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The induction of large unilamellar vesicle fusion by cationic polypeptides: the effects of mannitol, size, charge density and hydrophobicity of the cationic polypeptides

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Polylysine and lysine-based copolymers induced fusion of large unilamellar vesicles only in media containing at least 0.4 M mannitol. In the absence of mannitol, polylysine and certain lysine-based copolymers also containing acidic amino acids were not able to induce fusion. Fusion, in the presence of mannitol, was induced at nanomolar concentrations of the polycations. Excess polymer caused reduced rate and extent of fusion. In the presence of 100 mM NaCl the effective concentration range of the polycations was narrower. Kinetic analysis determined that salt increased the aggregation constant C_{11} while reducing the fusion constant f_{11}. Addition of polylysine in excess resulted in smaller C_{11}. Short polylysine (3500) was less effective on a molar basis than a long one (37 000). Copolymers were more effective than polylysine due to higher aggregation potential. Copolymers were also more effective in promoting Ca^{2+}-induced fusion in the absence of mannitol, their greater efficiency resulting from substantially larger fusion potential, without a greater rate of leakage. Preincubation of the vesicles with the polycations for less than 20 s resulted in faster fusion rates, while longer preincubations caused slower fusion rates. Addition of polycations to the preincubated mixture enhanced the fusion rates, indicating that the polycations were not available, rather than the vesicles being not susceptible to fusion. The effect of preincubation suggests two phases in the binding of the polycations to the vesicles; a fast phase of partial binding and a slower phase resulting in complete binding. The addition of millimolar concentrations of pyrophosphate or sulphate provided a fine control of the effective polycation concentration and its effect on fusion.

Introduction

Since the early work of Hammes and Schullery [1] that, indirectly, described liposome fusion induced by polylysine, liposome fusion induced by polycations has been demonstrated for basic polypeptides and poly-amino acids of various sizes [2–7], polymyxin B [4,5], melittin [8], cytochrome *c* [3] and polyamines [9]. Most of the work focused on negatively charged small unilamellar vesicles. When compared with Ca^{2+} or Mg^{2+}, the potency of these polycations was remarkably greater in terms of the concentrations needed to induce fusion [4,5,8]. Polyamines were able to promote Ca^{2+}-induced fusion of LUV by lowering the cation's threshold concentration. Polyamines in-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; N-NBD-PE, *N*-(7-nitro-1,2,3-benzoxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine (egg yolk).

duced fusion of acidic liposomes when phosphatidylethanolamine was included in them [9]. Similarly, polylysine was shown to promote fusion of acidic liposomes with Ca^{2+} as the inducer [10]. Polylysine facilitated Ca^{2+}-induced fusion when Ca^{2+} concentrations an order of magnitude lower were used. Polycations such as polylysine may be of great importance as inducers of membrane fusion, since they may pro-mote liposome–cell interactions, e.g., for the introduction of macromolecules into cells or membrane proteins into target cell membranes. However, so far no induction of fusion by polylysine has been recorded in liposome–cell systems.

In the present work the requirement for high concentrations of non-electrolytes, e.g., mannitol or sucrose, is demonstrated. The effect of size, charge density and hydrophobicity of polylysine and its copolymers is described. It appears that copolymers are at least as effective as polylysine of the same molecular weight, both in inducing fusion and in promoting Ca^{2+}-induced fusion. From the preincubation experiments we determined the existence of two phases of polylysine binding. The effects of the various polycations and salt are discussed in context with the mechanism of polycation-induced fusion.

Materials and Methods

Materials

Phosphatidylcholine (PC), cardiolipin, cholic acid and all the polylysine (3500, 14000, 37000 M_r) and the copolymers species (Lys, Ala 1 : 1, 37000; Lys, Ala 3 : 1, 37000; Lys, Phe 1 : 1, 40000; Lys, Ala, Glu, Tyr 5 : 6 : 2 : 1, 52000 M_r) were purchased from Sigma Chemicals Co. N-NBD-PE and N-Rh-PE were purchased from Avanti Polar Lipids. The detergent Ammonyx LO was purchased from Onyx Corp. 5(6)-Carboxyfluorescein was purchased from Eastman and was further purified according to Ralston et al. [11]. The poly-amino acids were suspended in buffer and kept in small aliquots at −18°C. None of the aliquots were refrozen, since degradation occurred during repetitive thawing. Cholic acid was further purified by twice recrystallizing it from ethanol [12]. All phospholipids were assayed for purity on thin layer chromatography and proved to be at least 99% pure. Phospholipid concentration was determined by the concentration of phosphate [13]; thereafter, liposome concentration was given as lipid phosphorus content.

Liposome preparation

The NBD/Rh resonance energy transfer fusion assay [14] was used to monitor membrane fusion. LUV, composed of PC : cardiolipin (1 : 1 molar ratio), were prepared with or without 0.5 mol% of each fluorescent probe, by the reverse-phase evaporation technique [15], followed by extrusion through 0.4 and 0.2 μm polycarbonate membranes [16]. LUV were prepared in 0.6 M mannitol/0.1 mM EDTA/5 mM Hepes (pH 7.4), from here on referred to as buffer 1 (no salt), or in 0.4 M mannitol/0.1 M NaCl/0.1 mM EDTA/5 mM Hepes (pH 7.4), from here on referred to as buffer 2 (with salt), or in 100 mM NaCl/5 mM Hepes (pH 7.4)/0.1 mM EDTA for the Ca^{2+}-induced fusion experiments.

