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CATIONIC POLYPEPTIDE-INDUCED FUSION OF ACIDIC LIPOSOMES

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Fusion of acidic liposomes was induced by Mg^{2+}, Ca^{2+}, polylysine and polymyxin B. The extent of fusion and the concomitant change in liposome permeability induced by divalent cations depended on the concentration of liposomes in the suspension as well as on the cation concentration. In contradistinction, the extent of fusion and the change in permeability induced by the polypeptides depended only on the polycation concentration. The difference in the pattern of interaction, between the liposomes and the various cations, is a result of different binding affinities. The binding of the polypeptides to the liposomes, in contrast to divalent cations, is practically irreversible. The potential of polylysine to induce fusion of acidic phosphatidylethanolamine-devoid liposomes was used to demonstrate that in order to obtain fusion, both membranes involved must be susceptible, at least to a certain degree, to fusion by the proper inducer. When lysophosphatidylcholine substituted for phosphatidylcholine in phosphatidylethanolamine-rich acidic liposomes, extensive polylysine-induced fusion was obtained without concomitant spillage of the liposome contents.

Introduction

Fusion of membranes has attracted, for the last decade, the attention of many groups [1]. Most of the work performed on model systems; i.e. liposome fusion, was devoted to divalent cation-induced fusion; the reason being its possible relevance to cellular mechanisms, e.g. exocytosis which depends on the presence of Ca^{2+} [2]. Aspects, investigated, included specificity of cations and their relative efficiency in inducing fusion [3,4], the specificity of membrane components in promoting fusion, e.g. the inhibitory effects of phosphatidylinositol as compared to phosphatidic acid [5], the importance of phosphatidylethanolamine in promoting fusion of system with a low content of acidic components [5–7].

In addition, extensive study was conducted on the physical changes that divalent cations exerted on membranes, e.g. phase transition and phase separation [8], the shift from bilayer to hexagonal arrangement [9,10], or the appearance of lipidic particles [11]. The relevance of the mentioned phenomena to fusion has been thoroughly discussed. Effects similar to those observed with divalent cations, e.g phase separation, have been observed when cationic polypeptides such as polylysine [12] or polymyxin B [13] were used instead of divalent cations. These basic polypeptides have also been used very efficiently in inducing fusion of acidic liposomes [14,15].

In the present work the effects of these cationic polypeptides on liposomes are compared to those of divalent cations. Cationic polypeptides exhibited practically irreversible binding to acidic liposomes. As a consequence the extent of their effect on liposomes depended only on their concentration and not on that of the liposomes.

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Materials and Methods

Calf thymus DNA, cardiolipin, lysophosphatidylcholine and phosphatidylcholine (type VII E) were purchased from Sigma Chemical Co. Polylysine (30–70 kDa) and polymyxin B sulfate were purchased from Chemalog. 6-Carboxyfluorescein was purchased from Eastman and further purified according to Ralston et al. [16]. Phosphatidylethanolamine was purified from soybean phospholipids as described [17]. Phosphatidylpropane-1,3-diol was synthesized from phosphatidylcholine essentially as described [18]. With the addition of an equal volume of propane-1,3-diol to the reaction mixture. All phospholipids were checked for purity on thin-layer chromatography. Phospholipid concentration was determined by total ashing, hydrolysis in 0.5 M HCl and phosphate analysis [19]. The concentration of both phospholipids and liposomes was expressed as mM P_i. Photosynthetic pigment extract was prepared from spinach leaves as described [20]. The chlorophyll content and the ratio of chlorophylls were determined according to Arnon [21].

Fusion assay. Liposomes, of the desired phospholipid mixture, were prepared with or without the addition of 2% (w/w) spinach pigment extract as described [15]. The concentration of pigmented liposomes was 12.5 mM P_i and that of non-pigmented liposomes was 25 mM P_i. The buffer used throughout the work was 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes, pH 7.6), 128 mM KCl and 32 mM NaCl. The fusion assay was carried out as recently described [15], the only modification being the chelation of polylysine by a sonicate of calf thymus DNA (10 mg/ml) instead of by poly(aspartic acid) or poly(glutamic acid).

