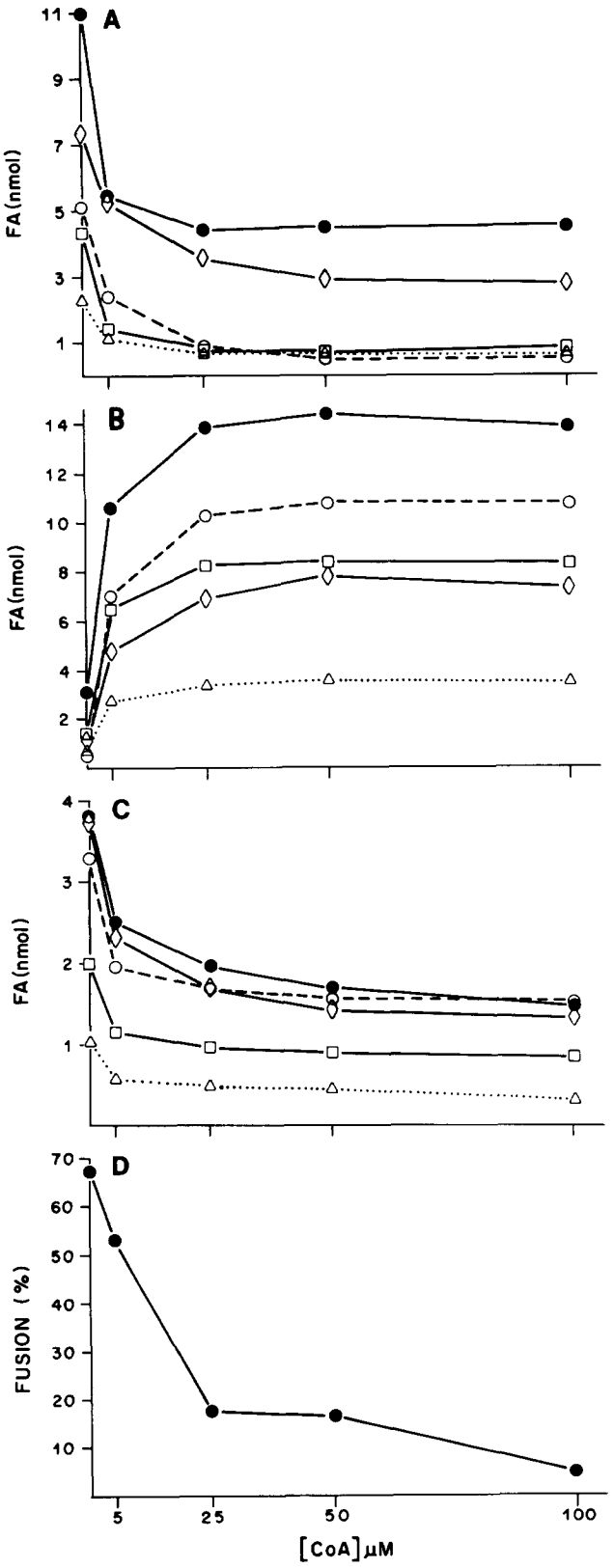


Fig. 4. Effect of delipidated BSA on the accumulation of free fatty acids and on GTP-dependent membrane fusion. Stripped rough microsomes (150 μg membrane protein) were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM GTP, and 0.5 mM CMP in the absence or presence of 10 mg/ml delipidated BSA. Following incubation for different time periods the microsomes were centrifuged (10000 rpm, 30 min at 4°C in a Sorvall RC-2 centrifuge using a Sorvall SS-34 rotor, Dupont, Willmington, DE, USA) to produce a pellet containing membrane and a supernatant with or without BSA. Gas chromatography (see Material and methods) was employed to compare total free fatty acids accumulated in the membrane pellet in the absence of BSA (○——○, A) to those accumulated in the two fractions after incubation in the presence of BSA (●——●, membrane pellet and ×——×, supernatant in A). Additional samples were fixed and processed for morphometric analysis of membrane fusion (○——○, fusion in the absence of BSA, ●——●, fusion in the presence of BSA in B) as in the legend to Fig. 3. Amounts of free fatty acids are expressed in nanomoles and amounts of membrane fusion as percent values. The results shown are representative of three separate experiments.



mal membranes (Fig. 4A) and most of the fatty acids were recovered in the supernatant fraction after incubation, presumably complexed to BSA (Fig. 4A). Analysis of membrane fusion by morphometric quantitation revealed a greater than 50% inhibition of membrane fusion in the presence of BSA (Fig. 4B). In the absence of BSA, the accumulation of linoleic and arachidonic acid had the highest correlation with membrane fusion ($r = 0.921$ and $r = 0.937$, respectively).

Stripped rough microsomes were also incubated in the presence of 500 μM GTP, 500 μM CMP, 2 mM ATP and varying concentrations of CoA. Increasing concentrations of CoA led to the appearance of a new lipid which co-migrated with a triacylglycerol lipid standard as indicated after separation and analysis by thin-layer chromatography (data not shown). The amounts of free fatty acid, diacylglycerol and triacylglycerol lipids were quantitated by gas chromatography. An inverse correlation was observed between decreasing amounts of free fatty acids and increasing amounts of triacylglycerols (Fig. 5A and B). Fatty acid analysis of accumulated triacylglycerols revealed an unusual enrichment in arachidonic acid (Fig. 5B). Diacylglycerol also decreased as a function of the increasing CoA concentrations but at a less rapid rate (Fig. 5C). Analysis of membrane fusion by morphometric measurement revealed a steady decrease in amount of fusion (Fig. 5D).

