Uptake of liposomes containing the photoprotein obelin by rat isolated adipocytes

Adhesion, endocytosis or fusion?

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1. The uptake of liposomes containing the photoprotein obelin by rat isolated adipocytes was investigated with the aim of producing liposome–cell fusion, enabling obelin to be introduced into the cytoplasm of intact cells. 2. Incubation of liposomes containing obelin with rat isolated adipocytes resulted in a time-dependent uptake of entrapped obelin by the adipocytes. The uptake by adipocytes (at 2 h) of liposomes prepared from phosphatidylcholine, phosphatidylcholine + phosphatidylserine (molar ratio 4:1) and phosphatidylcholine + *N*-octadecylamine (molar ratio 4:1) was approx. 6, 10 and 10% of original entrapped obelin per g dry wt. of adipocytes respectively. 3. During incubation with adipocytes some of the liposomes became permeable to Ca^{2+} ions, resulting in stimulation of obelin luminescence from within the liposomes. This increase in permeability to Ca^{2+} seemed to be the result of the interaction of liposomes with the cell membrane. 4. Approx. 50% of liposome uptake could be inhibited by cytochalasin B (500 μM). This was consistent with this uptake being the result of endocytosis. The remaining uptake was probably the result of adhesion of liposomes to the cell membrane. 5. In an attempt to detect the presence of cytoplasmic obelin, after incubation of adipocytes with liposomes, a method of causing a rapid rise in cell-membrane permeability to Ca^{2+} was developed in which an anti-cell antibody–complement reaction occurred at the cell membrane. There was no detectable transfer of active obelin into the cell cytoplasm. 6. After incubation of liposomes with adipocytes in the absence of bovine serum albumin, obelin luminescence from a small proportion of liposomes increased (approx. 1.5%) in response to anti-(5'-nucleotidase) antibody plus complement. 7. It was concluded that under the conditions of these experiments, (a) no detectable transfer (<0.1%) of liposome-entrapped obelin to the adipocyte cytoplasm had occurred, (b) an increase in liposome permeability to Ca^{2+} occurred during incubation with adipocytes, (c) at least 50% of liposome uptake by adipocytes was the result of endocytosis, presumably into secondary lysosomes, and the remaining uptake was apparently the result of loose attachment of liposomes to the cell surface, and (d) in the absence of bovine serum albumin, a portion of at least one surface antigen, the ectoenzyme 5'-nucleotidase, was transferred from the adipocyte membrane to the liposome membrane.

Fusion of pure phospholipid vesicles (liposomes) with the cell membrane has been proposed as a method for introducing into the cell cytoplasm impermeant molecules and ions (Tyrrell *et al.*, 1976; Pagano & Weinstein, 1978). Although some cases of liposome–cell fusion have been reported (Weinstein *et al.*, 1977; Barenholz *et al.*, 1978; Theoharides & Douglas, 1978; Gutman *et al.*, 1979), few studies

have been able to quantify the amount of substance originally entrapped within the liposome that became cytoplasmic. Besides fusion with the cell membrane, endocytosis and adhesion of liposomes to the cell surface can occur (Martin & MacDonald, 1976; Weinstein *et al.*, 1977; Pagano & Takeichi, 1977; Hoekstra *et al.*, 1978). An aim of the present study therefore was to examine quantitatively the interac-

tion of liposomes with cells and in particular to locate liposome-entrapped substances within the cell.

The fraction of liposomal material transferred to the cytoplasm may be as low as 5×10^{-5} (Weinstein *et al.*, 1977). An intra-liposomal marker that can be detected in very small quantities was therefore required. Obelin has been incorporated into liposomes (Dormer *et al.*, 1978a), which are highly impermeable to Ca^{2+} and appear to be taken up by isolated rat adipocytes in a time-dependent manner (Dormer *et al.*, 1978b). Although it was possible to detect the uptake of less than 10^{-5} of the original intra-liposomal material, the location of obelin within the cell was not determined. The transfer of obelin, a Ca^{2+} -activated photoprotein, to the adipocyte cytoplasm would allow for the first time changes in cytoplasmic Ca^{2+} to be monitored in small mammalian cells exposed to physiological and pharmacological stimuli (Campbell *et al.*, 1979a; Ashley & Campbell, 1979). Rat adipocytes were used because of their hormone-sensitivity and the possibility that intracellular Ca^{2+} ions may be involved in their responses to hormones.

