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Roles of Lipids and Proteins in the Ca^{2+}-PO_4-Induced Aggregation of Cytoskeleton-Free Erythrocyte Vesicle Membranes†

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ABSTRACT: The roles of lipids and proteins in Ca^{2+}-PO_4-induced membrane aggregation were investigated. Cytoskeleton-free vesicles derived from intact human and rabbit erythrocytes ($H_E$ves and $R_E$ves, respectively) were employed as a model system. The $H_E$ves and $R_E$ves have a simplified membrane protein composition [band 3 proteins and glycoproteins PAS-1, -2, and -3 ($H_E$ves)] and normal lipid composition. Optimal experimental conditions for pH, [PO_4], and [$CaCl_2$] were determined for quantitatively examining the dynamics and extent of $H_E$ves and $R_E$ves aggregation, measured turbidimetrically. The aggregation process was found to be quite sensitive to small changes in pH and [PO_4] and much less sensitive to [$CaCl_2$]. The roles of membrane proteins in vesicle aggregation were examined by selectively modifying the proteins enzymatically. The roles of lipids were studied by using sonicated lipid vesicles [small unilamellar vesicles (SUVs)] made from Dodge ghost lipid extracts. Enzymatic treatment with trypsin, chymotrypsin, or Pronase had no effect on either the rates or the extent of vesicle aggregation (2-min incubation period). Neuraminidase treatment reduced both factors by approximately 20%. SUVs aggregated with Ca^{2+}-PO_4 in a way which depended on the PO_4/lipid ratio. Together the results suggest the following: (1) PO_4 is associated with the vesicle surface, involving the membrane lipids; (2) the vesicle + PO_4 incubation time component of the PO_4 effect is eliminated by enzymatically modifying the vesicle membrane proteins; (3) qualitative, rather than quantitative, properties of sialic acid containing molecules affect vesicle aggregation; and (4) with the exception of the incubation time effect, membrane proteins seem neither to promote nor to inhibit Ca^{2+}-PO_4-induced $H_E$ves or $R_E$ves aggregation.

The aggregation and fusion of membranes are two of the most basic processes in biology, being fundamentally involved in such phenomena as exocytosis, plasmogamy, and karyogamy. Of particular interest are the possible roles of membrane lipids and proteins in these processes. Recently, researchers have turned to the use of erythrocytes and/or erythrocyte ghosts as a model system for examining membrane aggregation and fusion. Although erythrocytes do not normally fuse in nature, their relative simplicity, ready availability, and well-characterized chemical composition strongly recommend their use. As a result, it has been demonstrated that various chemical agents, such as oleoylglycerol (Quirk et al., 1978; Blow et al., 1979), lysolecithin (Lucy, 1976), calcium-phosphate (Zakai et al., 1976, 1977; Majumdar & Baker, 1980), poly(ethylene glycol) (Ahkong et al., 1975; Knutton, 1979a,b), and inactivated Sendi virus (Peretz et al., 1974; Volsky & Loyter, 1978; Knutton, 1979a,b; Lalazar & Loyter, 1979; Sekiguchi et al., 1981), will induce the aggregation and fusion of erythrocyte membranes.

These studies, however, still suffer from a number of problems in data interpretation, due mainly to the still large heterogeneity in their membrane composition, especially proteins, and the difficulty in distinguishing between phenomena involving cytoskeleton–membrane interactions and those involving the lipids and/or proteins of the membrane proper. To help alleviate these problems, we have developed a simple method for obtaining large ($\simeq$0.5-1.0 μm), cytosk-

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eleton-free vesicles from human and rabbit erythrocytes (Leonards & Ohki, 1983). Using these vesicles, we have undertaken a systematic examination of membrane aggregation and fusion.

In the present study, we examine the roles of the membrane proteins and lipids in the Ca^{2+}–PO_4-induced aggregation of erythrocyte vesicles isolated from both human and rabbit erythrocytes. Vesicle aggregation is examined first because, although membrane aggregation and fusion can be considered as independent phenomena, aggregation is a necessary prerequisite for membrane fusion. Ca^{2+}–PO_4 was chosen because its effects on membrane aggregation and fusion must be mediated by changes which occur in the properties of the existing erythrocyte vesicle components, rather than by exogenous molecules intercalating into the membrane. In addition, by comparing the results obtained with both human and rabbit erythrocyte vesicles under identical conditions, it may be possible to gain insight into the influence of those membrane components which are different in these two cell types, such as sialic acid content (Fischer et al., 1979) etc., on vesicle membrane aggregation. Parts of the results obtained in this study were presented previously (Leonards & Ohki, 1982).

Materials and Methods

Fresh human blood was supplied by the Veterans Administration Hospital, Buffalo, NY. Fresh rabbit blood was obtained by ear puncture using Sequester-Sol (Cambridge Chemical Products, Inc.) as an anticoagulant. In both cases, the blood was used within 2–3 h and *not* cooled below room temperature before use.

Human erythrocyte vesicles ($H_E$ves) and rabbit erythrocyte vesicles ($R_E$ves) were produced and isolated according to the method of Leonards & Ohki (1983). Briefly, erythrocytes were repeatedly washed by centrifugation at room temperature in isotonic solution (150 mM NaCl) buffered with 10 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) (NaT_R^-, pH 7.4) followed by washing in Na–Tricine buffer containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF) (NaT_R^+, pH 7.4). The erythrocyte pellets (5–6 mL) were then diluted with NaT_R^+ to 40 mL and incubated at 45 °C. The large cytoskeleton-free vesicles were produced from the incubating erythrocytes by "budding". This was accomplished by titrating the cell suspensions with ethylenediaminetetraacetic acid (EDTA) and $CaCl_2$. The budding and vesiculation process was monitored microscopically. The titration procedure involved the addition of EDTA and $CaCl_2$ in increments, separated by 15-min incubation periods. Once approximately 50% of the cells were observed to be forming buds, EDTA and $CaCl_2$ addition was stopped, and the cell suspension was incubated another 15 min. At this point, the buds had been pinched off from the cells ("mother cells") and were free as large vesicles in the suspension. The vesicles could then be easily separated from the remaining intact cells, mother cells, etc. by sucrose gradient centrifugation.

