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Influence of liposome charge on the association of liposomes with Kupffer cells in vitro. Effects of divalent cations and competition with latex particles

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We studied the interaction of large unilamellar liposomes carrying different surface charges with rat Kupffer cells in maintenance culture. In addition to ^{14}C-labeled phosphatidylcholine, all liposome preparations contained either ^{3}H-labeled inulin or ^{125}I-labeled bovine serum albumin as a non-degradable or a degradable aqueous space marker, respectively. With vesicles carrying no net charge, intracellular processing of internalized liposomes caused nearly complete release of protein label into the medium in acid-soluble form, while phospholipid label was predominantly retained by the cells, only about one third being released. The presence of the lysosomotropic agent, ammonia, inhibited the release of both labels from the cells. At 4°C, the association and degradation of the vesicles were strongly reduced. These results are very similar to what we reported on negatively charged liposomes (Dijkstra, J., Van Galen, W.J.M., Hulstaert, C.E., Kalicharan, D., Roerdink, F.H. and Scherphof, G.L. (1984) Exp. Cell Res. 150, 161–176). The interaction of both types of vesicles apparently proceeds by adsorption to the cell surface followed by virtually complete internalization by endocytosis. Similar experiments with positively charged vesicles indicated that only about half of the liposomes were taken up by the endocytic route, the other half remaining adsorbed to the cell-surface. Attachment of all types of liposomes to the cells was strongly dependent on the presence of divalent cations; Ca^{2+} appeared to be required for optimal binding. Neutral liposomes only slightly competed with the uptake of negatively charged vesicles, both at 4°C and 37°C, whereas negatively charged small unilamellar vesicles and negatively charged latex beads were found to compete very effectively with the large negatively charged liposomes. Neutral vesicles competed effectively for uptake with positively charged ones. These results suggest that neutral and positively charged liposomes are largely bound by the same cell-surface binding sites, while negatively charged vesicles attach mainly to other binding sites.

Introduction

Phospholipid vesicles, after systemic administration, are largely cleared by the liver and spleen [1,2]. In liver, considerable amounts of the vesicles are recovered in the non-parenchymal cell fraction [3,4] of which the Kupffer cells are almost exclusively responsible for this uptake [4–6]. In a recent study, we demonstrated that the main mechanism of interaction of liposomes with Kupffer cells in maintenance culture is adsorptive endocytosis [7]. After internalization, the liposomal constituents were degraded within the lysosomal system, as was concluded from morphological observations and the effects of lysosomotropic amines [7,8]. The degradation products of radiolabeled liposome-encapsulated albumin were almost completely released by the cells [8]; water-soluble products aris-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

ing from degradation of radio-labeled liposomal phospholipid were substantially reutilized by the cells for de novo synthesis of cellular phosphatidylcholines [9].

Our previous investigations were confined to negatively charged vesicles. In this paper we report on the uptake and degradation of neutral and positively charged liposomes by cultured Kupffer cells. In an attempt to gain more insight into the details of the interaction mechanism, we compared the effects of divalent cations on the interaction of differently charged vesicles with Kupffer cells and investigated if and to what extent the different vesicle populations mutually compete for binding and uptake.

Materials and Methods

Materials. Egg-yolk phosphatidylcholine (Sigma, type V-E) was labeled in the choline moiety with [^{14}C]methyliodide according to Stoffel [10] and purified as described before [7]. Phosphatidylserine was isolated from bovine brain [11] and converted to the sodium salt [12]. Bovine serum albumin (Sigma, fraction V) was labeled with sodium [^{125}I]iodide according to the method of Greenwood et al. [13]. All radiochemicals were obtained from the Radiochemical Centre, Amersham. Cholesterol (type CH-S), stearylamine, EGTA, Hepes, trypsin (from bovine pancreas, type III), latex beads (0.45 μM) and carboxylated latex beads (0.45 μm) were all purchased from Sigma. Prior to use, the suspensions of latex beads were dialyzed for 48 h against three changes of 10 mM Hepes/135 mM NaCl buffer (pH 7.4).

Isolation and culture of Kupffer cells. The Kupffer cells were isolated from female Wistar rats (170–210 g) by pronase digestion of the liver and purified by centrifugal elutriation according to Knook and Sleyster [14]. $(1.7–2)\cdot 10^6$ cells per 35-mm petri dish were kept in maintenance culture in 1.5 ml culture medium and used for uptake experiments after 2 or 3 days. Further details of cell isolation and culture were as described previously [7].