The effect of CoA and ATP on membrane structure was also examined. Incubation of SRM with 500 μM GTP, 500 μM CTP and 2 mM ATP led to extensive membrane fusion and the formation of large membrane-bound fusion products (Fig. 6A). Incubation in the same medium minus ATP yielded similar results (data not shown). Incubation of SRM in the presence of 500 μM GTP, 500 μM CTP, 2 mM ATP and 1 mM CoA led to dramatic changes in membrane structure (Fig. 6 B). As judged by the size of the membrane-

Fig. 5. Effect of ATP and increasing concentrations of coenzyme A, (CoA), on accumulation of free fatty acids, on triacylglycerol and diacylglycerol fatty acid compositions and on GTP-dependent membrane fusion. Stripped rough microsomes (150 μg membrane protein) were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM GTP, 0.5 mM CMP, 2 mM ATP and varying concentrations of CoA at 37°C for 120 min. Lipids were extracted and analyzed by gas chromatography as described in Materials and methods. Equivalent samples were fixed and processed for morphometric analysis of membrane fusion indicated in Materials and methods. Amounts of free fatty acids (A) are expressed as nanomoles, amounts of fatty acids in triacylglycerol and diacylglycerol (B and C) are expressed as nanoequivalents and amounts of fusion (D) as percent values. ●———●, palmitic acid; ◇———◇, stearic acid; △ ··· △, oleic acid; □ ——— □, linoleic acid; ○– – –○, arachidonic acid. The biochemical results shown are representative of three separate experiments. The morphometric results are representative of nine separate experiments.

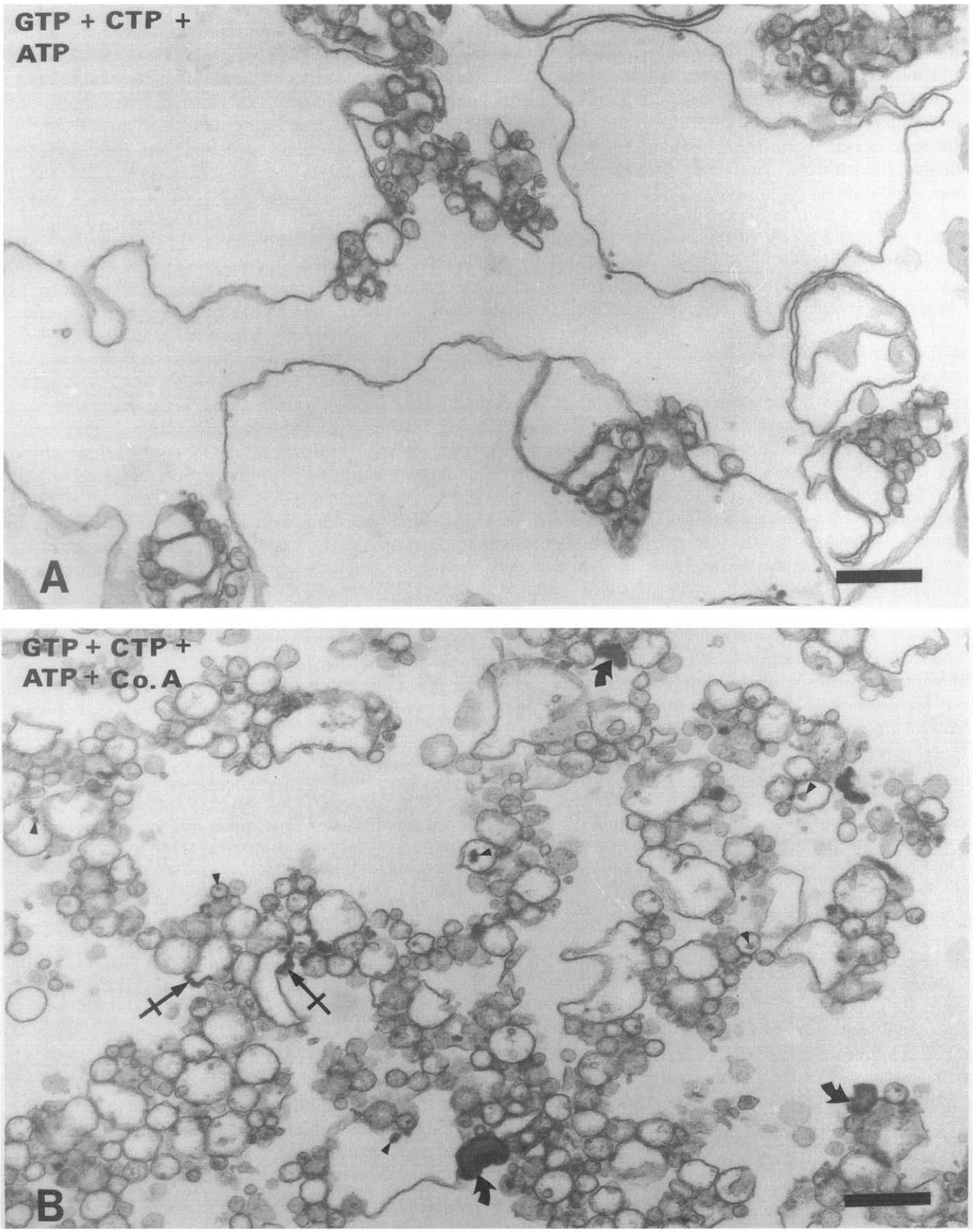
bound vesicles membrane fusion was significantly inhibited under the above stated conditions (Fig. 6B). The same incubation conditions which caused an inhibition in membrane fusion also led to the appearance of electron-dense structures both within and without the boundaries of the vesicles (arrows and arrowheads, Fig. 6B). The electron-dense structures had an amorphous content and varied both in size and location. They were predominantly small (20–80 nm) and round within the vesicles (arrowheads, Fig. 6B) and varied in shape outside the vesicles. The extra-vesicular electron-dense structures were always closely associated with the periphery of both fused and unfused vesicles. They often formed small caps at the cytosolic side of the vesicles (crossed arrows, Fig. 6B) or were large (0.1–1 μm in diameter) and round or reniform (curved arrows, Fig. 6B). Similar results were obtained when either CDP or CMP were substituted for CTP (data not shown). When GTP was omitted from the incubation medium membrane fusion did not occur and extra-vesicular electron-dense structures were much smaller (< 0.2 μm, data not shown).