Experimental

Materials

Chemicals. Phosphatidylserine (bovine) and phosphatidylcholine (egg) were obtained from Lipid Products, South Nutfield, Surrey, U.K. *N*-Octadecylamine (stearylamine) was obtained from ICN, K & K Laboratories, Plainview, NY, U.S.A. EGTA was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Cytochalasin B was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. [^3H]Inulin (25 nCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100 (scintillation grade) was obtained from BDH Chemicals, Poole, Dorset, U.K. All other chemicals were AnalaR grade from BDH Chemicals.

Obelin. The Ca^{2+} -activated photoprotein was extracted and purified from the hydroid *Obelia geniculata* as previously described (Campbell & Dormer, 1975, 1978; Campbell *et al.*, 1979b).

Complement and antisera. Guinea-pig serum was separated from blood collected from ether-anaesthetized animals by cardiac puncture and was used as a source of complement. Pooled guinea-pig serum was stored at -70°C . Heat-inactivated serum was produced by warming the serum at 56°C for 30 min.

Rabbit anti-(rat erythrocyte) antisera and rabbit and sheep anti-(5'-nucleotidase) antisera were kind gifts from Dr. J. P. Luzio (Department of Clinical

Biochemistry, University of Cambridge) and were prepared as described by Richardson & Luzio (1980). The antisera in the presence of guinea-pig serum as a source of complement were able to release approx. 50% of the total lactate dehydrogenase from isolated adipocytes. All antisera were stored at -20°C .

Preparation of liposomes

Liposomes, either neutral, negative or positive, composed of phosphatidylcholine, phosphatidylcholine + phosphatidylserine, (molar ratio 4:1) or phosphatidylcholine + *N*-octadecylamine (molar ratio 5:1) respectively, containing obelin were prepared by a modification of the method of Dormer *et al.* (1978a). The dried phospholipids were initially resuspended in 0.2 ml of Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid)-buffered saline containing obelin, before addition of a further 1.8 ml of Tes-buffered saline and sonication. This procedure resulted in approximately three times the amount of entrapped obelin compared with that obtained by initially resuspending the phospholipids in 2 ml of obelin-containing solution. Extra-liposomal obelin was inactivated by addition of CaCl_2 (0.1 mM) for 15 min, the only active obelin remaining being entrapped within Ca^{2+} -impermeable liposomes. Under conditions used for incubation, in the absence of adipocytes, 80–100% of entrapped obelin remained active for at least 2 h at 25°C .

Assay of active obelin

Free obelin was assayed by addition of a saturating Ca^{2+} concentration, as described previously (Campbell, 1974; Campbell & Dormer, 1975). Cell-associated obelin or liposomal obelin was assayed by addition of CaCl_2 (25 mM) containing Triton X-100 (0.5%, v/v).

Preparation of adipocytes

Adipocytes were prepared by collagenase digestion of rat epididymal fat-pads as described by Newby *et al.* (1975). Cells were incubated in modified Krebs–Ringer Hepes buffer, containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 , 1.3 mM- CaCl_2 , 2% (w/v) bovine serum albumin and 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], adjusted to pH 7.4 with NaOH. Cell viability was determined under the conditions of liposome incubation, by monitoring hormone sensitivity to adrenaline and insulin (Siddle & Hales, 1974) and measuring release of lactate dehydrogenase from the cells or cellular ATP (Siddle *et al.*, 1973).

Measurement of uptake of liposomes by adipocytes

Samples of adipocytes (approx. 100 mg dry wt.; about 2.5×10^6 cells), in 1 ml of Krebs–Ringer

Hepes buffer were incubated with liposomes (approx. 2.8–5.6 mg of phospholipid) at 24–26°C (above the transition temperature of phospholipids used). At the end of the incubation period, cells were washed four times by centrifugation at 500 *g* for 2 min and resuspension in 2.5 ml of fresh Krebs–Ringer Hepes buffer, to remove free liposomes. By using an extracellular marker, [³H]inulin, it was shown that this procedure left less than 0.01% of the extracellular fluid. The cells were assayed for associated obelin as described above.