Liposomes. Large unilamellar vesicles were prepared in 10 mM Hepes/135 mM NaCl buffer (pH 7.4) according to Szoka and Papahadjopoulos [15] as described in more detail before [7]. The vesicles were composed of phosphatidylcholine, cholesterol and phosphatidylserine in a molar ratio of 4 : 5 : 1 (negatively charged vesicles), of phosphatidylcholine and cholesterol in a molar ratio of 5 : 5 (neutral vesicles) or of phosphatidylcholine, cholesterol and stearylamine in a molar ratio of 4.5 : 4.5 : 1 (positively charged vesicles). For preparation of stearylamine-containing liposomes, the buffer capacity was increased to 30 mM Hepes/110 mM NaCl (pH 7.4). The liposomes contained phosphatidyl[^{14}C]choline in addition to encapsulated [^{3}H]inulin or [^{125}I]iodoalbumin when indicated. The vesicle preparations were sized by extrusion through 0.4-μm polycarbonate filters (Unipore, Bio-Rad) [16] and the vesicles were separated from non-entrapped material by gel filtration [7]. The calculated average diameter of the vesicles was about 260 nm, corresponding with an average number of $7.5\cdot 10^8$ vesicles per nmol of total lipid [7]. Small unilamellar vesicles consisting of phosphatidylcholine, cholesterol and phosphatidylserine (molar ratio of 4 : 5 : 1) were prepared by bath sonication (Branson, type B-220) of a suspension of 20 μmol total lipid in 1 ml buffer for 2 h at 15–20°C. Contaminating multilamellar vesicles were removed by sedimentation in a SW 50 rotor (Beckman) for 2 h at 30000 rpm. Small unilamellar vesicles containing 50 mol% cholesterol have a diameter of about 40 nm [17], which corresponds with a calculated number of $3\cdot 10^{10}$ liposomes per nmol of total lipid [18].

Incubations. All incubations were performed in duplicate in 1 ml incubation medium consisting of culture medium without antibiotics or serum [7]. Usually the liposomes were added as concentrated suspensions in buffer after a 15-min preincubation of the cells in incubation medium. For the competition experiments, the liposomes were premixed with the competing vesicles or latex beads in 1 ml incubation medium and the suspensions were added after removal of the preincubation medium. In those cases where the effects of ammonia or EGTA on uptake of liposomes were investigated, these substances were already present during the preincubation. Upon the addition of Ca^{2+} or Mg^{2+} (as 0.25 M solutions of $CaCl_2$ or $MgCl_2$) during incubation of EGTA-containing dishes, 0.5 M NaOH was added to maintain medium pH. For uptake studies at 4°C with cells treated with pro-

teolytic enzymes, the dishes were incubated at 37°C for 30 min in incubation medium containing the indicated amounts of trypsin or pronase. Subsequently, cold incubation medium was added after extensive washing with cold phosphate-buffered saline and, 15 min later, the liposome suspension was added. Medium replacement during incubations and the termination of incubations were done as described before [8]. The amounts of label associated with the cells or present in the medium were determined for each label individually and expressed as nmol of total liposomal lipid per mg of cell protein. The protein content of the dishes amounted to 100–140 μg after 2 or 3 days in maintenance culture.

Other methods. Degradation products of ^{125}I-labeled albumin were detected in the incubation medium as radioactivity in the supernatant after precipitation in 10% (v/v) trichloroacetic acid (final concn.) with 1 mg/ml unlabeled albumin as a carrier. Radioactivities were measured as described previously [7]. Protein was determined by the method of Lowry et al. [19] in 1% sodium dodecyl sulfate. When the samples contained latex beads, these particles were first removed by sedimentation in an Eppendorf table centrifuge. Phospholipid phosphorus was estimated according to Ames and Dubin [20].

Results

Effects of divalent cations and proteolytic treatment on uptake of negatively charged liposomes

The effect of Ca^{2+}, Mg^{2+} and EGTA on the uptake of negatively charged liposomes by Kupffer cells is shown in Fig. 1. Figs. 1A and B show that the uptake of both the encapsulated [^{3}H]inulin and the membranous [^{14}C]phosphatidylcholine labels was substantially reduced in the presence of EGTA. Addition of an excess of Mg^{2+} or Ca^{2+} to EGTA-containing dishes resulted in no effect with Mg^{2+} but in a rapidly restored uptake to control levels with Ca^{2+}. Fig. 1C shows the $^3H/^{14}C$ ratios of the cells. The isotopic ratio in the control incubation slightly decreased initially and then increased during prolonged incubation to approx. 40% over the value of the liposomes before incubation. The initial decrease is partly explained by cell-induced leakage of liposomal contents during binding of vesicles to the cells; the subsequent rise

