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ION SPECIFICITY IN FUSION OF PHOSPHATIDIC ACID-PHOSPHATIDYLCHOLINE MIXED LIPID VESICLES

MEI-JUNE LIAO and JAMES H. PRESTEGARD

Chemistry Department, Yale University, New Haven, CT 06520 (U.S.A.)

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Summary

Fusion of phosphatidylcholine-phosphatidic acid mixed lipid vesicles has been studied under the influence of the divalent Ca^{2+}, Mg^{2+}, Cd^{2+}, and Ba^{2+}, which range in size from much smaller to much larger, than Ca^{2+}. Fusion has also been studied under the influence of Eu^{3+}, which has a similar radius but different charge. The effects of these ions are reflected in the different degrees of fusion determined by changes in vesicle size, and in the varying fusion rates monitored by ^{1}H-NMR spectroscopy. Cd^{2+}, which has an ionic radius similar to Ca^{2+}, exhibits the same effect on fusion as Ca^{2+}, while other ions show lower efficiencies. This suggests that the vesicle fusion intermediate has a geometry ideally suited to the binding of Ca^{2+} or Cd^{2+}.

Introduction

Recent efforts in our laboratory, as well as the laboratories of others, have been directed at the development and characterization of the phosphatidylcholine-phosphatidic acid-Ca^{2+} system as a suitable model for membrane fusion [1—3]. The system is unusual in that, with an appropriate ratio of Ca^{2+} to phosphatidic acid and phosphatidic acid to phosphatidylcholine, fusion of vesicles stops after a small number of events rather than continuing to very large nonvesicular structures [1]. It is also unusual in that fusion is accompanied by the retention of a large fraction of internal contents [1].

Ca^{2+} may be intimately involved in both of these characteristics. Ca^{2+} is known to cross the bilayer during fusion, to occupy internal phosphatidic acid

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

sites. Saturation of these sites inhibits fusion [4]. Thus, transbilayer movement may be the process which stops fusion after a small number of events. It is also possible that Ca^{2+} bridging of bilayers, before and during fusion, provides a junction that retards leakage of internal contents. Since specific structural complexes are likely to mediate these processes, one might expect some degree of specificity for Ca^{2+} over other multivalent ions. A more detailed study of ion requirements is clearly in order.

Recently, attention has been given to the specificity for divalent metal ions in many natural cellular processes in which membrane fusion occurs [5]. In a number of processes related to exocytosis or secretion, a high specificity for Ca^{2+} is exhibited. For example, in the experiments of Miledi [6] in which Ca^{2+} was microinjected into the terminus of the squid axon and transmitter release recorded, neither Mg^{2+} nor Mn^{2+} was as effective as Ca^{2+}. Schudt et al. [7,8] have also demonstrated that the requirement of Ca^{2+} in myoblast fusion is specific with other ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+}, Cd^{2+}, Li^{+} and La^{2+} not substituting for Ca^{2+}. There are, however, processes with less specificity. Okada and Murayama [9] for example, found that in virus-induced cell fusion, Mn^{2+}, Sr^{2+} and Ba^{2+} can substitute for Ca^{2+} with only moderate variation in efficiency. Lipid bilayer model systems developed recently for membrane fusion also reveal different levels of ion selectivity on thermotropic behavior and permeability properties [10]. An investigation of the ion specificity in vesicle fusion for our model system should, therefore, assist in relating the role of Ca^{2+} in fusion of phosphatidic acid-phosphatidylcholine vesicles to more fundamental bilayer properties and to properties of natural fusion systems.

Ionic properties such as ionic radius and ionic charge may govern selectivity. That charge and size may influence fusion efficiency is already suggested by data on Ca^{2+}-stimulated fusion of the phosphatidylcholine-phosphatidic acid system. First, fusion is not induced until a level of Ca^{2+} has been reached which has been suggested to be sufficient to neutralize charge on the bilayer-bound phosphatidic acid. Second, binding constants appear to be very high leading eventually to phosphatidic acid-Ca^{2+} complex of well defined 1 : 1 stoichiometry [11—13]. Such well defined stoichiometry is usually accompanied by geometric restraints.

