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Rapid Report

Accumulation of polyunsaturated free fatty acids coincident with the fusion of rough endoplasmic reticulum membranes

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The accumulation of polyunsaturated free fatty acids (PUFAs) was observed coincident with GTP-dependent fusion of liver rough microsomes. Whereas 0.5 mM NADPH led to a parallel reduction (> 50%) in membrane fusion and PUFA accumulation, indomethacin (50 μM) either had little effect or slightly augmented both processes. CTP was observed to stimulate accumulation of PUFAs and diacylglycerol (DAG). Therefore PUFAs may be relevant for GTP-dependent membrane fusion and together with DAG may play a role in fusion stimulated in the presence of CTP.

Recently while comparing the effects of GTP on phospholipid metabolism and on fusion of rough endoplasmic reticulum (RER) membranes cell-free incubation conditions were found which promoted 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 (PI) [1]. This observation and the previous demonstration that polyunsaturated free fatty acids (PUFAs) can promote membrane fusion using natural membranes [2–4] prompted us to examine for the presence of phospholipase activity in RER membranes. This paper describes evidence for this activity and furthermore using electron microscope stereology the GTP-dependent fusion of RER membranes is compared quantitatively with that of PUFA accumulation.

Stripped rough microsomes (SRM) were prepared from rat liver homogenates as previously described [5]. The cell-free membrane fusion assay was carried out by morphometric measurement of embedded and sectioned membranes using electron microscopy [6].

Endogenous lipids from SRM were extracted by a modification of the Schact procedure [7] as outlined by Smith and Wells [8]. The organic extract was used fresh or was stored no longer than 12 h at 4°C under nitrogen gas before use.

For identification of lipids, aliquots (10 μl) of lipid extracts were spotted using a Drummond Microdispenser (Drummond Scientific Co., Broomall, PA, U.S.A.) 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 either of the following solvent mixtures, chloroform/methanol/water (140: 60: 10, v/v) [9] or *n*-hexane/diethyl ether/acetic acid (70: 30: 1, v/v) [10]. 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. Polyunsaturated 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 concentrations of the PUFA

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standard employed (arachidonic acid, 0.5 to 8 μg) (data not shown). Corrections for variations in lipid extractions were carried out using absorbance data for lipid spots which were observed not to vary under the experimental conditions employed.

We looked for the endogenous formation of PUFAs in SRM incubated in the absence or presence of medium containing 100 mM Tris-HCl (pH 7.4), 0.5 mM GTP and 2 mM $MnCl_2$. When the extracted lipids were analyzed by thin-layer chromatography and the lipids detected with 10% phosphomolybdic acid a distinct difference was observed in the amount of lipid which co-migrated with arachidonic acid (Fig. 1). More of this product was accumulated in the presence of medium (Fig. 1). Control experiments indicated that saturated free fatty acid and PUFA standards had similar chromatographic mobilities in the solvent systems used (data not shown). However, since only the PUFA standards (e.g. linoleic, linolenic and arachidonic acid) were stained with 10% phosphomolybdic