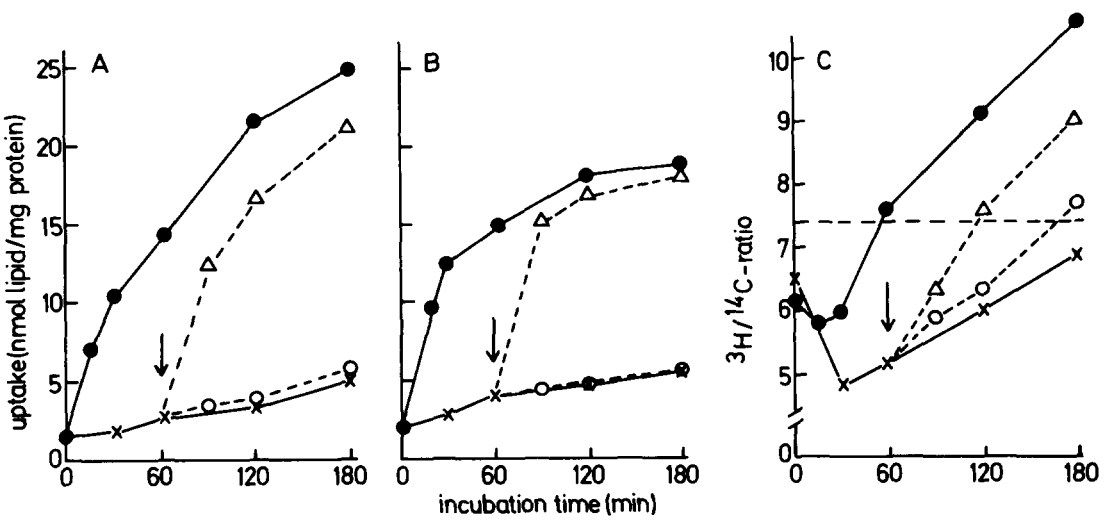

Fig. 1. Effect of divalent cations on uptake of negatively charged large unilamellar vesicles by Kupffer cells at 37°C. Negatively charged liposomes (80 nmol of total lipid) containing [^{14}C]phosphatidylcholine and encapsulated [^{3}H]inulin as markers were incubated at 37°C for the times indicated with $1.8 \cdot 10^6$ Kupffer cells in Dulbecco's modified Eagle's medium (1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}), without further additions (●———●) or with 5 mM EGTA (×——×). After 60 min incubation (arrow), 5 mM Ca^{2+} (△- - - - -△) or 5 mM Mg^{2+} (○- - - - -○) were added to a number of the EGTA-containing dishes. Liposome uptake, either calculated from ^{3}H label (A) or from ^{14}C label (B), was expressed as nmol of total lipid per mg of cell protein. (C) $^3H/^{14}C$ ratios were calculated from the cell-associated radioactivities. The dashed horizontal line represents the isotopic ratio of the liposomes at the time of addition corrected for 2.5 % non-entrapped inulin.

is due to release of degraded lipid label from the cells [7,8]. In the present of EGTA, the initial drop in isotopic ratio was more pronounced, suggesting a more extensive leakage of inulin from the vesicles upon contact with the cells. The slow increase of the $^{3}H/^{14}C$ ratio after 30 min incubation in the presence of EGTA suggests that the small amount of vesicles that become cell-associated is only slowly internalized. Addition of Mg^{2+} to EGTA-containing dishes slightly stimulated the rise in isotopic ratio, while Ca^{2+} restored the ratio increase to control rates.

In Fig. 2 the effect of EGTA on, specifically, vesicle binding to the cell surface [7,8] can be observed, since these experiments were done at 4°C. The association of both labels, which at this temperature was already substantially diminished, was reduced to negligible levels when EGTA was present. As at 37°C, Ca^{2+} restored binding to control levels, indicating that the EGTA effect is reversible; however, of the liposomes adsorbing to the cells at 4°C, only about 40% could be detached with EGTA (results not shown). Pretreatment of the cells at 37°C with 0.05% trypsin or pronase also led to a drastic decrease of liposome binding at 4°C (Fig. 2A, B). The $^{3}H/^{14}C$ ratio of the control incubations at 4°C dropped, as usual, precipitously upon exposure of the liposomes to the cells, but remained constant during prolonged incubation (Fig. 2C). This shows, as reported before [7], that cell-surface induced leakage of inulin also occurs at 4°C during the attachment step but ceases once the liposomes are bound to the cells. Like at 37°C (Fig. 1), the presence of EGTA initially caused a more pronounced drop in isotopic ratio. After proteolytic treatment the isotopic ratio continued to decline slowly after the initial drop, most probably as a result of continued leakage of inulin from the vesicles under these conditions.

Uncharged liposomes

The considerable effects of Ca^{2+} and EGTA on liposome uptake, suggesting involvement of charged groups, led us to an investigation of the effect of liposome charge on the binding and uptake processes. Figs 3A and B show the time-dependent association of lipid and inulin label of