Permeability assay. Liposomes were prepared as described [14] in a buffer containing also 40 mM 6-carboxyfluorescein. After sonication the samples were passed through a Sephadex G-50 column (1×25 cm) to remove free dye. The assay for changes in permeability was carried out as follows: 1.25 μmol P_i liposomes were incubated in varying volumes (0.1–2.5 ml) with or without 1.5 μmol Mg^{2+} or Ca^{2+} and 50 μg polylysine. After 30 min of the divalent cations were chelated by 2-fold excess of EDTA and polylysine by 2-fold excess of calf thymus DNA sonicate. The change in fluorescence was recorded after adjusting the volume of each sample to 2.5 ml (excitation 491 nm emission 510 nm). All fluorimetric measurements were carried out in an MPF-44B Perkin-Elmer spectrofluorimeter.

Results

The extent of fusion, induced by various cations, applied at fixed charge ratio, was determined as a function of the liposome concentration. The extent of divalent cations-induced fusion varied both with the cation and liposome concentration (Fig. 1 a,b). Almost no fusion was detected at liposome concentrations lower than 1 mM even at a charge ratio of 10. In contradistinction, the extent of the polypeptide-induced fusion (polylysine and polymyxin B) depended only on the polycation concentrations (Fig. 1 c,d). Essentially the same results were obtained for a variety of acidic phospholipids (unpublished results).

An identical pattern of interaction between liposomes and cations was observed when the change in liposome permeability was assayed (Fig. 2). Polylysine induced a similar extent of leakage regardless of liposome concentration while the effect of divalent cations increased practically from 0 to 100% over the range of liposome concentration used (0.5–12.5 mM). The effect of Ca^{2+} was greater than that of Mg^{2+} (Fig. 2).

The use of polylysine combined with the chlorophyll dilution assay sheds light on another aspect of membrane fusion. In order to detect fusion between two different populations of liposomes, liposomes known to undergo extensive fusion [7] were prepared containing the pigment extract. These liposomes were incubated with non-pigmented liposomes consisting of phosphatidylcholine and cardiolipin (7 : 3), a lipid composition known to undergo only limited fusion if at all [14,15]. The addition of polylysine to this mixed suspension induced fusion between the two populations (Fig. 3). When the fusible liposomes were in excess an increase in fusion extent was recorded. On the other hand, in the presence of Ca^{2+} almost no fusion was observed, regardless of the type of liposomes being in excess (Fig. 3).