Since the formation of electron-dense structures in the presence of ATP and CoA occured coincident with the accumulation of triacylglycerols it was suggested that these might be intra- and extra-vesicular stores for accumulated triacylglycerols. To test this possibility microsomal lipids were stained with OsO_4. Stripped rough microsomes were incubated in the presence of GTP, CMP, ATP and CoA to generate electron-dense structures. They were then treated with OsO_4 to stain lipids, and embedded in Epon. Thin sections were analyzed without further staining with heavy metals, and these revealed dense osmiophilia associated with morphological structures resembling, in form and location, the electron-dense, amorphous structures described above (Fig. 7). Osmiophilia was also observed in association with small round particles within vesicles (not shown). The lipidic nature of the electron dense structures was also examined by lipid solvent extraction of fixed microsomes. Stripped rough microsomes were incubated in the presence of GTP, CMP, ATP and CoA to permit formation of electron-dense structures. They were then filtered onto Millipore membranes (Millipore, Bedford, MA, USA) and treated by fixation in 1.5% glutaraldehyde alone or by double fixation in 1.5% glutaraldehyde plus 1% OsO_4. Following fixation the microsomes were extracted with chloroform/methanol (2:1) and then processed for analysis by electron microscopy. Lipid solvent extraction caused disappearance of electron-dense structures after fixation with glutaraldehyde but not after double fixation including OsO_4 (data not shown). Thus the electron dense structures generated in the presence of ATP and CoA probably represent lipid droplets formed coincident with synthesis of triacylglycerols.

Cytidine nucleotides provoked the accumulation of both DAG and UFAs and this occured coincident with the formation of multilamellar membrane structures (Fig. 2B). It was thus suggested that both DAG and UFAs may be associated with the multilamellar membrane structures. If so it was reasoned that the addition of varying concentrations of CoA in the presence of conditions which promote the formation of multilamellar membrane structures should lead to the appearance of lipid droplets in association with these same structures. Microsomes were therefore incubated using conditions which promoted the formation of multilamellar membrane structures (Fig. 8A–C, 0 μM CoA). Addition of ATP and increasing concentrations of CoA (1–5 μM) led to the appearance of elements which exhibited characteristics intermediate between that of multilamellar membrane structures and that of electron-dense, amorphous structures (Fig. 8A–C, 1 and 2 μM CoA). Only lipidic droplets were observed after incubation in the presence of 5 μM CoA (Fig. 8A–C, 5 μM CoA). Electron-dense, amorphous structures (lipid droplets) were enumerated for six separate experiments and expressed per 500 μm of membrane. The number of electron dense structures increased with increasing concentrations of CoA (Table 1). At 5 μM CoA there were no more multilamellar membrane structures (Figs. 8A–C, 5 μM CoA) and the number of electron-dense, amorphous structures was maximal (Table 1). The increase in number of electron-dense, amorphous structures occured coincident with decreasing accumulations of DAG and UFAs and increasing accumulations of triacylglycerols (Fig. 5).

The back reaction of cholinephosphotransferase [44,45] as well as that of phosphatidylinositol synthase [46] were shown to be stimulated in the presence of cytidine nucleotide and to lead to the accumulation of diacylglycerol. We have examined for evidence of a similar back reaction using SRM. Microsomes were incubated under fusion conditions in the presence of [2-^{14}C]cytidine-5′-monophosphate at 37°C for 120 min. Lipids were then extracted and separated by thin-layer chromatography. Radioactivity was associated with a single spot that co-migrated with purified CDP-diacylglycerol (Fig. 9A and B). The aqueous extract of this experiment was also analyzed by thin-layer chromatography. A single radioactive spot was observed to co-migrate with CDP-choline (data not shown).

Since diacylglycerol and monoacylglycerol lipase activity were shown associated with rat liver microsomes [47] we looked for similar enzyme activity in SRM in order to try and explain the accumulation of free fatty acids during GTP-stimulated membrane fusion. Stripped rough microsomes were incubated in the presence of exogenously added 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol at 37°C for 120 min. Lipids were then extracted and separated by thin-layer chromatography.


GTP + CTP +
ATP
A
GTP + CTP +
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B

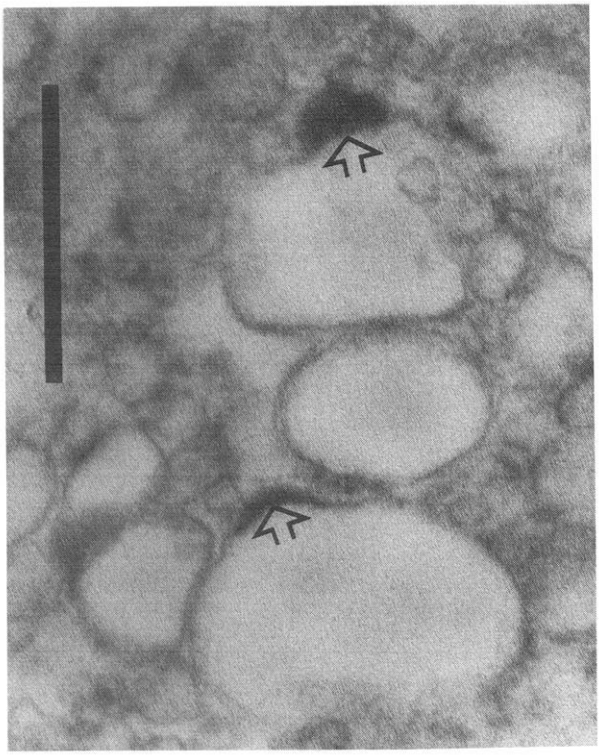

Fig. 7. Osmiophilia of electron-dense, amorphous structures formed in the presence of ATP and CoA. Stripped rough microsomes were incubated in buffer containing 0.5 mM GTP, 5 mM $MgCl_2$, 0.5 mM CMP, 2 mM ATP and 1 mM CoA, 120 min at 37°C. The microsomes were then filtered onto Millipore membranes (Millipore, Bedford, MA, USA) and treated with a 1% OsO_4 solution on ice for 60 min. They were then processed for electron microscopy and analysis of thin sections was done without further staining. Arrows point to large osmiophilic structures located at the periphery of large membrane-bound fusion products. Bar, 0.5 μm.