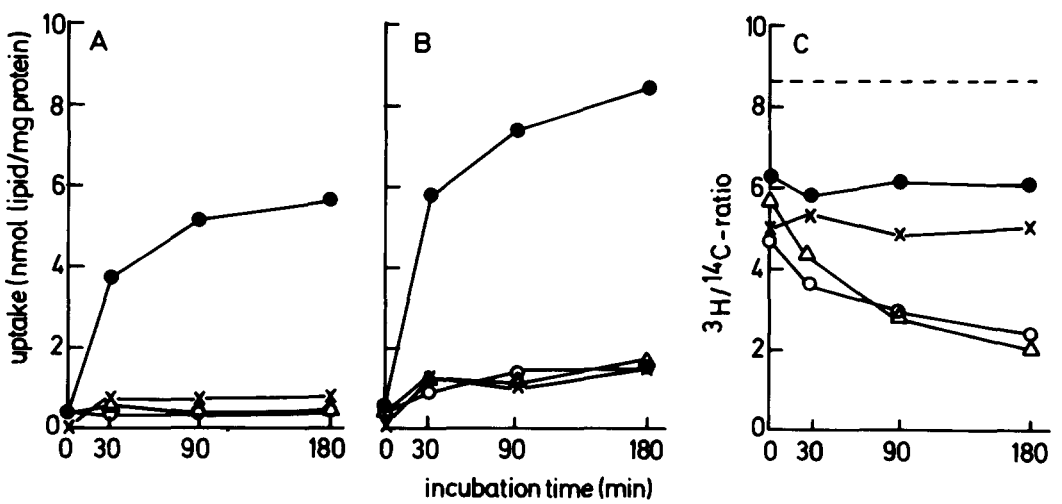

Fig. 2. Effect of proteolytic treatment and divalent cations on binding of negatively charged large unilamellar vesicles at 4°C. $1.8 \cdot 10^{6}$ Kupffer cells were incubated at 4°C for the times indicated with negatively charged liposomes (68 nmol total lipid) containing [^{14}C]phosphatidylcholine and entrapped [^{3}H]inulin as markers; without addition or treatment of the cells (●———●), after pretreatment of the cells with 0.05% trypsin (△———△), after pretreatment of the cells with 0.05% pronase (○———○), or in the presence of 5 mM EGTA (×———×), Liposome uptake, either calculated from ^{3}H label (A) or from ^{14}C label (B), was expressed as nmol of total lipid per mg of cell protein. (C) $^{3}H/^{14}C$ ratios were calculated from the cell-associated labels. The dashed line represents the isotopic ratio of the liposomes at the time of addition corrected for 3% non-entrapped inulin.

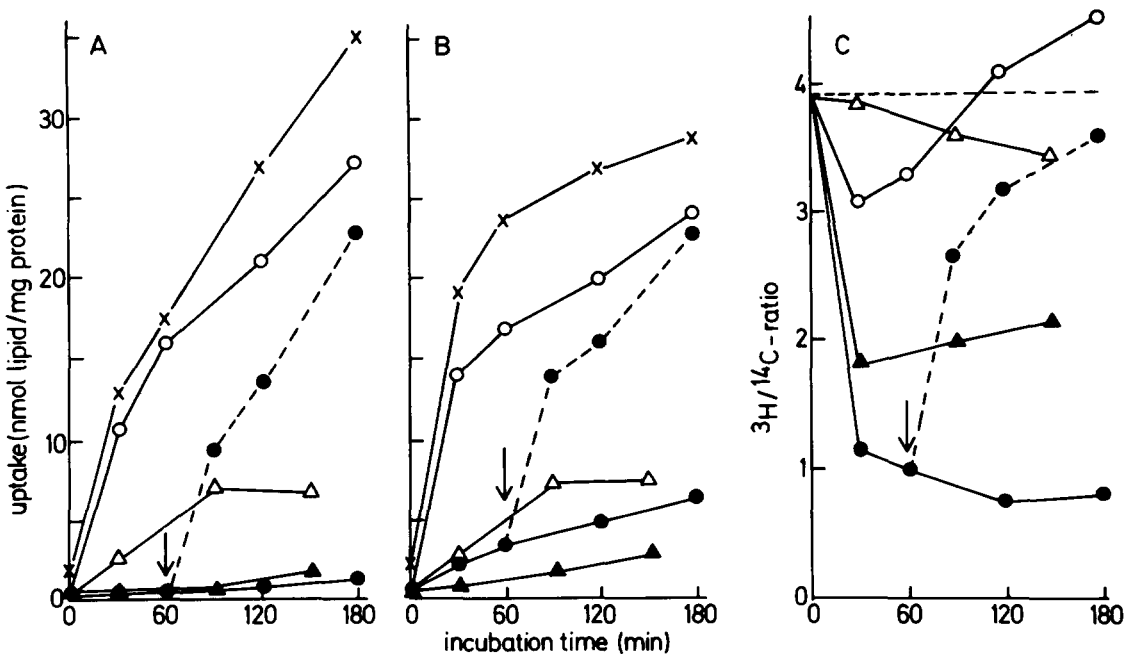

Fig. 3. Uptake of neutral large unilamellar vesicles by Kupffer cells at 4 and 37°C. Effects of divalent cations. Kupffer cells ($1.8 \cdot 10^6$) were incubated for the times indicated with uncharged liposomes (65 nmol total lipid) containing [^{14}C]phosphatidylcholine and entrapped [^{3}H]inulin as markers; at 37°C in normal medium (○——○), at 37°C in the presence of 5 mM EGTA (●——●), at 4°C in normal medium (△——△), or at 4°C in the presence of 5 mM EGTA (▲——▲). After 60 min incubation (arrow) 5 mM Ca^{2+} was added to a number of dishes incubated at 37°C in presence of EGTA (●- - - - -●). In addition, cells were incubated at 37°C with negatively charged liposomes (65 nmol total lipid) containing [^{14}C]phosphatidylcholine and [^{3}H]inulin (×——×). Liposome uptake, either calculated from ^{3}H label (A) or from ^{14}C label (B), was expressed as nmol of total lipid per mg of cell protein. (C) $^3H/^{14}C$ ratios were calculated from the cell-associated radioactivities of the neutral liposomes. The dashed horizontal line represents the isotopic ratio of the neutral liposomes at the time of addition corrected for 4% non-entrapped inulin.

uncharged liposomes with the cells, both at 37°C and 4°C. As for negatively charged vesicles [7], association was strongly reduced at 4°C. To enable direct comparison, negatively charged liposomes were also incubated, at 37°C, with the same Kupffer cell culture; these vesicles were taken up to slightly higher extents than the neutral ones. Also, for the neutral vesicles, the presence of EGTA drastically diminished the association of lipid and inulin label at both temperatures. Again, the effect of EGTA was overcome by an excess of Ca^{2+} (37°C, Fig. 3A, B; 4°C, not shown). The $^3H/^{14}C$ ratio curves, for the control incubations, revealed similar characteristics as were found for negatively charged vesicles: an initial drop followed by a gradual increase. At 4°C the ratio only slowly declined during incubation. The effect of EGTA on the decrease of the ratios at 37 and 4°C was much more pronounced than in the analogous experiments with negatively charged vesicles (Figs. 3C vs. 1C and 2C). At 37°C, the ratio, after a steep drop, remained relatively constant in presence of EGTA during further incubation and increased only if an excess of Ca^{2+} was added.