We have, therefore, chosen to study fusion under the influence of ions which have the same charge as Ca^{2+} but span a range of sizes from much smaller, to much larger, than Ca^{2+}. These are Mg^{2+} at 0.065 nm, Cd^{2+} at 0.097 nm and Ba^{2+} at 0.135 nm. They may be compared to Ca^{2+} at an ionic radius of 0.099 nm. We have also studied the influence of an ion which has a similar radius but different charge, Eu^{3+} at 0.095 nm and +3 charge. Choice of ion might be expected to influence both the degree and rate of fusion. The degree of fusion (number of fusion steps) is reflected in the ultimate size of the vesicle product. As in the past, we will use gel permeation chromatography to monitor size distributions of vesicle products. Rates of fusion could be monitored by the same technique, extracting and inhibiting fusion of aliquots periodically in time. Because of the quantity of data involved we have, however, employed a NMR technique which allows continuous monitoring. ^{1}H-NMR linewidths increase monotonically with vesicle size, and respond only slightly to vesicle aggregation in these mixed

phosphatidylcholine-phosphatidic acid systems. Increases in linewidth or the reciprocally related amplitude can, therefore, be used to rapidly monitor rates of fusion.

Materials and Methods

Materials. Phosphatidylcholine was extracted from egg yolks and phosphatidic acid was synthesized from it by the action of partially purified phospholipase D, from cabbage, on sonicated phosphatidylcholine vesicles. Isolation, digestion and purification procedures are derived from the works of Papahadjopoulos and Miller and have been described in detail elsewhere [1]. 2-(*N*-Morpholino)ethanesulfonic acid, Mes, used as a buffering agent, was purchased from Calbiochem (San Diego, CA). Sepharose 2B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

Vesicle preparation. Vesicles were prepared by sonication at 6% (w/v) total lipid in a pH 6.0 ± 0.1 buffer containing Mes (10 mM), NaCl (100 mM) and ^2H_2O (10%). A Branson Model E bath sonicator thermostatically controlled at 30 ± 5°C was employed. Sonication was continued until no further changes in sample absorbance were apparent, usually 4 h. Precautions were taken to maintain samples under a nitrogen atmosphere. Products were examined by TLC and found to exhibit less than 5% degradation. Fusion of vesicles was induced by adding the solutions prepared from phosphatidylcholine and phosphatidic acid in 2 : 1 mol ratio, to an equal volume of solution containing di- or trivalent cations at concentrations sufficient to achieve cation to phosphatidic acid mol ratios ranging from 0.2 to 1.2. For samples to be analyzed by gel permeation chromatography, the mixture was incubated at 37 ± 1°C for 30 min and then a slight molar excess of EDTA was added.

Vesicle samples for kinetic studies were prepared as above except at 8% (w/v) total lipid and in an unbuffered solution at p^{2}H 6.4 ± 0.2. Mixing with divalent cation solutions was carried out in a stop-flow cell and incubation occurred in the spectrometer at 37 ± 2°C. The stop-flow cell is of a simple design using parallel driven syringes connected to a 'Y' junction, constructed of Teflon tubing mounted at the top of an NMR tube. Prior to mixing, the solutions reside in excess tuning just above the NMR tube in a high-magnetic field region where spin equilibration takes place. Turbulent passage through the 'Y' junction effects mixing.

Vesicle size analysis. Vesicle sizes of samples incubated for a 30 min period terminated with EDTA addition were determined from elution volumes of a Sepharose 2B column. Lipid concentration was monitored using a Waters Associates Differential Refractometer. The maximum of the elution peak has been converted to a vesicle size using the equations of Ackers [14]. Sizes reported correspond only to this point. Attention should, however, be given to the fact that distributions are broad.

Changes in vesicle properties for kinetic samples were monitored using ^{1}H-NMR. Spectra were obtained on a Bruker HX270 spectrometer operating in the pulse Fourier transform mode and using quadrature detection. Acquisition began 1 s after mixing vesicle and divalent ion solutions in the stop-flow cell. In a typical experiment, one free induction decay resulting from a 90° pulse,

requiring 0.4 s and using 4096 data points was accumulated for each spectrum. Ten such free induction decays were successively stored on disk in an automated mode at 10 s intervals, followed by four free induction decays at 50 s intervals and five at 300 s intervals. These data were exponentially weighted and transformed to the frequency domain, prior to analysis.

