Phospholipids as Adjuncts for Calcium Ion Stimulated Release of Chromaffin Granule Contents: Implications for Mechanisms of Exocytosis†

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ABSTRACT: Structure–function relationships for the lipid component of chromaffin granules isolated from the bovine adrenal medulla have been investigated by employing ^{31}P nuclear magnetic resonance (NMR), freeze–fracture, and spectrophotometric techniques. Two aspects have been studied in detail, namely, the structural preferences of lipids in the isolated granule membrane and derived liposomal model membrane systems as well as the influence of exogenous lipid (in the form of sonicated vesicle systems) on the Ca^{2+}-stimulated release of granule contents. It is shown that at least 90% of endogenous granule membrane phospholipids assume a liquid-crystalline bilayer configuration at physiological temperatures. Liposomal dispersions of total granule lipid also exhibit bilayer structure, consistent with a structural role of phospholipids in vivo. Incubation of intact isolated granules in the presence of up to 10 mM Ca^{2+} does not induce significant release of contents above background levels. However, it is shown that incubation of granules in the presence of sonicated phospholipid systems which undergo structural transitions in the presence of Ca^{2+} can cause immediate and total release of granule contents at Ca^{2+} levels of 2 mM or more. This behavior is attributed to disruption of granule membrane integrity due to fusion of the vesicle systems with the chromaffin granules. Direct evidence for such fusion is obtained by freeze–fracture electron microscopy. On the basis of this information and with the assumption that the inner leaflet of the adrenal cell plasma membrane is composed predominantly of phosphatidylethanolamine and phosphatidylserine, a mechanism of Ca^{2+}-stimulated exocytotic release of catecholamines in vivo is proposed.

The chromaffin granules of the adrenal medulla act as storage vesicles for catecholamines which they concentrate to high levels (0.55 M) in association with ATP (0.125 M). The major biochemical processes associated with these granules involve the active uptake of adrenaline and noradrenaline (Casey et al., 1977) and their subsequent release to the extracellular medium as required. This latter process, which is triggered by the presence of Ca^{2+} (Douglas, 1975), is thought to occur via exocytosis involving fusion of the granule with the surrounding plasma membrane as an intermediary step.

In this work, we have approached the problem of exocytotic release of chromaffin granule contents from two points of view. First, it is of interest to ascertain whether the dynamic organization of lipid in this specialized membrane is significantly different from that observed in other biological membranes, and whether such differences can be correlated to functions such as fusion. In particular, it is important to characterize the bilayer or nonbilayer preferences of the endogenous lipid and the influence of Ca^{2+} on these preferences, given suggestions that nonbilayer lipid configurations occur as intermediaries in fusion of model (Verkleij et al., 1979) and biological (Cullis & Hope, 1978) membrane systems and that Ca^{2+} can trigger formation of such structures in certain lipid systems (Cullis et al., 1978; Cullis & Verkleij, 1979). Second, from the point of view of efficient extracellular release of catecholamines, it is obviously advantageous if granule–plasma membrane (as opposed to granule–granule) fusion is preferentially stimulated by the presence of Ca^{2+}. We have therefore investigated the influence of exogenous lipid (which may approximate the inner monolayer composition of the chromaffin cell plasma membrane) on Ca^{2+}-stimulated release of chromaffin granule contents.

It is shown by ^{31}P NMR techniques that endogenous chromaffin granule membrane phospholipids are primarily in the bilayer configuration at 37 °C, consistent with a recent spin-label electron spin resonance (ESR) study (Fretten et al., 1980). The presence of up to 10 mM Ca^{2+} does not markedly affect the preference for bilayer structure. Further, Ca^{2+} at concentrations up to 10 mM does not cause appreciable release of granule contents in the intact system. However, incubation of granules in the presence of sonicated model membrane systems of phosphatidylserine (PS)–phosphatidylethanolamine (PE) (1:3), pure PS, pure cardiolipin (CL), and "inner monolayer" erythrocyte lipids together with the subsequent introduction of 2 mM or more Ca^{2+} can result in complete release. All these model systems have the property that the introduction of Ca^{2+} results in changes in the structural preferences of the lipids which are accompanied by vesicle precipitation. In the case of PE–PS, CL, and inner monolayer systems, Ca^{2+} induces a bilayer to hexagonal (H_{II}) transition, whereas for pure PS vesicles Ca^{2+} induces formation of crystalline "cochleate" structures (Papahadjopoulos et al., 1974). In contrast, PC–PS and "outer monolayer" erythrocyte model membrane lipid systems which do not undergo such transitions in the presence of Ca^{2+} are ineffective for inducing release. On the basis of these observations, and with the postulation that the inner monolayer of the plasma membrane of chromaffin cells contains predominantly PE and PS, a mechanism for the Ca^{2+}-stimulated release of catecholamines in vivo is suggested.

Materials and Methods

Sucrose (grade I) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Phosphatidylcholine (PC) was isolated from the total lipids of soybeans. Phosphatidylethanolamine and phosphatidylserine were obtained from soya PC, employing the base-exchange capacity of phospholipase D (Comfurius & Zwaal, 1977). Phospholipase D was obtained from savoy cabbage according to established techniques (Kates & Sastry, 1969). The phospholipids were purified by employing silicic acid and

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0006-2960/82/0421-4583$01.25/0

(carboxymethyl)cellulose column chromatography, arriving at white compounds which were at least 99% pure as indicated by thin-layer chromatography (TLC). Phosphatidylserine was converted to the sodium salt as indicated elsewhere (Hope & Cullis, 1980). Individual classes of erythrocyte lipids were isolated and purified by using low-pressure liquid chromatography as described elsewhere (Hope & Cullis, 1979). Cardiolipin (bovine heart) was obtained from Sigma (St. Louis, MO).

