Biochimica et Biophysica Acta, 774 (1984) 296–300
Elsevier

BBA Report

BBA 70171

Ca^{2+}-INDEPENDENT, PROTEIN-MEDIATED FUSION OF CHROMAFFIN GRANULE GHOSTS WITH LIPOSOMES

MICHAL BENTAL [a], PETER I. LELKES [a,*], JANNY SCHOLMA [b], DICK HOEKSTRA [b] and JAN WILSCHUT [b,**]

[a] *Department of Membrane Research, The Weizmann Institute of Science, P.O.B. 26, Rehovot 76100 (Israel) and* [b] *Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen (The Netherlands)*

(Received March 15th, 1984)

Key words: Chromaffin granule; Exocytosis; Liposome; Phospholipid vesicle; Membrane fusion; Ca^{2+}

We have investigated the interaction between isolated membrane vesicles from chromaffin granules and large unilamellar phospholipid vesicles (liposomes). Mixing of membrane lipids has been monitored continuously, utilizing the fluorescence resonance energy transfer assay described by Struck et al. ((1982) Biochemistry 20, 4093–4099). To demonstrate coalescence of the internal vesicle volumes the transfer of colloidal gold from the liposomes to the interior of the granule membrane vesicles has been examined. Efficient fusion of the liposomes with the granule membranes was observed. Significant fusion occurred in the absence of Ca^{2+}, although the extent of interaction was enhanced in its presence. The sensitivity of the interaction to pretreatment of the granule membranes with trypsin showed the fusion reaction to be a protein-mediated process.

The hormones of the adrenal medulla, adrenaline and noradrenaline, are stored within secretory granules in the chromaffin cells. The release of granular contents occurs by compound exocytosis [1]. This process involves attachment of the secretory vesicle to the cellular plasma membrane and subsequent fusion of the vesicle membrane with the plasma membrane. In addition, granules fuse with one another, thus forming channels to the cell exterior. An in vitro, cell free model of the system would contribute significantly to the study of the molecular mechanisms of exocytosis, which, as yet, are largely unresolved. In search of such a model, we have investigated the interaction of isolated membrane vesicles of chromaffin granules (chromaffin granule ghosts) with phospholipid vesicles (liposomes). We demonstrate efficient interaction between the two vesicle populations, and present evidence that the nature of this interaction is a protein-dependent fusion process.

To unequivocally establish fusion between two vesicle populations, both the mixing of membrane components and the coalescence of the aqueous intravesicular contents should be demonstrated. The first process was monitored using the resonance energy transfer (RET) assay described by Struck et al. [2]. This assay allows continuous monitoring of the decrease in energy transfer efficiency between two non-exchangeable [2,3] fluorescent lipid analogues, *N*-NBD-PE (the donor)

* Present address: Laboratory of Cell Biology and Genetics, NIADDK, National Institutes of Health, Bldg. 4 Rm. 312, Bethesda, MD 20205, U.S.A.

** To whom correspondence should be addressed.

Abbreviations: Chol, cholesterol; CL, cardiolipin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LUV, large unilamellar vesicles; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine; RET, resonance energy transfer.

and *N*-Rh-PE (the acceptor), as they dilute into a non-labeled membrane. The mixing of vesicle contents was demonstrated by the transfer of colloidal gold from the liposomes [4,5] to the internal space of the chromaffin granule ghosts.

Chromaffin granules were isolated from bovine adrenal medulla by means of isotonic density gradient centrifugation [6]. Ghosts were prepared from intact granules by lysis in 5 mM Hepes (pH 7.4), and three subsequent washes in the same buffer. The ghosts (> 99.5% free of granular catecholamines) were resuspended in 0.15 M KCl/10 mM Hepes (pH 7.4). Chromaffin granule ghost preparations, obtained by a similar procedure of hypotonic lysis, have been shown to contain only 11% of inside-out resealed vesicles [7]. Liposomes (large unilamellar vesicles, LUV) were prepared in the KCl/Hepes buffer from a CL/PC/Chol (molar ratio, 3:2:5) mixture by reverse phase evaporation and sized to 0.1 μm by extrusion through Unipore polycarbonate membranes (BioRad) as described before [8]. All phospholipids were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Cholesterol was from Sigma.