Preparation of adipocyte membrane vesicles

Isolated adipocytes were homogenized in 150 mM-KCl/5 mM-MgCl₂/20 mM-Tris, adjusted to pH 7.4 with HCl, by passing the cells through a steel mesh (Millipore, 13 mm Swinnex, mesh approx. 150 μ m) 20 times; the homogenate was centrifuged at 500 *g* for 5 min and the supernatant centrifuged at 10 000 *g* for 30 min at 4°C (Sorvall RC-5 Superspeed), in order to remove cell debris and nuclei, and most of the mitochondria, respectively. The final supernatant was centrifuged at 100 000 *g* for 60 min at 4°C (MSE Superspeed 50) to produce a crude membrane pellet, which was resuspended in homogenization medium plus EGTA (10 mM) and washed twice to remove EGTA and contaminating Ca²⁺. The membrane suspension was sonicated in the presence of obelin (approx. 10⁹ luminescent counts) for 15 s with an MSE ultrasonicator tuned for maximum amplitude, and passed down a gel-filtration column (32 cm $\times$ 1.5 cm; Sephadex G-75) equilibrated in Ca²⁺-free Krebs–Ringer Hepes buffer (Westwood, 1979). Membrane vesicles produced by this method entrapped approx. 0.01% of the original obelin within relatively Ca²⁺-impermeable membranes.

Results and discussion

Effects of liposome lipid composition on the uptake of entrapped obelin by adipocytes

It has been reported (Stendahl & Tagesson, 1977) that the uptake of liposomal material by cells is influenced by the net surface charge on the liposomes used. The initial experiments were designed therefore to investigate the effects of liposome surface charge on liposome uptake by adipocytes (Fig. 1). Since in the presence of external Ca²⁺ the cellular uptake of obelin within neutral liposomes appeared to be diminished (Dormer *et al.*, 1978b), the initial experiments were performed in the absence of external Ca²⁺ with EGTA (1 mM) present in the medium.

After incubation for 120 min, 5.6% of obelin within neutral liposomes was associated with the fat-cells. The uptake by the cells of obelin within charged liposomes was approximately twice that within neutral liposomes. The percentage uptake of

Fig. 1. Effect of liposome surface charge on the uptake of liposome-entrapped obelin by adipocytes

Three types of liposomes, with the phospholipid compositions shown in the Experimental section, were prepared; negative liposomes entrapped approx. 1.2×10^7 luminescence counts, positive liposomes approx. 0.65×10^7 and neutral liposomes approx. 0.12×10^7 . Each type of liposome was incubated with isolated adipocytes at approx. 25°C in Ca²⁺-free Krebs–Ringer Hepes buffer with EGTA (1 mM) added. After the incubation period the adipocytes were washed and the cell-associated obelin was assayed. Each point is the mean of two separate experiments. The data are expressed as percentage of total liposome obelin taken up per g dry wt. of adipocytes: (a) negative liposomes, (b) positive liposomes, (c) neutral liposomes.

total entrapped material of both types of charged liposomes was similar. However, more obelin was taken up by adipocytes from negatively charged liposomes, because negatively charged liposomes were less Ca²⁺-permeable and entrapped approx. 3 times more obelin (Dormer *et al.*, 1978a). Consequently negatively charged liposomes were used throughout the course of this investigation.

Isolated rat adipocytes often contain a small quantity of triacylglycerol from broken cells. In order to test that the uptake of entrapped obelin required intact cells, a comparison of the uptake of the three charged liposomes by intact and homogenized cells was made (Table 1). The association of liposomes with broken cells was approximately one-third of that with intact cells. It was estimated that in most experiments less than 20% of the cells were broken. It was concluded therefore that more than 90% of the liposome-entrapped obelin was associated with intact cells.

Table 1. *Effect of incubation of liposomes with intact adipocytes and cell homogenates*

A comparison was made of the uptake of three different types of liposomes by intact cells or adipocyte homogenates. Adipocyte homogenate was prepared by passing a suspension of isolated adipocyte in Krebs–Ringer HEPES buffer 20 times through a fine metal mesh (Millipore, Swinnox 13 mm, mesh approx. 150 μ m), to produce a cell-free fat cake. Liposomes were incubated with either intact cells or homogenates at 25°C and associated obelin was assayed as described in the Experimental section. The data are expressed as percentage of total entrapped obelin taken up per g dry wt. of adipocytes or cell homogenates. Each point is the mean of two experiments, and the ranges are shown.