Intracellular processing of neutral vesicles was examined with liposomes labeled with [^{14}C]phosphatidylcholine and encapsulated [^{125}I]iodoalbumin. Fig. 4 shows the same discrepancy between protein and lipid label uptake as we reported before for negatively charged vesicles [8]. Also, with the neutral vesicles, ammonia was able to raise the association of protein label several-fold to levels comparable with those of lipid label uptake. The association of lipid label was also slightly stimulated by ammonium chloride. The inhibitory effect of ammonium chloride on the degradation of liposomal constituents becomes even more pronounced when, after 60 min of incubation, the

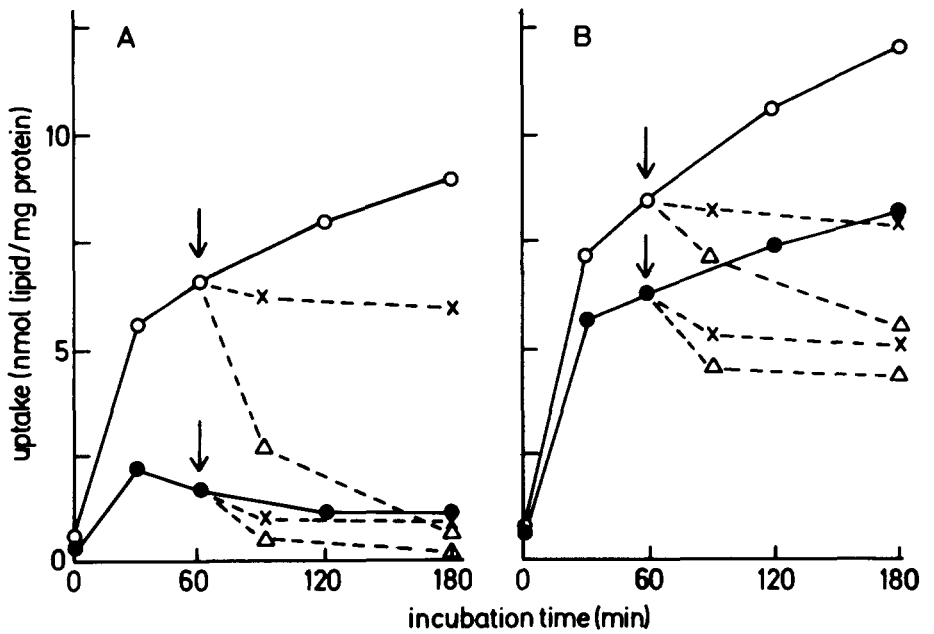

Fig. 4. Effect of NH_4Cl on uptake and release of cell-associated liposomal lipid and protein labels during interaction at 37°C with neutral large unilamellar vesicles. Neutral liposomes (75 nmol of total lipid) containing [^{14}C]phosphatidylcholine and entrapped [^{125}I]iodoalbumin as markers were incubated at 37°C for the time indicated with $1.7 \cdot 10^6$ Kupffer cells in the presence (○———○) or absence (●———●) of 10 mM NH_4Cl. After 60 min (arrow) the medium of a number of dishes was replaced by a liposome-free medium containing 10 mM NH_4Cl (×------×) or no NH_4Cl (△-----△). Liposome uptake was expressed as nmol of total lipid per mg cell protein and was calculated from ^{125}I label (A) or from ^{14}C label (B).

medium is replaced by a liposome-free medium. Without re-addition of ammonia, about 30% of the lipid and about 90% of the protein label was released by the cells during continued incubation, irrespectively of the presence or absence of ammonia during the preceding uptake period. When ammonia was re-added after the medium change, both lipid and protein label maintained nearly constant levels for several hours. In the absence of ammonia between 80 and 90% of the protein label released into the incubation medium is acid-soluble, while in its presence only about half is released in acid-soluble form after medium change (not shown).