Chromaffin granules were isolated from fresh bovine adrenal glands obtained from a local abattoir, employing a slightly modified version of the methods of Smith & Winkler (1967). In particular, for large preparations, homogenization of the adrenal medullas was more conveniently carried out by employing 40–100 mesh sand (Guena, 1974). The large granule fraction was suspended in 0.3 M buffered sucrose [10 mM Hepes, pH 7.0, and 1 mM ethylenediaminetetraacetic acid (EDTA)]. In the majority of experiments, the large granule fraction rather than highly purified granules was employed due to the osmotic fragility of the purer preparations. The specific activity of the mitochondrial marker enzyme, malate dehydrogenase, in the large granule fraction was approximately one-third of that in the initial homogenate.

Chromaffin granule membranes were prepared by lysing in 5 mM Hepes (pH 7.0) and three freeze–thaw cycles followed by centrifugation at 27000*g* in an SS-34 rotor for 30 min. This was repeated 4–5 times until the absorbance at 265 nm of the supernatant after centrifugation was less than 5% of the value obtained after the first lysis.

Two types of calibration experiments were performed in order to quantify the amount of chromaffin granule membrane phospholipid contributing to the ^{31}P NMR signal observed. Initially, the CG:PC ratios (R_{PC}^{CG}) of the integrated signal intensities (recorded sequentially under exactly similar experimental conditions) from a standard sample of egg yolk PC and the chromaffin granule membrane sample were obtained. This was subsequently compared first to the ratio obtained from phospholipid phosphorus assays of the standard egg PC sample and the granule membrane sample. Second, R_{PC}^{CG} was compared to the ratios of the egg PC standard and the chromaffin granule ^{31}P NMR signal intensities after the addition of 0.4 mL of Triton X-100 to both samples. This was sufficient detergent to solubilize both membrane systems, giving rise to translucent dispersions and a narrow, symmetric ^{31}P NMR spectral feature. In the case of the ratios obtained from phospholipid phosphorus assays and after solubilization with detergent, it may be presumed that all the chromaffin granule phospholipid contributes to the R_{PC}^{CG} ratio obtained. Thus, comparison of the ratios obtained by employing the intact granule membrane to those obtained via the phospholipid phosphorus assay and after detergent treatment gives a measure of the amount of phospholipid not detected in the intact granule membrane.

Phospholipid phosphorus and water-soluble phosphorus determinations for both the intact granules and isolated membranes were performed by precipitation in 10% trichloroacetic acid (Cl_3CCOOH) and subsequent centrifugation (27000*g*, 15 min). Water-soluble phosphorus was determined by phosphorus assay (Böttcher et al., 1961) of the supernatant, whereas the lipid phosphorus and protein (Lowry et al., 1951) were assayed by employing the washed pellet dissolved in 0.5 N NaOH. The protein to phospholipid ratios (weight to weight, assuming an average phospholipid molecular weight of 800) for the intact granules and the isolated membranes were 6.25 and 1.0, respectively. This is consistent with the values obtained by other workers (Winkler et al., 1970). The water-soluble phosphorus to phospholipid phosphorus ratios (mole per mole) of the intact granules and isolated membranes were found to be 5.0 and 0.08, respectively.

Total lipids were extracted (Bligh & Dyer, 1959) from the large granule fraction, and the phospholipid composition was obtained by two-dimensional TLC followed by visualization by iodine vapor and assay of phospholipid phosphorus in the various spots. The composition on a mole percent basis was found to be 8.1% phosphatidylserine, 10.1% lysophosphatidylcholine, 15.1% sphingomyelin, 35.7% phosphatidylethanolamine, and 31% phosphatidylcholine, consistent with literature values (de Oliveira-Filgueiras et al., 1979).

The phospholipid model systems were obtained by mixing appropriate quantities of lipid in chloroform. The chloroform was then evaporated under a stream of nitrogen and by subsequent storage under vacuum for 2 h. The lipid was hydrated in the buffered sucrose solution and sonicated intermittently (30-s sonication followed by 30-s intervals in an ice–water bath) by employing a tip sonicator. Sonication was continued until the dispersion became optically clear (approximately 5 min).

Release of granule contents after various experimental protocols was conveniently followed spectrophotometrically by monitoring the absorbance at 265 nm, where the contents of the chromaffin granules (ATP, protein, catecholamines) absorb quite strongly (Edwards et al., 1974; Morris et al., 1977). Typically, chromaffin granules (0.8–1.2 mg of protein, 40–50 μL of the large granule fraction) in buffered sucrose were incubated in the presence of sonicated vesicles, Ca^{2+}, etc. at 25 °C for 15 min in a total volume of 1.0 mL. Subsequently, this mixture was diluted to 4.0 mL with cold buffered sucrose and centrifuged (12000*g*, 15 min, 4 °C). The supernatant was assayed for release of chromaffin granule contents by monitoring the absorbance at 265 nm as well as by protein and catecholamine determinations (see below). Controls contained appropriate amounts of NaCl instead of $CaCl_2$ in order to minimize osmotic differences.