During the process of bud formation, the discoid erythrocyte became spherical and then began to form large buds. Usually only one bud was formed per cell, two buds were more infrequent, and three buds per cell were rather rare. It should be noted that this entire budding and vesiculation process occurs without hemolysis.

Enzymatic modification of the erythrocyte vesicle membrane proteins was carried out with (1) trypsin (EC 3.4.21.4) (Sigma, type XI) according to the procedure of Elgsaeter & Branton (1974), (2) chymotrypsin (EC 3.4.21.1) (Sigma, type II) by using the procedure of Siegel et al. (1980), (3) neuraminidase (EC 3.2.1.18) (Sigma, type V) according to the method of Nigg et al. (1980), and Pronase (Calbiochem, grade B) as described by Snow et al. (1981), with the modification that NaT_R^- (pH 7.4) was used instead of Na–Tris (pH 7.4).

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was carried out according to the method of Fairbanks et al. (1971), as modified by Steck & Yu (1973). Gels were stained with Coomassie Blue or periodic acid–Schiff base (PAS) as described by Fairbanks et al. (1971). Protein bands were identified according to the nomenclature of Steck (1974) by using ghosts obtained according to Dodge et al. (1963) and purchased molecular weight marker proteins (Boehringer Mannheim GmbH-Biochemica) as standards. Gels were scanned by using a Beckman spectrophotometer equipped with a linear transport system. Peak areas for each band were determined by weighing cutouts of scan tracings. Protein was measured with the Bio-Rad protein assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Lipids were extracted from Dodge ghosts of human erythrocytes with chloroform/methanol (2:1 v/v) and the nonlipid contaminants removed according to the method of Radin (1969). Phosphate content was measured according to Bartlett (1959). Lipids were stored in chloroform/methanol (2:1 v/v) under N_2 at −20 °C until used.

Unilamellar lipid vesicles were prepared from the lipids isolated from "Dodge" ghosts of human erythrocytes according to standard methods. Aliquots of the isolated lipids in chloroform/methanol (2:1 v/v) were dried first under N_2 and then under vacuum. The sample was resuspended (2.5 μmol of phospholipid/mL) in a buffer consisting of 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) + 100 mM NaCl + 0.05 mM EDTA, pH 7.0, vortexed for 10 min, and sonicated in a bath-type sonicator (Heat Systems, Ultrasonics) under N_2 at 30 °C until clear. The vesicle suspensions were then centrifuged at 100000*g* for 1 h and the supernatants used for aggregation experiments.

Vesicle aggregation was followed by measuring the turbidity of vesicle suspensions with a Hitachi (100-60) spectrophotometer, equipped with a temperature control cell housing connected to a water bath/pump (Neslab Instruments). The turbidity of $H_E$ves and $R_E$ves suspensions was monitored at 500 nm. This wavelength was chosen to minimize interference from hemoglobin absorption. Isolated lipid vesicle suspensions were monitored at 400 nm (a wavelength in the visible range which gave maximum light scattering of the sonicated vesicles), after determining that the results obtained at 400 and 500 nm were qualitatively the same.

To ensure that the vesicle concentrations of control and enzymatically treated $H_E$ves and $R_E$ves were equivalent, a standard curve was generated relating the initial turbidity of untreated vesicle suspensions to vesicle concentration (milligrams of protein per milliliter). Experiments were conducted at vesicle concentrations corresponding to the linear portion of the curve, with both control and enzymatically treated samples being plotted as a function of their initial turbidities, rather than their final protein concentrations. An equal quantity of vesicles was then added to the reference cuvette to balance the relative optical density (OD) at zero. To maintain internal consistency, a portion of a vesicle preparation to be enzymatically treated was removed and used as an additional control sample during aggregation experiments.

All vesicle aggregation experiments were carried out under standardized conditions. This was especially important because of the sensitivity of the aggregation process to the effects of

temperature, pH, and phosphate concentration. The conditions used in these experiments were the following: (1) cuvettes containing 2 mL of buffer solution (NaT_R^- + 0.05 mM EDTA, at the desired pH) were equilibrated to the desired temperature (usually 30 °C) for 10 min; (2) aliquots of the vesicle preparations were added and incubated for 5 min; (3) aliquots of concentrated (0.2 M) phosphate solutions, made from premixed solutions of mono- and dibasic sodium phosphate to give the same pH as the buffer solution upon dilution, were added and the samples incubated for the time indicated; and (4) aliquots of concentrated (1.0 M) $CaCl_2$ were added to give the desired final Ca^{2+} concentration in the test sample. Vesicle aggregation was monitored continuously for at least 30 min, by using an Omniscribe recorder (Houston Instruments) interfaced to the spectrophotometer, and in many cases also checked by plotting the digital readout values obtained in 2-min increments.

Results

Vesicle Composition. Both $H_E$ves and $R_E$ves have markedly simplified membrane protein compositions, as characterized by $NaDodSO_4$ gel electrophoresis (Leonards & Ohki, 1983). In addition to the absence of cytoskeleton-associated proteins, the only membrane proteins detected were band 3 region polypeptides and glycoproteins (PAS-1, -2, and -3 in $H_E$ves, three or four glycoproteins in $R_E$ves). These proteins maintain their native orientation within the membrane, and the vesicles, which are right side out, maintain their cytoplasmic integrity. The lipids of the vesicles are qualitatively the same as those of intact erythrocytes, although they do have a slightly increased cholesterol content (no diacylglycerols are present) (Leonards & Ohki, 1983).

Establishing Optimal Conditions for Examining Ca^{2+}–PO_4-Induced $H_E$ves and $R_E$ves Aggregation. Previous studies of Ca^{2+}–PO_4-induced membrane aggregation and/or fusion have been conducted under conditions where the total [Ca^{2+}], [PO_4], pH, or temperature was reported (Zakai et al., 1976, 1977; Majumdar & Baker, 1980; Fraley et al., 1980). In order to provide a basis for comparing the results obtained in the present study to those previously published, and to determine the optimal conditions for studying $H_E$ves and $R_E$ves aggregation, we examined the effects of pH, [Ca^{2+}], [PO_4], and order of ion addition on vesicle aggregation.