Results

Let us consider first the vesicle size distributions which result when vesicle preparations are incubated in the presence of various ions. These distributions were studied using Sepharose 2B column chromatography with fusion of 90 mM lipid samples being initiated as described in the Materials and Methods section. Since sizes of initial vesicle preparations vary somewhat (±10 Å), the results are presented in Fig. 1 as a change in vesicle size. For divalent ion-treated samples, the size dependence on ion concentration is in all cases sigmoidal. The sigmoidal curves show increases in size with ion concentrations

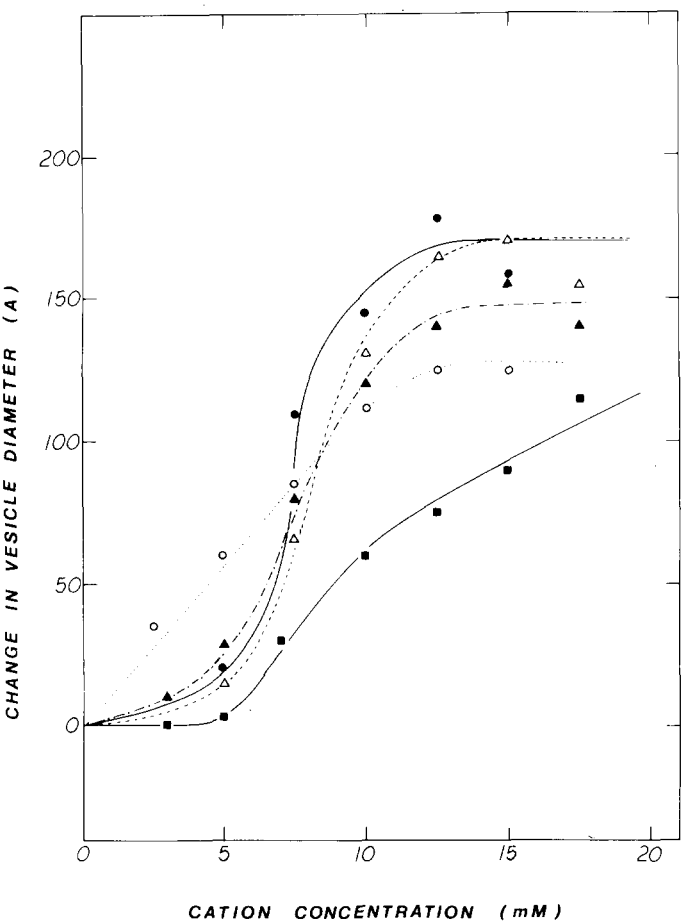

Fig. 1. Changes in vesicle size as a function of added cation concentrations; ●———●, Ca^{2+}; △- - - - -△, Cd^{2+}; ▲· — · — ·▲, Mg^{2+}; ○· · · · · ·○, Eu^{3+}; ■———■, Ba^{2+}. Size changes are taken from the maximum in Sepharose elution profiles and have an accuracy of ±10%.

beginning at points which represent approx. 0.3 : 1 cation/phosphatidic acid ratio. Size changes plateau at approx. 1 : 1 cation/phosphatidic acid ratio. Initiation and plateau points have previously been correlated with outside surface charge neutralization and phosphatidic acid binding site saturation, respectively [1,4]. In the case of the trivalent cation, Eu^{3+}, the initiation point is less well defined and shifted to a substantially lower ion concentration. A naive view of total charge neutralization at pH 6 would suggest that the initiation point should shift to lower stoichiometries by the ratio of Ca^{2+}/Eu^{3+} charges, i.e., to 0.2 : 1. The observed point is lower than this. The surface potential which may influence the onset of aggregation is, however, not a simple function of Eu^{3+}/phosphatidic acid ratio, and may be influenced by the entire ionic environment. It is also possible that the result sheds some doubt on a simple charge neutralization interpreation of the fusion initiation point.