The Tb-dipicolinic acid fusion assay [17] was used to confirm all the results of the NBD/Rh resonance energy transfer assay, so as to exclude artefacts of molecular transfer and any other non-fusion membrane mixing. LUV were prepared as previously described [10] in buffers 1 and 2.

Fusion assay

All measurements were carried out at 26°C in an SLM 4800 spectrofluorometer. In the NBD/Rh resonance energy transfer fusion assay, labelled and unlabelled liposomes were mixed, the unlabelled being in a 2–4-fold excess, and were added last to the thermally controlled, stirred fusion mixtures in the fluorometer. Liposome concentration was routinely 50 μM lipid phosphorus unless otherwise mentioned. Membrane mixing was monitored by the dequenching of NBD fluorescence indicating a decrease in the resonance energy transfer efficiency due to dilution of the probes upon fusion. The system was calibrated by comparing the readings with those after the addition of detergent, 0.1% Ammonyx LO. Ammonyx LO was the detergent of choice for the NBD/Rh resonance energy transfer assay, since it caused no quenching of the fluorescence such as Triton X-100 or cholate did. Excitation and emis-

sion wavelengths were 450 nm and 530 nm, respectively, with a 520 nm cut-off filter in the path of the emission beam.

The Tb-dipicolinic acid complex was excited at 276 nm and the fluorescence was measured at 545 nm after passing through a Corning 3-68 cut-off filter (> 530 nm). All measurements were carried out at 26°C. Measurements were carried out at liposome concentrations of 50 μM lipid phosphorus, unless otherwise mentioned (for more details see Ref. 10).

Preincubation with polycations

Unlabelled LUV were preincubated for given intervals of up to 90 s with polycations in the cuvette. Subsequently, labelled LUV were added and the change in the fluorescence at 530 nm was recorded. Another aliquot of polycations was added when a marked inhibition of fusion was observed.

Analysis of fusion kinetics

The analysis followed a mass action kinetic model [18–21], which views the overall fusion reaction as a sequence of two gross steps: (1) aggregation, whose rate constant, C_{11}, has the units $M^{-1} \cdot s^{-1}$; and (2) fusion, i.e., membrane destabilization and merging, whose rate constant, f_{11}, has the unit s^{-1}. Dissociation of aggregates with a rate constant, D_{11} (s^{-1}), was explicitly considered.

At the second stage, more accurate numerical calculations were carried out, which enabled an extension up to a fluorescence change of 40% [22]. In these calculation deaggregation was explicitly accounted for. All the data presented in Tables II and III were obtained from the extended calculations.

Leakage of liposome contents

LUV were prepared as above, also containing 20 mM 5(6)-carboxyfluorescein. After passing through a Sephadex G-75 column to remove untrapped 5(6)-carboxyfluorescein the vesicles were treated as in fusion experiments. The change in the fluorescence of 5(6)-carboxyfluorescein due to its dilution upon leaking out of the vesicles was monitored; excitation and emission wavelengths were 493 nm and 516 nm, respectively [23].

Results

The addition of polylysine, as well as its copolymers, of any size to LUV, composed of PC : cardiolipin 1 : 1 (molar ratio) in relatively low osmotic saline solutions, e.g., 0.1 M NaCl, induced appreciable aggregation as observed by a gradual turbidity increase. However, the massive aggregation was not followed by fusion (Fig. 1). Only the poly(Lys, Ala) species induced fusion (see Table III). If, on the other hand, LUV were prepared in at least 0.4 M mannitol or sucrose and the reactions were carried out in similar solutions, the addition of polylysine as well as the other cationic polymers resulted in fusion, as observed by a change in resonance energy transfer or an increase in Tb-dipicolinic acid fluorescence (Fig. 1). Having shown that the effect was not exclusive to mannitol, the rest of the experiments were carried out with mannitol.

In order to elucidate the role of mannitol in enhancing the effect of polylysine, its effect was

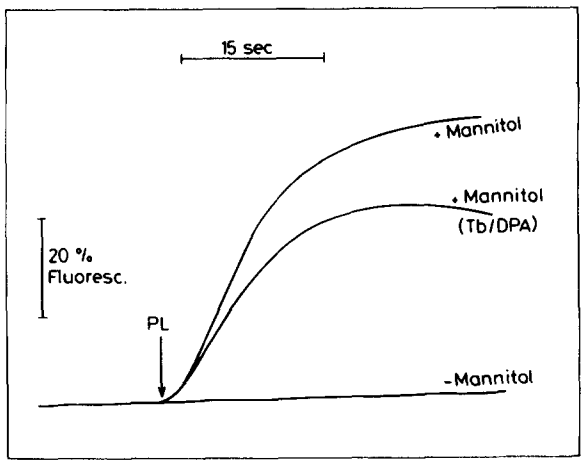

Fig. 1. Polylysine-induced fusion of LUV. 50 μM LUV (labelled/unlabelled, 1 : 2) were incubated at 26°C in buffers containing 0.1 mM EDTA/5 mM Hepes (pH 7.4) and either 100 mM NaCl (− mannitol) or 0.6 M mannitol (+ mannitol). The fluorescence of the N-NBD-PE/N-Rh-PE couple at 530 nm was recorded before and after the addition of 135 nM polylysine (37000). 50 μM LUV, prepared for the Tb-dipicolinic acid fusion assay in mannitol-containing buffer (+ mannitol; Tb/DPA), were incubated with 135 nM polylysine (37000). The change in the fluorescence at 545 nm was recorded.

