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Interaction of RGD Liposomes with Platelets

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The interactions of platelets and liposomes with the tripeptide arginine-glycine-aspartic acid (RGD) as a surface ligand (RGD liposomes) were studied. The results suggest that the presence of the RGD ligand on the liposomes results in receptor-mediated mixing of lipid and aqueous contents of the liposomes and platelets. Mixing of the lipid and aqueous contents of RGD liposomes with platelets is approximately 4–9 times and 3–4 times greater than that for unlabeled liposomes, respectively. Measurements of the cytoplasmic free Ca^{2+} concentration in platelets, $[Ca^{2+}]_i$, show that the RGD liposomes have little effect on $[Ca^{2+}]_i$. © 1996 Academic Press, Inc.

Recently, liposome-platelet interactions have focussed on the phagocytosis of liposomes by platelets and the role of platelets in clearing liposomes from the circulation (1-5). In order to enhance liposome-platelet interactions, we prepared arginine-glycine-aspartic acid (RGD)-conjugated liposomes. A family of extracellular matrix adhesion molecules including fibronectin, vitronectin, and fibrinogen, which act on many different cell types, have been demonstrated to contain the adhesive tripeptide RGD (6). Cell surface receptors of platelets bind to the RGD sequence of small peptides in an activation-independent manner (14). In this paper, we present the mixing of the lipid and aqueous contents of RGD-liposomes with platelets, and the changes in cytoplasmic free Ca^{2+} concentration of platelets in contact with the liposomes.

MATERIALS AND METHODS

Control liposome preparation. Negatively charged liposomes (DMPG liposomes) were made with dimyristoyl phosphatidylglycerol (DMPG), dimyristoyl phosphatidylcholine (DMPC) and cholesterol (CHO) in the molar ratio 1:6:3. Neutral liposomes (DMPC liposomes) were made with DMPC and CHO in the molar ratio 2:1. The liposomes were prepared by reverse-phase evaporation (8) in Hepes buffer (5 mM Hepes, 5 mM sodium acetate, pH 7.4) and the liposome suspensions were passed through a series of Nucleopore polycarbonate membrane filters of subsequently decreasing pore size (in the order of 1 μm, 0.6 μm, 0.4 μm, and 0.2 μm) set in an extruder (Lipex Biomembranes, Vancouver). Phospholipid was quantitated as lipid phosphorus by the method of Bartlett (9).

Preparation of RGD liposomes. N-3-(2-dithiopyridyl) propionyl phosphatidyl ethanolamine (DTP-PE) was synthesized according to the procedure described by Kinsky et al (7). For the preparation of RGD-liposomes, 4 mol% of DTP-PE was incorporated in the liposome bilayers (DTP-PE liposomes). After filtration through polycarbonate membranes, GRGDSPC peptide was added to DTP-PE liposome preparations and incubated overnight at room temperature. Thereafter, the concentration of the released pyridine-2-thione was determined spectrophotometrically at 343 nm (molar extinction coefficient 8.08×10^3 $M^{-1}cm^{-1}$). This concentration is equivalent to the concentration of 2-pyridyl disulfide residues substituted in DTP-PE-liposomes by GRGDSPC peptide. RGD-liposome preparations were dialysed in Hepes buffer. Efficiency of peptide-liposome conjugation was 42.6 ± 5.5 %. For the lipid mixing measurement determined by the resonance energy transfer (RET) assay, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidyl ethanolamine (N-NBD-PE) and N-(lissamine Rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) were incorporated in the liposome bilayers. For the measurement of the mixing of aqueous contents, liposomes containing the pH-sensitive dye pyranine (pyranine-liposomes) were prepared in 35 mM pyranine and unencapsulated pyranine was separated from liposomes by gel filtration on a Sephadex G-75 column (12).

Preparation of Fura-2-loaded platelets. Platelets were isolated from bovine blood that was collected into an acid-

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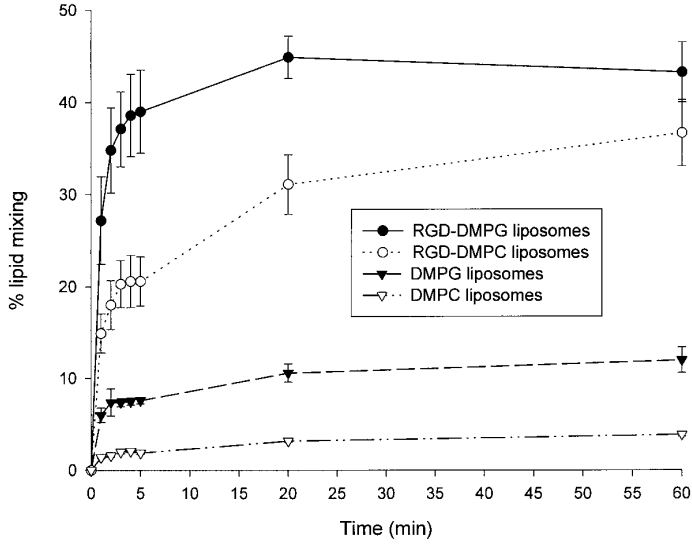