Radioactivity was detected in association with lipid products that co-migrated with diacylglycerol and free fatty acids (Fig. 9C and D).

4. Discussion

4.1. Polyunsaturated free fatty acids are required for GTP-dependent membrane fusion

We have modulated the content of unsaturated free fatty acids in SRM and observed coincident changes in GTP-dependent membrane fusion. Our data confirms the previous demonstration of a close correlation between unsaturated free fatty acid accumulation and GTP-dependent membrane fusion [35] and further identifies endogenous linoleic acid and arachidonic acid as the most likely co-effectors of membrane fusion. Together with the previous demonstration that exogenously added polyunsaturated free fatty acids can stimulate fusion of natural cellular membranes [36,48–50] these results support the suggestion that polyunsaturated free fatty acids are important for membrane fusion.

GTP does not stimulate polyunsaturated free fatty acid accumulation in endoplasmic reticulum membranes [35] but it does require small amounts of these endogenous lipid constituents to promote membrane fusion (this paper). Physiological concentrations of CMP on the other hand can stimulate accumulation of diacylglycerol, CDP-diacylglycerol as well as polyunsaturated free fatty acids but not membrane fusion. The combination of GTP and elevated amounts of polyunsaturated free fatty acids greatly enhances membrane fusion. We suggest that GTP may regulate the interaction of components required for the proper juxtaposition of adjacent membranes while polyunsaturated free fatty acids, by virtue of their physical properties, may work subsequent to membrane docking permitting membrane disruption and coalescence (Fig. 10).

4.2. Fatty acylation, diacylglycerol and lysophospholipids are not involved in the fusion of RER membranes

Fatty acylation was previously shown to promote fusion of transport vesicles with Golgi cisternae [51] but is not important in our fusion model. This is because fusion occurs in the absence of ATP and CoA which are required for the generation of acyl-CoA necessary for fatty acylation. Rather than promoting GTP-dependent fusion of RER membranes the presence of ATP and CoA actually inhibited this process (Figs. 5D and 6B, see also Ref. 52). This may suggest that our fusion system, which is a homotypic fusion system, is independent of NSF the (N)EM-(S)ensitive (F)usion protein and does not require fatty acylation.

Although diacylglycerol has been implicated in membrane fusion [53] this lipid is thought to be less important for fusion of rough microsomal membranes

Fig. 6. Effect of ATP and CoA on the structure of stripped rough microsomes. Stripped rough microsomes were incubated in buffer containing 0.5 mM GTP, 5 mM $MgCl_2$, 0.5 mM CTP, 2 mM ATP in the absence (A) or presence (B) of 1 mM CoA for 120 min at 37°C. Incubation in the absence of CoA permitted extensive fusion and the production of large membrane-bound fusion products (A). Incubation in the same medium plus CoA inhibited membrane fusion as indicated by the absence of very large membrane-bound fusion products and led to the appearance of electron-dense, amorphous structures both inside and outside the microsomal vesicles. Arrowheads indicate small round electron-dense structures within the vesicles. Crossed arrows point to electron-dense caps and curved arrows to large electron-dense masses at the periphery of vesicles. Bars, 0.5 μm.

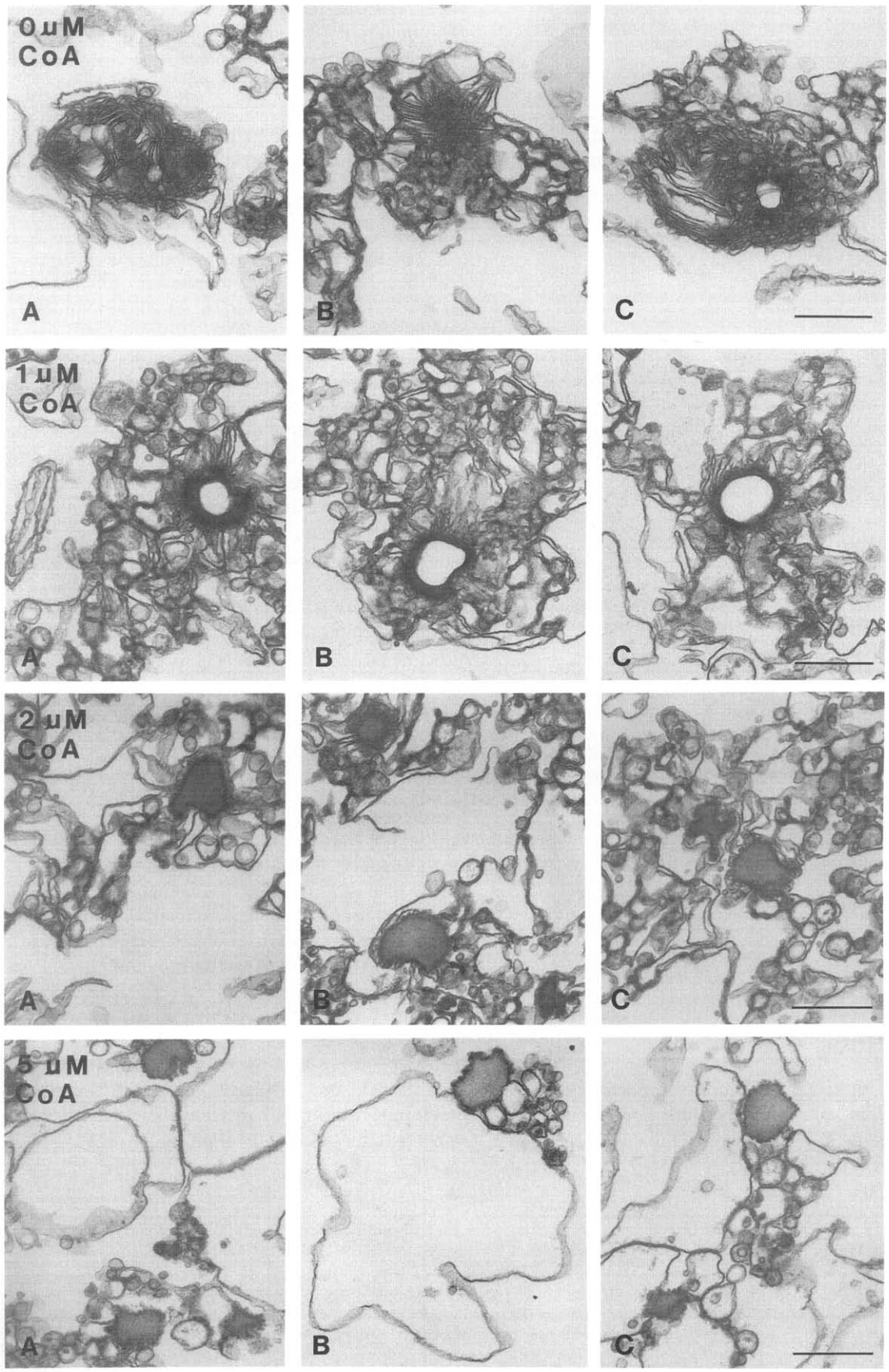
0 µM CoA
A
B
C
1 µM CoA
A
B
C
2 µM CoA
A
B
C
5 µM CoA
A
B
C