The exchange of phosphatidylcholine for lyso-

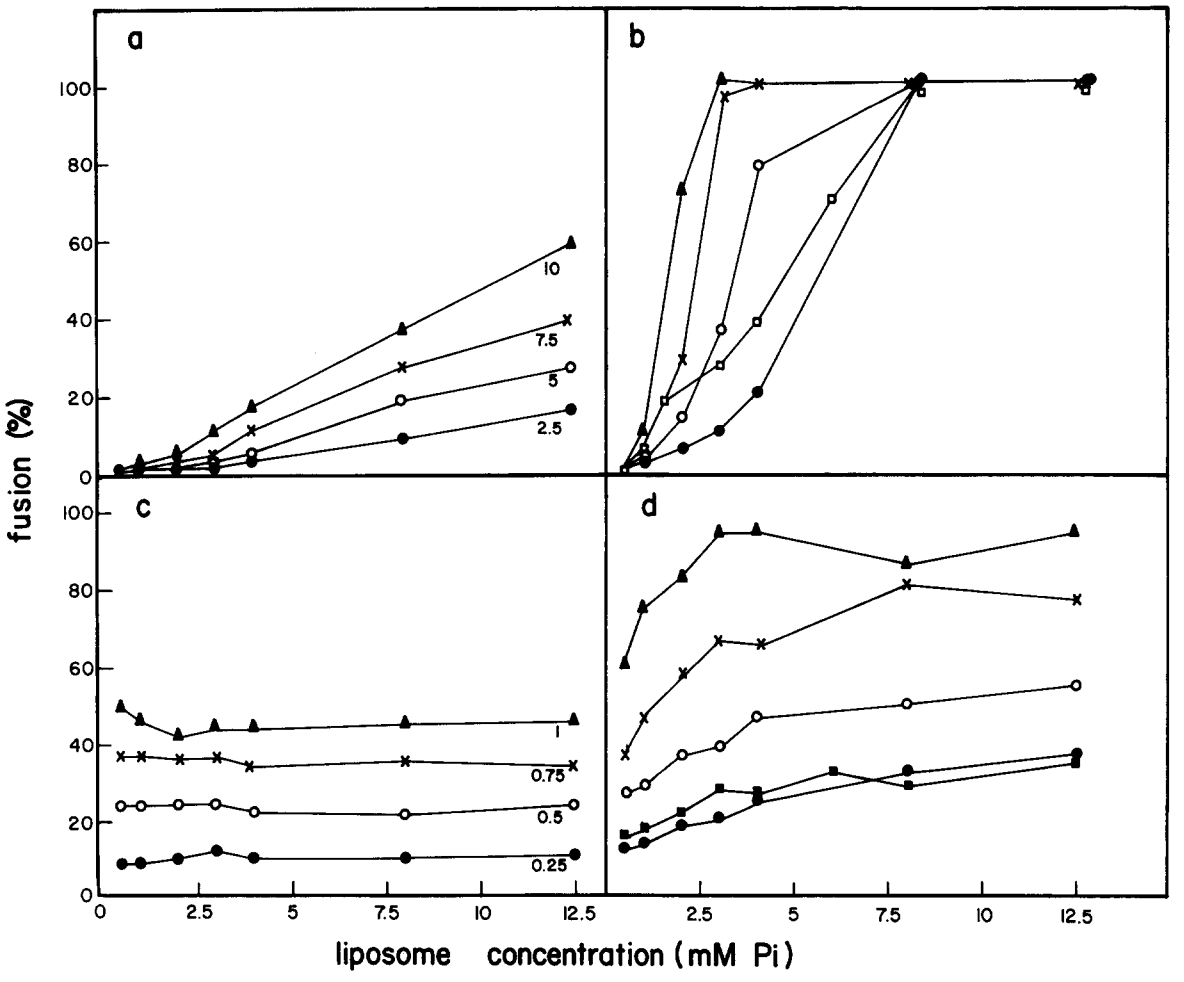

Fig. 1. The effect of liposome concentration on cation-induced fusion at fixed charge ratios. Liposomes (0.125 μmol P_i) composed of 2% spinach pigment extract and phosphatidylcholine/phosphatidylethanolamine/cardiolipin at molar ratios of 5 : 2 : 3 (a, c) or 1 : 6 : 3 (b, d), respectively, were mixed with 10-fold excess of similar non-pigmented liposomes and fixed amounts of $CaCl_2$ (a, b, at charge ratios of 2.5, 5, 7.5, 10), polylysine (c, d, at charge ratios of 0.25, 0.5, 0.75, 1.0) and $MgCl_2$ (b, □, at a charge ratio of 5). Liposomes consisting of 2% spinach pigment extract and phosphatidylcholine/phosphatidylethanolamine/phosphatidylpropane-1,3-diol at a molar ratio of 1 : 6 : 3 were mixed with 10-fold excess of similar non-pigmented liposomes and fixed amounts of polymyxin B (d, ■, at a charge ratio of 0.25). Each of the above mixtures was incubated for 1 h at room temperature in various final volumes i.e. at varying liposome concentrations, yet maintaining the charge ratios fixed. After 1 h incubation $CaCl_2$, $MgCl_2$, and polylysine were chelated and the extent of fusion was determined in each sample.

phosphatidylcholine in phosphatidylethanolamine-rich liposomes practically abolished their capacity to undergo Ca^{2+}-induced fusion (unpublished results). On the other hand, polylysine induced fusion of such liposomes to an extent similar to that of phosphatidylcholine-containing liposomes (Fig. 4). However, the increase in the permeability of liposomes containing lysophosphatidylcholine, induced by polylysine was remarkably lower than the comparable increase in phosphatidylcholine-containing liposomes (Ref. 14, Fig. 4).

Discussion

The efficiency of polycations in inducing fusion of acidic liposomes has already been shown, by several methods to be, on a charge ratio basis,