The amount of protein released was measured by assaying (Lowry et al., 1951) 1.0 mL of the supernatant. The catecholamine release was determined according to a spectrophotometric assay (von Euler & Hamberg, 1949) on 1.0 mL of the supernatant. Briefly, this involved addition of 1.0 mL of 1 M acetic acid buffer (pH 6.0) containing 50 μL of 10% sodium dodecyl sulfate ($NaDodSO_4$) and 0.2 mL of 1 N iodine solution to 1.0 mL of the supernatant. After 10 min, 0.2 mL of 0.5 M sodium thiosulfite was added, and the absorbance at 530 nm was read immediately. The blank was 1.0 mL of buffered sucrose in both determinations.

A reproducible background level of about 25% release was observed in all controls, in agreement with other workers (Hillarp et al., 1953; Morris et al., 1977). Therefore, release of contents was expressed after subtracting the background, and total release corresponded to lysis in 1.0 mL of 10 mM Hepes (pH 7.0) followed by a freeze–thaw cycle and dilution to 4.0 mL with buffered sucrose as in the above protocol.

The ^{31}P NMR experiments were performed on a Bruker WP 200 NMR spectrometer, operating at 81 MHz for phosphorus, which was equipped with proton decoupling and temperature control. Accumulated free induction decays were obtained for up to 2000 transients by employing an 11-μs 90° pulse and 0.8-s interpulse time.

Freeze–fracture was performed on the undiluted 1.0-mL samples described above. Glycerol was added as a cryoprotectant to a final concentration of 25%, and the samples were

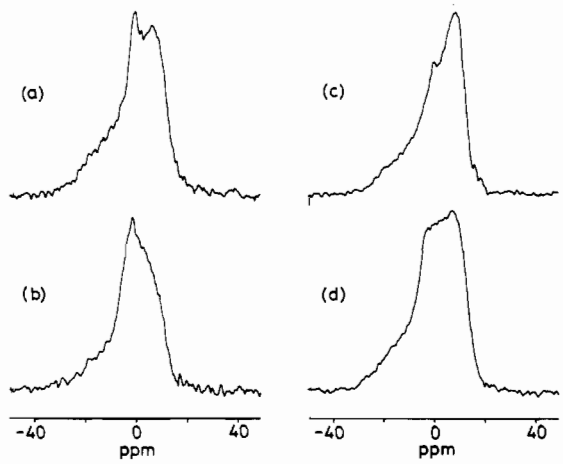

FIGURE 1: ^{31}P NMR spectra (81.0 MHz) at 37 °C arising from the following: (a) isolated chromaffin granule membranes; (b) isolated chromaffin granule membranes in the presence of 10 mM $CaCl_2$; (c) liposomes composed of lipids extracted from chromaffin granule membranes; (d) same as (c) but in the presence of 10 mM $CaCl_2$. All preparations contained 10 mM Tris–HOAc (pH 7.2) and, where $CaCl_2$ was not present, 2 mM EDTA. The solutions used for the biological membrane contained 10% D_2O, whereas that of the liposomes contained 90% D_2O. 0 ppm refers to the resonance position of sonicated PC vesicles.

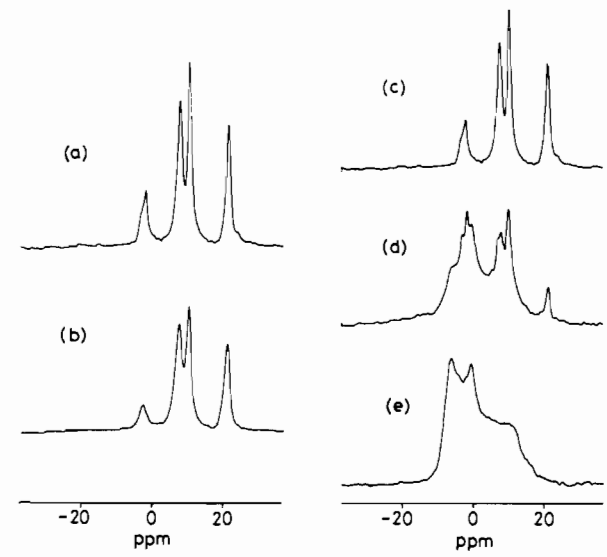

FIGURE 2: ^{31}P NMR spectra (81.0 MHz) at 37 °C obtained from the following: (a) intact chromaffin granules; (b) granules incubated at 37 °C for 15 min in the presence of 10 mM $CaCl_2$; (c) same as (a); (d) chromaffin granules incubated in the presence of PE–PS (3:1) vesicles (R = 4.0, for definition of R, see below) for 10 min at 37 °C followed by introduction of $CaCl_2$ to a concentration of 10 mM, after which the incubation at 37 °C was continued for an additional 5 min; (e) same as (d) with the exception that R = 6.0. R is defined as the molar ratio of exogenous (vesicular) PE–PS phospholipid to endogenous (chromaffin granule) phospholipid. The buffered sucrose medium (see Materials and Methods) was employed throughout. For other details, see Materials and Methods.

immediately frozen in liquid Freon and stored under liquid nitrogen. Freeze–fracture was done according to standard procedures by employing a Balzer's apparatus, and replicas were viewed by employing a Philip's 400 electron microscope.