FIG. 1. Extent of lipid mixing of liposomes with platelets. Total amount of liposome lipid, 0.1 μmol/ml; platelet concentration, 1×10^8/ml; mean of 4 experiments in Hepes buffer at room temperature, pH 7.40, with the standard error.

citrate-dextrose anticoagulant (6:1, v/v). Isolated platelets were incubated with 5 μM Fura-2 AM for 1 hr at 37°C, in Tyrode buffer, pH 7.35. At the end of the incubation time, the platelets were washed with Tyrode buffer by centrifugation twice (183 $\times$ g for 15 min at 37 °C). Fura-2 loaded platelets were resuspended in Tyrode buffer and the concentration was determined by the Coulter Cell Counter (Coulter Electronic, Hialeah, FL).

Fluorescence measurements. The RET assay continuously monitors the reduction of energy transfer between N-NBD-PE and N-Rh-PE as the two probes diffuse from the labeled liposomes into the platelet membranes (10). The increase of the N-NBD-PE fluorescence was measured in a mixture of fluorescence-labeled liposomes and platelets in Hepes buffer. The excitation and emission wavelengths were 465 nm and 530 nm, respectively. The initial fluorescence of the solution was taken as the zero level. The level of infinite dilution (100% fluorescence) was obtained after disruption of the liposomes and platelets with 1% (w/v) Triton X-100.

For the measurement of changes in the intracellular platelet calcium concentration after contact with the liposomes, fluorescence measurements of a Fura-2 loaded platelet suspension, mixed with the liposomes in Hepes buffer, were made using a Perkin-Elmer Intracellular Biochemistry Application (ICBC) system with magnetic stirring. The changes in fluorescence were monitored continuously at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. The cytoplasmic free calcium concentration, $[Ca^{2+}]_i$, was calculated as described by Tsien (11).

For the measurement of the mixing of aqueous contents, fluorescence excitation spectra were measured at 510 nm emission, after incubation of pyranine-liposomes with platelets at 37 °C for various times. Pyranine exhibits two major fluorescence maxima depending on pH. The peak at 403 nm is maximal at low pH, while the peak at around 455 nm is maximal at high pH. The fluorescence at 413 nm is relatively pH independent and is used to standardize the concentration of pyranine associated with the cell. The fraction of liposomes taken up by platelets and delivered to an acidic (pH 6.0) environment was calculated using the 455/413 nm ratio and the following equation (4):

$$\text{fraction phagocytosed} = (R_{\text{pH 7.4}} - R_{\text{measured}}) / (R_{\text{pH 7.4}} - R_{\text{pH 6.0}})$$

where R is the 455/413 nm ratio.

RESULTS AND DISCUSSION

Lipid mixing of RGD liposomes with platelets. Fig. 1 shows the extent of lipid mixing of liposomes with platelets. Lipid mixing of RGD-DMPC liposomes and RGD-DMPG liposomes with platelets is approximately 9 times and 4 times greater than that for unlabeled liposomes (DMPC and DMPG liposomes), respectively. It has been shown that primary platelet aggrega-

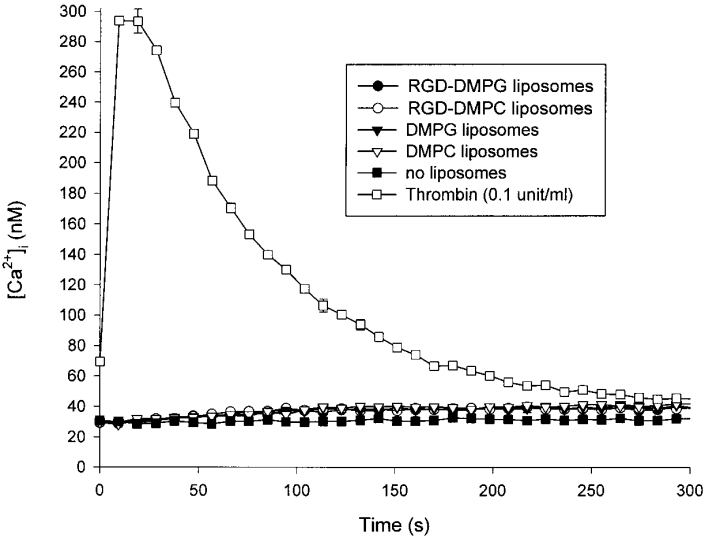

FIG. 2. Changes in $[Ca^{2+}]_i$ in the presence of liposomes. Total amount of liposome lipid, 0.1 μmol/ml; platelet concentration, 1×10^8/ml; mean of 3 experiments in Hepes buffer/1 mM Ca^{2+} at room temperature, pH 7.40, with the standard error.