For the RET assay, 0.8 mol% each of *N*-NBD-PE and *N*-Rh-PE were incorporated in the liposomal bilayer. Fluorescence (λ_{ex} 465 nm, λ_{em} 530 nm) was monitored continuously using a Perkin-Elmer MPF43 fluorimeter, with a thermostatically controlled, magnetically stirred cell holder, and a cut off filter (> 520 nm) between sample and emission monochromator. Triton X-100 (0.5%, v/v) was added to the liposomes to determine the fluorescence at infinite probe dilution. This value represents 70% of the maximal NBD fluorescence, since Triton reduces NBD fluorescence by 30% (Ref. 3).

Fig. 1 (trace a) shows the fluorescence development observed upon addition of chromaffin granule ghosts to liposomes, labeled with *N*-NBD-PE and *N*-Rh-PE, at 37°C. An increase of the NBD fluorescence was seen, reflecting the dilution of the fluorescent probes into the chromaffin granule membrane. The reaction reached a plateau after approx. 5 min. The subsequent addition of Ca^{2+} induced a further dilution of the fluorescent lipids. The initial spontaneous reaction was not affected by the presence of 10 mM EGTA in the medium

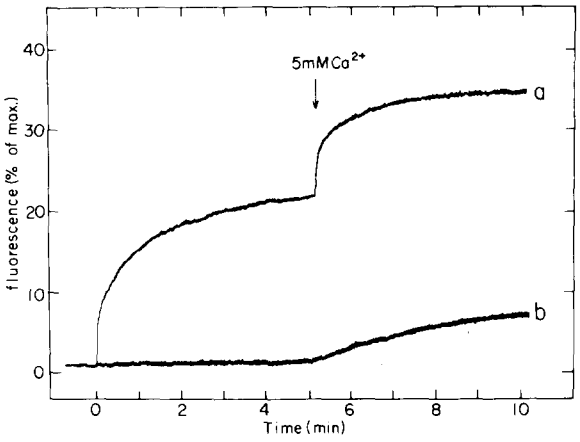

Fig. 1. Interaction of fluorescently labeled liposomes with chromaffin granule ghosts. Large unilamellar vesicles (25 μM lipid phosphorus; CL/PC/Chol/*N*-NBD-PE/*N*-Rh-PE, molar ratio 3:2:5:0.08:0.08) were equilibrated at 37°C in 0.15 M KCl/10 mM Hepes (pH 7.4). At time zero, chromaffin granule ghosts (100 μM lipid phosphorus) were added to the cuvette. After 5 min, $CaCl_2$ (0.2 M) was added to a final concentration of 5 mM. (a) Untreated chromaffin granule ghosts, (b) chromaffin granule ghosts pretreated with trypsin (0.5 mg/ml) during 10 min at 37°C. The fluorescence scale is calibrated such that the residual fluorescence of the liposomes is taken as the zero level and the value after addition of Triton X-100 (0.5%, v/v), corrected for the effect of Triton on the NBD fluorescence, as 100%.

(not shown). Addition of Ca^{2+} before or immediately after the addition of granule ghosts resulted in the same final fluorescence intensity as in the case of Ca^{2+} addition after 5 min (not shown). The extent of the spontaneous reaction at 37°C was around 22%, and that of the Ca^{2+}-induced reaction approx. 13% of the fluorescence intensity at infinite probe dilution.

In order to investigate the possible involvement of chromaffin granule protein component(s) in the interaction, granule ghosts were pretreated with trypsin. At a trypsin concentration of 0.5 mg/ml, the spontaneous reaction was completely abolished (Fig. 1, trace b), while the Ca^{2+}-induced reaction was also strongly inhibited. Granule ghosts simultaneously pretreated with trypsin and a 2-fold higher concentration of trypsin inhibitor showed the same reaction as untreated ghosts (see Fig. 1, trace a). These results indicate that the spontaneous interaction is completely dependent on protein components in the granule membrane, while

the Ca^{2+}-induced enhancement of the interaction is, at least in part, protein-mediated as well.