assayed in the system of Ca^{2+}-induced fusion. As summarized in Table I, mannitol amplified the inhibiting effect of NaCl (0–100 mM) by increasing the $CaCl_2$ threshold concentration from 9 to 14 to 20 mM. However, in the presence of polylysine the $CaCl_2$ threshold concentration dropped from 0.5 (without mannitol) to 0.2 (with mannitol and NaCl). A substantial reduction in the fusion rate occurred when 25 mM NaCl was added to already fusing LUV (with 12 mM $CaCl_2$) in 0.6 M mannitol. Addition of polylysine, at this point, enhanced the fusion rate as previously described [10].

The effective concentration of the polycations was in the nanomolar range and corresponded to charge ratios of 0.1–10 (polylysine/cardiolipin on the outer layer; assuming equal distribution of cardiolipin in the membrane). The addition of polycations in excess caused a decrease in the overall fusion rate and extent (Fig. 2). When 0.1 M NaCl substituted for equiosmolar mannitol the effective concentration range of the polycations was appreciably narrower (Fig. 2); significant inhibition was observed already at polylysine amounts corresponding to a charge ratio of 5. Maximal fusion rates and extent were obtained at charge ratios of 2–3 with polylysine and 0.7–2 with the copolymers, independent of the concentration of LUV. Only short polylysine (3500) was insensitive to salt additions.

TABLE I

EFFECT OF MANNITOL AND NaCl ON $CaCl_2$ THRESHOLD CONCENTRATION

100 μM LUV (PC/cardiolipin 1:1 molar ratio) were prepared and assayed for Ca^{2+}-induced fusion by the NBD-Rh resonance energy transfer assay in various buffer combinations with or without 57 nM polylysine 37000 (an amount corresponding to a charge ratio of 0.5). For each case the threshold $CaCl_2$ concentration, i.e., the lowest $CaCl_2$ concentration required to induce fusion, was determined.

NaCl (M)	Mannitol (M)	$CaCl_2$ threshold concentration (mM)	
		without polylysine	with polylysine
0.1	–	9.0	0.5
0.1	0.4	20.0	0.2
0.05	0.5	14.0	–
–	0.6	7.0	0.1

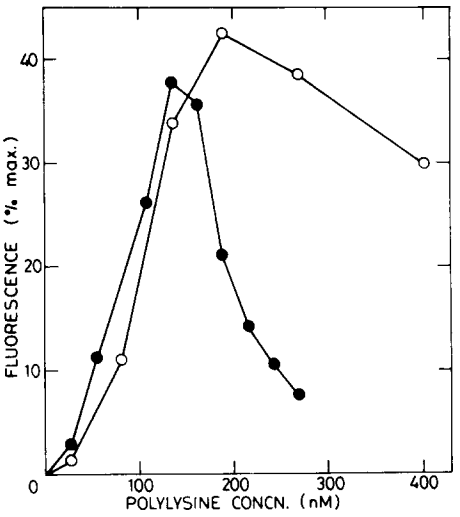

Fig. 2. The effect of salt on polylysine-induced fusion of LUV. 50 μM LUV (labelled/unlabelled, 1:4) were added to buffers 2 (●) or 1 (○) also containing various amounts of polylysine (37000). The change in the fluorescence at 530 nm was recorded and the relative change was calculated after 60 s.

The effect of size and charge density on fusion

It is apparent from Fig. 3 that copolymers with smaller charge densities could induce fusion at lower concentrations, the effectivity being:

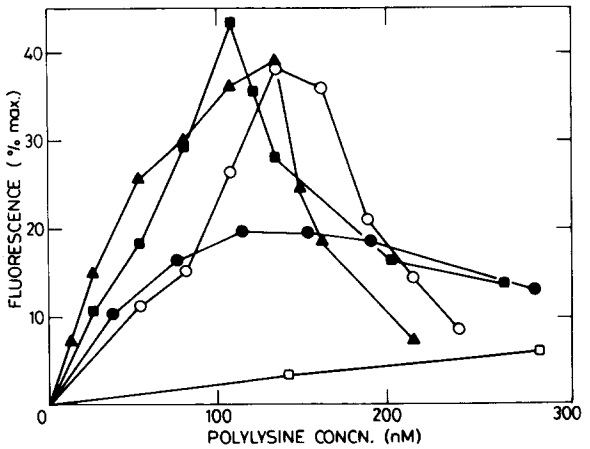

Fig. 3. The effect of polycation size and charge density on LUV fusion. 50 μM LUV were added to buffer 2 also containing various amounts of polylysine (37000; ○), polylysine (3500; □), poly(Lys, Ala) (1:1; 37000; ▲), poly(Lys, Ala) (3:1; 37000; ■) and poly(Lys, Ala, Glu, Tyr) (5:6:2:1; 52000; ●). The change in the fluorescence at 530 nm was recorded and the relative change was calculated after 60 s.

