Synergism between Mellitin and Phospholipase A_2 from Bee Venom: Apparent Activation by Intervesicle Exchange of Phospholipids[†]

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ABSTRACT: Mellitin, a cationic amphiphilic peptide, has an apparent activating effect on interfacial catalysis by phospholipase A_2 (PLA2) of bee venom on zwitterionic vesicles of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine (POPC) and on anionic vesicles of 1,2-dimyristoylglycero-*sn*-3-phosphomethanol (DMPM), as well as on covesicles of POPC/DMPM (3:7). On the other hand, mellitin-induced increase in the rate of pig pancreatic PLA2 is seen only on anionic vesicles. Interfacial kinetic protocols and spectroscopic methods show that the activation is due to enhanced substrate replenishment resulting from intervesicle exchange of zwitterionic or anionic phospholipids through vesicle−vesicle contacts established by mellitin. It is shown that as the hydrolysis on POPC vesicles progresses, due to a high propensity of bee PLA2 for binding to the product containing zwitterionic vesicles, most of the enzyme in the reaction mixture is trapped on few vesicles that are initially hydrolyzed, and thus reaction ceases. Under these conditions, mellitin promotes substrate replenishment by direct exchange of the products of hydrolysis from the enzyme-containing vesicles with the substrate present in excess vesicles which have not been hydrolyzed. Pig PLA2 has poor affinity for POPC vesicles, and the affinity is only modestly higher in the presence of low mole fractions of the products of hydrolysis; therefore, the enzyme is not trapped on those vesicles. Biophysical studies confirm that the phospholipid exchange occurs through stable intervesicle contacts formed by low mole fractions of mellitin, without transbilayer movement of phospholipids or fusion of vesicles. At high mole fraction (>1.5%) mellitin induces leakage in POPC vesicles and does not form additional contacts. In POPC/DMPM vesicles, the contacts are formed even at high mole fractions of mellitin. Changes in intrinsic tryptophan fluorescence of mellitin indicate that bound mellitin exists in at least two different functional forms depending on the lipid composition and on the lipid:peptide ratio. A model is proposed to accommodate amphiphilic mellitin as a transmembrane channel or an intervesicle contact.

Mellitin, a basic peptide with 26 residues in a amphiphilic sequence with most charges at the C-terminus (GIGAVLKVLT[10]TGLPALISWI[20]KRKRQQ), is the major constituent of honeybee venom. Mellitin belongs to a class of α-helical peptide toxins that have attracted a great deal of interest due to their antibacterial and hemolytic activities, generally attributed to their ability to perturb the barrier function of membranes (Saberwal & Nagaraj, 1994; Cornut et al., 1993; Boman, 1995; Mancheño et al., 1996). Although mellitin binds to zwitterionic interfaces (Levin et al., 1982; Schwarz & Beschiaschvili, 1989), biophysical studies have shown that the affinity is considerably higher (>100-fold) for (co)dispersions of anionic phospholipids. At high mole fractions, mellitin induces leakage of trapped solutes from vesicles (Benachir & Lafleur, 1995,1996), fusion (Morgan et al., 1982; Eytan & Almary, 1983), and phase changes (Verma & Wallach, 1976; Dufourc et al., 1986; Dempsey et al., 1989; Batenburg et al., 1988). Concerns about the complications caused by the presence of phospholipase A_2 (PLA2)[1], invariably present as an impurity in commercial samples, are now reasonably resolved by the use of synthetic or carefully purified preparations of mellitin.