In the first series of experiments, the optimum pH for monitoring aggregation was determined by measuring the turbidities (OD_{500}) of $H_E$ves suspensions as a function of [Ca^{2+}] at pH 7.4, 7.6, 7.8, and 8.0. Both the [vesicles] (≃60 μg of protein/mL) and [PO_4] (2 mM) were kept constant. The [Ca^{2+}] was increased in increments every 2 min by injecting aliquots of a 1.0 M $CaCl_2$ solution, mixing, and recording the OD observed at each 2-min interval. It should be noted that the OD_{500} values obtained at each interval do not represent equilibrium values but were arbitrarily chosen for internal consistency. Typical results are shown in Figure 1. They indicate that the maximum turbidity obtained, over the [Ca^{2+}] range tested, increased as the pH of the suspension was raised. At pH 8.0, however, the turbidity of the sample decreased after the [Ca^{2+}] was raised beyond a certain level (25 mM). This decrease was not a consequence of the settling of the vesicles, suggesting that structural changes may be occurring in the vesicles themselves at these [Ca^{2+}] values.

In addition to a pH effect, Figure 1 indicates that the length of time the vesicle suspensions were incubated with PO_4 before Ca^{2+} addition affected the extent of vesicle aggregation. In each case, the turbidity of the suspension was greater when the vesicles were incubated with PO_4 for 30 min before Ca^{2+}

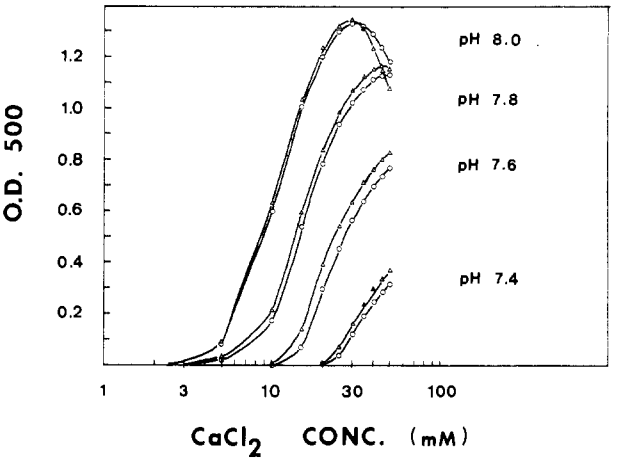

FIGURE 1: Turbidity (OD_{500}) of $H_E$ves suspensions in 2 mM PO_4 at different pH values as a function of [$CaCl_2$] (vesicle concentration ≃60 μg of protein/mL): (○) 2-min incubation period, vesicles + PO_4, before initial $CaCl_2$ addition; (△) 30-min incubation period, vesicles + PO_4, before initial $CaCl_2$ addition.

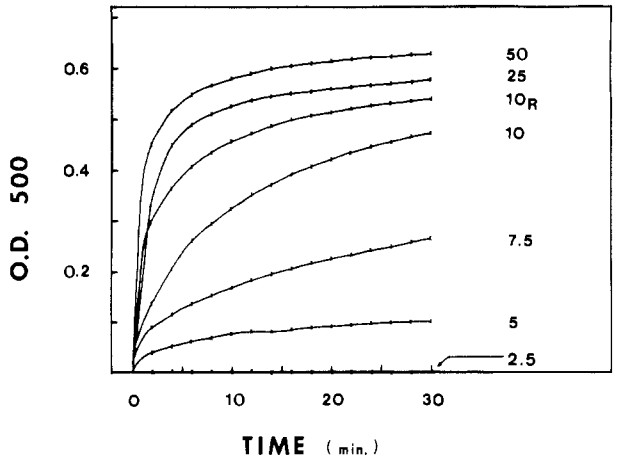

FIGURE 2: Turbidity (OD_{500}) of $H_E$ves suspensions (pH 7.8) in 2 mM PO_4 + indicated [$CaCl_2$] as a function of time. Vesicles were incubated with PO_4 for 2 min before $CaCl_2$ addition. Vesicle concentration was ≃45 μg of protein/mL. 10_R, $CaCl_2$ added 2 min before PO_4.

addition, rather than 2 min, although the difference becomes relatively small at pH 8.0. Control experiments conducted under the same conditions, but in the absence of vesicles, did not display this effect. Other control experiments, conducted in the presence of either vesicles + Ca^{2+} or vesicles + PO_4 only, demonstrated no change in turbidity from base-line values. From these results, we determined that the turbidity values obtained at pH 7.8 would be sufficiently sensitive to small environmental changes, while still being within the optimum OD range of the spectrophotometer. Possible complications in data analysis, due to unknown gross structural changes occurring in the vesicles, would also be avoided at this pH.

In a second series of experiments, the effects of varying the relative [PO_4] and [Ca^{2+}] ratios on vesicle suspension turbidities were examined. The effects of changing the [Ca^{2+}] on both the extent and kinetics of vesicle suspension turbidity increases are shown in Figure 2. For these experiments, the [PO_4] was kept constant at 2 mM. The results indicate that there is a threshold [Ca^{2+}], between 2.5 and 5 mM, below which no change in turbidity occurs. After this threshold value is crossed, there is a rapid increase in both the rate and extent of sample turbidity. The relative difference between 25 and 50 mM Ca^{2+}, however, is much less than that between 7.5 and 10 mM Ca^{2+}, indicating that the effect of increasing the [Ca^{2+}] approaches a limiting value. The curves obtained for 10 mM