tion is dependent on the binding of fibrinogen to platelet membrane GPIIb-IIIa (13). Efficient binding of large GPIIb-IIIa ligands, such as fibrinogen or the anti-GPIIb-IIIa monoclonal antibody, requires platelet activation by agonists such as ADP or thrombin. In contrast, small fibrinogen-mimetic ligands, such as RGD-containing synthetic peptides, can bind to GPIIb-IIIa in an activation-independent manner (14). For receptor-mediated platelet activation, a stimulatory signal is transmitted from the outside to the inside of the cell through the plasma membrane. The signal is then transferred to so-called second messengers, which induce a variety of cellular responses (15). One of the common second messengers is calcium, which is released from internal stores into the cytoplasm and/or is taken up from the external medium (16). Therefore, elucidation of the platelet activation related to intracellular calcium levels is important in designing non-thrombogenic materials. In order to determine the thrombogenicity of the liposomes, fluorescence measurements of Fura-2-labeled platelets mixed with liposomes were made. The changes in the cytoplasmic free calcium concentration in platelets, $[Ca^{2+}]_i$, in the presence of 1 mM external Ca^{2+}, are shown in Fig. 2. The changes in $[Ca^{2+}]_i$ stimulated by 0.1 unit/ml thrombin were also examined as a control. $[Ca^{2+}]_i$ goes from a resting level of approximately 30 nM up to 300 nM after stimulation of platelets by thrombin. On the other hand, the increase in $[Ca^{2+}]_i$ in the presence of RGD-liposomes is similar to both the $[Ca^{2+}]_i$ increase for unlabeled liposomes, and the increase observed in the absence of liposomes, suggesting that lipid mixing of RGD-liposomes and platelets is induced by the binding of liposome surface RGD ligand to GPIIb-IIIa without causing platelet activation.

In vitro uptake of liposomes by bovine platelets. Fig. 3 shows the percentage of liposomes taken up by bovine platelets. Uptake of RGD-liposomes is 3-4 times greater than that for unlabeled liposomes. In order to determine whether phagocytosis of the liposomes contributes to the changes in fluorescence of pyranine, pyranine-liposomes were incubated with platelets in the absence of Ca^{2+} and the fluorescence excitation spectra were measured, since it has been reported that the addition of EDTA inhibits the phagocytosis of particles by platelets (17). As shown in Fig. 3, the percentage of phagocytosed RGD-liposomes is significantly reduced in the absence of Ca^{2+}, suggesting that the fluorescence changes of pyranine-liposomes

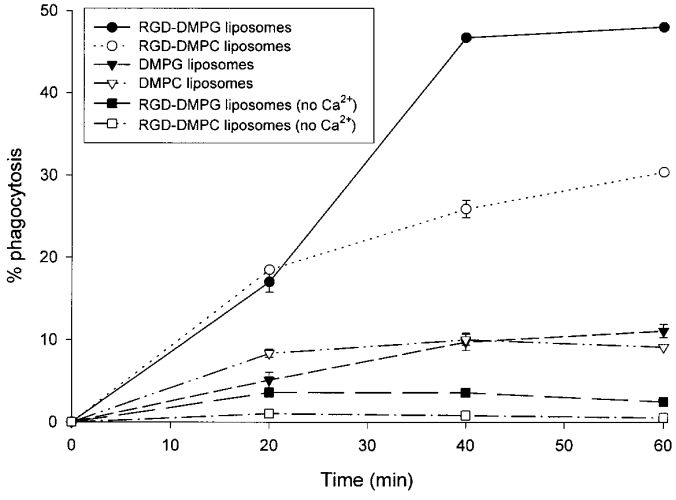

FIG. 3. Percentage of liposomes taken up by platelets. Total amount of liposome lipid, 0.1 μmol/ml; platelet concentration, 1×10^8/ml; mean of 3 experiments in Hepes buffer at room temperature, pH 7.40, with the standard error.

are mainly due to phagocytosis of the liposomes. These results indicate that the phagocytosis of RGD-liposomes by platelets takes place in the early stages of the liposome-platelet interaction, and that the liposomes are decomposed in an acidic environment.

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