Many questions remain about the basis for the function of mellitin. In bee venom, mellitin is secreted with a phospholipase A_2; therefore, the possibility of functional interactions and kinetic synergism is intriguing. In fact, several groups have reported the effect of mellitin on the rate and extent of hydrolysis of phospholipid vesicles by PLA2 (Argolias & Pisano, 1985; Mollay & Kreil, 1973; Yunes et al., 1977; Nishiya, 1991). Until recently, it was not possible to characterize such kinetic effects, because factors governing interfacial catalysis could not be adequately controlled under most commonly used assay conditions. Having demonstrated that interfacial catalysis by PLA2 can be analytically described in terms of the primary interfacial rate and equilibrium parameters (Berg et al., 1991; Jain et al., 1995; Yu et al., 1997), we undertook kinetic characterization of the effects of mellitin on PLA2. In this paper, it

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[1] Abbreviations: cmc, critical micelle concentration; DC_7PC, 1,2-diheptanoylglycero-*sn*-3-phosphocholine; DMPM, 1,2-dimyristoylglycero-*sn*-3-phosphomethanol; DTPE-DNS, *N*-dansylated 1,2-ditetradecylglycero-*sn*-3-phosphoethanolamine; DTPC, 1,2-ditetradecylglycero-*sn*-3-phosphocholine; DTPM, 1,2-ditetradecylglycero-*sn*-3-phosphomethanol; K_{SV}, Stern−Volmer quenching constant; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine; PLA2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphocholine; POPC-ether, 1-hexadecyl-2-(1-octadec-9-enyl)-glycero-*sn*-3-phosphocholine; PxB, polymyxin B; PyPM, 1-hexadecanoyl-2-(1-pyrenedecanoyl)glycero-*sn*-3-phosphomethanol; R18, octadecyl rhodamine; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoyl phosphatidylethanolamine; SUV, small unilamellar vesicles.

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is shown that the apparent activation of PLA2 by mellitin is due to its ability to mediate substrate replenishment on the enzyme-containing vesicles by direct intervesicle exchange of phospholipids with excess vesicles. Although the overall magnitude of the effect depends on the nature of the interface and the source of PLA2, the most dramatic effect is seen for the hydrolysis of zwitterionic vesicles by bee PLA2. Biophysical characterization of the mellitin-induced exchange shows that low mole fractions of mellitin establish contacts between vesicles and promote a direct intervesicle exchange of phospholipids between the outer monolayers of the vesicles in contact.

EXPERIMENTAL PROCEDURES

Bee venom PLA_2 was from Boehringer Manheim. Sources of other PLA_2 are as described (Jain et al., 1991c). For most studies, we used mellitin purified from bee venom (Sigma) by the published procedure (Habermann & Reiz, 1965) and further purified on CM-cellulose to yield a fraction that contained less than 0.01% PLA2 activity by weight as determined by kinetic analysis in the scooting mode on DMPM vesicles under the pseudo-first-order conditions (Jain et al., 1986a−d). For some studies and for numerous controls, we also used a mellitin preparation, purified by ion exchange chromatography after reductive (by dithiothreitol) denaturation of PLA2, kindly provided by Professor Michael Gelb (Seattle); it contained no detectable (<0.005%) PLA2. Samples of mellitin from Sigma or Calbiochem contained varying amounts (up to 1% by weight) of PLA2 and were found to be unsatisfactory for some of the studies. A molar extinction coefficient of 5700 M^{-1} cm^{-1} was used to calculate the mellitin concentration. The concentration of mellitin in reaction mixtures is expressed as mole percent in total phospholipid. The underlying assumption that substantially all mellitin is in the interface under our experimental conditions is supported by results in Figure 11, which show that the affinity of mellitin for anionic and zwitterionic interface is high. Pyrene-labeled phospholipids (pyPM) and R18 were from Molecular Probes. POPC, NBD-, and rhodamine-phospholipids labeled at the amino group of phosphatidylethanolamines were from Avanti. POPC-ether was from Calbiochem. DMPM (Jain et al., 1986c) and DTPE-DNS (Jain & Vaz, 1987) were synthesized as described. Dithionite (sodium hydrosulfite) was from Mallinckrodt Chemical Works (St. Louis).