Results

As indicated in the introduction, a first priority was to establish the dynamic structural organization of the endogenous lipid of the chromaffin granule membrane. As shown elsewhere (Cullis & de Kruijff, 1979), ^{31}P NMR is a useful technique for determining the bilayer or nonbilayer preferences of phospholipids in model and biological membranes. Briefly, in large (diameter >2000 Å) bilayer systems, phospholipids in the liquid-crystalline bilayer phase exhibit broad asymmetric ^{31}P NMR signals with a low-field shoulder, whereas hexagonal (H_{II}) phase phospholipids give ^{31}P NMR signals with reversed asymmetry which are narrower by a factor of 2. Finally, phospholipids in phases such as inverted micellar, cubic, or rhombic exhibit narrow symmetric signals.

The ^{31}P NMR spectra arising from the isolated chromaffin granule membranes, as well as model (liposomal) systems consisting of hydrated preparations of the total extracted lipid, are indicated in Figure 1. Two features are apparent. First, at 37 °C, a large majority of the endogenous granule membrane lipids evidence ^{31}P NMR spectra characteristic of the bilayer phase. This is with the exception that a small (<5%) component gives a signal characteristic of isotropic motional averaging which may arise from small membrane fragments or lipids in other structures allowing isotropic averaging. Second, the model membrane liposomal systems exhibit very similar bilayer spectra, consistent with a structural role of phospholipids in the intact membrane. Finally, the addition of Ca^{2+} to these systems results in little change in the biological membrane spectra and in the appearance of a relatively small (<10%) component, possibly arising from phospholipid in the hexagonal (H_{II}) phase, in the model system composed of the isolated total lipids. In order to show what fraction of the endogenous chromaffin granule membrane phospholipids is actually detected, we calibrated the observed intensity of the ^{31}P NMR signal for the intact granule membranes against a known amount of egg phosphatidylcholine liposomes as described under Materials and Methods. The ratios of chromaffin granule membrane phospholipid signal intensity to that of the egg PC standard were 0.35 ± 0.02 for the intact systems, 0.36 ± 0.02 as determined by phospholipid phosphorus assays, and 0.37 ± 0.02 as determined after solubilization with Triton X-100. These results indicate that more than 90% of the endogenous chromaffin granule phospholipids contribute to the observed ^{31}P NMR signal.

The second stage of the research was to establish the influence of exogenous lipid and Ca^{2+} on chromaffin granule release. This was first approached by employing ^{31}P NMR techniques, as summarized in Figure 2. This figure indicates the spectra obtained from intact chromaffin granules (large granule fraction) incubated at 37 °C for 15 min in the absence of Ca^{2+} (Figure 2a) and in the presence of 10 mM Ca^{2+} (Figure 2b). These preparations were subsequently concentrated for ^{31}P NMR studies by centrifugation. In both cases, the dominant features of the ^{31}P NMR spectra arise from ATP. It may be noted that these spectra are equivalent to previously published (Seeley et al., 1977) ^{31}P NMR spectra of intact granules. The presence of ATP in the spectra of Figure 2b demonstrates that the presence of 10 mM Ca^{2+} does not induce release of granule contents, in agreement with other studies (Edwards et al., 1974). Against this background, the results implicit in Figure 2c–e are dramatic. These spectra were obtained from granules incubated in the presence of varying amounts of PE–PS (3:1) vesicles (10 min, 37 °C) to which Ca^{2+} was added subsequently and incubation continued for an additional 5 min. Defining R as the molar ratio of added exogenous (vesicular) phospholipid to endogenous (chromaffin granule) phospholipid, it is clear that for R = 6.0 (Figure 2e) relatively complete release of granule contents has occurred as indicated by the absence of ATP. In addition, a new spectral component is apparent in the region of −7 ppm which coincides with the position of the low-field peak arising

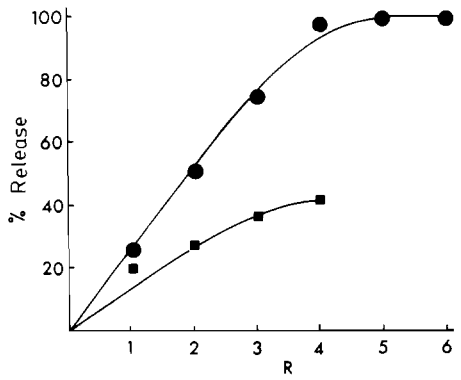

FIGURE 3: Release of chromaffin granule contents after incubation in the presence of Ca^{2+} and increasing amount of exogenous PE–PS phospholipid as assayed by spectrophotometric techniques (see Materials and Methods): (●) incubation in the presence of PE–PS vesicles (3:1) (15 min) where 5 mM $CaCl_2$ was introduced after 10 min; (■) incubation in the presence of PE–PS (3:1) vesicles (15 min) where 5 mM $CaCl_2$ was present in the granule suspension prior to introduction of the vesicles. Buffered sucrose was employed throughout.

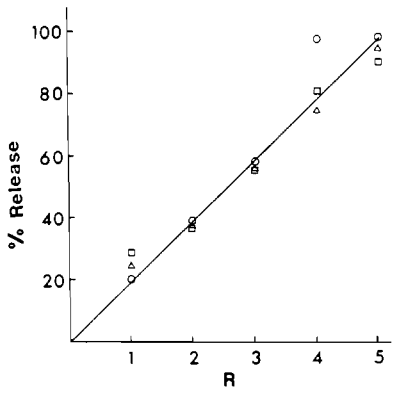

FIGURE 4: Correlation of the amount of release of the chromaffin granule contents in the presence of exogenous PE–PS (3:1) vesicles with subsequent addition of $CaCl_2$ as determined by monitoring the absorbance at 265 nm (△), the amount of protein released (○), and the amount of catecholamine released (□). For details, see Materials and Methods.