Table 1
Effect of coenzyme A on the formation of lipid droplets. Lipid droplets (electron-dense, amorphous structures) were identified after incubation of stripped rough microsomes in the presence of coenzyme A. Stripped rough microsomes were incubated in the presence of GTP (500 μM), CMP (500 μM) and increasing concentrations of CoA for 120 min. Membranes were then fixed and processed for electron microscopy and membranes were measured by morphometry as described in Materials and methods. Lipid droplets were counted and their sums expressed per 500 μm of incubated membranes. Data are derived from six separate experiments.

Coenzyme A (μM)	Total membrane (μm)	Lipid droplets per 500 μm membrane
1	868.0 ± 179.8	7.7 ± 2.5
2	708.4 ± 149.4	10.4 ± 4.8
3	653.4 ± 106.2	17.4 ± 2.7
5	528.8 ± 65.5	19.9 ± 2.1

for the following reasons: (1) fusion can occur without significant accumulation of diacylglycerol (e.g., in the presence of GTP and Mg^{2+}, and absence of CMP); and (2) fusion can be inhibited by the presence of delipidated bovine serum albumin which presumably affects only free fatty acid pools and not diacylglycerol.

We have also looked for lysophospholipids in our system since these constituents have previously been implicated in membrane fusion [54]. Addition of exogenous radiolabeled-phosphatidylcholine (*L*-3 phosphatidylcholine, 1,2-di[1-^{14}C]palmitoyl) to RER membranes incubated under fusion conditions did reveal a small amount, < 5% of the metabolic label associated with lysophospholipid compared to > 95% associated with diacylglycerol and free fatty acid (Lavoie, C. and Paiement, J., unpublished observations). Addition of exogenous lysophospholipids (lysophosphatidylcholine or lysophosphatidylinositol, at concentrations varying between 50 and 200 μM) to RER membranes incubated in the presence of GTP led to complete inhibition of fusion (Lavoie, C. and Paiement, J., unpublished observations). Our results confirm those of Chernomodik et al. [55] and suggest that it is highly unlikely that lysophospholipids are involved in GTP-dependent fusion of RER membranes.

4.3. The mechanism of action of CMP and possible physiologic role of unsaturated fatty acids

Our studies on the metabolism of radioactive lipid precursors by SRM have shown that membranes produce labeled CDP-diacylglycerol and labeled CDP-choline from [2-^{14}C]cytidine-5′-monophosphate, and unesterified [^{14}C]arachidonic acid from 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol. Our data is consistent with the possibility that CMP promotes the back reac-

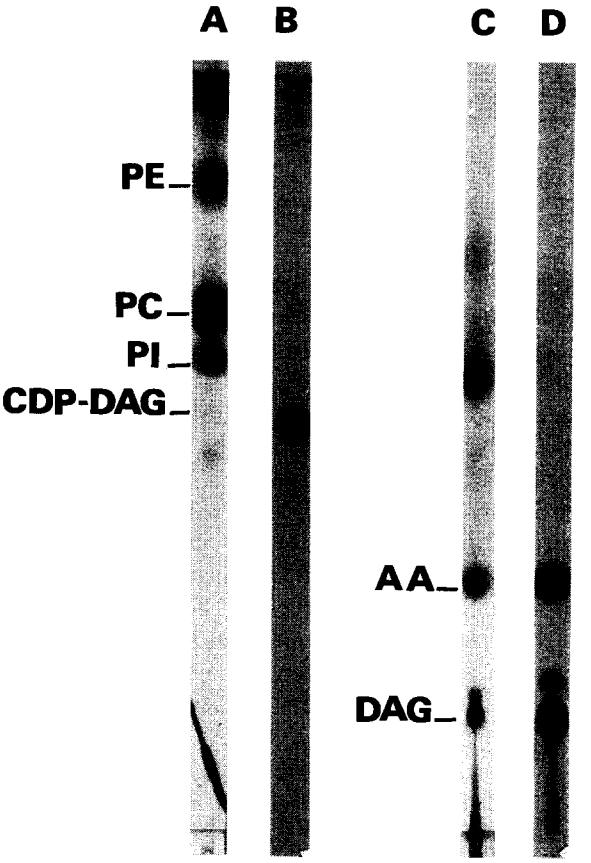

Fig. 9. Potential substrates involved in the metabolic pathway yielding diacylglycerol and free fatty acids. In A and B stripped rough microsomes were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM GTP, 0.5 mM CMP and 1.324 nM [2-^{14}C]cytidine-5′-monophosphate (spec. act. 60.4 mCi/mmol) at 37°C for 120 min. Lipids were then extracted and separated by thin-layer chromatography using chloroform/methanol/water/acetic acid (50:28:8:4, v/v) stained with 10% phosphomolybdic acid (A) or visualized by radioautography (B). In C and D, stripped rough microsomes were incubated in buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM GTP and 22.7 μg 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol (spec. act. 55.6 mCi/mmol) at 37°C for 120 min. Lipids were then extracted and separated by thin-layer chromatography using hexane/diethyl ether/acetic acid (70:30:1, v/v) stained with 10% phosphomolybdic acid (C) or visualized by radioautography (D). The positions of migration of lipid standards are shown to the left of the results showing stained lipids. AA, arachidonic acid; CDP-DAG, CDP-diacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Results shown are representative of three independent experiments.