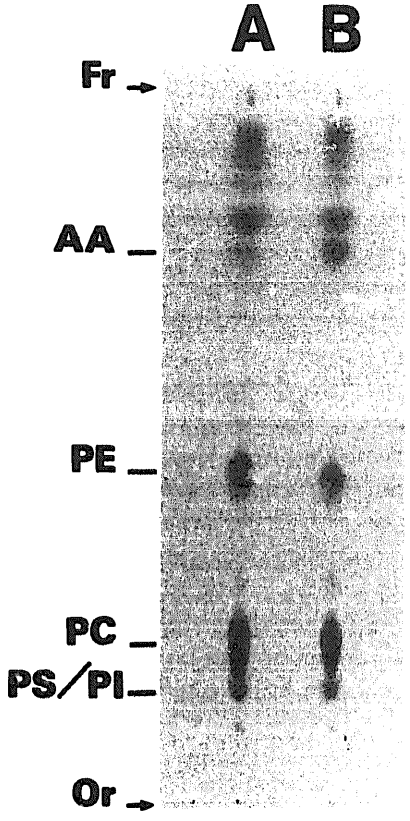

Fig. 1. Identification of lipids in lipid extracts of SRM by thin-layer chromatography. Stripped rough microsomes (200 μg of membrane protein) were incubated for 120 min at 37 °C in the absence (A) or presence (B) of medium (100 mM Tris-HCl (pH 7.4) plus 0.5 mM GTP and 2 mM $MnCl_2$). Lipids were extracted and separated by thin-layer chromatography using chloroform/methanol/water (140:60:10, v/v). The positions of migration of phospholipid and fatty acid standards are indicated to the left of the results. Lipid standards were obtained from Sigma (St-Louis, MO, U.S.A.) and Serdary (London, Canada). AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Fr, front of solvent; Or, origin.

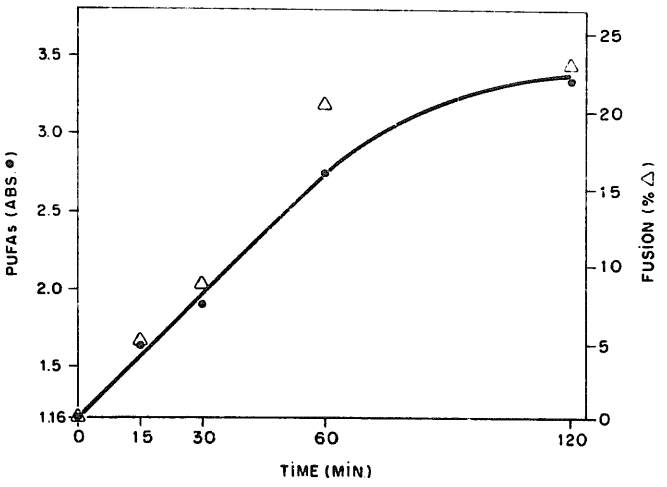

Fig. 2. Relationship of accumulation of PUFAs and membrane fusion in the presence of GTP. Stripped rough microsomes (200 μg membrane protein) were incubated in the presence of 2 mM $MnCl_2$ and 0.5 mM GTP at 37 °C for varying periods of time. Lipids were extracted and analysed as described in Fig. 1. Duplicate samples were fixed and processed for morphometric analysis of membrane fusion as previously described [6]. Amount of PUFAs is expressed in relative absorbance units (ABS, •) and amount of membrane fusion is expressed in percent values (%, △) obtained by comparing membrane lengths of fused vesicles with those of unfused vesicles [6]. The data is derived from one of three experiments which yielded similar results and calculated to a straight line with a coefficient of correlation of $r = 0.989$, with a significance of $P < 0.01$.

acid we concluded that the differences in the amount of lipid observed in SRM under different experimental conditions were primarily due to variations in amounts of PUFAs.

The kinetics of PUFA accumulation were examined using medium containing Tris-HCl (pH 7.4), 0.5 mM GTP and 2 mM $MnCl_2$. Polyunsaturated free fatty acid accumulation was linear for the first 30 min and started to plateau after 60 min (data not shown). The effect of cations was examined. In the presence of Tris-HCl (pH 7.4), 0.5 mM GTP none of the cations examined (e.g. Mg^{2+}, Mn^{2+}, Ca^{2+}) stimulated PUFA accumulation to any significant degree at concentrations varying between 0 and 10 mM (data not shown). Polyunsaturated free fatty acid accumulation occurred in the presence of 5 mM EGTA suggesting Ca^{2+} independence (data not shown).

We next wanted to examine the relation between accumulation of PUFAs and GTP-dependent membrane fusion. In three separate experiments we observed a time dependent increase in the accumulation of PUFAs and a coincident increase in amount of membrane fusion as assayed by morphometric analysis (Fig. 2). Linear regression analysis of the three results defined a high correlation between accumulation of PUFAs and increases in the amount of membrane fusion. The coefficients of correlation varied between $r = 0.912$ and $r = 0.989$ and were found to be statistically significant with P values equal to or less than

0.05 as evaluated by the Student's *t*-test. Since PUFA accumulation and membrane fusion exhibit similar kinetic properties we suggest that GTP-dependent membrane fusion may be facilitated by PUFA accumulation.