poly(Lys, Ala) 1 : 1 > poly(Lys, Ala) 3 : 1 > polylysine. All of the above-mentioned polymers are of the same molecular weight. The copolymer (Lys, Ala, Glu, Tyr 5 : 6 : 2 : 1, 52000) which has an even smaller charge density (1/3 of polylysine, if the negative charges of glutamic acid are not accounted for) was still more effective than polylysine. If these results were presented as a function of the charge ratio (Fig. 4), the superiority of the copolymers was even more pronounced. Among polylysine species there was hardly any difference between long and short polylysine (Fig. 4). Polylysine, (3500) did not exhibit a significant decrease in fusion rates, such as the other polycations showed, at higher concentrations, corresponding to charge ratios up to 10.

Leakage of vesicle contents

The leakage of LUV contents was monitored by the dequenching of 5(6)-carboxyfluorescein [23]. Addition of any polycation to LUV resulted in loss of LUV contents. The leakage exhibited an optimum (Fig. 5) at polycation-to-LUV ratios similar to those observed in the fusion experiments. Exclusion of salt from the fusion medium resulted in greater leakage. Copolymers, as a rule, caused greater leakage than polylysine. The copolymers poly(Lys, Ala, Glu, Tyr) 5 : 6 : 2 : 1; (Fig. 5) and poly(Lys, Phe) 1 : 1 induced leakage at an extent much greater than any of the other polycations.

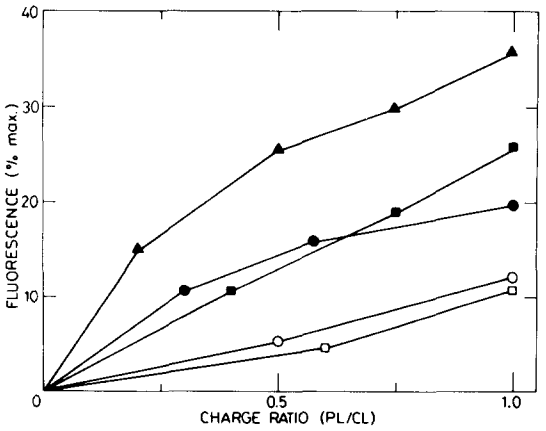

Fig. 4. Fusion potential of polycations as a function of charge ratio. The amounts of the various polycations used in Fig. 3 were translated to charge ratios (polymer/cardiolipin (PL/CL) of the outer liposome layer). The results obtained for Fig. 3 were drawn as a function of the various charge ratios up to theoretical total neutralization, i.e., charge ratio = 1.

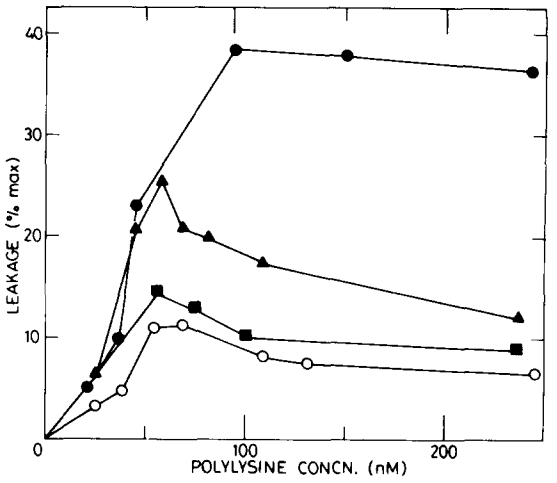

Fig. 5. The effect of polycations on leakage of LUV contents. LUV were prepared with 20 mM (5(6)-carboxyfluorescein entrapped in them as described in Materials and Methods. 25 μM LUV were added to buffer 2 also containing various amounts of polylysine (37 000; ○), poly(Lys, Ala) (1 : 1; ▲), poly(Lys, Ala, Glu, Tyr) (5 : 6 : 2 : 1; ●) and to buffer 1 also containing various amounts of polylysine (37000; ■). The changes in the 5(6)-carboxyfluorescein fluorescence were recorded and the relative change was calculated after 36 s.

Promotion of Ca^{2+}-induced fusion by polycations

All the polycations promoted Ca^{2+}-induced fusion (without mannitol). No fusion was detected with less than 0.5 mM $CaCl_2$. All the polymers had a concentration optimum, the order of the efficiency being: poly(Lys, Ala, Glu, Tyr) 5 : 6 : 2 : 1 > poly(Lys, Ala) 1 : 1 > poly(Lys, Ala) 3 : 1 > polylysine (14000) > polylysine (35000) > polylysine (3500).

The efficiency of the various polycations was tested comparing fusion and leakage rates (Fig. 6). Copolymers had, as a rule, larger fusion/leakage ratios, i.e., they promoted less leakage per fusion event. Among the polylysines polylysine (14000) had the highest ratio and polylysine (3500) the lowest, i.e., promoted greatest extent of leakage per fusion event.