Fig. 8. Effect of increasing concentrations of CoA on the structure of stripped rough microsomes. Stripped rough microsomes were incubated in the presence of 0.5 mM GTP, 5 mM $MgCl_2$, 0.5 mM CMP, 2 mM ATP and increasing concentrations (0–5 μM) of CoA for 120 min at 37°C. Microsomes were then fixed and processed for electron microscopic analysis as described in Materials and methods. Three representative examples of the membranes incubated at each CoA concentration are shown (A–C). Bars, 0.5 μM.

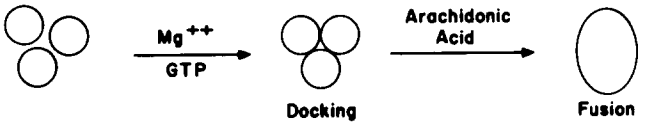

Fig. 10. Model for the action of GTP and arachidonic acid in the fusion of rough endoplasmic reticulum membranes.

tion of both cholinephosphotransferase [44,45] and phosphatidylinositol synthase [46] leading to the accumulation of diacylglycerol and CDP-diacylglycerol, respectively. Since liver microsomes are known to contain an active CDP-diacylglycerol hydrolase [56] we believe that CDP-diacylglycerol from the back reaction of phosphatidylinositol synthase will further be hydrolyzed by this enzyme resulting in the release of diacylglycerol. Both diacylglycerol and monoacylglycerol lipase activities are involved in the release of free fatty acids from diacylglycerol (Fig. 9). This latter data agrees with the previous demonstration of both these enzymes in liver microsomes [47]. Since diacylglycerol does not accumulate to a significant extent in the absence of CMP but free fatty acids do (Figs. 1 and 3) we cannot exclude the possibility that a phospholipase A-type reaction also occurs in our membranes. In either case accumulation of free fatty acids, in particular linoleic and arachidonic acid, occurs most efficiently and has dramatic effects on GTP-dependent membrane fusion.

Membrane fusion is important for membrane traffic in interphase cells as well as for organelle reconstitution in dividing cells [17]. Accumulation of unsaturated fatty acids, particularly arachidonic acid, was demonstrated during stimulus-secretion response [57–65]. Whether such lipids are required for GTP-stimulated phenomena in vivo is worthy of consideration.

4.4. Polyunsaturated free fatty acids and divers GTP-dependent functions of membranes

GTP stimulates CDP-DG formation [32,33], enhances core glycosylation [30], induces membrane permeability changes [26,27], promotes protein synthesis [29] as well as calcium release [21,28]. It also stimulates the formation of multilamellar membrane structures and large lipidic droplets from stripped rough microsomes (this paper). The one thread that could possibly link several of these apparently disparate functions may be polyunsaturated free fatty acids. Indeed polyunsaturated free fatty acids, particularly arachidonic acid, have been implicated in membrane permeability changes [54,66], were found to promote calcium release [67,68] were observed to stimulate membrane fusion [36,49,50] and were shown to be associated with cell secretion [57–65]. GTP may actively displace some constituents permitting membrane destabilization by polyunsaturated free fatty acids and thereby activating a variety of physical and chemical changes in membranes which could influence diverse metabolic events.

4.5. Membrane transformations coincident with changes in lipid stores

In the presence of GTP the cytidine nucleotides (5′-CTP, 5′-CDP or 5′-CMP) provoked the formation of complex membrane aggregates termed multilamellar membrane structures (Fig. 2B and Figs. 8A–C, 0 μM CoA). This occurred in the presence of concentrations ($\approx$ 500 μM) known to be physiological for these nucleotides [69,70]. The presence of both nucleotides, GTP plus one of the cytidine nucleotides was required to produce multilamellar membrane structures. Although formed using physiological conditions of incubation, such structures are not normal since they do not exist in hepatocytes. Additional factors in the hepatocyte cytoplasm may limit their formation. For example, the presence of attached ribosomes could influence such a process. Consistent with this suggestion is the observation that rough microsomes did not permit formation of multilamellar membrane structures (Paiement, J., unpublished observations).

Incubation of SRM in the presence of GTP, CMP, ATP plus increasing concentrations of CoA led to the accumulation of increasing amounts of triacylglycerols and decreasing amounts of UFAs. The membranes provided the source of enzymes and substrates. We have therefore avoided the use of labeled exogenous phospholipids and analyzed by quantitation all the accumulated UFAs and triacylglycerols. This permitted the preservation of the substrate-enzyme relationship in the natural membrane environment and also allowed analysis of membrane structure by electron microscopy. Electron microscopy indicated the production of electron-dense, amorphous structures coincident with triacylglycerol synthesis. The electron-dense structures were shown to be extractable by lipid solvents in the absence of fixation with OsO_4 and to be highly osmiophilic. These properties are identical to those previously described for hepatic intracellular osmiophilic droplets [71]. Based on these observations we hypothesize that the electron-dense, amorphous structures represent lipid pools enriched in triacylglycerols and that the majority of triacylglycerols accumulate in association with the cytosolic surface of the incubated membranes, as would be predicted knowing that CDP-diacylglycerol acyltransferase is an enzyme active at the cytosolic surface of the endoplasmic reticulum membrane [72]. The morphological studies on the effects of CoA revealed an apparent transformation of the multilamellar membrane structures into lipid droplets. Quantitation of the number of structures at different concentrations of CoA revealed an increase in number of lipid structures as expressed per unit membrane at

the higher concentrations of CoA. These results are thought to reflect a reduction in actual membrane mass due to the transfer of membrane lipid (diacylglycerol and fatty acids) to the droplets containing newly synthesized lipid and situated outside the vesicles. Triacylglycerols can also accumulate within the ER lumen as a complex with lipoproteins, phosphatidylcholine and cholesterol [73,74]. Whether intralumenal triacylglycerol accumulation occurred in our model membrane system and what might be the physical state of such triacylglycerols remains to be determined.