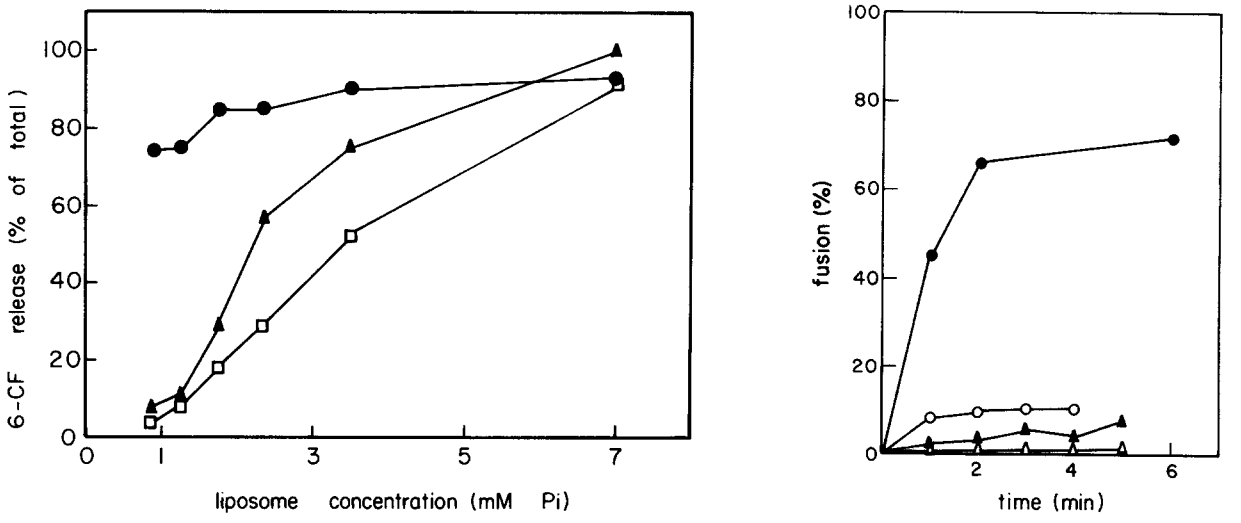

Fig. 2. The effect of liposome concentration on cation-induced leakage of contents. Liposomes composed of phosphatidylcholine/phosphatidylethanolamine/cardiolipin at a molar ratio of 1 : 6 : 3 were prepared in a buffer containing 40 mM 6-carboxylfluorescein. After passage through a Sephadex G-50 column, the liposomes were incubated at varying final lipid concentration for 30 min with $MgCl_2$ (□) or $CaCl_2$ (▲) at a charge ratio of 7.5, and with polylysine (●) at a charge ratio of 1. After the chelation of the cations the change in the fluorescence due to leakage of the entrapped dye was determined.

Fig. 3. Fusion of mixed population of liposomes. Pigmented liposomes consisting of phosphatidylcholine and cardiolipin (7 : 3, ●, ▲), were incubated with 10-fold excess of non-pigmented liposomes consisting of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (1 : 6 : 3) in the presence of $CaCl_2$ (▲, charge ratio 10) or polylysine (●, charge ratio 1). Pigmented liposomes consisting of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (1 : 6 : 3, ○, △) were incubated with 10-fold excess of non-pigmented liposomes, consisting of phosphatidylcholine/cardiolipin (7 : 3) in the presence of $CaCl_2$ at a charge ratio of 10 (△) or polylysine at a charge ratio of 1 (○). At various intervals the extent of fusion was determined.

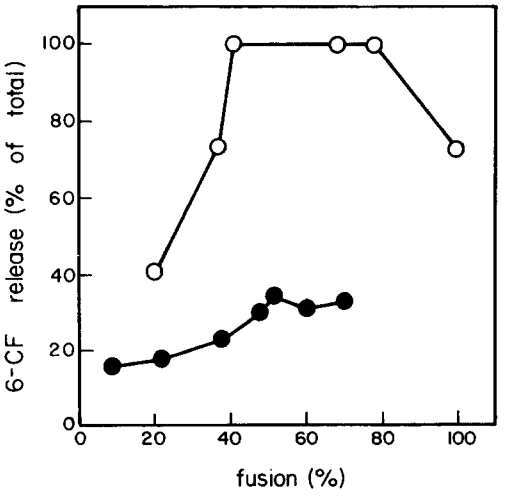

Fig. 4. The effect of lysophosphatidylcholine on the extent of fusion and the leakage of contents induced by polylysine. Pigmented liposomes, non-pigmented liposomes, non-pigmented liposomes and liposomes entrapping 40 mM 6-carboxyfluorescein were prepared consisting of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (○) or lysophosphatidylcholine/phosphatidylethanolamine/cardiolipin (●) at a molar ratio of 2 : 5 : 3. Pigmented liposomes were incubated for 30 min with non-pigmented liposomes in the presence of varying amounts of polylysine. 6-Carboxyfluorescein-containing liposomes were incubated for 30 min with similar amounts of polylysine. After the chelation of the polylysine the extent of fusion and the extent of leakage of the liposomes was determined in each sample.

about an order of magnitude greater than that of divalent cations [14,15]. However, the difference in the potential to induce fusion is not constant. As seen in Fig. 1 the difference was much more pronounced at low lipid concentrations. Thus, if fusion must be induced at low liposome concentrations, polylysine should be favored over divalent cations for its much greater fusogenic potential.