Kinetic analysis of LUV fusion

A mass-action kinetic model [10,18–22] was used to analyse the fusion reaction induced by the

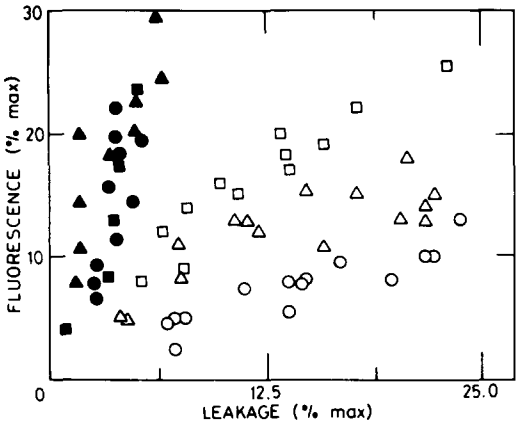

Fig. 6. The effect of polycation size and species on fusion and leakage. 50 μM LUV were injected into suspensions containing 8 mM $CaCl_2$ and varying amounts of polylysine (35000; △), polylysine (14000; □), polylysine (3500; ○), poly(Lys, Ala) (3:1; ●), poly(Lys, Ala) (1:1; ■) and poly(Lys, Ala, Glu, Tyr) 5:6:2:1; ▲). Fusion and leakage of contents were assayed. Tb-dipicolinic acid assay was used for the polylysine studies (fusion given as the corrected values as in Ref. 10). The NBD-Rh resonance energy transfer fusion assay and the 5(6)-carboxyfluorescein leakage assay were used for the copolymer studies.

various polycations (Table II). The most outstanding observation is that the fusion constants of all the polycations – 0.025–0.065 s^{-1} – are one order of magnitude smaller than those found for Ca^{2+}-induced fusion (Table III and Refs. 10, 24, 25). In the salt-containing buffer both C_{11} and f_{11}, of the copolymers, were consistently larger than those of polylysine. There was an increase in f_{11} values and a concomitant decrease in C_{11} in medium without salt (buffer 1). If the amount of polycation were not reduced proportionally with lower vesicle concentrations, so as to maintain a fixed charge ratio, C_{11} values were even smaller. The aggregation and fusion constants of the short polylysine (3500) did not differ significantly with the various treatments, which was in accordance with its insensitivity to salt or to its application in excess.

Table III summarizes the kinetic analysis of the polycation-promoted fusion with 8 mM $CaCl_2$. Among the polylysine species the medium size polymer (14000) had the highest C_{11} value and polylysine (3500) the lowest. All the copolymers, exhibiting greater promotion capacity, had lower C_{11} but remarkably higher f_{11} values. Manipulation of the copolymer concentration affected almost exclusively the aggregation potential (Table III, C_{11} values of Lys, Ala, Glu, Tyr).

Preincubation of LUV with polycations

Preincubation of LUV with polycations, at

TABLE II

KINETIC ANALYSIS OF POLYCATION-INDUCED FUSION

LUV (PC/cardiolipin 1:1 molar ratio) were incubated in buffers 2 (+) or 1 (−) at concentrations of 100, 20 and 10 μM lipid phosphorus with fixed amounts (given concentration) or with varying (var.) amounts of polycations, corresponding to the given charge ratios (polycation/cardiolipin). The fixed concentration was equal to that applied in the fixed charge ratio sets when run with 100 μM liposomes. The data obtained, using the NBD-Rh resonance energy transfer fusion assay, were analysed as described under Materials and Methods. All values are the best fitted within a 10% experimental error. PL, polylysine; L:A, poly(Lys, Ala) 1:1 or 3:1 molar ratio, respectively; L:A:G:T, poly(Lys, Ala, Glu, Tyr) 5:6:2:1.

Polycation species	Polycation concn. (nM)	Charge ratio	C_{11} ($M^{-1}\cdot s^{-1}$)	f_{11} (s^{-1})	D_{11} (s^{-1})	0.1 M NaCl included
PL 37000	var.	2.5	$5.0\cdot10^8$	0.025	0.35	+
PL 37000	270	var.	$3.3\cdot10^8$	0.035	0.3	+
PL 37000	var.	2.5	$4.0\cdot10^8$	0.06	0.35	−
PL 37000	270	var.	$3.0\cdot10^8$	0.065	0.3	−
L:A, 3:1	var.	1.9	$6.0\cdot10^8$	0.036	0.4	+
L:A, 1:1	var.	1.0	$6.5\cdot10^8$	0.038	0.38	+
L:A:G:T	var.	1.3	$1.0\cdot10^9$	0.008	0.25	+
PL 3500	var.	6.3	$7.5\cdot10^8$	0.032	0.3	+
PL 3500	var.	6.3	$8.0\cdot10^8$	0.05	0.2	−
PL 3500	7140	var.	$8.5\cdot10^8$	0.05	0.25	−
PL 3500	var.	2.5	$8.0\cdot10^8$	0.035	0.25	+

TABLE III

KINETIC ANALYSIS OF POLYCATION-PROMOTED LIPOSOME FUSION

LUV were incubated at concentrations of 100, 20, 10, 5 μM lipid phosphorus with 8 mM $CaCl_2$ and given concentrations of various polylysine (PL) species and lysine-based copolymers (Lys, Ala = L : A; Lys, Ala, Glu, Tyr 5 : 6 : 2 : 1 = L : A : G : T). Polycation concentration was changed in proportion to LUV concentrations so as to maintain fixed polycation-to-LUV ratios. The data collected, using NBD-Rh resonance energy transfer fusion assay, were analysed employing a mass action kinetic model in its extended form. All values are the best fitted within a 10% experimental error.