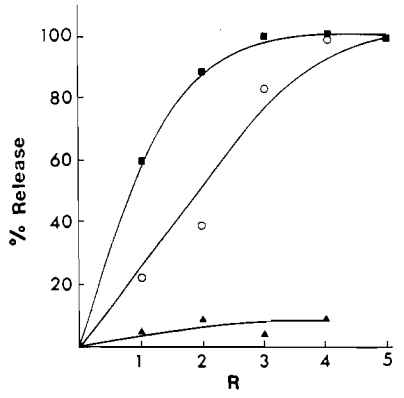

FIGURE 5: Ca^{2+}-stimulated release of chromaffin granule contents after incubation with vesicles of various compositions: (○) incubation in the presence of pure soya PS vesicles (15 min) where 5 mM $CaCl_2$ was introduced after 10 min; (■) incubation in the presence of pure cardiolipin vesicles (15 min) where 5 mM $CaCl_2$ was introduced after 10 min; (▲) incubation in the presence of PC–PS (3:1) vesicles (15 min) where 5 mM $CaCl_2$ was introduced after 10 min.

from phospholipids in the hexagonal (H_{II}) phase (Cullis & de Kruijff, 1978).

The appearance of a hexagonal (H_{II}) phase phospholipid component is not unexpected on the basis of the behavior of the PE–PS (3:1) vesicles in the presence of Ca^{2+}. The addition of Ca^{2+} to PE–PC (3:1) vesicles (which induces immediate precipitation of the vesicle suspension) triggers formation of the hexagonal (H_{II}) phase, as indicated by ^{31}P NMR (results not shown). This is consistent with previous results obtained for bilayer liposomal PE–PS systems (Tilcock & Cullis, 1981) where the presence of Ca^{2+} also triggers precipitation and H_{II} phase formation.

In order to further characterize this ability of exogenous lipid to act as an adjunct for Ca^{2+}-stimulated release of granule contents, we performed spectrophotometric assays as indicated under Materials and Methods. The results obtained are summarized in Figure 3, which shows that the presence of PE–PS (3:1) vesicles at a concentration corresponding to a model membrane phospholipid to chromaffin granule membrane phospholipid ratio (R) of 5 (mol/mol) results in complete release when Ca^{2+} is added. This contrasts strongly with the results obtained when either PE–PS vesicles or Ca^{2+} is employed separately. No release for Ca^{2+} concentrations as high as 10 mM was observed. This clearly establishes a requirement for both PE–PS vesicles and Ca^{2+} as indicated by the ^{31}P NMR results. Further, the amount of release is sensitive to the order in which these agents are added to the chromaffin granule preparation. Less release is observed if the granules are incubated with Ca^{2+} prior to incubation with PE–PS (3:1) vesicles. This behavior is interpreted as arising from Ca^{2+}-induced precipitation of the sonicated vesicles to form the hexagonal H_{II} phase before the vesicles can interact with the granule membranes.

The assay for chromaffin granule release by monitoring the absorbance at 265 nm may be criticized on the basis that it is relatively nonspecific. Assays for release of granule protein and catecholamines were therefore performed as indicated under Materials and Methods. As indicated in Figure 4, assays for release employing A_{265}, protein determination, and catecholamine determinations gave equivalent results. Thus, as the A_{265} assay was the most convenient, it was employed in all subsequent experiments.

The mechanism whereby PE–PS (3:1) vesicles act as adjuncts for Ca^{2+}-stimulated release of chromaffin granule contents (Figures 2–4) is of particular interest. It may be suggested that the chromaffin granule release results from Ca^{2+}-induced fusion of the vesicles with the granule membrane. Lysis may then occur as a result of the fusion event itself, or the presence of "nonbilayer" lipid in the granule membrane which can then no longer support bilayer structure. As we have indicated elsewhere (Tilcock & Cullis, 1981), the addition of Ca^{2+} to PE–PS systems results in a structural segregation of the PS component into crystalline (presumably cochleate) (Papahadjopoulos et al., 1975) regions, allowing the PE to revert to the H_{II} phase it prefers in isolation. Questions then arise as to whether it is the ability of Ca^{2+} to induce crystalline cochleate structures, or hexagonal H_{II} phase organization (or both), which is related to the lytic event.

These questions were approached by testing the ability of vesicles composed of pure phosphatidylserine and pure cardiolipin to act as adjuncts for Ca^{2+}-stimulated releases. Whereas Ca^{2+} induces formation of a crystalline, apparently anhydrous cochleate structure for phosphatidylserine dispersions (Papahadjopoulos et al., 1975; Hope & Cullis, 1979), the addition of Ca^{2+} to cardiolipin model systems triggers formation of the hexagonal (H_{II}) phase (Rand & Sengupta, 1972; Cullis et al., 1978). As shown in Figure 5, both PS and CL vesicles are effective adjuncts for Ca^{2+}-stimulated release of chromaffin granule contents.