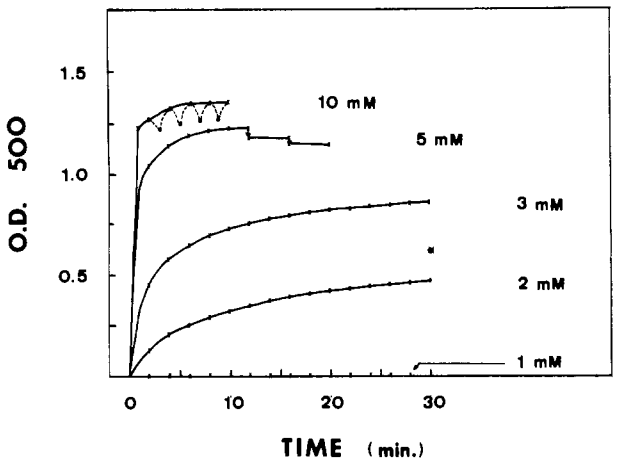

FIGURE 3: Turbidity (OD_{500}) of $H_E$ves suspensions (pH 7.8) at the indicated [PO_4] + 10 mM $CaCl_2$ as a function of time. Broken curves for 10 mM PO_4 indicate settling of cuvette contents. Vesicle concentration was ≃45 μg of protein/mL; the asterisk indicates the value obtained for 2 mM PO_4 + 50 mM $CaCl_2$ from Figure 2.

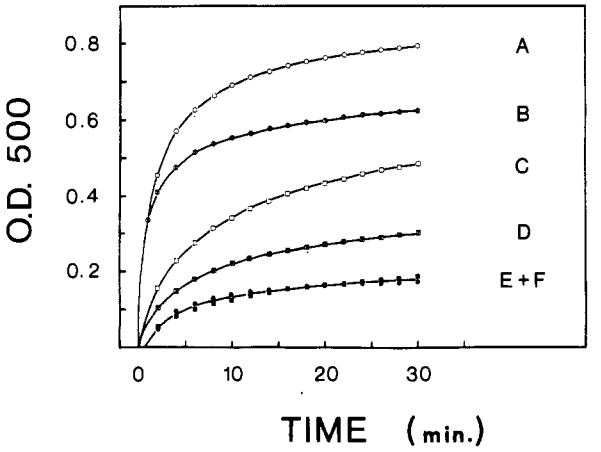

FIGURE 4: Apparent and actual aggregation of $H_E$ves induced by Ca^{2+}–PO_4 as a function of time (pH 7.8). Curve A, 3 mM PO_4 + 10 mM $CaCl_2$ + $H_E$ves; curve B, 3 mM PO_4 + 10 mM $CaCl_2$ only; curve C, 2 mM PO_4 + 10 mM $CaCl_2$ + $H_E$ves; curve D, 2 mM PO_4 + 10 mM $CaCl_2$ only; curve E (■), OD_{500} = $H_E$ves – control (curve C – curve D); curve F (●), OD_{500} = $H_E$ves – control (curve A – curve B). Vesicle concentrations ≃45 μg of protein/mL.

Ca^{2+} allowed both the kinetics and the turbidity levels of the sample to be accurately determined; 10 mM Ca^{2+} was, therefore, employed in our further studies of vesicle aggregation.

In the previous experiments, PO_4 was added to the vesicle suspension before Ca^{2+}. To ascertain if the order of ion addition affected the results obtained, experiments were conducted with the order of addition reversed. The results for the case of 2 mM PO_4 and 10 mM Ca^{2+} are also shown in Figure 2. A comparison of the curve observed for PO_4 and then Ca^{2+} (curve 10) to that for Ca^{2+} and then PO_4 (curve 10_R) indicates that there is indeed a difference. However, as discussed below, this difference was found to be due to an increase in the background Ca^{2+}–PO_4 only values and not vesicle aggregation itself.

In reciprocal experiments, the effects of altering the [PO_4] on both the extent and kinetics of vesicle suspension turbidity increases were examined. The results are shown in Figure 3. The [Ca^{2+}] was kept constant at 10 mM in these experiments. The experiments indicate a threshold [PO_4], between 1 and 2 mM, below which no turbidity change occurs. Above this threshold level, there is a rapid increase in the rate and extent of suspension turbidity. At 5 mM PO_4, however, there is a decrease in OD_{500} after approximately 12 min. Since this decrease was not reversible by additional mixing, it indicates that changes, other than the settling of the cuvettes' contents, are also occurring in the suspension. When the [PO_4] was increased to 10 mM, there was an immediate flocculation and settling of sample contents which could be reversed by mixing over the first few minutes. The flocculation overwhelmed any other changes occurring in the sample, and the experiments were therefore terminated after only a few minutes. On the basis of these results, we determined that 2–3 mM PO_4 was in the optimum phosphate concentration range for examining $H_E$ves and $R_E$ves aggregation.

A comparison of the data presented in Figures 2 and 3 indicates that the turbidity of the sample was significantly more sensitive to changes in [PO_4] than in [Ca^{2+}]. This is demonstrated by the asterisk in Figure 3, which corresponds to the OD_{500} value obtained (Figure 2) with 2 mM PO_4 + 50 mM $CaCl_2$ after 30 min. This sensitivity to [PO_4] was found to be responsible for the increase noted in Figure 2 (curves 10 and 10_R). In the first case, Ca^{2+} was added to a previously mixed and dispersed PO_4 solution. However, when the order of addition was reversed, the result was a very high, albeit temporary, local [PO_4], and thus a higher OD_{500} value. To avoid complications in interpreting the data obtained, PO_4 was added and mixed prior to Ca^{2+} injection.

To this point, the results obtained have been described in terms of the absolute turbidities of the sample suspension. The presentation of the results in this format coincides with that of the previously cited reports for comparative purposes. It should be noted, however, that the data presented above are consequences of two distinct and superimposed phenomena. One is the change in turbidity due to the aggregation of the vesicles themselves. The second is the increase in OD_{500} due to the formation of Ca^{2+}–PO_4 complexes in solution (Robertson, 1973; Boskey & Posner, 1977; Eanes & Meyer, 1977). To separate these two phenomena, we conducted parallel experiments under identical conditions, but in the absence of vesicles. Assuming simple additivity, these background turbidity curves were then subtracted from the experimental curves (obtained in the presence of vesicles) to estimate the actual changes in turbidity due to vesicle aggregation (designated ΔOD_{500}). Figure 4 illustrates the results found for such experiments. When vesicle suspensions contained 3 mM PO_4 + 10 mM Ca^{2+} (A), the apparent degree of vesicle aggregation was significantly greater than that (C) seen with 2 mM PO_4 + 10 mM Ca^{2+}. However, when the background curves were subtracted, the curves obtained for both 2 and 3 mM PO_4 (B and D) were found to be essentially identical (E and F). These results indicate that a proper evaluation of vesicle aggregation must include corrections for changes in background turbidity. Consequently, all further experiments were evaluated in terms of ΔOD_{500} values. In addition, as will be demonstrated later, it is necessary to maintain the [PO_4] in a range where its concentration in solution is not significantly depleted by PO_4 association with the vesicle membrane surface.