Positively charged liposomes

Uptake of lipid and inert inulin label from positively charged liposomes at 37 and 4° C is shown in Fig. 5. In contrast to the results obtained with negatively charged [7] or neutral (previous section) vesicles, the association of both labels at 4°C was only slightly lower than at 37°C. Also, the effect of EGTA on the uptake was less pronounced for the positive vesicles, both at 4 and 37°C. The rates and extents of label association in the presence of EGTA were about equal at 37 and 4°C; the effect of EGTA could, again, be overcome by addition of excess Ca^{2+} (for 37°C, Fig. 5; for 4°C, not shown). The effect of ammonia on liposome processing was again examined with liposomes labeled with [^{14}C]phosphatidylcholine and encapsulated [^{125}I]iodoalbumin. From Fig. 6 it is clear that ammonia increases the extent of association and retention of both labels, although the effect was much less pronounced than with neutral (Fig. 4) or negatively charged vesicles [8]. Removal of liposomes, followed by continued incubation in the absence of ammonia caused the release of large amounts of protein and lipid label. Unexpectedly, addition of ammonia at the medium change failed to achieve complete label retention, as we found for the neutral vesicles (Fig. 4). The explanation follows from the observation that, in the absence of ammonia, as much as 50–75% of the protein

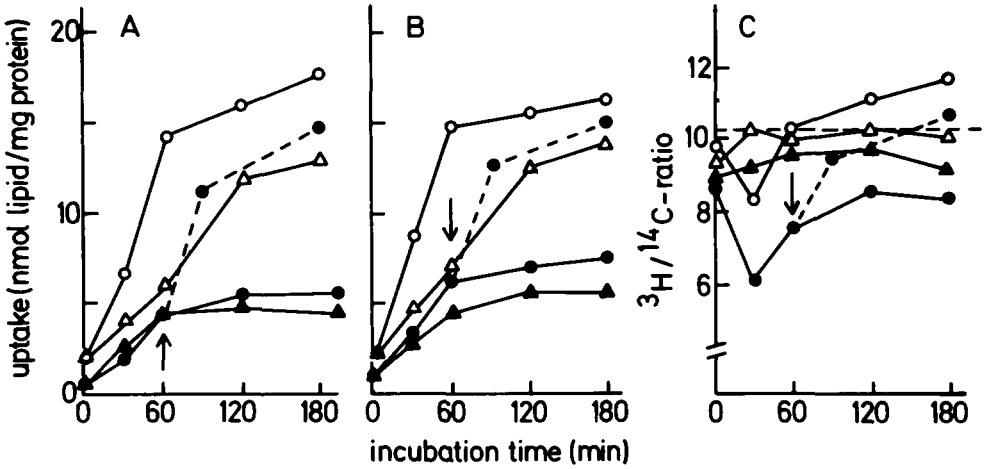

Fig. 5. Uptake of positively charged large unilamellar vesicles by Kupffer cells at 4 and 37°C. Effects of divalent cations. $1.9 \cdot 10^6$ Kupffer cells were incubated for the times indicated with positively charged liposomes (65 nmol total lipid) containing [^{14}C]phosphatidylcholine and entrapped [^{3}H]inulin as markers; at 37°C in normal medium (○———○), at 37°C in the presence of 5 mM EGTA (●———●), at 4°C in normal medium (△———△), or at 4°C in the presence of 5 mM EDTA (▲———▲). After 60 min (arrow) 5 mM Ca^{2+} was added to a number of dished incubated at 37°C in the presence of EGTA (●- - - - -●). Liposome uptake, either calculated from ^{3}H label (A) or from ^{14}C label (B), was expressed as nmol of total lipid per mg of cell protein. (C) $^3H/^{14}C$ ratios were calculated from the cell-associated radioactivities. The dashed horizontal line represents the isotopic ratio of the liposomes at the time of addition corrected for 5% non-entrapped inulin.