		Obelin (% of total entrapped/g dry wt. of adipocytes or cell homogenate)						
Approx. time (min)	Liposome charge ...	Intact cells			Cell homogenate			
		Positive	Negative	Neutral	Positive	Negative	Neutral	
10		2.12 $\pm$ 1.20	1.33 $\pm$ 0.80	1.5 $\pm$ 0.5	0.75 $\pm$ 0.50	0.44 $\pm$ 0.05	0.5 $\pm$ 0.1	
30		2.42 $\pm$ 1.10	2.27 $\pm$ 1.60	2.12 $\pm$ 0.30	0.72 $\pm$ 0.20	0.69 $\pm$ 0.40	0.85 $\pm$ 0.20	

Effect of extracellular Ca^{2+} on uptake of entrapped obelin

It has been reported that Ca^{2+} may be necessary for fusion of membranes (Papahadjopoulos *et al.*, 1975; Duzgune & Ohki, 1977). It was therefore important to examine the effects of external Ca^{2+} on entrapped obelin uptake by the cells. The presence of external Ca^{2+} (1 mM) decreased the uptake of active obelin at 2 h by approx. 90% compared with the uptake observed in the presence of external EGTA (1 mM) (Fig. 2). However, when liposomes were prepared containing both obelin and EGTA (1 mM), external Ca^{2+} (1 mM) decreased the uptake of active obelin by only 40%. These results were interpreted as showing a loss of active photoprotein when liposomes associated with cells in the presence of external Ca^{2+} , owing to an increased permeability of liposomes to Ca^{2+} .

Since this should result in an increase in obelin luminescence, the experiment was repeated while the cells were in front of the photomultiplier tube, with the result shown in the next section.

Investigation of liposome permeability to Ca^{2+} during incubation with cells

An increase in luminescence on addition of liposomes to cells was detectable, but not when liposomes were added to medium alone (Fig. 3). It was possible that this increase in obelin luminescence could be the result of liposome lysis allowing slow release of obelin in the medium. This possibility was ruled out by the observation that EGTA entrapped within the liposomes markedly decreased this increase in obelin luminescence (Fig. 3); even if all the EGTA were released, the concentration in the external medium (<50 μ M) would be insufficient to protect obelin in the presence of Ca^{2+} (1 mM).

Further evidence for an increase in permeability of liposomes to Ca^{2+} was obtained by studying the liposomes after incubation with the cells. After incubation of liposomes with adipocytes in the

Fig. 2. *Effect of extracellular Ca^{2+} and intraliposomal EGTA on uptake of liposome-entrapped obelin by adipocytes*

Liposomes, prepared containing either obelin alone or obelin plus EGTA (1 mM), were incubated with adipocytes in Krebs–Ringer HEPES buffer, either with CaCl_2 (1 mM) or Ca^{2+} -free with EGTA (1 mM). (a) Liposomes containing obelin alone entrapped 1.6×10^7 luminescence counts/ml and liposomes containing obelin plus EGTA entrapped 3.6×10^7 luminescence counts/ml. After the incubation period, the adipocytes were washed and cell-associated obelin was assayed. The time course of liposome uptake is shown: O, liposomes containing obelin alone incubated with adipocytes in Krebs–Ringer HEPES buffer containing CaCl_2 (1 mM); ▲, liposomes containing obelin and EGTA (1 mM) incubated with adipocytes in Krebs–Ringer HEPES buffer containing CaCl_2 (1 mM); ●, liposomes containing obelin incubated with adipocytes in Krebs–Ringer HEPES buffer containing EGTA (1 mM). Each point represents the mean of three determinations and the bars represent the S.E.M.

Fig. 3. Effect of adding liposomes to adipocytes on the Ca^{2+} -permeability of the liposomes

Samples of liposomes, diluted in 0.5 ml of Krebs–Ringer HEPES buffer containing CaCl_2 (mM), were injected into a 0.5 ml adipocyte suspension (approx. 200 mg of adipocytes/ml) in the same medium placed in front of the photomultiplier tube. Both types of liposome, prepared containing either obelin alone or obelin plus EGTA (1 mM), entrapped approx. 10^7 counts/100 μl of liposomes. After the time period, Triton X-100 (10%, v/v) was added to stimulate all remaining entrapped obelin and the percentage consumption of obelin was calculated. Stimulation of luminescence required the presence of extracellular Ca^{2+} ions. ●, Liposomes containing obelin were injected into a cell suspension; ▲, liposomes containing obelin plus EGTA were injected into a cell suspension; △ and ○, liposomes containing obelin plus EGTA and obelin alone respectively injected into Krebs–Ringer HEPES buffer containing no adipocytes. The results show the points taken from a single representative experiment.

absence of Ca^{2+} and in the presence of EGTA (1 mM), infranatant liposomes were removed. The permeability to Ca^{2+} of these liposomes was tested by the addition of Krebs–Ringer HEPES buffer containing Ca^{2+} (1 mM). The addition of Ca^{2+} to infranatant liposomes, which had previously been incubated with adipocytes for 60 min, caused an immediate stimulation of obelin luminescence. Approx. 0.1% of the liposome-entrapped obelin was stimulated within the first 10 s after addition of Ca^{2+} . However, the rate of this luminescence was too slow to be due to free obelin, thereby excluding the possibility of liposome lysis having occurred. There was no detectable increase ($<10^{-3}\%$) in luminescence when Ca^{2+} was added to liposomes incu-

bated under similar conditions but without adipocytes. It was therefore concluded that the incubation of liposomes with cells leads to an increase in liposome permeability to Ca^{2+} .