Polycation species	Fusion without $CaCl_2$	Polycation concn. (nM)	Charge ratio	C_{11} ($M^{-1} \cdot s^{-1}$)	f_{11} (s^{-1})	D_{11} (s^{-1})
PL 35000	–	57	0.5	$5.5 \cdot 10^8$	0.35	1.0
PL 14000	–	142	0.5	$8.5 \cdot 10^8$	0.33	0.85
PL 3500	–	570	0.5	$4.5 \cdot 10^8$	0.7	0.6
L : A, 1 : 1	+	54	0.25	$2.1 \cdot 10^8$	1.9	0.85
L : A : G : T	–	36	0.17	$2.1 \cdot 10^8$	2.0	0.85
L : A : G : T	–	7.2	0.034	$7.5 \cdot 10^7$	2.5	0.7

[a] Polycation concentration added per 100 μM P_i liposomes.

sub-optimal concentrations (see Fig. 3), revealed dependence on the duration of the preincubation. Preincubation of up to 20 s in buffer 2 or 12 s in buffer 1 resulted in increased fusion rates (Fig. 7). Longer preincubation resulted in reduced fusion rates. The shorter period of enhancement and the greater inhibition at longer times was characteristic of buffer 1. Supra-optimal concentrations of polycations (see Fig. 3) produced a smaller effect, especially in long periods of preincubation, The effect of preincubation was also smaller, in longer periods, with the low charge density polycation (Lys, Ala,Gly,Tyr, 5 : 6 : 2 : 1; Fig. 1).

The effect of multivalent anions on fusion and leakage

The addition of PP_i or sulphate to the reaction mixture altered the rate and extent of fusion (Fig. 8). With sub-optimal amounts of polycations (see Fig. 3) the multivalent anions reduced the rate and extent of fusion, while with supra-optimal amounts they enhanced it (Fig. 8). A fusion reaction mixture containing 50 μM LUV and 540 nM poly(Lys, Ala) 3 : 1 exhibited no fusion, but when 1.4 mM PP_i was added the rate and extent of fusion were similar to that induced by 270 nM poly(Lys, Ala) 3 : 1. If the anions were added prior to the LUV and were allowed to mix with the polycations, their influence was markedly larger (Fig. 8). Similar effects of enhancement and inhibition induced by the addition of multivalent anions were observed also in leakage determina-

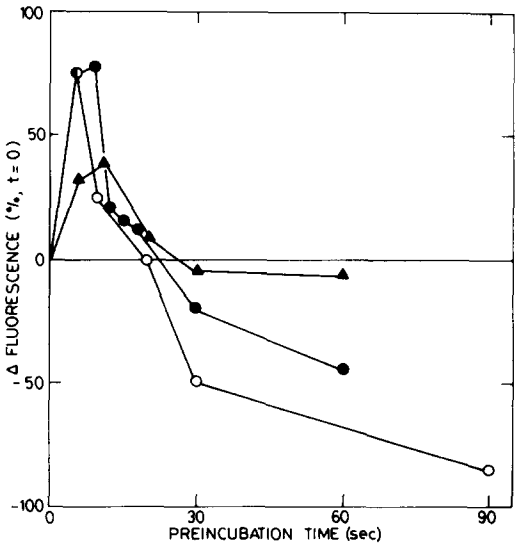

Fig. 7. The effect of preincubation on polycation-induced fusion. 40 μM unlabelled LUV were preincubated for various time intervals with 110 nM polylysine (37000) in buffer 1 (○) or buffer 2 (●) and with 75 nM poly(Lys, Ala, Glu, Tyr) (5 : 6 : 2 : 1) in buffer 2 (▲). The change in the fluorescence at 530 nm was recorded after the addition of 10 μM labelled liposomes. The change in the fluorescence, relative to LUV fusion without preincubation, was calculated after 9 s.

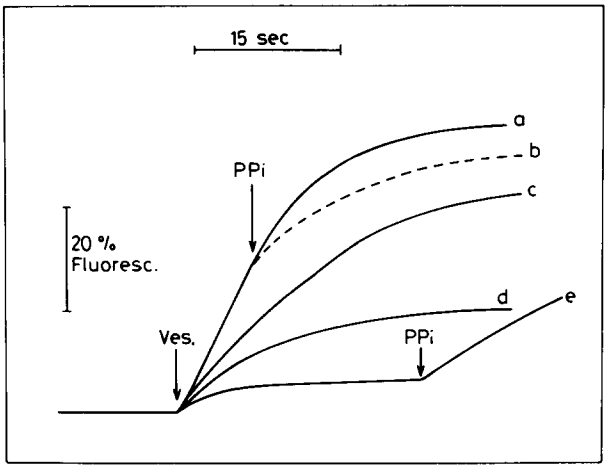

Fig. 8. The effect of pyrophosphate on polycation-induced fusion. 50 μM LUV were added to buffer 2 also containing 100 nM (a, b) or 300 nM (e) polylysine (37000). In (b) 2.5 mM PP_i were added after 8 s, and in (e) 2.5 mM PP_i were added after 30 s of fusion. Alternatively, 100 nM polylysine (37000) were added to 50 μM LUV already mixed with 2.5 mM PP_i (c) or 50 μM LUV were added to 100 nM polylysine 37000 already mixed with 2.5 mM PP_i (d). The changes in the fluorescence at 530 nm were recorded.