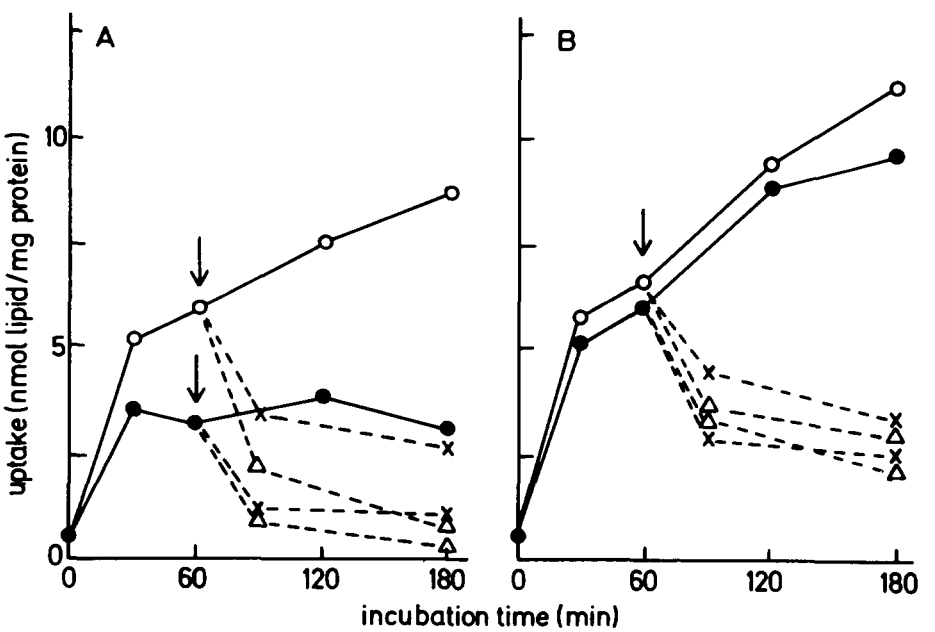

Fig. 6. Effects of NH_4Cl on uptake and release of cell-associated liposomal lipid and protein labels during interaction of positively charged unilamellar vesicles with Kupffer cells at 37°C. Kupffer cells ($1.8 \cdot 10^6$) were incubated at 37°C for the times indicated with positively charged liposomes (75 nmol total lipid) containing [^{14}C]phosphatidylcholine and encapsulated [^{125}I-iodoalbumin as markers in the presence of 10 mM NH_4Cl (○———○) or in the absence of NH_4Cl (●———●). After 60 min (arrow) the medium of a number of dishes was replaced by a liposome-free medium containing 10 mM NH_4Cl (×- - - - -×) or no NH_4Cl (△- - - - -△). Liposome uptake was expressed as nmol of total lipid per mg of protein and calculated from ^{125}I label (A) or from ^{14}C label (B).

label appearing in the medium was acid-insoluble (not shown). This suggests that a major fraction of the radioactivity, released into the medium after medium change, represents intact vesicles which were not internalized but, rather, had remained adsorbed on the cell surface.

Competition for uptake by unlabeled liposomes and latex beads

To investigate the possibility that vesicles of different charges interact, at least in part, with different cell-surface structures, we estimated the effect of neutral vesicles on the uptake of negatively charged vesicles. Fig. 7A, B shows that, both at 4 and 37°C, even a large excess of neutral liposomes reduced the uptake of negatively charged ones only moderately. At equal vesicle-lipid concentration, i.e., 50 nmol per dish, the reduction in uptake of the labeled liposomes did not exceed 10%. In view of our observation (Fig. 3) that rate and extent of uptake of neutral vesicles are of the same order of magnitude as those for negatively charged ones, this lack of severe competition is suggestive of the existence of different binding sites for the two vesicle types. Negatively charged small unilamellar vesicles (mean diameter 0.04 μm), however, competed very efficiently with large vesicles of the same lipid composition. At equal lipid concentrations the association was reduced to about 50% at 37°C and even to 20% at 4°C. Fig. 7 also shows that this competitive effect is not specific for liposomes. Increasing amounts of negatively charged latex beads (0.45 μm) also competed very efficiently for uptake with the liposomes at both temperatures. In the presence of $3.8 \cdot 10^{10}$ latex beads, a number which roughly equals the number of liposomes in 50 nmol of total lipid [7], the association of the vesicles was reduced to 50% of the control at 4°C and to 40% at 37°C, when association was calculated from lipid label uptake. At 37°C, there is a substantial discrepancy between the ^{3}H and ^{14}C labels. This is most probably due to partial release of liposomal contents as is indicated by the decreased $^{3}H/^{14}C$ ratios of the cell-associated labels in the presence of latex beads (not shown). Apparently, the com-

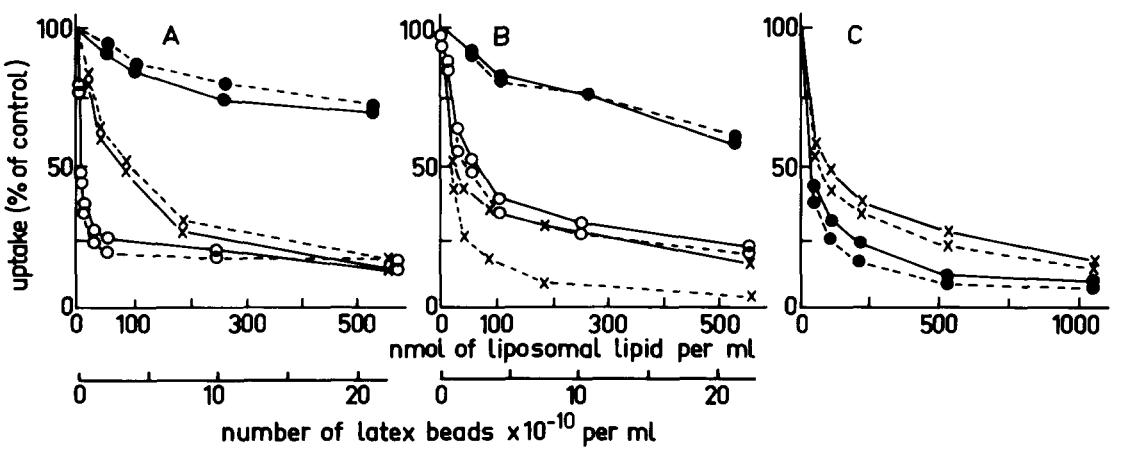

Fig. 7. Uptake of negatively and positively charged labeled liposomes; competition with different types of unlabeled liposomes and with latex beads. (A and B) $1.7 \cdot 10^6$ Kupffer cells were incubated at 4°C (A) or at 37°C (B) with negatively charged large unilamellar liposomes containing [^{14}C]phosphatidylcholine and entrapped [^{3}H]inulin as markers for 90 min in the presence of indicated amounts of unlabeled neutral large unilamellar liposomes (●), unlabeled negatively charged small unilamellar liposomes (○) or negatively charged (0.45 μm) latex beads (×). Uptake of the labeled liposomes was expressed as a percentage of the amounts taken up during a control incubation without competing liposomes or latex beads and calculated from ^{14}C label (———) or from ^{3}H label (- - - - -). (C) $1.9 \cdot 10^6$ Kupffer cells were incubated for 90 min with positively charged large unilamellar liposomes (40 nmol of total lipid) containing [^{14}C]phosphatidylcholine and entrapped [^{3}H]inulin as markers, in the presence of the indicated amounts of unlabeled neutral large unilamellar liposomes at 4°C (●) or at 37°C (×). Uptake of the labeled liposomes was expressed as a percentage of the amounts taken up during a control incubation in the absence of competing neutral liposomes and calculated from ^{14}C label (———) or from ^{3}H label (- - - - -).