Roles of Membrane Proteins and Lipids in Ca^{2+}–PO_4-Induced Aggregation of $H_E$ves and $R_E$ves. To provide a reference base line for these studies, experiments were first conducted with untreated $H_E$ves and $R_E$ves. These experiments were evaluated by examining the ΔOD_{500} values obtained at the 30-min time point as a function of vesicle concentration, under various conditions. The results are shown in Figure 5. There is a linear relationship between the extent of vesicle aggregation and the concentration of vesicles, over

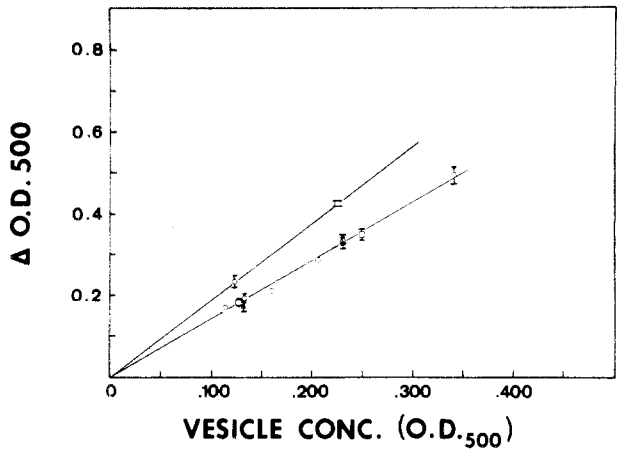

FIGURE 5: ΔOD_{500} for Ca^{2+}–PO_4-induced aggregations of $H_E$ves and $R_E$ves as a function of vesicle concentration (pH 7.8, 27 °C). Lower line, vesicles incubated 2 min with PO_4 before 10 mM $CaCl_2$ addition. Upper line, vesicles incubated 1 h with PO_4 before 10 mM $CaCl_2$ addition. (□) $R_E$ves + 2 mM PO_4; (○) $H_E$ves + 2 mM PO_4; (△) $H_E$ves + 3 mM PO_4. Points are the average of at least two samples; standard deviations (vertical bars) are indicated where three or more samples were tested. An OD_{500} of 0.100 was found for a vesicle concentration of ≃35 μg of protein/mL. [See Leonards & Ohki (1983) for $NaDodSO_4$ gel electrophoresis patterns of $H_E$ves and $R_E$ves.]

the range tested. The slopes of these lines depended on the length of time the vesicles were incubated in the presence of PO_4 before Ca^{2+} addition. When vesicle suspensions were incubated for 2 min, the lower line was obtained. When the incubation time was increased to 1 h, the upper line was obtained. Other experiments (not shown), having incubation times between these two, indicated that most of the incubation period effect occurred within the first 15–20 min and that the 1-h value represents a stable maximum.

Although the lipid compositions of human and rabbit erythrocytes are relatively similar (Nelson, 1972), their sialic acid contents are quite different (Fischer et al., 1979). To determine if these, or other, compositional differences might affect vesicle aggregation, experiments were conducted to compare $H_E$ves with $R_E$ves. The results, illustrated in Figure 5, show that there was no difference in vesicle aggregation between $H_E$ves and $R_E$ves. Even the incubation period effect was found to be the same.

To confirm that the ΔOD_{500} values obtained for 2 and 3 mM PO_4 (Figure 4) represented a true equivalence in vesicle aggregation, and not merely a coincidence of the vesicle concentration used, experiments were conducted by employing both PO_4 concentrations at different vesicle concentrations. The results, also shown in Figure 5, demonstrate that vesicle aggregation is indeed equivalent in 2 and 3 mM PO_4. These results indicate that the extent of vesicle aggregation is determined by the concentration of vesicles in the sample, over this range of $[PO_4]$.

Membrane Proteins. The potential roles of the membrane proteins in vesicle aggregation were studied by selectively modifying these proteins enzymatically and determining the effects of the modifications on the aggregation process. The enzymes employed in this series of experiments were chymotrypsin, trypsin, and Pronase. We have previously demonstrated that the enzyme molecules do not enter the vesicles (Leonards & Ohki, 1983). Consequently, the sites of enzymatic action are restricted to cleavage sites exposed on the exterior surface of the vesicle membrane. For chymotrypsin, which is able to attack both band 3 proteins and glycophorin at the exterior surface of the erythrocyte membrane (Steck,

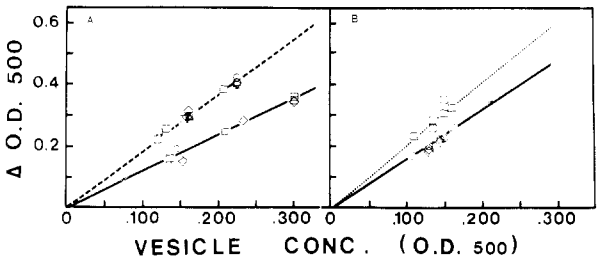

FIGURE 6: ΔOD_{500} for Ca^{2+}–PO_4-induced aggregation of $H_E$ves or $R_E$ves ± enzymatic treatment as a function of vesicle concentration (2 mM PO_4 + 10 mM $CaCl_2$, pH 7.8). (□) $H_E$ves or $R_E$ves minus enzyme treatment (control); (○) $H_E$ves or $R_E$ves + enzyme treatment (incubated 2 min with PO_4 before $CaCl_2$ addition); (◇) $H_E$ves or $R_E$ves + enzyme treatment (incubated 40 min with PO_4 before $CaCl_2$ addition). An OD_{500} of 0.100 was determined at a vesicle concentration of ≃35 μg of protein/mL. (A) In the presence or absence of chymotrypsin: (—) 25 °C; (---) 30 °C. (B) Neuraminidase at 30 °C: (···) without neuraminidase; (—) with neuraminidase.