Because PUFAs are major substrates for lipid peroxidation in endoplasmic reticulum membranes [11] we next determined the effect of the presence of NADPH, a cofactor in lipid peroxidation, on accumulation of PUFAs and on GTP-dependent membrane fusion. Stripped rough microsomes were incubated in the presence of Tris-HCl (pH 7.4), 0.5 mM GTP, 5 mM $MgCl_2$ and 0.5 mM NADPH. This led to significant inhibition of accumulation of PUFAs and coincident inhibition of membrane fusion (Table I). In contrast when membranes were incubated in the presence of 0.5 mM GTP, 5 mM $MgCl_2$ and indomethacin, a cyclooxygenase inhibitor, either at 10 μM or 50 μM PUFA accumulation and GTP-dependent membrane fusion were not inhibited (Table I). The inhibitor of the lipoxygenase pathway of arachidonic acid metabolism, nordihydroguaiaretic acid at either 10 or 50 μM, was also observed to have little effect on either PUFA accumulation or membrane fusion (data not shown). Although these studies are consistent with PUFAs being involved in GTP-dependent membrane fusion they suggest that metabolites of arachidonic acid per se are probably not implicated.

The formation of PUFAs was stimulated by CTP but not by any of the following, ATP, GTP, ITP, UTP or GTPγS (Table II). Although GTP has been implicated in the activation of phospholipase activity in some membrane systems [12,13] we were unable to confirm this using SRM (Table II). ATP had a slight inhibitory effect and the presence of both CTP and GTP yielded similar results to CTP alone (Table II).

When examined in greater detail CTP was observed to be able to promote the formation of an additional lipid which co-migrated with diacylglycerol (data not shown). We studied this further by using membranes pre-labeled *in vivo* by injecting [U-^{14}C]arachidonic acid (from New England Nuclear, Boston, MA) into the portal vein of rats 15 min before killing for purification of rough microsomes. *In vivo*-labeled SRM were incubated in medium containing 2 mM $MnCl_2$ in the absence or presence of CTP. CTP was observed to stimulate the accumulation of radiolabeled lipid which comigrated with an arachidonic acid standard (Fig. 3). A [U-^{14}C]arachidonic acid-labeled lipid with the chromatographic mobility of standard diacylglycerol was also formed in the presence of CTP (Fig. 3). We conclude that CTP in addition to being able to stimulate PUFA accumulation can lead to the appearance of diacylglycerol. Such lipids have been shown to express fusogenic properties [2–4,14] and thus could be candidates involved in the fusion of RER membranes stimulated in the presence of CTP [1].

The phospholipase activity we describe resembles that previously observed in liver microsomes by Lumb and Allen [15] based on kinetics of activity and Ca^{2+} independence at neutral pH. It is unlikely to be the calcium-dependent phospholipase A_2 previously described in various rat liver subcellular fractions [16,17] since we observed PUFA accumulation both in the absence of calcium and in the presence of 5 mM EGTA. A more detailed characterization of this activity especially using *in vivo*-labeled membranes is required not only because it may lead to a better under-

TABLE I

Effect of NADPH or indomethacin on accumulation of PUFAs and on GTP-dependent membrane fusion

Stripped rough microsomes (150 μg membrane protein) were incubated in the presence of 0.5 mM GTP, 5 mM $MgCl_2$ and in the absence or presence of either 0.5 mM NADPH or indomethacin for 120 min at 37 °C. Lipids were extracted and separated by thin-layer chromatography using *n*-hexane diethyl ether acetic acid (70:30:1, v/v). Purified arachidonic acid was used to generate standard curves for the experiments and these were used to convert absorbance values into μg units. Membrane fusion was assayed in duplicate samples as previously described [6]. Values indicated in parentheses represent data obtained from a separate experiment using membranes from a different fractionation experiment.

Incubation condition	Polyunsaturated free fatty acids (μg)	Fusion index (%)
Control	2.37	19.7
	(2.20)	(36.2)
+ NADPH	1.12	0
	(0.95)	(0)
+ Indomethacin (10 μM)	2.83	23.8
	(2.02)	(27.6)
+ Indomethacin (50 μM)	3.76	30.8
	(2.25)	(35.0)

TABLE II

Effect of different nucleotides on the accumulation of PUFAs

Stripped rough microsomes (150 μg membrane protein) were incubated in the presence of 5 mM $MgCl_2$ and one of the following nucleotides at 0.5 mM; ATP, CTP, GTP, ITP, UTP or GTPγs. Incubations were carried out 120 min at 37 °C. Lipids were then extracted and analyzed as described in Table I. n.d., not determined.