Effect of cytochalasin B on uptake of entrapped obelin by adipocytes

Liposomes can be taken up by some cells by endocytosis (Martin & MacDonald, 1976; Pagano & Takeichi, 1977; Hoekstra *et al.*, 1978). The effect of cytochalasin B, a potent inhibitor of endocytosis (Allison & Davies, 1974), on liposome uptake by adipocytes was therefore investigated.

Preincubation of adipocytes with cytochalasin B (1–500 μM) produced an inhibition of liposome uptake, in both the absence and the presence of extracellular Ca^{2+} (Fig. 4). Maximum inhibition was achieved at 500 μM -cytochalasin B, when approx. 50% of the uptake was inhibited in the presence of either external Ca^{2+} or external EGTA. This inhibition could not be explained by cell breakage, since no significant depletion of cell ATP nor any increased release of lactate dehydrogenase was caused by cytochalasin B.

Fig. 4. Effect of cytochalasin B on liposome uptake by adipocytes

Adipocytes were preincubated for 5 min with cytochalasin B (1–500 μM) dissolved in dimethyl sulphoxide (final concn. 0.1%) before addition of (i) liposomes containing obelin alone in the presence of extraliposomal EGTA (1 mM) or (ii) liposomes containing obelin plus EGTA (1 mM) in the presence of extraliposomal Ca^{2+} (1 mM). Liposomes and cells were then incubated for a further 30 min, at the end of which free liposomes were washed away and cell-associated obelin was assayed. Liposome uptake was expressed as a percentage of uptake in the absence of cytochalasin B; (a) Krebs–Ringer HEPES buffer; (b) Krebs–Ringer HEPES buffer plus 0.1% dimethyl sulphoxide. Each point is the mean of three determinations and the bars represent the S.E.M.

The concentrations of cytochalasin B used in these experiments were considerably higher than those used by other workers for inhibiting endocytosis in other cell types (Silverstein *et al.*, 1977). This may be due to the inhibitor, which is lipophilic in nature, accumulating in the triacylglycerol of the adipocyte. The inhibition by cytochalasin B of liposome uptake was consistent with 50% of the uptake by the adipocytes being due to endocytosis. It has previously been shown that isolated adipocytes can endocytose small particles (Barnett & Ball, 1960; Cushman, 1970). The uptakes of liposomes by other cell types have also been decreased by inhibitors of endocytosis (Pagano & Weinstein, 1978).

Location of cell-associated obelin after homogenization and centrifugation

If the cytochalasin B-insensitive uptake of liposomes (approx. 50% of the total) was the result of liposomes fusing with adipocyte membranes, thereby releasing obelin into the cell cytoplasm, a consequence of this would be the presence of free obelin in the supernatant of a cell homogenate centrifuged at 100 000 *g* for 60 min. Free liposomes do not sediment at 100 000 *g*; however, it was hoped to distinguish them from free obelin in the 100 000 *g* supernatant by two procedures: (i) Ca^{2+} (1 mM) does not significantly stimulate obelin luminescence in liposomes unless a detergent is also present, and (ii) liposomes can be separated from free obelin by gel filtration (Dormer *et al.*, 1978a). After liposome incubation, adipocytes were homogenized in 0.25 M-

sucrose/10 mM-Tes/1 mM-EGTA, pH 7.4, and centrifuged at 100 000 *g* for 60 min. A sample of the 100 000 *g* supernatant was retained for gel filtration, and in the remainder the Ca^{2+} -activated luminescence was measured in the presence and absence of a detergent, Triton X-100 (10%, v/v). Stimulation of luminescence after the addition of Triton X-100 indicated obelin within Ca^{2+} -impermeable membranes. The total obelin activity in the cell homogenate was approx. 85% of that originally associated with intact cells. When obelin was added to a cell homogenate, approx. 95% was recovered after centrifugation.