A tendency for size changes to plateau at high ion concentration is apparent in Fig. 1 for all ions except Ba^{2+}. For convenience of discussion, we will define a limiting size for all ions as that observed at 15 mM. The limiting sizes are easy to rationalize in terms of the average number of fusion events. Since the diameter of the fusion product resulting from a two vesicle fusion will increase by $\sqrt{2}$ and all preparations start at approximately the same size (280 ± 10 Å), the limiting sizes can be converted to average numbers of fusions. These are 1.5 ± 0.1, 1.5 ± 0.1, 1.3 ± 0.1, 1.1 ± 0.1 and 0.8 ± 0.1 for Ca^{2+}, Cd^{2+}, Mg^{2+}, Eu^{3+} and Ba^{2+}, respectively. It is clear that Cd^{2+} has the same capacity as Ca^{2+} in stimulating vesicle fusion while other ions have less capacity. Ba^{2+} is particularly interesting in that the data in Fig. 1 reveal not only the smallest changes in size but also an incomplete sigmoidal curve. No plateau is seen within the range of Ba^{2+} concentrations investigated and, one would anticipate further fusion at higher Ba^{2+} concentration. Since we have demonstrated a fusion rate dependence on ion concentration, it is possible that the Ba^{2+} curve is reflecting a slower fusion rate rather than a direct dependence of vesicle size distributions on the nature of the ion used. Differences in ion specificities not apparent in final size distribution might also occur as differences in fusion rates among various ions. We will, therefore, proceed to describe the results of fusion kinetic studies using a variety of ions.

Our previous results have shown that ^{1}H-NMR is a useful technique for studying vesicle fusion [15]. The linewidth of the acyl chain methylene resonance is sensitive to changes in vesicle size but relatively insensitive to vesicle aggregation. In fact, the linewidth increases in a linear fashion with vesicle diameter over the range 250—600 Å. Since total intensity of NMR resonances is conserved, amplitudes and widths of Lorentzian lines are inversely related. To the extent that lines from vesicles can be approximated as Lorentzian, widths and amplitudes can provide equivalent information. We have chosen to use amplitudes in this study. Considering only the early time points, the fusion process may be considered to be a transformation from n vesicles of initial size to $n-m$ vesicles of initial size and $m/2$ vesicles of a size $\sqrt{2}$ larger. Under these conditions, the fusion rate is linearly related to the change in peak amplitude [15].

Fig. 2 shows the effect of various ions on the change in methylene amplitude as a function of time. Vesicle solutions 111 mM in total lipid and having phos-

phatidylcholine/phosphatidic acid in a 2 : 1 ratio were mixed with equal volumes of cation solutions, at a level of 0.8 : 1 with respect to phosphatidic acid. This ratio approaches the point at which rates and product distributions remain relatively independent of fraction of phosphatidic acid complex and yet avoids large particulate materials produced at much higher cation levels. It is apparent