It is of interest to extend these observations to model systems which may more closely approximate the composition of the

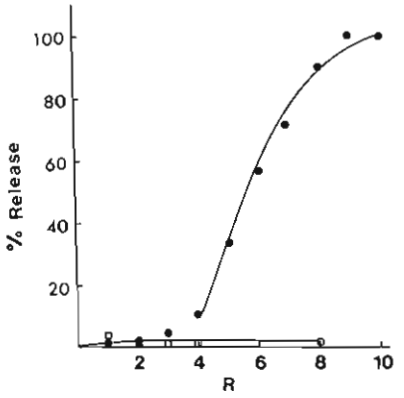

FIGURE 6: Release of chromaffin granule contents after incubation with vesicles composed of erythrocyte lipids: (□) incubation in the presence of outer monolayer lipids (15 min) where 5 mM $CaCl_2$ was introduced after 10 min; (●) incubation in the presence of inner monolayer lipids (15 min) where 5 mM $CaCl_2$ was introduced after 10 min. *R* is defined as the molar ratio of exogenous (vesicular) lipid to endogenous (chromaffin granule) phospholipid.

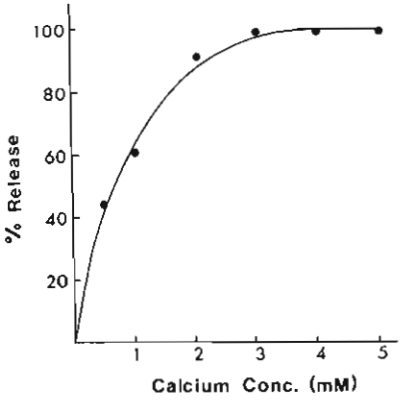

FIGURE 7: Effect of calcium concentration on the release of chromaffin granule contents incubated in the presence of PE–PS (3:1) vesicles to obtain a ratio *R* of exogenous (vesicular) phospholipid to endogenous (chromaffin granule) phospholipid of 4.0 for 15 min where the $CaCl_2$ was added after 10 min.

inner monolayer of the adrenal cell plasma membrane. On the assumption that the transbilayer distribution of phospholipids in this membrane is similar to that observed for the erythrocyte (the only plasma membrane well characterized in this regard), we examined the ability of vesicles composed of inner monolayer erythrocyte phospholipids [47 mol % PE, 28 mol % PS, 15 mol % PC, and 10 mol % sphingomyelin containing equimolar cholesterol with respect to phospholipid; see Zwaal et al. (1977)] to induce release. This protocol also assumes that the outer monolayer of the vesicle systems composed of inner monolayer phospholipids reflects the original lipid composition. This may be slightly different due to asymmetric lipid distribution in sonicated systems (Berden et al., 1975). It has been shown elsewhere that the inner monolayer model system (partially) adopts the hexagonal (H_{II}) phase in the presence of Ca^{2+}, whereas outer monolayer systems remain in a bilayer organization (Hope & Cullis, 1979). As indicated in Figure 6, the inner monolayer system can act as an adjunct for Ca^{2+}-stimulated release of granule contents, whereas the outer monolayer system does not. It may be noted that somewhat larger ratios of exogenous (vesicular) inner monolayer phospholipid to endogenous (granule) phospholipid are required than for the other adjunct systems illustrated here, a situation which we tentatively attribute to the instability of the sonicated inner monolayer vesicles, as indicated by an increasingly cloudy dispersion obtained as a function of time after sonication.

In all the above experiments, excess Ca^{2+} (5 mM) was used so that the limiting factor was the amount of phospholipid vesicles. When the amount of Ca^{2+} was made limiting, it was found that approximately 1 mM Ca^{2+} was required to induce effective release of the chromaffin granule contents (Figure 7) when PE–PS vesicles were employed as adjuncts. This value corresponds with the concentration of Ca^{2+} required to induce formation of the hexagonal (H_{II}) phase in analogous PS–PE liposomal systems (Tilcock & Cullis, 1981).

As indicated above, we attribute the ability of PE–PS and other vesicle systems to act as adjuncts for Ca^{2+}-stimulated release of granule contents to arise from fusion of the model systems with the granules. In order to place this hypothesis on a firmer foundation, we studied the PE–PS (3:1) vesicle–chromaffin granule systems by employing freeze–fracture techniques. Figure 8 shows the influence of 2 and 5 mM Ca^{2+} on the chromaffin granule (Figure 8a–c) and PE–PS (3:1) vesicle systems (Figure 8d–f). It may be noted that the