Our results with ATP and CoA raise questions as to whether the CMP effect would not lead in vivo to triacylglycerol formation rather than fusion. This is certainly possible and whether the reaction goes in the opposite direction to favor fusion will probably be determined by the availability of required cofactors and enzymes within specific microenvironments of the membrane which in turn may be affected by the physiological state of the cell at the time of accumulation of fusogen.

4.6. Signal transduction and the endoplasmic reticulum

A noteworthy observation was the accumulation of diacylglycerol coincident with accumulation of free fatty acids (Fig. 3). Studies have shown that the accumulation of diacylglycerol in rat hepatocytes is stimulated by vasopressin [75,76], epinephrine, angiotensin II, and ATP [76]. Composition analysis of diacylglycerol fractions revealed two possible sources, one enriched in stearic and arachidonic acid, suggesting derivation mainly from phosphoinositides and another fraction enriched in palmitic and oleic acids suggesting a derivative of phosphatidylcholine [76]. Our results considered in the context of the literature cited above prompt the question of whether a discrete ER polyphosphoinositol signalling system may exist which is distinct from the well-known plasma membrane-located system. Evidence for such a system has previously been shown in association with nuclei [77]. Although we have not studied extensively the formation of diacylglycerol and fatty acids from phosphatidylcholine, the back reaction of cholinephosphotransferase has previously been implicated in response to a cellular stimulus [78] and has been demonstrated using microsomes in vitro [44,45]. The extent to which phospholipid metabolism (formation of diacylglycerol and arachidonic acid) in the endoplasmic reticulum may play a role in signal transduction during cell activation merits further analysis.

5. Acknowledgements

We thank Dr. John Silvius (Biochemistry, McGill University) for relevant comments on the manuscript, Dr. E. Vance (Biochemistry, University of Alberta) for relevant discussion on lipid metabolism and Drs. Philippe Crine and Michel Bouvier (Biochemistry, Université de Montréal) for use of the LKB Ultra Scan XL laser densitometer. We also thank Line Roy, Anne Guénette and Sylvie Cordeau for expert technical assistance and Sylvie Magnan for typing the manuscript. Supported by the Medical Research Council of Canada (MRCC) and the Cancer Research Society Inc. C.L. was recipient of studentships from the MRCC and the FCAR (attributed via the Groupe de recherche en transport membranaire, Université de Montréal).

6. References

[1] Porter, K.R. (1961) In The Cell II (Brachet, J. and Mirsky, A.E., eds), pp. 621–675, Academic Press, New York.
[2] Farquhar, M.G. and Rinehart, J.F. (1954) Endocrinology 54, 516–541.
[3] Ishikawa, A. (1965) J. Cell Biol. 24, 369–385.
[4] Higgins, J.A. (1974) J. Cell Biol. 62, 635–646.
[5] Jingami, H., Brown, M.S., Goldstein, J.L., Anderson, R.G.W. and Luskey, K.L. (1987) J. Cell Biol. 104, 1693–1704.
[6] Cardell, R.R., Michaels, J.E., Hung, J.T. and Cardell, E.L. (1985) J. Cell Biol. 101, 201–206.
[7] Pacifici, M. and Iozzo, R.V. (1988) J. Biol. Chem. 263, 2483–2492.
[8] Booth, C. and Koch, G.L.E. (1989) Cell 59, 729–737.
[9] Terasaki, M., Chen, L.B. and Fujiwara, K. (1986) J. Cell Biol. 103, 1557–1568.
[10] Lee, C. and Chen, L.B. (1988) Cell 54, 37–46.
[11] Kachar, B. and Reese, T.S. (1988) J. Cell Biol. 106, 1545–1552.
[12] Houliston, E. and Elinson, R.P. (1991) J. Cell Biol. 114, 1017–1028.
[13] Schmitt, H.D., Puzicha, M. and Gallwitz, D. (1988) Cell 53, 635–647.
[14] Porter, K.R. and Machado, R.D. (1960) J. Biophys. Biochem. Cytol. 7, 167–180.
[15] Franke, W.W. (1977) Biochem. Soc. Symp. 42, 125–135.
[16] Zeligs, J.D. and Wollman, S.H. (1979) J. Ultrastruct. Res. 66, 53–77.
[17] Warren, G. (1993) Annu. Rev. Biochem. 62, 323–348.
[18] Dabora, S.L. and Sheetz, M.P. (1988) Cell 54, 27–35.
[19] Vale, R.D. and Hotani, H. (1988) J. Cell Biol. 107, 2233–2241.
[20] Paiement, J., Beaufay, H. and Godelaine, D. (1980) J. Cell Biol. 86, 29–37.
[21] Dawson, A.P. (1985) FEBS Lett. 185, 147–150.
[22] Watkins, J.D., Hermanowski, A.L. and Balch, W.E. (1993) J. Biol. Chem. 268, 5182–5192.
[23] Balch, W.E. (1990) Trends Biochem. Sci. 15, 473–477.
[24] Hall, A. (1990) Science 249, 635–640.
[25] Goud, B. and McCaffrey, M. (1991) Cell Biol. 3, 626–633.
[26] Godelaine, D., Beaufay, H., Wibo, M. and Ravoet, A.-M. (1983) J. Cell Biol. 97, 340–350.
[27] Nicchitta, C.V., Joseph, S.K. and Williamson, J.R. (1987) Biochem. J. 248, 741–747.
[28] Gill, D.L., Ueda, T., Chueh, S.-H. and Noel, M.W. (1986) Nature (London) 320, 461–464.
[29] Connolly, T. and Gilmore, R. (1989) Cell 57, 599–610.
[30] Godelaine, D., Beaufay, H. and Wibo, M. (1977) Proc. Natl. Acad. Sci. USA 74, 1095–1099.
[31] Paiement, J. and Bergeron, J.J.M. (1983) J. Cell Biol. 96, 1791–1796.