The distribution of cell-associated obelin between the 100 000 *g* supernatant and pellet and the requirement for Triton X-100 in the stimulation of Ca^{2+} -dependent light emission are shown in Table 2. The results of two different incubation conditions, in the presence of either extracellular Ca^{2+} or extracellular EGTA, are presented. Approx. 50% of the obelin in the 100 000 *g* supernatants of either type of cell homogenate could be stimulated by Ca^{2+} (1 mM) alone, consistent with this fraction being free cytoplasmic obelin. However, this obelin could be separated from free obelin by gel filtration on a Sephadex G-75 column (Fig. 5). Treatment of the 100 000 *g* supernatant with Triton X-100 in the absence of Ca^{2+} resulted in this obelin being eluted with the free obelin (Fig. 5). It was concluded that approx. 50% of the obelin in the 100 000 *g* supernatant was within liposomes impermeable to Ca^{2+} . Most of the remainder was not free obelin released into the cell cytoplasm as a result of fusion, but was

Table 2. *Distribution of cell-associated obelin after homogenization and centrifugation*

Adipocytes were incubated either with liposomes containing obelin and 1 mM-EGTA (liposomes type 1) in an incubation medium containing Ca^{2+} (1 mM) or with liposomes containing obelin alone (liposomes type 2) in an incubation medium containing no added Ca^{2+} and EGTA (1 mM), for 60 min. After washing, the cells were homogenized in 0.25 M-sucrose, 10 mM-Tes, 1 mM-EGTA, adjusted to pH 7.4 with NaOH, by 20 passes through a fine steel mesh (Millipore, 13 mm Swinnex, mesh approx. 150 μm). The cell homogenate was centrifuged at 100 000 *g* for 60 min and the supernatant, pellet and fat-cake were assayed for obelin stimulated either (a) by the addition of Ca^{2+} alone or (b) by Triton X-100 (10%, v/v), after the addition of Ca^{2+} . Column (a) shows the quantity of obelin stimulated by the addition of CaCl_2 (25 mM) expressed as a percentage of the total obelin in the cell homogenate. Column (b) shows the quantity of obelin stimulated by Triton X-100 (10%, v/v) after the addition of CaCl_2 (25 mM) expressed as a percentage of total obelin in the cell homogenate. Column (c) shows the total obelin in the cell fraction expressed as a percentage of total obelin in the cell homogenate and is the sum of the obelin stimulated by Ca^{2+} alone and the obelin stimulated by the addition of Triton X-100 after Ca^{2+} (a + b). Results shown are the means $\pm$ S.E.M. for three experiments.

Ca ²⁺ concn. in cell incubation medium Liposomes	Obelin (% of total in cell homogenate)					
	1 mM			Less than 1 mM (EGTA = 1 mM)		
	1			2		
	(a) Ca ²⁺	(b) Triton	(a) + (b) = (c) Total	(a) Ca ²⁺	(b) Triton	(a) + (b) = (c) Total
Pellet (100 000 <i>g</i>)	8.9 $\pm$ 13.9	40.8 $\pm$ 6.3	49.7	10.2 $\pm$ 3.1	23.3 $\pm$ 3.2	33.5
Supernatant (100 000 <i>g</i>)	13.5 $\pm$ 6.0	19.7 $\pm$ 8.1	33.2	29.7 $\pm$ 2.7	31.5 $\pm$ 8.7	61.2
Fat cake	8.5 $\pm$ 4.4	8.6 $\pm$ 5.3	17.1	3.9 $\pm$ 0.5	1.9 $\pm$ 3.1	5.8

Fig. 5. Gel filtration of 100000 g supernatant of a cell homogenate after incubation of adipocytes with liposomes

A 2 ml sample of 100000 g supernatant prepared as described in Table 2 was eluted through a Sephadex G-75 column (32 cm $\times$ 1.5 cm) at 4°C. The column was pre-equilibrated in the homogenization medium, and 2.8 ml fractions were collected and assayed for obelin by the addition of CaCl_2 (25 mM) or CaCl_2 (25 mM) plus Triton X-100 (1%, v/v). Infranant liposomes after the incubation period (□) and free obelin (■) were eluted. Supernatant obelin was eluted (△), or collected and treated with Triton X-100 (1%, v/v) 10 min before re-elution (▲). Data shown are from a single representative experiment where liposomes containing obelin alone were incubated with adipocytes in the presence of extraliposomal EGTA (1 mM). A similar result was obtained when liposomes containing both obelin and EGTA were incubated with adipocytes in the presence of Ca^{2+} (1 mM). Results are expressed as percentage of total obelin activity added to the column.

probably within liposomes which had become highly permeable to Ca^{2+} . In view of the large acid phosphatase activity in the 100000 g supernatant (approx. 40% of the total activity in the homogenate), it was possible that this fraction of obelin that was stimulated by Ca^{2+} alone originated from broken secondary lysosomes. This conclusion was supported by the fact that preincubation of cells with cytochalasin B (500 μM) decreased this fraction of obelin in the 100000 g supernatant by approx. 50%. These results highlighted the difficulty of using cell fractionation to locate substances taken up from liposomes by cells.