1978), the result should be an extensive alteration of the vesicle surface. Trypsin, like chymotrypsin, attacks both band 3 proteins and glycophorin. However, the cleavage site for trypsin on the band 3 protein is located on the cytoplasmic side of the plasma membrane (Steck, 1978). Consequently, trypsin should modify the glycophorin but not the band 3 protein molecules in $H_E$ves. Pronase, which is a mixture of numerous proteolytic enzymes, should cause the maximum alteration of the proteins exposed at the external membrane surface. It could therefore be used to expand and confirm the results obtained with chymotrypsin and trypsin. In all cases, $NaDodSO_4$ gel electrophoresis confirmed that the membrane proteins were modified as expected. To ensure internal consistency, control experiments were always conducted with the same buffer solutions by using an untreated portion of the vesicle preparation.

The results obtained from experiments measuring vesicle aggregation (ΔOD_{500}), ± chymotrypsin treatment, as a function of vesicle concentration, are shown in Figure 6A. The data obtained indicate that chymotrypsin treatment had no observable effect on the extent of vesicle aggregation for either $H_E$ves or $R_E$ves when the PO_4 incubation time was 2 min. There was also no effect on the rate of vesicle aggregation over the 30-min period monitored (data not shown). However, in contrast to experiments with untreated vesicles, the effect of PO_4 incubation time was eliminated by chymotrypsin treatment (Figure 6A). The ΔOD_{500} values obtained for $H_E$ves ± trypsin treatment and $H_E$ves ± Pronase treatment gave results identical with those obtained in the presence or absence of chymotrypsin treatment.

To determine if the effects of the PO_4 incubation period were distinct from those of temperature, experiments were conducted at 25 °C (solid line) and 30 °C (broken line). As Figure 6A demonstrates, increasing the incubation temperature from 25 to 30 °C did increase the extent of vesicle aggregation (slope increased). Yet, at both temperatures, the effect of PO_4 incubation time was eliminated, indicating that these are distinct phenomena.

The three enzyme preparations employed in the preceding sections modified the proteins of the vesicle membrane by cleaving the amino acid backbone of the molecules. The vesicle surface, however, is also known to contain a substantial quantity of carbohydrates, especially sialic acid molecules, linked to both proteins and lipids. It was previously observed that the quantitative differences between the sialic acid contents of $H_E$ves and $R_E$ves did not affect vesicle aggregation (Figure 5). It is still possible that the presence or absence of

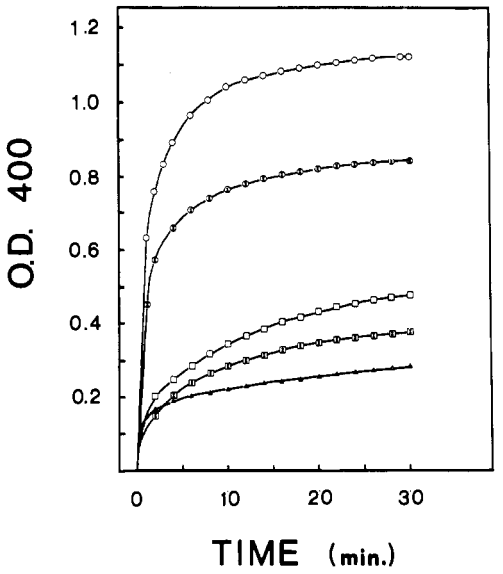

FIGURE 7: Ca^{2+}–PO_4-induced aggregation of lipid vesicles as a function of time. The vesicles were made from the isolated lipid extracts of Dodge ghosts obtained from human erythrocytes (cholesterol/phospholipid ratio = 0.83, pH 7.8, 30 °C). Incubation time of vesicles with PO_4 before $CaCl_2$ addition = 2 min. Note: 10 μL of lipid vesicles = 12.5 nmol of phospholipid. (× in open box) 2 mM PO_4 + 10 mM $CaCl_2$ only; (□) 2 mM PO_4 + 10 mM $CaCl_2$ + 10 μL of lipid vesicles; (△) 2 mM PO_4 + 10 mM $CaCl_2$ + 20 μL of lipid vesicles. Note that curve (△) is below the control curve (× in open box). (⊗) 3 mM PO_4 + 10 mM $CaCl_2$ only; (○) 3 mM PO_4 + 10 mM $CaCl_2$ + 20 μL of lipid vesicles. Note that by increasing the [PO_4] from 2 to 3 mM the aggregation of a 20-μL sample of lipid vesicles is now above the control curve (⊗). Note: Addition of $CaCl_2$ only to a lipid vesicle suspension did not cause any aggregation (OD_{400} at 30 min = 0.0).

specific sialic acid residues on select cellular components could be involved in the aggregation process. To examine this possibility, $H_E$ves were treated with neuraminidase, and the effects of this treatment on vesicle aggregation were determined.

The ΔOD_{500} values obtained for $H_E$ves ± neuraminidase treatment are shown in Figure 6B. The results indicate that neuraminidase treatment reduced the extent of vesicle aggregation of vesicle suspensions incubated for 2 min with PO_4 before Ca^{2+} addition (approximately 20%). The rates of vesicle aggregation were also reduced (data not shown). The effect of the PO_4 incubation period was also eliminated. These results are in marked contrast to those observed for chymotrypsin, trypsin, and Pronase treatments, where only the effect of the PO_4 incubation period was removed. Control experiments involving vesicle preparations carried through all of the steps of neuraminidase treatment, but minus the enzyme, did not display these effects, confirming that the reduced levels and rates of $H_E$ves aggregation were a direct consequence of neuraminidase activity.