Nucleotide	Polyunsaturated free fatty acids (μg)		
	Expt. 1	Expt. 2	Expt. 3
None	3.91	3.00	2.06
ATP	2.92	2.72	1.34
CTP	6.08	5.37	3.40
GTP	3.24	2.49	1.91
ITP	3.24	2.59	2.11
UTP	3.65	2.55	2.06
GTPγs	3.18	1.73	1.61
GTP+CTP	n.d.	5.33	3.43

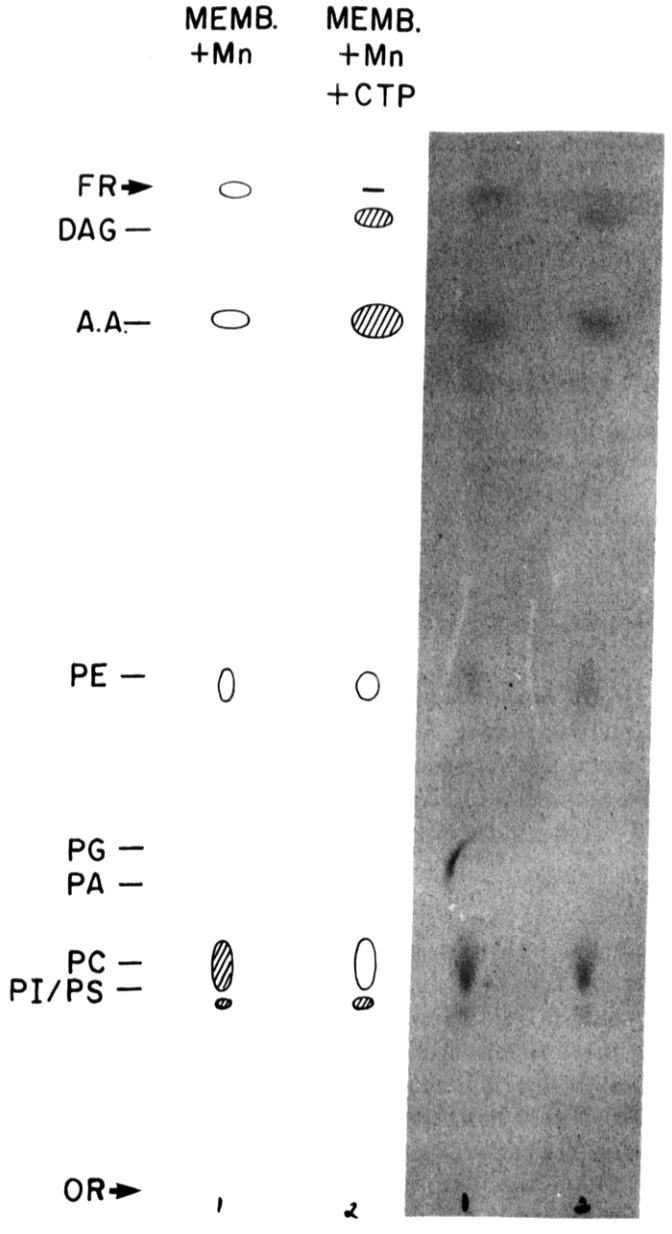

Fig. 3. Effect of incubation of SRM in the presence of CTP on the lipid profile observed after extraction and analysis by thin-layer chromatography. Endoplasmic reticulum membranes were labeled *in vitro* as described by Valtersson et al. [28] by injection of [U-^{14}C]arachidonic acid (56 mCi/m mol, 12 μCi injected into the portal vein of three rats). After 15 min animals were killed and microsomes prepared. Stripped rough microsomes (200 μg membrane protein) were incubated for 120 min at 37 °C in the absence or presence of 3 mM CTP and 2 mM $MnCl_2$. Lipids were extracted and analyzed as described in the legend to Fig. 1. Radioautographic detection was done as previously described [1]. The positions of migration of phospholipid and fatty acid standards are indicated to the left of the results. AA, arachidonic acid; DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; Fr, front of solvent; Or, origin. This experiment was repeated three times with similar results.

standing of the mechanism of fusion of RER membranes but also because it could provide information relevant to previously described changes in membrane permeability induced in the presence of PUFAs [18,19] or GTP [20,21]. Further studies could also provide information relevant to previously described calcium release by membranes in the presence of PUFAs [22–24] or GTP [25–27].