Vesicles. Small unilamellar vesicles (SUVs) of POPC, POPC-ether, or POPC/DMPM (3:7) alone or with the fluorescent probes NBD-PE, Rh-PE, DTPE-DNS, or pyPM were prepared by evaporation of a mixture of the lipids in $CHCl_3/CH_3OH$ (2:1 v/v). The dried film was hydrated and then sonicated in a bath-type sonicator (Lab Supplies, Hickesville, NY, Model G112SPIT) above the gel−fluid transition temperature until a clear dispersion was obtained (typically 2−4 min). R18 (6 mol %) was incorporated in the outer monolayers of preformed vesicles by adding an aliquot of vesicles to a tube with a film of R18 (formed from a stock solution in ethanol), mixing, and incubating for 60 min in the dark. Vesicles were annealed for 1 h above their transition temperature.

Vesicles Labeled in the Inner Monolayer. Vesicles of POPC/DMPM (3:7) or POPC containing 0.6% NBD-PE incorporated in inner and outer monolayers were incubated in 0.2 mL of 10 mM Tris (pH 8.0) with 19 mM dithionite to selectively modify the NBD-PE groups present in the outer monolayer. After 5 min, the reaction mixture was diluted with more buffer to a final volume of 1.5 mL, with 54.4 μM lipid and 2.4 mM dithionite. At this point, all the outer membrane lipid has reacted with dithionite. Vesicles were used for fusion studies immediately after dilution.

Kinetic Studies in the Scooting Mode. Protocols for monitoring the reaction progress for the hydrolysis of vesicles by PLA2 in the scooting mode have been described elsewhere (Berg et al., 1991; Jain et al., 1991a, 1986a). Typically, hydrolysis was monitored on a Radiometer pH-stat titration assembly with the stirrer speed of about 2000 rpm. The reaction mixture containing the indicated amount of vesicles in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl solution was equilibrated at pH 8.0 in a stream of nitrogen. The reaction was initiated by adding the enzyme solution, typically 2−30 pmol of PLA2 in 1−10 μL of water. The number of substrate molecules present in the outer monolayer of the vesicles (N_s) was obtained from the extent of hydrolysis under the conditions where there was at most 1 enzyme per enzyme-containing vesicle. The total amount of substrate available in the reaction mixture for the hydrolysis by PLA2 was obtained by adding excess enzyme, so that there was at least 1 enzyme on every vesicle. Typically, about 65% of the total substrate was accessible for hydrolysis by the enzyme. As shown elsewhere (Berg et al., 1991), this is expected only if the lipid in the outer monolayer of vesicles is hydrolyzed, if the lipid present in the inner monolayer of vesicles does not flip to the outer layer, and if the vesicles remain intact even after all the substrate in the outer monolayer is hydrolyzed by PLA2. Results reported in this paper show that these conditions are satisfied in the presence of PLA2 and low concentrations of mellitin. To extend the initial linear (zero-order) phase of the reaction progress, 6 nmol (0.9 mol %) of PxB was added before or after initiating the reaction with enzyme (Jain et al., 1991b; Cajal et al., 1995, 1996a,b).

Fluorescence Measurements. Spectroscopic measurements were carried out on an AB-2 spectrofluorimeter (SLM-Aminco) with constant stirring. All spectral manipulations were done with the software provided with the instrument. Typically, the slit-widths were kept at 4 nm each and the sensitivity (PMT voltage) was set for the buffer blank to 1% for the Raman peak corresponding to the same excitation wavelength. All readings were corrected for any background or dark current contributions.

(i) Lipid Transfer Assay. The exchange of lipid between vesicles on the addition of mellitin was assessed by monitoring transfer of pyrene-labeled phospholipids from covesicles of pyPM/POPC (7:3) or from vesicles of pyPC (1.7 μM) to a 125-fold excess of unlabeled phospholipid vesicles as acceptor. Fluorescence emission was monitored at 395 nm (with excitation at 346 nm) corresponding to the monomer emission. δF is defined as the relative increase in fluorescence intensity $(F - F_o)/F_o$, where F_o and F are the intensities without and with mellitin, respectively. Mellitin did not have any effect on the fluorescence emission of pyrene vesicles.