Membrane Lipids. To ascertain the potential roles of the membrane lipids in this process, the aggregation of small unilamellar vesicles (SUVs), made from Dodge ghost lipid extracts of human erythrocytes, was examined. We previously reported that the lipid compositions of $H_E$ves and Dodge ghosts are qualitatively similar (Leonards & Ohki, 1983). Quantitatively, the $H_E$ves are slightly enriched in their cholesterol content (cholesterol/phospholipid ratio of 0.94 in $H_E$ves vs. 0.83 in Dodge ghost lipid extracts of human erythrocytes). The results obtained with SUVs of Dodge ghost lipids should, therefore, be applicable for initial studies on the roles of $H_E$ves lipids in vesicle aggregation.

Examples of the data observed in experiments measuring the changes in OD_{400} as a function of time for such SUV preparations are shown in Figure 7. The results obtained suggest a significant interaction between PO_4 and SUV lipids which seems to be quite sensitive to the lipid to PO_4 ratio in the suspension. In the lower three curves, experiments were conducted with 2 mM PO_4. (×) in an open box refers to the control experiment (Ca^{2+} + PO_4 only). When the lipid concentration was 12.5 nmol/mL phospholipid (□), the OD_{400} values obtained were above control values over the entire time course of the experiment, indicating the the SUVs were aggregating. However, when the lipid concentration was doubled, to 25 nmol/mL phospholipid (△), the OD_{400} curves obtained were below the control curves. (Statistical analysis of multiple experiments confirmed that the observed curves were significantly different.) In addition, the OD_{400} values observed for control experiments conducted in the presence of the SUVs + Ca^{2+} only (no PO_4) remained at zero, eliminating the possibility of direct Ca^{2+}-induced vesicle aggregation. These results suggest that the apparent concentration of PO_4 in the sample was reduced below that of the control samples at the higher lipid concentration. One possible explanation for this effect would be that a significant fraction of the PO_4 molecules were removed from the solution by associating with the lipid vesicle surface. If correct, it should be possible to compensate for this interaction by increasing the PO_4 concentration. To examine this possibility, experiments were repeated by using the higher lipid concentration (25 nmol/mL phospholipid) under the same conditions, but in the presence of 3 mM PO_4. The results obtained are shown in the upper 2 curves of Figure 7 [(× in open circle) Ca^{2+} + PO_4 only]. The top curve (○), containing the same concentration of lipid as the bottom curve (△), is now substantially above the control curve (⊗), indicating extensive vesicle aggregation. Thus, the factors giving rise to the bottom curve (△) can be compensated for by increasing the [PO_4] in the sample.

Discussion

The experimental results obtained in this study can be divided into two sections. In the first section, the optimal values of pH, [PO_4], and [$CaCl_2$] were determined for quantitatively examining the extent and dynamics of $H_E$ves and $R_E$ves aggregation. In the second section, the possible roles of membrane proteins and lipids in the aggregation process were examined under these "standard conditions".

Previous studies on the Ca^{2+}–PO_4-induced aggregation and/or fusion of erythrocytes, erythrocyte ghosts, or synthetic lipid vesicles have concentrated upon pH or temperature effects and assumed the Ca^{2+}–PO_4 complex formation was constant under a variety of conditions (Zakai et al., 1976, 1977; Majumdai & Baker, 1980; Baker & Kalra, 1979; Fraley et al., 1980). To study these parameters, and to provide a comparative base line, we examined the effects of pH, [PO_4], [$CaCl_2$], and incubation time on $H_E$ves and $R_E$ves suspension turbidity. The results obtained indicate that vesicle aggregation is quite sensitive to the experimental conditions. The pH study demonstrates that large changes in vesicle suspension turbidity could occur over a relatively small pH range (7.4–8.0). In addition, the shifting of the turbidity curves to lower [$CaCl_2$] with increasing pH indicates that complex formation between Ca^{2+} and PO_4 is not constant but varies with pH. The effects of varying [PO_4] and [$CaCl_2$] at constant temperature and pH indicate that sample turbidity is more sensitive to [PO_4] than [$CaCl_2$].

Together these results indicate that two major conditions must be met to properly evaluate Ca^{2+}–PO_4–membrane interactions. First, experiments examining the effects of Ca^{2+}–PO_4 on membrane aggregation and/or fusion must take

into account the variation in Ca^{2+}–PO_4 complex formation occurring as a function of temperature and pH, in order to distinguish between factors which influence the physicochemical properties of the membranes themselves and their interactions from those directly affecting the formation of Ca^{2+}–PO_4 complexes (Robertson, 1973; Boskey & Posner, 1977; Eanes & Meyer, 1977). Second, because of the relative greater sensitivity of the membranes to changes in $[PO_4]$, experiments should be conducted under conditions where $[PO_4]$ is carefully controlled, kept below concentrations which cause the nonspecific breakdown of the membrane but above those where the association of PO_4 molecules with the vesicle surface could significantly alter the $[PO_4]$ in solution. In the present study, both of these conditions were met for $H_E$ves and $R_E$ves by using 2 and 3 mM PO_4 and by correcting the data obtained for background Ca^{2+}–PO_4 complex formation under the specific conditions being tested. In addition, the time dependence of the changes observed under these standard conditions enabled us to measure the dynamics of the interactions as well as their end products.

In earlier studies (Zakai et al., 1976, 1977; Majumdar & Baker, 1980; Baker & Kalra, 1979), it has been suggested that Ca^{2+}–PO_4 may be inducing membrane aggregation and/or fusion by modifying the erythrocytes' spectrin–actin cytoskeletal network and inducing the lateral aggregation of membrane proteins. While Ca^{2+}–PO_4-induced modifications in the spectrin–actin cytoskeletal network of erythrocytes are quite likely, such effects are ruled out in the present study since both the $H_E$ves and $R_E$ves are cytoskeleton free (Leonards & Ohki, 1983) yet undergo extensive vesicle aggregation. It should be noted that this does not preclude the possibility that membrane proteins are aggregated to form protein-free areas for membrane fusion but suggests that such aggregation phenomena may be directly induced at the membrane surface.