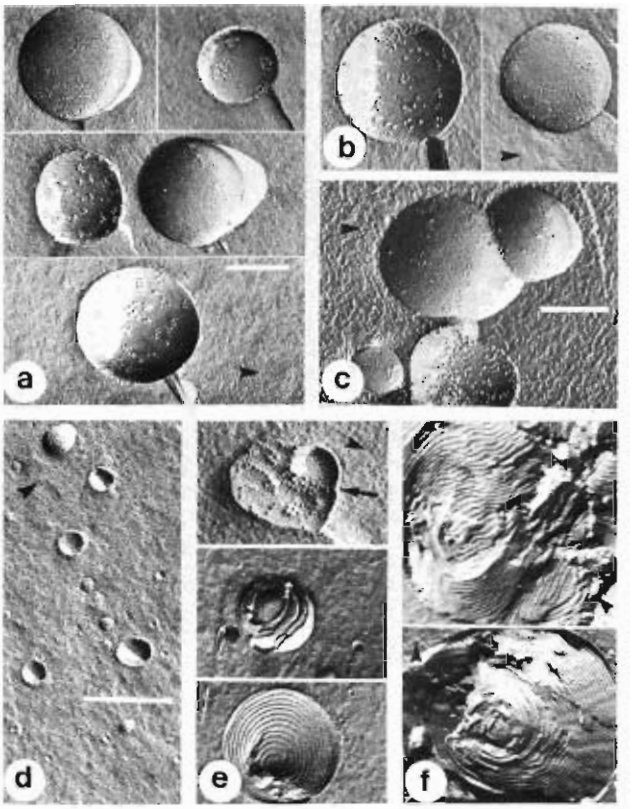

FIGURE 8: Freeze–fracture micrographs of the following: (a) chromaffin granules in the absence of Ca^{2+}, showing both convex (EF) and concave (PF) fracture faces; (b) chromaffin granules in the presence of 2 mM Ca^{2+}; (c) chromaffin granules in the presence of 5 mM Ca^{2+}; (d) sonicated PE–PS (3:1) vesicles in the absence of Ca^{2+}; (e) PE–PS (3:1) vesicles in the presence of 2 mM Ca^{2+} (the arrow indicates rows of lipidic particles); (f) PE–PS (3:1) vesicles in the presence of 5 mM Ca^{2+}. The white bars represent 200 nm, and the direction of shadowing is indicated by the arrowhead in each micrograph.

presence of 2 mM Ca^{2+} (Figure 8b) does not result in significant changes in granule distribution or size whereas 5 mM Ca^{2+} produces aggregation but not fusion (fusion is defined by the observation of larger fully rounded systems). This is consistent with the results of other workers (Edwards et al., 1974; Dahl et al., 1979) who observe aggregation (without mixing of internal compartments) at Ca^{2+} concentrations less than 10 mM and some fusion (appearance of larger rounded structures) at higher Ca^{2+} levels, although Ekerdt et al. (1981) do report fusion at lower Ca^{2+} levels. In the PE–PS (3:1)

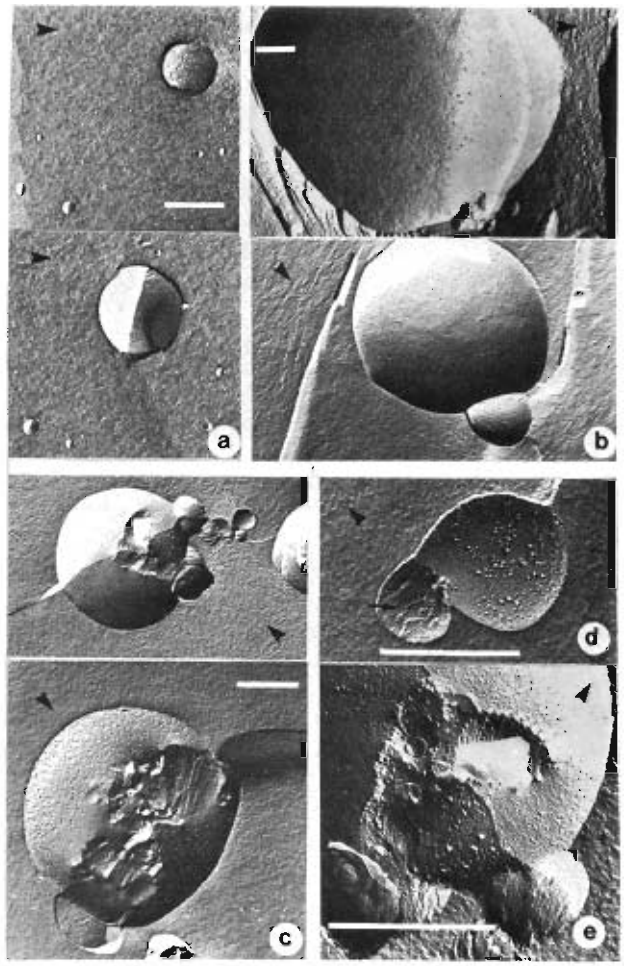

FIGURE 9: Freeze–fracture micrographs of the following: (a) chromaffin granules in the presence of sonicated PE–PS (3:1) vesicles where the ratio of chromaffin granule lipid to exogenous phospholipid is 1 to 4 and where the sample was prepared in the absence of Ca^{2+}; (b) the same preparation as (a) but incubated in the presence of 2 mM Ca^{2+}; (c) the same preparation as (a) but incubated in the presence of 5 mM Ca^{2+}; (d) a micrograph at higher magnification depicting the interaction between chromaffin granules and exogenous PE–PS (3:1) vesicular lipid after incubation in the presence of 2 mM Ca^{2+} (upper portion); (e) same as (d) but in the presence of 5 mM Ca^{2+}. Arrows indicate particles which exhibit characteristics of lipidic particles. The white bars represent 400 nm, and the direction of shadow is indicated by the arrowhead in each micrograph.

vesicle systems on the other hand, 2 mM levels of Ca^{2+} result in larger systems containing lipidic particles, some regions of hexagonal (H_{II}) structure (not shown), and also some spiral structures similar to cochleate PS–Ca^{2+} domains (Papahadjopoulos et al., 1975). These may be attributed to the ability of Ca^{2+} to segregate PS into crystalline domains in these mixed systems (Tilcock & Cullis, 1981). The presence of 5 mM Ca^{2+} (Figure 8f) results in large regions of hexagonal (H_{II}) phase, in agreement with the ^{31}P NMR results of Figure 2, as well as regions characteristic of cochleate structure.