(ii) Release of Self-Quenching. Lipid mixing induced by mellitin was monitored by measuring the release of self-quenching of 6 mol % R-18 incorporated in the outer monolayer of POPC or POPC/DMPM vesicles. These vesicles (1.7 μM) were mixed with unlabeled vesicles in

1:125 mole ratio, and the fluorescence increase at 577 nm (excitation 556 nm) upon the addition of mellitin was recorded. The change in fluorescence was calculated as $[F - F_o]/[F_{max} - F_o]$, with F_o and F corresponding to the fluorescence intensities before and after the addition of mellitin, and F_{max} as the fluorescence after total dequenching calculated by disruption of the vesicles with 3 mM deoxycholate.

(iii) Lipid Mixing by RET. Vesicles of POPC or POPC/DMPM containing 0.3% of NBD-PE and 0.3% of Rh-PE (21.8 μM) were mixed with 50-fold excess of unlabeled vesicles. Fluorescence from rhodamine was monitored at 592 nm (excitation 460 nm) to minimize contribution from NBD fluorescence. The change in fluorescence was calculated as δF. Readings were corrected for the effect of mellitin on the fluorescence RET of POPC-labeled vesicles (< 15%); no correction was needed in POPC/DMPM-labeled vesicles.

(iv) Dithionite-Induced Quenching Assay to Determine the Accessibility of Phospholipids in SUV Vesicles. Reaction of SUVs containing NBD-PE with dithionite selectively eliminates the fluorescence signal derived from NBD present in the outer leaflet by a reduction reaction (McIntyre & Sleight, 1991; Hoekstra et al., 1993), unless of course the vesicles become leaky. An aliquot of POPC/DMPM or POPC vesicles doped with 0.6% NBD-PE was added to 1.5 mL of 10 mM Tris (pH 8.0) saturated with nitrogen, with constant stirring (lipid concentration = 110 μM). When a stable baseline was achieved (usually less than 60 s after the addition), the reaction was started by adding dithionite from a stock solution to a final concentration of 10 mM. The time-dependent decrease in the fluorescence at 535 nm was recorded for 800 s (resolution = 1 s). Excitation wavelength was set at 460 nm. Stock solutions of freshly prepared 1.44 M dithionite in 0.5 M Na_2CO_3 (pH 11) buffer saturated with nitrogen gas were stored at 0 °C and used within 1 h.

(v) Inner Monolayer Lipid Mixing by RET. A 1:1 mixture of vesicles containing 0.6% Rh-PE and of asymmetrically labeled NBD-PE vesicles, where the NBD groups in the outer monolayer were chemically quenched by reaction with dithionite, was used to monitor mixing of phospholipids in the inner monolayers by mellitin (lipid concentration = 110 μM). The probes were codispersed with POPC/DMPM or with POPC. Excitation was at 460 nm, and emission was 592 nm. The change in fluorescence was calculated as $[F - F_o]/[F_{max} - F_o]$, where F_{max} is the fluorescence after total mixing of inner monolayer lipids, measured with covesicles containing 0.3 mol % of each of the probes at the same total bulk lipid concentration, after dithionite reaction.

Quenching Experiments. Changes in tryptophan fluorescence spectra of mellitin on the addition of quencher were recorded at 330 nm (excitation = 285 nm), in 10 mM Tris at pH 8.0 and 25 °C with constant stirring. Appropriate amounts of lipid vesicles were added to a solution of 5.2 μM mellitin; spectrum was recorded after equilibration (about 30 s). To monitor quenching of fluorescence signal by acrylamide, the quencher was added in increasing amounts from a 3.3 M stock solution in water (final concentration from 0 to 400 mM). Quenching results were analyzed according to the Stern−Volmer equation for static and collisional quenching (Georghiou et al., 1982); however, for practical reasons, we decided to use the slope of a linear fit