The results obtained in this study indicate that there is a vesicle membrane–PO_4 molecule interaction which seems to be comprised of at least two components. Moreover, the results suggest that the major component, which is responsible for vesicle aggregation, is a specific association of the PO_4 molecules with vesicle membrane lipids. This conclusion is supported by a number of lines of evidence. These include the following: (a) the finding of identical kinetics of vesicle aggregation exhibited by both $H_E$ves and $R_E$ves, even though their sialic acid content and glycoprotein compositions are different; (b) the observation of the same degree of aggregation for both $H_E$ves and $R_E$ves in 2 and 3 mM PO_4; (c) the lack of any affect of extensive enzymatic modification of the membrane proteins on the kinetics or extent of vesicle aggregation for samples incubated for 2 min with PO_4 before Ca^{2+} addition; and (d) the interactions between PO_4 and SUVs made from the lipids extracted from Dodge ghosts of human erythrocytes, which indicate a strong PO_4–membrane lipid association (Figure 7).

The second component of the PO_4–membrane interactions does seem to involve the membrane proteins, as evidenced by the influence of enzyme treatment on the vesicle + PO_4 incubation time effect. This influence, however, may be rather indirect and secondary since the results obtained for the 2-min vesicle + PO_4 incubation period were unaffected by trypsin, chymotrypsin, or Pronase treatment. [Freeze–fracture electron microscopy of the vesicles indicates that the membrane proteins, i.e., intramembranous particles, maintain their random distribution under these conditions (K. S. Leonards et al., unpublished results).]

Of the four enzyme treatments employed in this study, only neuraminidase treatment affected the major component of $H_E$ves aggregation induced by Ca^{2+}–PO_4, reducing both its rate and its extent by approximately 20%. However, since both $H_E$ves and $R_E$ves had identical aggregation kinetics, though different sialic acid contents, this effect cannot be merely due to the number of sialic acid residues present on the membrane surface. Instead the results suggest two other possibilities. One is that removal of sialic acid residues from the glycophorin molecules exposes a chemical group (perhaps carbohydrate) which itself inhibits Ca^{2+}–PO_4-induced vesicle aggregation. The second is that neuraminidase treatment is affecting vesicle aggregation by removing sialic acid residues from glycolipid components in the membrane. These two possibilities are being presently investigated.

The ability of Ca^{2+}–PO_4 to aggregate SUVs made from the lipids extracted from Dodge ghosts of human erythrocytes, in a way which seems to be strongly influenced by the PO_4/lipid ratio, suggests that PO_4 is interacting directly with the $H_E$ves and $R_E$ves membrane lipids. At this point, however, it is not known whether this association is a nonspecific lipid–PO_4 interaction or requires the presence of particular lipids at the membrane surface. We are presently investigating SUVs made from the different lipid components present in erythrocyte membranes to answer this question.

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Registry No. Calcium, 7440-70-2; phosphate, 14265-44-2; neuraminidase, 9001-67-6.

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Rapid Laser Flash Photoaffinity Labeling of Binding Sites for a Noncompetitive Inhibitor of the Acetylcholine Receptor†

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ABSTRACT: Photoaffinity labeling of the nicotinic acetylcholine receptor from *Torpedo marmorata* electric tissue was performed in the presence of cholinergic effectors in the millisecond to second time range by a combination of a stopped-flow apparatus and a high-energy pulse laser. The label applied was [^{3}H]triphenylmethylphosphonium, a lipophilic cation previously shown to be a specific blocker of the acetylcholine receptor ion channel. With the receptor in the resting state most of the label was incorporated into the α polypeptide chains. In the presence of agonists and antagonists increasing incorporation into the δ- and (less pronounced) the β-chain was observed. The time course of this increase had a half-life of about 0.4 s, being slower than receptor activation and channel opening. In the resting, active, and even rapidly desensitized state, the α polypeptide chains appear to be the primary targets of the photoaffinity reaction. The action spectrum of the photolabeling has a sharp maximum at λ = 270 nm and a small-side maximum at λ = 290 nm. It does not resemble the absorption spectrum of the label and may hint at amino acid side chains as the moieties activated by UV light causing the photolabeling. The effector specificity of the observed slow increase of label incorporation into the δ polypeptide chain was investigated. It does not prove that slow desensitization is the underlying event. The agonists acetylcholine and carbamoylcholine as well as treatment of receptor-rich membranes with phospholipase A_2 (but not phospholipase D) triggered labeling of δ, but antagonists such as D-tubocurarine and most conspicuously flaxedil had a similar effect.

The nicotinic acetylcholine receptor (AChR)[1] is present in the postsynaptic membrane in at least three different functional states, termed resting (with its ion channel closed), active (ion channel open), and inactive or desensitized [ion channel closed, affinity for agonists 2 orders of magnitude higher than in the resting state [for a recent review, see Changeux (1981)]]. The molecular mechanism of the transitions between these states is generally grossly described as conformational changes of the receptor protein, but no details are available as to the subunits or the part of the primary structure involved. It is well established now that the α polypeptide chains (M_r 40 000) contain the binding site for agonists and antagonists. But there is little information concerning the role of the other three polypeptide chains of the receptor complex (β, M_r 48 000; γ, M_r 60 000; δ, M_r 68 000). The δ-chain has been implied in high-affinity binding of noncompetitive blockers (NCBs) (Oswald & Changeux, 1981). NCBs are allosteric inhibitors of the AChR and its ion channel but at least some of them appear to be direct steric blockers of the channel as well (Heidmann et al., 1983). For elucidation of the blocking mechanisms of NCBs—the allosteric and the steric one—and for identification of their site of action, the following observations could be helpful: Several NCBs have been shown to react covalently with AChR upon UV irradiation of the receptor–NCB complex (Oswald & Changeux, 1981). For example, the lipophilic cation [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) has been shown to block reversibly the cation flux through the ion channel (Lauffer & Hucho, 1982) and to react during UV irradiation covalently with various receptor subunits depending on the receptor state (Muhn & Hucho, 1983). In the absence of cholinergic ef-

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[1] Abbreviations: [^{3}H]TPMP$^+$, [^{3}H]triphenylmethylphosphonium; AChR, nicotinic acetylcholine receptor; NCB, noncompetitive blocker; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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