tions. Pyrophosphate was more potent than sulphate both in inhibiting and in enhancing fusion.

Discussion

The fact that PC : cardiolipin LUV were able to fuse upon the addition of polylysine only in the presence of high concentrations of non-electrolytes such as mannitol or sucrose, might be attributed to their dehydrating capacity. Papahadjopoulos et al. [26] have already suggested that the formation of intermembrane dehydrated complexes was the driving force for fusion. This hypothesis is supported by observations concerning the dehydrating capacities of Ca^{2+} and Mg^{2+} [27] and the low hydration of phosphatidylethanolamine [28], known to promote fusion [4,5,9]. Reduction of the $CaCl_2$ threshold concentration in the polylysine-promoted fusion system (taking into account the low background level of polylysine-induced fusion) in the presence of mannitol (Table I) supports this claim. We cannot exclude the possibility that the effect of sugars arises from their capacity to replace water on the bilayer [29] or from the capacity of other polyalcohols to destabilize the membrane by intercalating between the phospholipid head groups, as suggested for glycerol [29].

A much more pronounced effect was observed in the presence of salt (Table I), resulting in a rather dramatic increase in the $CaCl_2$ threshold concentration. NaCl was shown to have interfered with Ca^{2+}-induced fusion [25] but the effects were smaller and differences of 20–30 mM NaCl were hardly noticeable. If fusion induced by 12–14 mM $CaCl_2$ could be almost completely stopped by 25 mM NaCl in the presence of mannitol, it follows that Na^+ was able to successfully compete with Ca^{2+} on binding to the negatively charged LUV. Ca^{2+} binds more tightly to acidic vesicles because of its valency, although it is much more hydrated than Na^+ [30]. However, upon the addition of mannitol or other non-electrolytes the ions become less hydrated, probably as a result of a lower dielectric constant [29]. The free energy barrier, for binding [31], of Na^+ becomes small enough to facilitate successful competition with Ca^{2+}. Similarly, the affinity of polylysine for the acidic vesicle should increase with mannitol. As a consequence, and together with a partial dehydration or destabilization of the membrane, this would lead to the induction of fusion by polylysine and other copolymers that could not do so in the absence of mannitol.

The effect of monovalent cations on membrane fusion has been thoroughly discussed [20,25,32]. On the one hand Na^{2+} neutralizes surface charges of the vesicles hence promoting vesicle aggregation, but on the other hand it competes with Ca^{2+} on binding therefore reducing the fusion potential as reflected in a smaller f_{11} value. Our kinetic analysis produced matching results: lower f_{11} and higher C_{11} values with 100 mM NaCl.

We suggest an additional interpretation. As seen in Fig. 2, the vesicles incubated in the medium containing salt fuse at a higher rate and to a greater extent than in medium without salt with small (sub-optimal) amounts of polycations. Since fusion can occur even with less than half the vesicle charges neutralized (charge ratio < 0.5), aggregation cannot take place unless the polycations can bind simultaneously to two vesicles. Lampe et

al. [33] have shown that polylysine induced aggregation of vesicles when used at low concentrations. At higher concentrations of polylysine aggregation was inhibited, in contrast to human myelin basic protein, that did not exhibit inhibition when applied in excess. The difference was attributed to different modes of binding; polylysine binding to two vesicles simultaneously ('trans') while human myelin basic protein bound to only one vesicle ('cis'), aggregation being achieved through protein-protein interactions.

Such 'trans' binding as opposed to 'cis' binding, only to one vesicle, can be favorable when binding of the polymer is weaker. Hammes and Schullery [1] have reported that the addition of salt to mixtures of acidic vesicles and polylysine weakened their binding to the vesicles. The lysine-based copolymers, that have a lower charge density to begin with, induce more aggregation, i.e., show higher C_{11} values, than polylysine of a similar size. This observation actually suggests that the efficiency of polylysine is reduced due to excess charge. Furthermore, the preincubation experiments (Fig. 6) revealed a longer preincubation interval, during which fusion was enhanced and a weaker inhibitory effect at longer preincubation ($t > 20$ s), in the salt-containing medium. While in the Ca^{2+}-induced fusion [27] the 'trans' conformation is the driving force for fusion per se, it is the cause for the great aggregating potential in the polycation system.