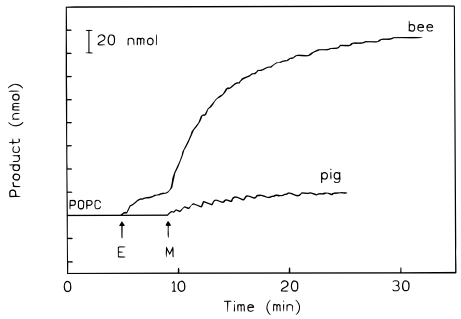

FIGURE 1: Effect of mellitin (6.1 nmol) on the reaction progress for the hydrolysis of small sonicated vesicles of POPC (520 nmol in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl at pH 8.0 and 23 °C) by PLA2 (18 pmol) from bee venom (top), or from pig pancreas (bottom). M, mellitin; E, PLA2.

of the Stern−Volmer plot for concentrations up to 150 mM, as criterion for mellitin accessibility to acrylamide: $F_o/F = 1 + K_{SV}[Q]$, where F_o and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher, and K_{SV} is the Stern−Volmer quenching constant. At this range of concentrations there is no deviation from linearity ($r^2 \geq 0.99$).

Light Scattering. The change in turbidity was measured as the change in the 90° scattered intensity at 360 nm and 1 nm slitwidths on a SLM-Aminco AB-2 spectrofluorimeter. The net change in the intensity of the scattered light is defined as $(I - I_o)$ where I_o and I are the intensity without and with mellitin, respectively. Vesicles (106 μM) were titrated with mellitin.

Size Exclusion Chromatography. HPLC was performed in a Dynamax Model SD-200 dual pump system with simultaneous recording on fluorescence and absorbance detectors. The Dynamax fluorescence detector model FL-2 (Rainin) was set at 285 and 340 nm (excitation and emission wavelengths, respectively), and the range was set at 10. The Dynamax absorbance detector (Model UV-1, Rainin) was set at 220 nm, range 0.5. Separations were carried out on a Hydropore-5™-SEC size exclusion chromatography column (Rainin), 5 μm particle size (4.6 mm × 25 cm), and an upper exclusion limit of 1×10^6 Da. The column was eluted isocratically with 0.1 M KH_2PO_4/0.1 M NaCl buffer (pH 7.0) at a flow-rate of 0.5 mL/min.

RESULTS

Effect of Mellitin on the Hydrolysis of POPC Vesicles by PLA2 from Different Sources. Probably the most dramatic demonstration of the effect of mellitin on the reaction progress by PLA2 is shown in Figure 1. During the initial burst of hydrolysis of POPC vesicles by bee venom PLA2, only a small fraction of the total substrate present in the reaction mixture is hydrolyzed. This is followed only by a very slow phase of hydrolysis for more than 1 h. If mellitin is added during the slow phase, a large increase in the rate of hydrolysis is observed, at the end of which the fraction of substrate hydrolyzed depends on the amount of mellitin added. On the other hand, as also shown in this figure, mellitin has only a modest effect on the hydrolysis of POPC by pig pancreatic PLA2. Although an apparent activating effect of mellitin has been reported before (Mollay & Kreil, 1974; Argiolas & Pisano, 1985), conditions for Figure 1 are chosen to dramatize the magnitude and differences. Experiments described below are designed to deconvolute the basis

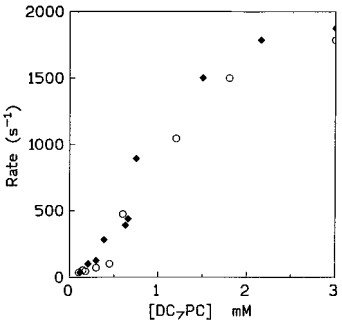

FIGURE 2: Rate of hydrolysis of DC_7PC by bee PLA2 (3.75 pmol, 0.94 nM) at varying concentrations of substrate in the presence (closed symbols) or absence (open symbols) of 0.7 μM mellitin.