We concur with the previous proposal that arachidonic acid and/or DAG maybe important for membrane fusion [2–4,14] and suggest that GTP may activate metabolic events upstream (e.g. initially during membrane recognition [29]) from those involved in direct membrane coalescence. CTP maybe able to bypass GTP-dependent events by virtue of its capacity to stimulate formation of both arachidonic acid and DAG.

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References

1 Jolicoeur, M., Kan, F.W.K. and Paiement, J. (1991) J. Histochem. Cytochem. 39, 363–372.
2 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. 136, 147–155.
3 Creutz, C.E. (1981) J. Cell Biol. 91, 247–256.
4 Meers, P., Hong, K. and Papahadjopoulos, D. (1988) Biochemistry 27, 6784–6794.
5 Paiement, J. and Bergeron, J.J.M. (1983) J. Cell Biol. 96, 1791–1796.
6 Paiement, J., Dominguez, J.M., Guénette, A. and Roy, L. (1991) Biochem. Biophys. Res. Commun. 176, 1494–1500.
7 Schact, J. (1981) Methods Enzymol. 72, 626–631.
8 Smith, C.D. and Wells, W.W. (1983) J. Biol. Chem. 258, 9368–9373.
9 Zahler, P., Reist, M., Pilarska, M. and Rosenheck, K. (1986) Biochim. Biophys. Acta 877, 372–379.
10 Diez, E. and Mong, S. (1990) J. Biol. Chem. 265, 14654–14661.
11 Sevanian, A. and Hochstein, P. (1985) Annu. Rev. Nutr. 5, 365–390.
12 Axelrod, J. (1990) Biochem. Soc. Trans. 18, 503–507.
13 Rubin, R.P., Withiam-Leitch, M. and Laychock, S.G. (1991) Biochem. Biophys. Res. Commun. 177, 22–26.
14 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.
15 Lumb, R.H. and Allen, K.F. (1976) Biochim. Biophys. Acta 450, 175–184.
16 Van Golde, L.M.G., Fleischer, B. and Fleischer, S. (1971) Biochim. Biophys. Acta 249, 318–330.
17 Aarsman, A.J., De Jong, J.G.N., Arnoldussen, E., Neys, F.W., Van Wassenaar, P.D. and Van den Bosch, H. (1989) J. Biol. Chem. 264, 10008–10014.
18 Lucy, J.A. (1974) FEBS Lett. 40, S105–S111.
19 Dawson, R.M.C., Irvine, R.F., Bray, J. and Quinn, P.J. (1984) Biochem. Biophys. Res. Commun. 125, 836–842.
20 Godelaine, D., Beaufay, H., Wibo, M. and Ravoet, A.-M. (1983) J. Cell Biol. 97, 340–350.

21 Nicchitta, C.V., Joseph, S.K. and Williamson, J.R. (1987) Biochem. J. 248, 741–747.
22 Messineo, F.C., Rathier, M., Favreau, C., Watras, J. and Takenaka, H. (1984) J. Biol. Chem. 259, 1336–1343.
23 Wolf, B.A., Turk, J., Sherman, W.R. and McDaniel, M.L. (1986) J. Biol. Chem. 261, 3501–3511.
24 Chan, K.-M. and Turk, J. (1987) Biochim. Biophys. Acta 928, 186–193.
25 Dawson, A.P. (1985) FEBS Lett. 185, 147–150.
26 Gill, D.L., Ueda, T., Chueh, S.-H. and Noel, M.W. (1986) Nature (London) 320, 461–464.
27 Henne, V. and Söling, H.-D. (1986) FEBS Lett. 202, 267–273.
28 Valtersson, C., Filipsson, L. and Dallner, G. (1986) J. Lipid Res. 27, 731–741.
29 Bourne, H.R. (1988) Cell 53, 669–671.