The dilution of the fluorescent probes into the granule membrane demonstrates the mixing of the lipids of the two vesicle populations. Although mixing of lipids could in principle occur through a process of exchange of individual lipid molecules, *N*-NBD-PE and *N*-Rh-PE have been shown to be non-exchangeable [2,3]. This suggests that the interaction observed involves vesicle-vesicle fusion. To further corroborate this, we studied the transfer of another non-exchangeable lipid marker, cholesteryl oleate [9], from liposomes to granule membranes. Liposomes containing cholesteryl [1-^{14}C]oleate and *N*-NBD-PE were incubated with chromaffin granule ghosts in the absence of Ca^{2+}. The ghosts were sedimented by centrifugation and washed twice. The radioactivity and NBD fluorescence were determined in the initial incubation mixture, the first supernatant and the final pellet. The results are shown in Table I. Both labels were transferred to the granule ghost pellet to the same extent, indicating that they behaved as parts of one unit. The amount of label left in the supernatant after the first centrifugation indicates that initially almost all of the liposomes associated with the granule ghosts, since in control experiments without granule ghosts no label was sedimented. However, a significant fraction of the granule-associated liposomes was loosely bound, and could be removed during the subsequent washings. Approx. 25–30% of the liposomes remained tightly bound to the granule membranes. It should be emphasized that this experiment, in principle, does not discriminate between mere liposome adhesion, lipid exchange or fusion. However, the simultaneous transfer of *N*-NBD-PE and another non-exchangeable lipid to the granule ghosts virtually rules out the possibility of exchange of individual lipid molecules, leaving adhesion of intact liposomes or fusion as possible mechanisms of interaction. The lipid mixing observed in the resonance energy transfer (RET) assay (Fig. 1) can not be reconciled with mere adhesion of liposomes. Therefore, the interaction must be a process of membrane fusion. Like the interaction observed in the RET assay, the lipid transfer in the experiment shown in Table I was also trypsin sensitive. Both

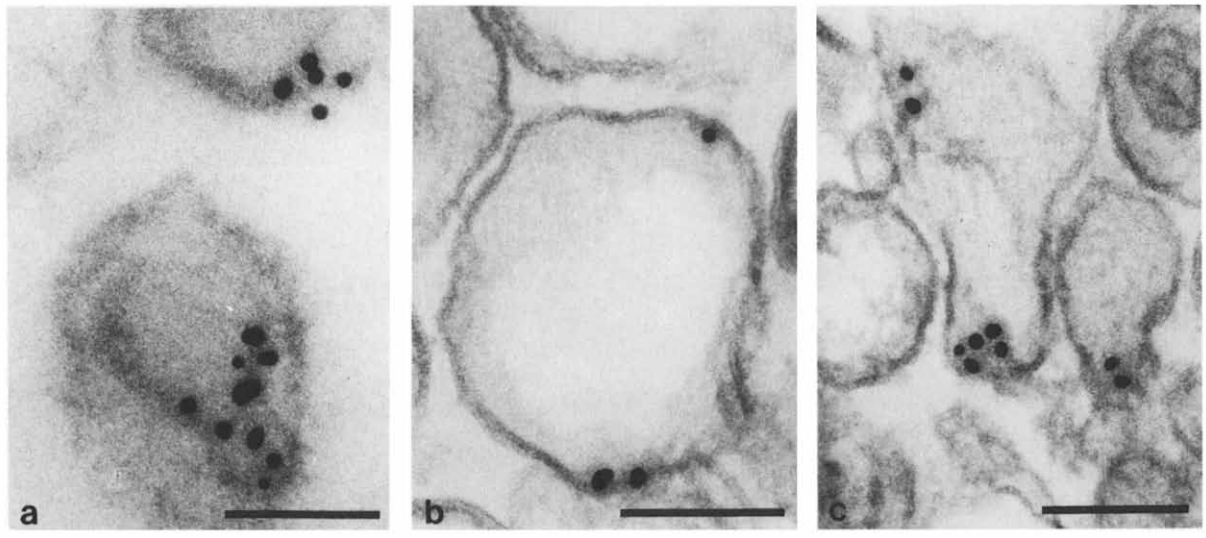

Fig. 2. Transfer of colloidal gold from liposomes to chromaffin granule ghosts. Large unilamellar vesicles (125 μM lipid phosphorus; CL/PC/Chol, molar ratio 3:2:5) containing colloidal gold were incubated with chromaffin granule ghosts (500 μM lipid phosphorus) in 0.15 M KCl/10 mM Hepes (pH 7.4). The membranes were pelleted (Eppendorf microfuge, 5 min, at 4°C) and prepared for thin section electron microscopy. (a) Gold-containing large unilamellar vesicles (sedimented by ultracentrifugation during 1 h at 105000 × *g* in a Beckman SW 50.1 rotor). (b) and (c), Fusion products formed during a 5 min incubation of large unilamellar vesicles with chromaffin granule ghosts at 37°C in the absence of Ca^{2+}. Bars represent 0.1 μm. Note the low-contrast, diffuse appearance of the liposomal membranes, allowing to distinguish them from the protein containing granule membranes.