Attempts to detect cytoplasmic obelin

Although the data presented above suggested that most of the uptake of liposome-entrapped obelin by cells was not the result of liposome-cell fusion, it was still possible that a small percentage fusion,

undetectable by these methods, might have occurred. A method was therefore required to detect the presence of small amounts of cytoplasmic obelin in the presence of a large fraction of non-cytoplasmic obelin. One approach was to use calcium ionophore A23187 to cause a rise in intracellular Ca^{2+} (Campbell & Dormer, 1978) and thus stimulate cytoplasmic obelin. Indeed, addition of ionophore A23187 to cells that had been incubated with liposomes containing obelin did cause an increase in obelin luminescence. However, ionophore A23187 would also stimulate obelin luminescence from liposomes adhering to the cell surface, and probably obelin within other intracellular compartments. From these experiments, it was therefore not possible to identify definitively any cytoplasmic obelin.

Another approach was to use agents, for example cyanide, that have been shown to release Ca^{2+} from mitochondria, causing a rise in cytoplasmic $[\text{Ca}^{2+}]$ (Baker *et al.*, 1971). However, the content of Ca^{2+} in mitochondria isolated from cells incubated with cyanide (5 mM) was found to decrease slowly, 50% of mitochondrial Ca^{2+} being released over 10 min, and was therefore unsuitable as a means of rapidly elevating cytoplasmic Ca^{2+} , to enable a small quantity of cytoplasmic obelin to be detected.

It has been shown, by using sealed pigeon erythrocyte 'ghosts' containing obelin, that the anti-cell antibody plus complement reaction at the cell membrane produces a very rapid rise in intracellular free Ca^{2+} (Luzio *et al.*, 1979; Campbell *et al.*, 1979b). The possibility that this method could be used to detect and quantify cytoplasmic obelin was therefore investigated.

Effect of antibody-complement reaction on obelin luminescence from adipocyte membrane vesicles

In order to establish the validity of using the anti-cell antibody-complement reaction as a means of stimulating obelin within the adipocyte cytoplasm without stimulating obelin within adhering liposomes, it was necessary to show that the reaction could occur on the adipocyte membrane, but not on the liposome membrane. Antisera to either rat erythrocytes or the ecto-enzyme 5'-nucleotidase caused an increase in obelin luminescence in membrane vesicles prepared from adipocyte membranes approx. 40 s after addition of complement (Fig. 6a). No effect was seen with non-immune serum. During the anti-cell antibody-complement reaction, approx. 40% of obelin was consumed; these results are consistent with this fraction of the vesicles originating from cell membrane with the surface antigens exposed to the external medium. Under these conditions no effect of antisera plus complement was observed on liposomes containing obelin.

Fig. 6. Effect of antibody plus complement on adipocyte membrane vesicles and liposomes after incubation with adipocytes

(a) Adipocyte membrane vesicles were prepared containing obelin, as described in the Experimental section. Vesicles were incubated either with (i) anti-(5'-nucleotidase) antibody (1/10 dilution) or (ii) non-immune serum (1/10 dilution) in 500 μ l of Krebs-Ringer Hepes buffer containing CaCl_2 (1 mM). At the end of the incubation period, the sample was transferred to the housing in front of the photomultiplier tube and guinea-pig serum (1/10 dilution) added (arrow 1; zero time on trace). At the end of the incubation period, Triton X-100 (1%, v/v; arrow 2) was added, causing stimulation of the remaining active obelin within the vesicles. With vesicles incubated with non-immune serum, 2% of obelin was consumed over the period, whereas with vesicles preincubated with antibody 39% of obelin was consumed. (b) Liposomes containing both obelin and EGTA were incubated with adipocytes in Krebs-Ringer Hepes buffer with Ca^{2+} (1 mM) but no bovine serum albumin for 15 min at 20°C. At the end of the incubation period, infranatant liposomes were separated and incubated with either (iii) non-immune serum or (iv) anti-(5'-nucleotidase) antibody (both 1/50 dilution) for 5 min. The sample was then transferred to the housing in front of the photomultiplier tube and guinea-pig serum (1/10 dilution) added (arrow 1; zero time on the trace). Liposomes incubated in similar conditions without adipocytes also failed to respond to the addition of complement after antibody. At the end of the incubation period Triton X-100 (1%, v/v; arrow 2)

Isolated adipocytes which had been incubated with liposomes containing obelin were then exposed to anti-cell antibody plus complement. Under all the conditions described above, no detectable effect of antibody-complement on obelin luminescence from the cells could be detected. It was therefore concluded that there was no detectable cytoplasmic obelin.