We therefore envisage two stages of the binding of the polymer to the vesicle (Fig. 9): (1) fast binding of a small fraction of the polymer which is closest to the vesicle surface and essentially tangent to it; and (2) slow binding of the rest of the polymer, 'unwinding' and adapting its position to the vesicle charges and curvature. The fact that after long preincubations (> 25 s) fusion could still be initiated by the addition of more polycations proved at the polycations, added during preincubation, were no longer available to form 'trans' complexes with the labelled vesicles added after preincubation, rather than the vesicles becoming insusceptible to fusion. Furthermore, the lack of substantial inhibition at long preincubation times with excess polycations supports our claim. In this case each polymer cannot complete its binding to the same vesicle because of the competition with the other polymer molecules. As a consequence part of the molecule remains free to form 'trans' complexes with additional vesicles.

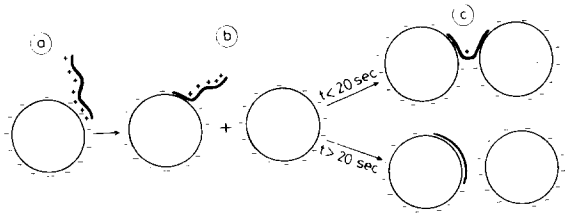

FIg. 9. Phases of polycation binding to negatively charged LUV. When polycations are added to negatively charged LUV (a) they form an immediate and tight, though partial, binding to the LUV (b). If more LUV are added the result depends on the time elapsed between the addition of the polycations and the LUV (c). Short preincubations (< 20 s) result in cross-linking between LUV, while longer preincubations (> 20 s) result in completion of binding to the same LUV.

Competition for binding to the same vesicle may also be the reason for the requirement for polycations in excess (charge ratio > 1) in order to obtain maximal fusion rate and extent. In addition, the fact that during the formation of the 'trans' complex, part of the polymer molecule bridges between the vesicles rather than binds to one of them should be considered. It may also be attributed to the fact that only half the polylysine charges are bound to the surface of the vesicle in a 'zig zag' manner [34]. The greater efficiency of the copolymers is evident from the comparison with respect to the charge ratio. With half the charge density of polylysine, poly(Lys, Ala) 1 : 1 induces greater aggregation and fusion when applied at the same concentration (i.e., at half the charge ratio). On the other hand, short polylysine (3500) was far less effective on a molar basis. The small molecule can much less efficiently form a stable 'trans' binding between two vesicles. In other words, there is a minimal size, larger than 3500, that is required to promote vesicle aggregation at low polymer concentration. Similarly, polylysine (14000; Table III) is more effective than longer or shorter species in promoting Ca^{2+}-induced fusion of similar LUV in solutions not containing mannitol.

The effect of copolymers on leakage was, as a rule, larger than that of polylysine and was less influenced by excess copolymer. Klibansky et al.

[35] demonstrated that basic copolymers were more effective than the corresponding basic polymer in destabilizing membranes, as observed in their greater potential to enhance digestion of phospholipids by phospholipases. Bach et al. [36] have reported that the interaction of cationic polymers with acidic vesicles was tighter with the more hydrophobic species, e.g., poly(Lys, Phe). We also found poly(Lys, Phe) to be the most effective inducer of fusion, with an f_{11} value an order of magnitude larger than any of the other polycations, but the results could not be confirmed by the Tb-dipicolinic acid assay due to massive leakage of contents. The combination of a molecule containing positive charges and lipophilic residues has been shown to be efficient in inducing fusion of acidic vesicles [4,7,37].

The observation that leakage induced by poly(Lys, Ala, Glu, Tyr) was the highest while its fusion potential was rather low suggests that fusion and leakage are not necessarily interdependent events, i.e., leakage is not necessarily a consequence of fusion. Poly(Lys, Ala, Glu, Tyr) is different from the other copolymers also in the way it promotes Ca^{2+}-induced fusion. The other copolymers show greater fusion potential, due to their amphipathic nature, both with and without Ca^{2+}. On the other hand, poly(Lys, Ala, Glu, Tyr) is extremely efficient only with Ca^{2+}. We therefore suggest that this copolymer enhances fusion by attracting Ca^{2+} ions to the membrane with its glutamate residues. As a result of the higher Ca^{2+} concentration near the bilayer, fusion proceeds at a much faster rate.

The use of multivalent anions to titrate polycation charges demonstrates the possibility to control the actual effective concentration of the polycation. The effects exerted by such low concentrations (up to 2.5 mM) cannot be attributed to changes in ionic strength. The ability of the multivalent anions to enhance fusion in the presence of excess polycations was related to their ability to titrate polycation charges, reducing its effective concentrations thus allowing 'intimate' contact between membranes followed by fusion and leakage of contents as discussed above. The greater efficiency of PP_i is attributed to its greater valency.

In conclusion, polycations induce aggregation of vesicles by forming 'trans' binding with two vesicles. In the presence of salt the binding to the bilayer is weaker and the 'trans' formation is established more readily at low polycation concentrations. This aggregation is followed by fusion if the reaction is carried out in a dehydrating medium containing high concentrations of nonelectrolytes. The extent of fusion is greater if the polymer contains hydrophobic residues and its charge density is smaller. When the polycation is applied in excess, 'intimate' contact between the two bilayers is smaller and thus fusion occurs at slower rates. Smaller charge density and hydrophobicity also affect Ca^{2+}-induced fusion.

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