TABLE I

SIMULTANEOUS TRANSFER OF *N*-NBD-PE AND CHOLESTERYL [1-^{14}C]OLEATE FROM LIPOSOMES TO CHROMAFFIN GRANULE GHOSTS

Chromaffin granule ghosts (500 μM lipid phosphorus) and LUV (125 μM lipid phosphorus) were incubated for 10 min at 37 °C in 0.15 M KCl/10 mM Hepes (pH 7.4). The samples were transferred to ice, and the ghosts pelleted by centrifugation (Eppendorf microfuge, 5 min, 4 °C). The pellets were washed twice with KCl/Hepes buffer. *N*-NBD-PE fluorescence and ^{14}C radioactivity were determined in the initial reaction mixture, in the first supernatant and in the final pellet. Liposomes were large unilamellar vesicles, CL/PC/Chol/*N*-NBD-PE (molar ratio 3:2:5:0.05). Cholesteryl [1-^{14}C]oleate (Radiochemical Centre, Amersham, U.K.) was incorporated in the liposomes as a trace amount (0.2 μCi/μmol total lipid). The liposomes were precentrifuged (Eppendorf microfuge, 15 min, 4 °C) and the supernatant was used for the experiment.

Sample	Label in supernatant (%)		Label in pellet (%)	
	^{14}C	NBD	^{14}C	NBD
Untreated granule ghosts	8.2	10.8	29.9	27.2
Trypsin-treated [a] granule ghosts	44.7	45.0	14.8	14.2

[a] Chromaffin granule ghosts were pretreated with trypsin (0.5 mg/ml) during 10 min at 37 °C.

initial binding of the liposomes to trypsin-pretreated ghosts and final association with the pellet were considerably reduced, but not completely abolished. The residual association under these conditions presumably represents adhesion of intact liposomes, fusion being completely inhibited as evidenced by the lack of probe dilution in the RET assay (Fig. 1).

Conclusive evidence that the interaction observed is indeed fusion was provided by the transfer of liposome-encapsulated colloidal gold into the aqueous space of the chromaffin granule ghosts. Gold-containing LUV were prepared according to the procedure of Hong et al. [4], and incubated with chromaffin granule ghosts in the absence of Ca^{2+}. The membranes were sedimented by centrifugation and prepared for thin section electron microscopy as described [4,5]. As shown in Fig. 2, the liposome-granule ghosts interaction resulted in the transfer of gold particles into the aqueous space of the ghosts. No free gold could be found in the samples. In control experiments where granule ghosts were incubated with free gold at a concentration 5-fold higher than that which would result from lysis of all the liposomes, either in the absence or presence of empty liposomes, no gold particles were observed inside the ghosts (not shown). Therefore, the presence of gold particles within the aqueous space of the ghosts could only be the result of ghost-liposome fusion.

At a lipid ratio of labeled liposomes to unlabeled granule ghosts of 1 : 4, as used in the RET assay, total mixing of the lipids in the system would result in the fluorescence reaching 80% of its value at infinite probe dilution. In the absence of Ca^{2+} the extent of fusion actually observed corresponds to about 22% of the probe fluorescence at infinite dilution (Fig. 1). The sub-optimal probe dilution could be due to incomplete lipid equilibration after extensive or even complete fusion of the liposomes. However, it is more likely that it is the result of incomplete fusion, since, assuming total lipid equilibration, the 22% probe fluorescence observed during the Ca^{2+}-independent reaction would correspond to 28% of the liposomes being fused with the granule membranes, which is in agreement with the results of the lipid transfer experiment shown in Table I.