The environment of any cytoplasmic obelin would be considerably different from that within membrane vesicles (Hodgkin & Keynes, 1957; Blaustein & Hodgkin, 1969) with Ca^{2+} -buffering systems, e.g. mitochondria and binding proteins (Brinley, 1978). However, if cytoplasmic Ca^{2+} were to respond with a similar time course to intravesicular Ca^{2+} , it can be calculated from knowledge of the detection limit for obelin that less than 0.1% of the cell-associated photoprotein could be within the cytoplasm.

Since Ca^{2+} is involved in a number of membrane-fusion events (Duzgune & Ohki, 1977; Papahadjopoulos *et al.*, 1975), it was possible that a large increase in cytoplasmic free Ca^{2+} occurred during liposome-cell fusion resulting in consumption of the obelin. However, this possibility seemed unlikely, since under conditions when obelin was protected from Ca^{2+} by the presence of EGTA within the liposomes, still no cytoplasmic obelin could be detected.

It could be argued that the remaining cytochalasin B-insensitive uptake of liposome obelin was the result of liposome adhesion to the cell surface without fusion occurring, as has been reported with other cell types (Huang *et al.*, 1976; Martin & MacDonald, 1976).

Transfer of adipocyte surface antigens to liposomes

It has been reported that liposome-cell fusion was inhibited by the presence of albumin in the incubation medium (Blumenthal *et al.*, 1977). Liposomes containing EGTA (1 mM) and obelin were therefore incubated with adipocytes in the absence of albumin and presence of external Ca^{2+} (1 mM). A small effect of both anti-erythrocyte and anti-5'-nucleotidase antibody plus complement reaction on obelin luminescence from the cells could be detected. However, it was found that a small proportion (1.5%) of the free liposomes after incubation with cells responded similarly to antibody plus complement (Fig. 6b).

was added, causing stimulation of the remaining obelin within the liposomes. With non-immune serum 0.1% of obelin was consumed, whereas with antibody 1.6% of obelin was consumed during the period. The ordinate is light intensity, measured in luminescent counts/s (c.p.s.).

This effect was able to account for the observed response of anti-cell antibody plus complement on the adipocytes after liposome incubation, owing to the presence of contaminating free liposomes or liposomes adhered to the cell surface. Liposomes incubated in similar conditions without adipocytes did not respond to anti-cell antibody plus complement. In view of the fact that anti-(5'-nucleotidase) antibody does not bind to erythrocytes (Newby *et al.*, 1975), it was concluded that a fraction of at least two membrane antigens had been transferred from some of the cells to some of the liposomes.

Liposomes have been previously used as a means of 'extracting' membrane proteins (Bouma *et al.*, 1977; Huestis, 1977) and also have been reported to take up cell-surface polypeptides (Bierbaum *et al.*, 1979). An important consequence of the insertion of membrane proteins may be the observed increase in Ca^{2+} permeability of the liposomes (Fig. 3).

Conclusions

It was concluded that the association of liposomes with isolated adipocytes caused an increase in the permeability of the liposome membrane to Ca^{2+} and that most of the uptake of liposomes was by endocytosis and adherence to the cell surface. Although no liposome-cell fusion could be detected, the use of obelin as an intraliposomal marker enabled the transfer of a small quantity of cell-membrane antigens to be detected. It would be interesting to investigate further the mechanism of this transfer and whether this phenomenon occurs in any physiological examples of membrane-membrane interaction.

A problem of considerable importance in cell physiology is the lack, at present, of a method for incorporating an indicator of free Ca^{2+} into small cells (Ashley & Campbell, 1979; Campbell *et al.*, 1979a). The results reported here suggest that it may be more fruitful to investigate the fusion of cells with biological membrane vesicles, such as erythrocyte 'ghosts', by using Sendai virus or other fusogens, rather than attempting to fuse directly pure phospholipid vesicles with cells.

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