petitive effect of the latex beads is closely associated with their negative charge, since unmodified, neutral latex beads of the same diameter did not reduce the association of the negatively charged liposomes at all (not shown). From Fig. 7C it is clear that the neutral vesicles competed effectively with the positively charged vesicles, at 4°C somewhat more strongly than at 37°C. At about equal lipid concentrations (50 nmol neutral vesicles) the association dropped to about 40–60% of the control value, suggesting that both vesicle types interact with the same binding sites at the cell surface.

Discussion

In this study we demonstrated that liposomes carrying no net charge or a positive charge are taken up by cultured Kupffer cells by a mainly endocytic mechanism which shares several characteristics with the uptake of negatively charged liposomes as previously reported [7,8]. Taking into account the fact that, of the positively charged vesicles, a substantial proportion remains adsorbed outside the cells without being internalized, while, much like the negatively charged vesicles, nearly all of the neutral ones that become cell-associated are internalized, our results indicate that the most avidly endocytosed liposomes are the negatively charged ones. This is in agreement with results obtained by others for the interaction of multilamellar or large unilamellar vesicles with other phagocytic cells [21–26]. From our binding experiments at 4°C we conclude that the adsorption of positive vesicles to the cell surface is as strongly dependent on bivalent cations (Ca^{2+}) as that of negatively charged or neutral vesicles. The decreased uptake at 37°C of all three types of vesicles in the presence of EGTA may, likewise, be explained as resulting from diminished binding, but we cannot exclude the possibility that extracellular bivalent cations are also involved in the internalization process per se. During phagocytosis of albumin-coated paraffin-oil particles or glutaraldehyde-treated erythrocytes by alveolar or peritoneal macrophages, the internalization phase of the process rather than the attachment phase was stimulated by Ca^{2+} or Mg^{2+} [27,28]. On the other hand, in receptor-mediated endocytosis of several proteins, the binding step was Ca^{2+}-dependent [29–35]. Moreover, ligand binding to isolated low-density lipoprotein and asialoglycoprotein receptors was also shown to require Ca^{2+} [36–38]. Conceivably, Ca^{2+} ions provide optimal receptor affinity, although recently the interesting possibility was suggested that Ca^{2+} also may control the number of receptors which are oriented at the exterior of the cell [37,39]. Other studies on liposome-cell interactions have resulted in inconsistent reports concerning Ca^{2+} or Mg^{2+} requirements. While some suggest that these ions have no effect at all [22], others report quantitatively varying degrees of influence [40–42] depending on cell type and liposome composition.

Although in our study we observed a strong Ca^{2+} requirement for binding and uptake, EGTA failed to remove adsorbed vesicles quantitatively from the cell surface; this implies that, unlike in studies on the receptor-mediated endocytosis of proteins [30,32–34], EGTA treatment of the cells cannot be applied to assess the fraction of cell-surface-bound vesicles. The very limited extent to which we found uncharged liposomes competing with negatively charged ones for uptake by Kupffer cells, together with the observation that labeled uncharged liposomes do become internalized by the cells only slightly less efficiently than negatively charged ones, suggests that uptake of these two vesicle types is not mediated by the same cell-surface structures. On the other hand, our results seem to indicate that positively charged and neutral vesicles do attach, for the greater part, to identical binding sites. Studies on liposome uptake by African green monkey kidney cells also led to the conclusion that neutral and negatively charged liposomes associate with different cell-surface structures [43]. Although the involvement of an endocytic process was clearly demonstrated in this work [44], the quantitative extent to which this mechanism contributed to the liposome uptake was not assessed. The strong competition we observed between negatively charged small and large liposomes suggests that these vesicles interact with identical binding sites. Recently, we reported on the ability of Kupffer cells to take up negatively charged small unilamellar vesicles [45]. The specific involvement of the negative charge of the liposomes is strongly suggested by our experiments with carboxylated latex beads as a competitive

agent. Fraley and co-workers suggested that negatively charged liposomes might be recognized by the so-called scavenger receptors [43], which bind molecules carrying multiple negative charges [46]. This probably does not apply to Kupffer cells in view of our observation that Ca^{2+} is involved in the attachment phase, whereas scavenger receptor-mediated uptake of acetylated- and malondialdehyde-treated low-density lipoprotein by non-parenchymal liver cells and cultured monocytes, respectively, was reported to be Ca^{2+}-independent [47,48]. Although detailed knowledge of the surface structures involved in liposomal binding awaits further experimentation, our observation that the binding capacity of Kupffer cells for liposomes is trypsin- and pronase-sensitive indicates the proteinaceous character of these structures.

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