In vitro interactions of chromaffin granules with target membrane vesicles, such as plasma membrane vesicles from chromaffin cells [10,11] or liposomes [12], and interactions among chromaffin granules themselves [13–17] have been studied extensively before. A role of membrane protein components in granule-granule interactions has been suggested by Morris et al. [13,14] and Ekerdt et al. [15]. The latter authors observed fusion between chromaffin granules, which was abolished by pretreatment of the granules with trypsin. The reaction (designated 'type I fusion'), the extent of which was limited, specifically required Ca^{2+} at low concentrations (10^{-7} to 10^{-4} M). With Ca^{2+} in the millimolar range Ekerdt et al. [15] observed more extensive fusion of the granules, in agreement with results of Morris et al. [13]. This (type II') fusion could also be induced by other divalent cations at millimolar concentrations. The Ca^{2+}-induced enhancement of the interaction between liposomes and granule membranes we observed (Fig. 1) as well as the Ca^{2+}-dependent interaction

between liposomes and chromaffin granules described by Nayar et al. [12] may well be equivalent to this 'type II' fusion. Ekerdt et al. [15] demonstrated that liposomes composed of granule membrane lipids also showed a 'type II' fusion reaction.

The most important result of the present study is the observation of a Ca^{2+}-independent fusion event between liposomes and chromaffin granule membranes. Although liposomes obviously differ considerably from the physiological target membrane for chromaffin granules, this observation may suggest that the involvement of Ca^{2+} in the 'type I' fusion between isolated chromaffin granules [15] and in the physiological process of catecholamine secretion [1] is not at the level of the fusion event itself, but at an earlier stage, such as the attachment of the granules to one another and to the cellular plasma membrane. This hypothesis would be consistent with observations of Creutz et al. [16,17]. They have demonstrated a role of Ca^{2+} in activating synexin, a cytosolic Ca^{2+}-binding protein from the adrenal medulla that facilitates chromaffin granule aggregation, but not fusion. A stimulation of membrane attachment by Ca^{2+}-activated synexin has also been reported for phospholipid vesicle systems, the enhanced rate of vesicle fusion under these conditions being specifically due to an increased rate of initial vesicle aggregation [18,19].

We wish to thank Professor Kurt Rosenheck and Dr. Gerrit Scherphof for their interest in the project, Ms. A. Zakaria for expert help in the preparation of the granules, Dr. Caesar Hulstaert and Mr. Ruby Kalicharan from the Center for Medical Electron Microscopy (University of Groningen) for carrying out the electron microscopy work. This investigation was supported by the European Molecular Biology Organization (short-term fellowship to J.W.) and by the Israel Academy of Sciences and Humanities, Basic Research Foundation (grant to P.I.L.).

References

1 Douglas, W.W. (1975) in Handbook of Physiology (Blaschko, H., Sayers, G. and Smith, A.D., eds.), Vol. VI, section 7, pp. 367–388, American Physiological Society, Washington, DC
2 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099
3 Hoekstra, D. (1982) Biochemistry 21, 2833–2840
4 Hong, K., Friend, D.S., Glabe, C.G. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 320–323
5 Straubinger, R.M., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1983) Cell 32, 1069–1079
6 Trifaro, J.M. and Dworkind, J. (1970) Anal. Biochem. 34, 403–412
7 Hunter, A., Waldron, K. and Apps, D.K. (1982) FEBS Lett. 144, 51–56
8 Wilschut, J., Düzgüneş, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021
9 Kamp, H.H., Wirtz, K.W.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 318, 313–325
10 Lelkes, P.I., Lavie, E., Naquira, D., Schneeweiss, F., Schneider, A.S. and Rosenheck, K. (1980) FEBS Lett. 115, 129–133
11 Konings, F. and De Potter, W. (1981) FEBS Lett. 126, 103–106
12 Nayar, R., Hope, M.J. and Cullis, P.R. (1982) Biochemistry 21, 4583–4589
13 Morris, S.J., Costello, M.J., Robertson, J.D., Südhof, T.C., Odenwald, W.F. and Haynes, D.H. (1983) J. Auton. Nerv. Syst. 7, 9–33
14 Morris, S.J., Südhof, T.C. and Haynes, D.H. (1982) Biochim. Biophys. Acta 693, 425–436
15 Ekerdt, R., Dahl, G. and Gratzl, M. (1981) Biochim. Biophys. Acta 646, 10–22
16 Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858–2866
17 Creutz, C.E. (1981) J. Cell Biol. 91, 247–256
18 Hong, K., Düzgüneş, N., Ekerdt, R. and Papahadjopoulos, D. (1982) Proc. Natl. Acad. Sci. USA 79, 4642–4644
19 Hong, K., Ekerdt, R., Bentz, J., Nir, S. and Papahadjopoulos, D. (1983) Biophys. J. 41, 31a