Figure 9 illustrates the influence of Ca^{2+} on chromaffin granules incubated with PE–PS (3:1) sonicated vesicles. Figure 9b depicts the situation after incubation with 2 mM Ca^{2+}. The large structures observed (5–10 times larger than the isolated granules) exhibit intramembrane particles on the concave (PF) face which are of a similar size as the PF particles of the intact granules, whereas on the convex (EF) face the particles are much less distinct, again corresponding to the situation for the intact granule (Figure 8a). These observations, together with the fact that fusion of the PE–PS (3:1) vesicle systems by 2 mM Ca^{2+} produces much different structures (Figure 8e), establish that the large structures arise, at least in part, from the granules. The density of intramembranous particles on the PF face of the large fused system of Figure 8b is approximately 360/μm^2, or ~40% of the PF particle density of the parent granules. This particle dilution may be attributed to the presence of exogenous lipid derived from the PE–PS (3:1) vesicles. In some cases (results not shown), the PF particle density of the large fused granules was not significantly different from that of normal granules, indicating some variability in the number of PE–PS vesicles accompanying granule–granule fusion.

Higher levels of Ca^{2+} (5 mM) lead to appreciable perturbation of the large fused systems as shown in Figure 9c. Patches of apparently H_{II} phase or cochleate lipid structure are observed to he intimately associated with the membrane of tbe fused granule system.

The nature of the particles and other features observed in the presence of 2 and 5 mM Ca^{2+} are of interest and are indicated at higher magnification in Figure 9d,e. In particular, the 2 mM Ca^{2+} micrograph of Figure 9d would appear to correspond to a large PE–PS (3:1) system fusing with a chromaffin granule system. This is suggested by the different nature of the particles observed in the fracture face. In the lipid system, the "lipidic particles" tend to line up in rows (see Figure 8e), whereas the intramembrane particles of the granule do not.

Discussion

The ^{31}P NMR results obtained for the isolated granule membrane and model systems composed of extracted lipid establish that the large majority (90% or more) of the endogenous phospholipid experiences the bilayer phase at physiological temperatures, in agreement with a recent ESR study (Fretten et al., 1980). This is consistent with a role of the lipid component which is primarily structural in nature, serving to maintain granule integrity. It sbould be noted that while this behavior appears logical and is similar to results obtained from other biological membranes, such as the erythrocyte ghost membrane (Cullis & de Kruijff, 1978, 1979), certain observations for organelle membranes indicate that such behavior cannot be assumed a priori. In the case of rat liver endoplasmic reticulum, for example, two laboratories have independently reported the occurrence of isotropic motional averaging for endogenous phospholipids (Stier et al., 1978; de Kruijff et al., 1978). The observation that Ca^{2+} does not induce major changes in the motional behavior of the isolated granule membrane phospholipids is consistent with the observed inability of up to 10 mM Ca^{2+} to increase release of intact granule contents above background levels.

The major result of this work concerns the observation that lipid vesicles which undergo structural transformations in the presence of Ca^{2+} can act as adjuncts for Ca^{2+}-stimulated release of chromaffin granule contents. This ability is associated with fusion of the vesicles with the granule, and lysis may result due either to the fusion event itself or as a result of the presence of nonbilayer lipid in the granule membrane. The common property of the phospholipid vesicles exhibiting this ability is that Ca^{2+} induces aggregation and formation of larger structures for the vesicle system in isolation. As both cardiolipin and phosphatidylserine systems are able to act as Ca^{2+} adjuncts, it would appear that the detailed nature of this larger structure (i.e., bexagonal H_{II} or cochleate) is not a determining factor. We suggest that the instability of the adjunct vesicles in the presence of Ca^{2+} is relieved on fusion either with each other or with the chromaffin granule membrane according to their proximity and that in the process of

embedding themselves in the granule membrane the integrity of that membrane is disrupted. It may be noted that the ability of the sonicated vesicles to induce fusion between chromaffin granules corresponds closely with the ability of (negatively charged) phosphatidylglycerol- (PG) and PS-containing vesicles to induce fusion between cultured cells in the presence of Ca^{2+} (Papahadjopoulos et al., 1973). The detailed mechanism involved in this process remains a matter for speculation, however.

The relationship between the results presented here and exocytotic release of chromaffin granule contents in vivo is not immediately obvious. However, in previous work (Hope & Cullis, 1979), we have demonstrated that model systems composed of erythrocyte lipids in the proportions found in the inner leaflet of the erythrocyte membrane adopt the H_{II} configuration in the presence of Ca^{2+}. Thus, if the inner monolayer of the adrenal cell plasma membrane has a similar lipid composition as the erythrocyte, the presence of Ca^{2+} will destabilize it in the sense that the preferred configuration for a large fraction of the lipid will be the H_{II} phase. As we have indicated elsewhere (Cullis et al., 1980), formation of H_{II} structure from previously bilayer systems appears to proceed as an *inter* bilayer event. Thus, the strain experienced by the inner monolayer of the plasma membrane in the presence of Ca^{2+} could be relieved by interacting with closely apposed granule membranes to form short "inverted" cylinders (H_{II} structure) or inverted micelles (lipidic particles; Verkleij et al., 1979) which would also provide intermediaries in the exocytotic event [for a detailed model, see Cullis et al. (1980)].

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