[32] Liteplo, R.G. and Sribney, M. (1980) Biochim. Biophys. Acta 619, 660–668.
[33] Jolicoeur, M., Kan, F.W.K. and Paiement, J. (1991) J. Histochem. Cytochem. 39, 363–372.
[34] Lanoix, J., Roy, L. and Paiement, J. (1989) Biochem. J. 262, 497–503.
[35] Lavoie, C., Jolicoeur, M. and Paiement, J. (1991) Biochim. Biophys. Acta 1070, 274–278.
[36] Kan, F.W.K., Jolicoeur, M. and Paiement, J. (1992) Biochim. Biophys. Acta 1107, 331–341.
[37] Paiement, J., Dominguez, J.M., Guénette, A. and Roy, L. (1991) Biochem. Biophys. Res. Commun. 176, 1494–1500.
[38] Schact, J. (1981) Methods Enzymol. 72, 626–631.
[39] Smith, C.D. and Wells, W.W. (1983) J. Biol. Chem. 258, 9368–9373.
[40] Diez, E. and Mong, S. (1990) J. Biol. Chem. 265, 14654–14661.
[41] Lepage, G. and Roy, C.C. (1986) J. Lipid Res. 27, 114–120.
[42] Glass, R.L. (1971) Lipids 6, 919–925.
[43] Gavino, G.R., Levy, E. and Gavino, V.C. (1992) J. Biochem. Cell Biol. 70, 224–227.
[44] Kanoh, H. and Ohno, K. (1973) Biochim. Biophys. Acta 326, 17–25.
[45] Baker, R.R. and Chang, H.-Y. (1984) Can. J. Biochem. Cell Biol. 62, 379–384.
[46] Hokin-Neaverson, M., Sadeghian, K., Harris, D.W. and Merrin, J.S. (1977) Biochem. Biophys. Res. Commun. 78, 364–371.
[47] Xia, T. and Coleman, R.A. (1992) Biochim. Biophys. Acta 1126, 327–336.
[48] Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. 136, 147–155.
[49] Creutz, C.E. (1981) J. Cell Biol. 91, 247–256.
[50] Meers, P., Ernst, J.D., Düzgünes, N., Hong, K., Fedor, J., Goldstein, I.M. and Papahadjopoulos, D. (1987) J. Biol. Chem. 262, 7850–7858.
[51] Pfanner, N., Glick, B.S., Arden, S.R. and Rothman, J.E. (1990) J. Cell Biol. 110, 955–961.
[52] Comerford, J.G. and Dawson, A.P. (1993) Biochem. J. 289, 561–567.
[53] Siegel, D.P., Banschbach, J., Alford, D., Ellens, H., Lis, L.J., Quinn, P.J. Yeagle, P.L. and Bentz, J. (1989) Biochemistry 28, 3703–3709.
[54] Lucy, J.A. (1974) FEBS Lett. 40, S105–S111.
[55] Chernomordik, L.V., Vogel, S.S., Sokoloff, A., Onaran, H.O., Leikina, E.A. and Zimmerberg, J. (1993) FEBS Lett. 318, 71–76.
[56] Sturton, R.G. and Brindley, D.N. (1977) Biochem. J. 162, 25–32.
[57] Kennerly, D.A., Sullivan, T.J., Sylvester, P. and Parker, C.W. (1979) J. Exp. Med. 150, 1039–1044.
[58] Stenson, W.F. and Parker, C.W. (1979) J. Clin. Invest. 64, 1457–1465.
[59] Walsh, C.E., Waite, B.M., Thomas, M.J. and DeChatelet, L.R. (1981) J. Biol. Chem. 256, 7228–7234.
[60] Garcia-Gil, M. and Siraganianl, R.P. (1984) J. Immunol. 136, 3825–3828.
[61] Frye, R.A. and Holz, R.W. (1984) J. Neurochem. 43, 146–150.
[62] Roldan, E.R.S. and Harrison, R.A.P. (1989) Biochem. J. 259, 397–406.
[63] Churcher, Y., Allan, D. and Gomperts, B.D. (1990) Biochem. J. 266, 157–163.
[64] Cockcroft, S. (1991) Biochem. J. 275, 127–131.
[65] Lelkes, P.I. and Pollard, H.B. (1991) In Membrane Fusion (Wilschut, J. and Hoekstra, D., eds.), pp. 511–551 Marcel Dekker, New York, pp. 511–551.
[66] Muramushi, N., Takagi, N., Muranishi, S. and Sezaki, H. (1981) Chem. Phys. Lipids 28, 269–279.
[67] Messineo, F.C., Rathier, M., Favreau, C., Watras, J. and Takenaka, H. (1984) J. Biol. Chem. 259, 1336–1343.
[68] Chan, K.-M. and Turk, J. (1987) Biochim. Biophys. Acta 928, 186–193.
[69] Kleineke, J., Düls, C. and Söling, H.-D. (1979) FEBS Lett. 107, 198–202.
[70] Plagemann, P.G.W. (1972) J. Cell Biol. 52, 131–146.
[71] Ashworth, C.T., Leonard, J.S., Eigenbrodt, E.H. and Wrightsman, F.J. (1966) J. Cell Biol. 31, 301–316.
[72] Coleman, R. and Bell, R.M. (1978) J. Cell Biol. 76, 245–253.
[73] Vance, J.E. and Vance, D.E. (1990) Experientia 46, 560–569.
[74] Gibbons, G. (1990) Biochem. J. 268, 1–13.
[75] Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716–5725.
[76] Bocckino, S.B., Blackmore, P.F. and Exton, J.H. (1985) J. Biol. Chem. 260, 14201–14207.
[77] Divecha, N., Banfić, H. and Irvine, R.F. (1991) EMBO J. 10, 3207–3214.
[78] Cornell, R. (1989) In Phosphatidylcholine Metabolism (Vance, D.E., ed.), CRC Press Inc., Boca Raton, pp. 47–64.