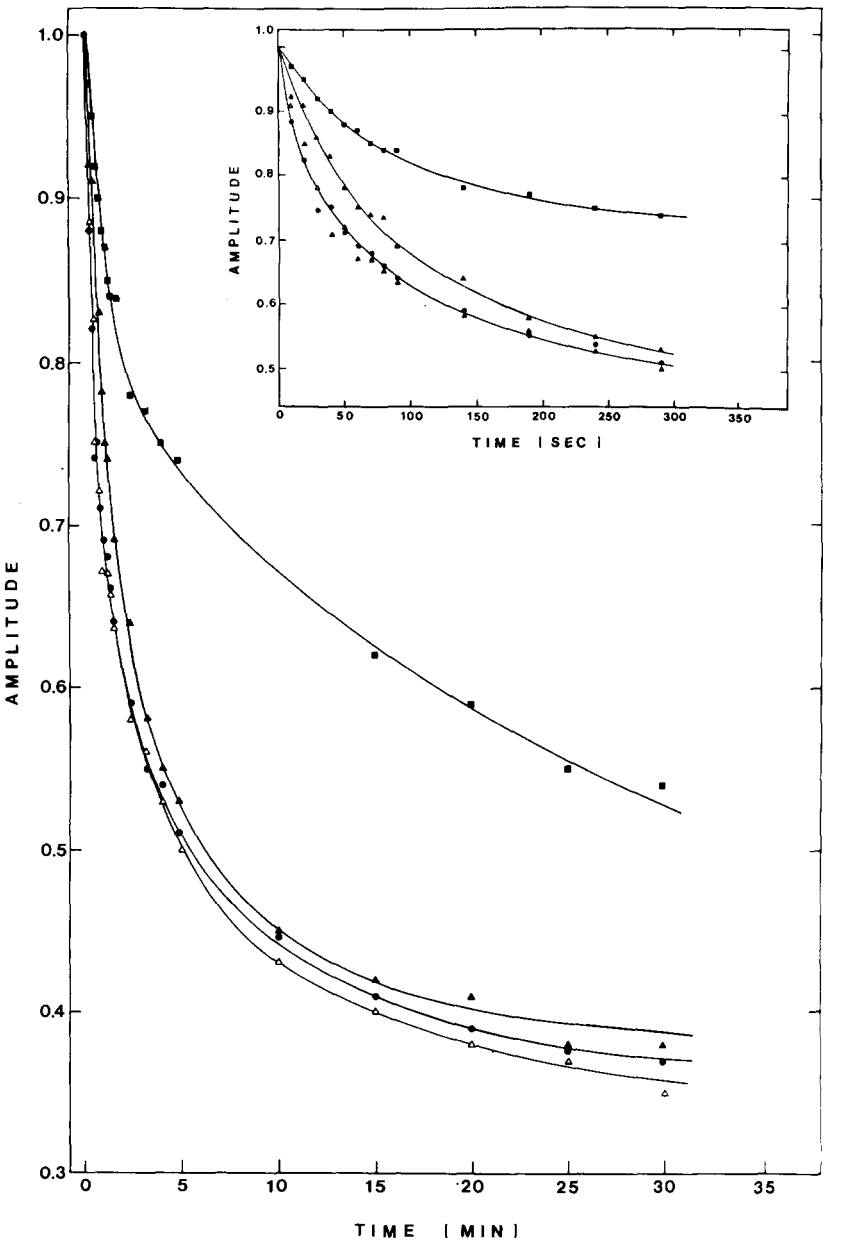

Fig. 2. The effect of various ions on the change in ^{1}H-NMR methylene amplitude as a function of time; ●———●, Ca^{2+}; △———△, Cd^{2+}; ▲———▲, Mg^{2+}; ■———■, Ba^{2+}. The insertion is an enlargement of the curves during the initial period.

from the data in Fig. 2 that the fusion process is biphasic with a rapid process accounting for 50—80% of the total change in methylene amplitude. An expansion of the early time points (insert in Fig. 2) allows comparison of the initial rates, 0.01 s^{-1} for Ca^{2+} and Cd^{2+}, 0.006 s^{-1} for Mg^{2+} and 0.003 s^{-1} for Ba^{2+}. Assuming second-order kinetics, this rapid process would be greater than 90% complete at 30 min for Ca^{2+}, Cd^{2+} and Mg^{2+}, but would be less than 80% complete for Ba^{2+}. Examination of the long time portion of the curves in Fig. 2 shows that existence of biphasic behavior has accentuated these differences. Fusion as stimulated by Ba^{2+} is far from complete at 30 min and the previously discussed fusion product size distribution for this ion is not representative of the final distribution. It is likely that limiting vesicle sizes are similar for all four divalent ions and only rates of fusion differ. Kinetics of fusion under the influence of Eu^{3+} were not investigated. It is possible however, that the decreased limiting size for this ion also reflects kinetics more than a qualitative difference in fusion.

Discussion

Given that the phosphatidic acid-Ca^{2+} complex involved in fusion of vesicles is of a well defined stoichiometry, and that this complex is likely to bridge vesicles in such a way that minimal leakage occurs during fusion, it is reasonable to seek a structural explanation of the differences in fusion rate under the influence of various cations studied here. The appropriate complex need not be the thermodynamically most stable form but may exist only in catalytic amounts. The structural properties of such an intermediate can be deduced by a systematic correlation of rates with ionic properties. In the series examined here ionic size has been varied in a systematic way. Ca^{2+} with an ionic radius of 0.099 nm shows a greater fusion rate than Mg^{2+} which is smaller, 0.065 nm, or Ba^{2+} which is larger, 0.135 nm. Cd^{2+} which is nearly identical in size, 0.097 nm, shows a fusion rate similar to Ca^{2+}. This would suggest that the complex intermediate in vesicle fusion, has a geometry ideally suited to the binding of Ca^{2+} or Cd^{2+}.