Since the phenomena of fusion and increase in permeability depend on the formation of cation-phospholipid complexes [22], the differences ob-

served, between the cationic polypeptides and divalent cations, must arise from a different character of binding. The fact that the effect of the polypeptides does not depend on liposome concentration suggests that their binding to the liposomes, over the liposome concentration range used, is practically irreversible. This is of course, in contrast to the reversible binding of divalent cations, which have binding constants in the mM range [23]. The fact that polylysine induced precipitation of acidic liposomes at a charge ratio of 1 while divalent cations did so only at charge ratios greater than 10 [14] indicate a much tighter, practically irreversible, binding of polylysine to the liposomes. Further support is obtained from comparing the potential of various cations to compete with Mn^{2+} on binding to liposomes. 20 μM polylysine released the same amount of Mn^{2+}, bound to liposomes, as 2 mM Ca^{2+} (unpublished results).

As already discussed elsewhere [14], the greater potential of polylysine may be attributed to the high local concentration of charge. The association of each charge with the bilayer increases the probability of the next charge on the same molecule to bind. Thus, although each lysine residue possessing a single charge is expected to have a binding constant lower than Na^{+}, which in itself is at least an order of magnitude lower than Ca^{2+}, the molecule as a whole binds practically irreversibly to the bilayer. Polymyxin B, although possessing only five charges at the pH used (in comparison to the 400 of a 50 kDa polylysine) is an extremely efficient inducer of fusion. It exhibits a similar pattern of influence as polylysine. It has already been reported that binding of polymyxin B to liposomes consisting of phosphatidic acid or phosphatidylglycerol, under pH and ionic strength conditions similar to ones used in the present work, was cooperative and almost irreversible even in the presence of Ca^{2+} in the same mM range [24]. The tight binding was attributed to the flat charged hydrophilic portion of the molecule facilitating good contact with the liposome surface. In addition, the hydrophobic tail penetrates the membrane and acts as a lipidic anchor. Hammes and Schuller [25] have shown that polylysine, at pH 7 upon mixing the phosphatidylserine liposomes, underwent a conformational change from a random coil to a helix. A similar effect was obtained upon neutralizing its charges at pH 10. This observation indicates that in the presence of negatively charged liposomes, the basic polypeptide binds tightly to the liposomes and as a consequence its charges are neutralized.

The results presented in Figs. 1 and 2 are seemingly contradictory to a report according to which fusion induced by Ca^{2+} depended on charge ratio and not on the cation concentration [5]. However, this is not the case since in the mentioned report [5] minimal and initial rates of fusion were assayed and in the present work the overall extent was determined.

As seen in Fig. 3 fusion depends both on the phospholipids constituting the liposomes as well as on the suitability of the inducer. Since Ca^{2+} cannot induce fusion of such vesicles with highly fusible ones, e.g. phosphatidylethanolamine-rich [7], even when the latter were in excess. In contrast, polylysine, known to induce limited extent of fusion in phosphatidylethanolamine-devoid liposomes [14,15] was able to induce appreciable fusion when the fusible liposomes were in excess since each event of fusion between a phosphatidylethanolamine-devoid and a phosphatidylethanolamine-rich liposome (which, under the experimental conditions, is the only kind of fusion detected) increased the phosphatidylethanolamine content in the phosphatidylethanolamine-devoid liposomes thus increasing their fusion potential.

Using lysophosphatidylcholine, which is known to promote cell fusion [28], but to inhibit cation-induced liposome/proteoliposome fusion [7], instead of phosphatidylcholine, leads to the construction of a system that undergoes relatively extensive fusion with minimal loss of liposome contents (Fig. 4). This mixture consisting of lysophosphatidylcholine, phosphatidylethanolamine and cardiolipin in a molar ratio of 2 : 5 : 3, respectively, may prove most suitable for the introduction of soluble substances into cells via fusion.

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