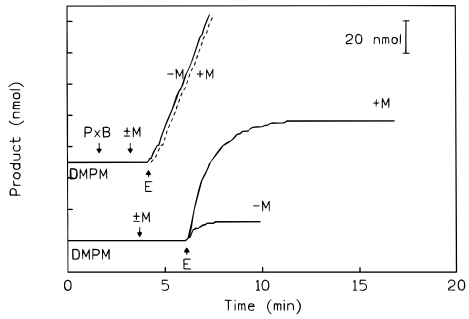

FIGURE 3: Effect of mellitin (1.4 nmol) on the reaction progress for the hydrolysis of DMPM SUVs (634 nmol) by PLA2 (15 pmol). (Top) Zero-order (substrate replenishment) conditions: PxB (6 nmol) was added to the DMPM vesicles followed by mellitin (dashed line) or without mellitin (solid line) and the reaction was initiated by addition of PLA2. (Bottom) First-order (substrate-limiting) conditions: Same as before but without PxB. Reaction was monitored in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl, pH 8.0. M, mellitin; E, bee or pig PLA2 (both exhibit the same behavior in this assay system with anionic phospholipid vesicles).

for the effects of mellitin on the kinetics of PLA2. Several possibilities are considered below. The first possibility is that mellitin has a direct activating effect on PLA2 trapped in the phase-separated products. The second possibility is that PLA2 is trapped in a few vesicles and mellitin promotes intervesicle exchange of the enzyme. The third set of possibilities examine whether mellitin promotes substrate replenishment by intervesicle exchange of the products with excess substrate by fusion or solubilization of vesicles or by direct intervesicle exchange.

Mellitin Does Not Have a Direct Effect on the Kinetic Properties of PLA2. The possibility that mellitin specifically activates the bee venom enzyme by direct interaction is inconsistent with the effect of mellitin on the hydrolysis of POPC vesicles by PLA2 from other sources. For example, PLA2 from bovine pancreas (type I), basic enzyme from *Agkistrodon halys Blomhoffi* (type II), and human synovial inflammatory exudates (type II) did not show any additional hydrolysis on the addition of mellitin under conditions comparable to those in Figure 1. On the other hand, PLA2 from *Crotalus adamentus* (type II) and *Naja nigricolas* (type I) showed additional hydrolysis on the addition of mellitin (results not shown). At the very least these results show that the "activating" effect of mellitin is not limited to the bee enzyme. This is a particularly important conclusion because the folding pattern for these three evolutionarily divergent types of secreted PLA2 are very different. Also there is a rather modest (<30%) homology although the architecture of the catalytic active site is similar (Scott & Sigler, 1994).

The possibility of a direct effect of mellitin on the kinetic parameters by PLA2 was examined in several different ways. For example, as shown in Figure 2, mellitin does not have a discernible effect on the dependence of the rate of hydrolysis on the concentration of DC_7PC. Not only does the maximum rate of hydrolysis remain the same but the overall shape of the curve also does not change significantly in the presence of mellitin. The shape of the curve in Figure 2 is noticeably different than that seen with pancreatic PLA2 (deHaas et al., 1971), which shows a sharp increase at the cmc, i.e. at 1.5 mM DC_7PC, rather than an increase seen at 0.3 mM with bee PLA2. Our observation that the increase in rate with the bee enzyme is seen below the cmc in the absence of mellitin is consistent with that reported by Tsai and co-workers (Lin et al., 1988). Collectively, these results show that not only does bee PLA2 not exhibit a large change in the rate of hydrolysis on the micellization of the bulk substrate but amphipathic mellitin does not have a significant influence on the turnover rate whether the substrate is monodisperse or micellized.

Mellitin Does Not Influence the Turnover Rate in the Scooting Mode. The time course of hydrolysis of anionic vesicles in the scooting mode is sensitive only to changes in the primary rate parameters at the interface, and anomalous effects can be readily deconvoluted (Jain et al., 1995). As shown in Figure 3 (top curves), the initial rate of hydrolysis of DMPM vesicles under zero-order conditions by bee PLA2 is not influenced by the presence of mellitin. These results were obtained under the conditions where the substrate replenishment between vesicles is rapid due to the presence of polymyxin B (Jain et al., 1991b; Cajal et al., 1996a,b), and thus the effective mole fraction of the substrate on the enzyme containing vesicle remains constant, $X_S = 1$. Comparable results were obtained with large fused vesicles in the absence of polymyxin B (results not shown). Such a lack of effect of mellitin on the highly processive interfacial catalytic turnover rate, v_o, is consistent with the results that show that mellitin does not influence the interfacial affinity for the active site of bee PLA2 for the substrate, products, active site-directed inhibitors, and calcium (Yu et al., 1997).