The catalytic complex is very likely a phase-separated region of phosphatidic acid having a full complement of bound ions. Such phase separations are known to occur in anionic lipid systems and many times exhibit lamellar phases. It seems reasonable to ask if such phases could offer a structural explanation for selectivity. Phosphatidylserine/phosphatidylcholine mixtures have been found to separate in bulk phase amounts, in the presence of Ca^{2+} but not in the presence of Mg^{2+} [16,17]. Phosphatidic acid/phosphatidylcholine mixtures have been found to separate in bulk phase amounts in the presence of Ca^{2+}, Ba^{2+}, Sr^{2+} and in the presence of high levels of Mg^{2+} [18].

Recent X-ray crystal structures of dimyristoylphosphatidylcholine [19] and dilauroylphosphatidylethanolamine [20,21] at low levels of hydration are likely to serve as reasonable models for bilayer structure and head-group distribution of these phase-separated complexes. The phosphatidylcholine structure shows two head-group geometries. The geometry expected for an ion complex is similar to that observed in the phosphatidylethanolamine structure. The phosphate groups in phosphatidylethanolamine are arranged in rows with the

phosphate-phosphate distance being 7.72 Å. Some disagreement as to the relevant phosphatidic acid-Ca^{2+} (or Cd^{2+}) stoichiometry exists in the literature. Both 1 : 1 and 2 : 1 complexes have been proposed [11—13,22—23]. Our own work using ^{31}P-NMR and differential scanning calorimetry to monitor titration shows that 1 : 1 complexes dominate for both ions at pH 6 and 0.1 M NaCl (unpublished data). With the 1 : 1 stoichiometry and assuming the ion to bridge adjacent bilayers in a manner similar to that postulated for the phosphatidylserine system [24], each Ca^{2+} or Cd^{2+} must be shared between at least two phosphates on adjacent bilayers and each phosphate must bind at least two Ca^{2+} or Cd^{2+}. If the ions are distributed along the same phosphate rows apparent in the crystal structure [21], a chain of ions would exist having 3.86 Å between ions. This is approximately the Cd^{2+}—Cd^{2+} distance in the crystals of ion-complexed phosphoglycerol derivatives, such as glycerophosphorylcholine cadmium chloride trihydrate [25].

Despite the possible existence of an ideal ion size, our data suggest that the geometry of the complex is not absolutely rigid. Larger ions such as Ba^{2+}, smaller ions such as Mg^{2+} and ions of different charge such as Eu^{3+} may be accommodated at the expense of distortion of normal bilayer structure.

There appears to be less contrast between Mg^{2+} and Ca^{2+} in the phosphatidic acid-phosphatidylcholine system than in other anionic lipid systems. Addition of Mg^{2+} to the phosphatidylserine and phosphatidylglycerol systems produces vesicle aggregation but not the cochleate cylinders which are produced by Ca^{2+} under similar conditions [10]. The inabilities of these anionic lipids to accommodate Mg^{2+} may be due to the significant steric hindrance between the more bulky head groups and the waters of hydration the Mg^{2+} is likely to carry [26]. Phosphatidic acid having more flexibility in head group conformation, however, could show a greater ability to accommodate a larger partially-hydrated Mg^{2+}. A partially-hydrated Mg^{2+} may have geometric demands similar to larger ions such as Ba^{2+}. We also cannot rule out the possibility that more diffuse association based largely on charge plays a partial role in the behavior of the phosphatidic acid system. Nevertheless, structural arguments give a reasonable explanation as to why complexes form more readily with Ca^{2+} or ions of similar size and charge, and why fusion occurs at higher rates with these ions.

It would be useful to compare our ion specificity to observations in natural fusions. Unfortunately, biological transformations are seldom compared under conditions where increased lengths of time or increased concentrations of cations are allowed to compensate for possible kinetic differences. Had we simply compared fusion at the end of a 5 min time period or at concentration less than 5 mM, large differences in fusion capacity of various ions would have been noted. It is possible that more than we suspect, cation specificities in biological transformations reflect quantitative differences in kinetics rather than qualitative difference in the intermediates formed.

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