Mellitin Promotes Hydrolysis under Substrate-Limiting Conditions. As shown in Figure 3 (bottom curves), the time course of hydrolysis of anionic vesicles of DMPM under substrate-limited conditions changes noticeably in the presence of mellitin. The apparent first-order reaction progress is due to the hydrolysis of a fraction of vesicles to which the enzyme is bound, and the substrate present in excess vesicles is not accessible to the bound enzyme. Thus, additional hydrolysis seen in the presence of mellitin added before or after the initiation of the reaction progress, is due to the exchange of the products of hydrolysis from the depleted vesicle with the substrate from the excess vesicles. Under these conditions, the same effect of mellitin is seen for pig PLA2.

Effect of Mellitin on the Hydrolysis of Zwitterionic Vesicles. One of the intriguing features of interfacial catalysis by PLA2 on zwitterionic vesicles is a complex shape of reaction progress (Upreti & Jain, 1980; Apitz-Castro et al., 1982). Independent experiments show that the affinity of PLA2 for zwitterionic vesicles is poor, and anionic additives, including the products of hydrolysis, enhance the

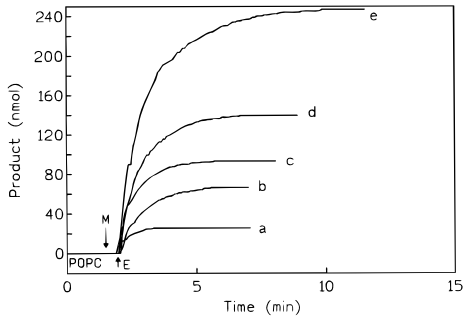

FIGURE 4: Dependence of the extent of hydrolysis of POPC (520 nmol) vesicles as a function of mellitin concentration. Mellitin (M) was added to the vesicles (4 mL) followed by bee PLA2 (E). Mellitin: (a) 0 nmol; (b) 0.55 nmol; (c) 0.83 nmol; (d) 1.66; (e) 4.16 nmol. Other conditions are as in Figure 1.

binding of PLA2 to the interface and thus shift the equilibrium from the aqueous phase to the interface (Jain & Berg, 1989). Since the "activating" effect of mellitin is seen on the hydrolysis of zwitterionic vesicles (for example, Figure 1), we further examined the possible effects of mellitin on the hydrolysis of POPC vesicles. As shown in Figure 4, mellitin added to POPC unilamellar vesicles induces additional hydrolysis by bee PLA2, and the extent of the extra hydrolysis depends on the amount of mellitin present; for example, with 0.1 mol % mellitin in phospholipid, approximately twice as much substrate is hydrolyzed by the same amount of enzyme. Such kinetic effects of mellitin, and their remarkable similarity to the results with polymyxin B in anionic vesicles (Cajal et al., 1995, 1996a,b), prompted us to explore the possibility that the apparent activating effect of mellitin is seen when the reaction progress is limited by the substrate replenishment on the enzyme-containing vesicles. A difference between the behavior of bee and pig pancreatic PLA2 on POPC vesicles (Figure 1) and a similarity on anionic DMPM vesicles (Figure 3) or covesicles of POPC/DMPM (3:7) (not shown, but similar to Figure 3) suggests that the clue for the difference must lie in the factors that modulate reaction progress for the hydrolysis of zwitterionic POPC vesicles.

Mellitin Does Not Induce Lipid Flip-Flop or Vesicle Solubilization. The fraction of POPC in SUV vesicles that is hydrolyzed by bee PLA2 with increasing amounts of mellitin is shown in Figure 5 (Top). The extent of hydrolysis increases until a plateau is reached at around 1−1.5 mol % mellitin, and about 65% of the total substrate present in the reaction mixture is hydrolyzed. These results indicate that, at ∼1.5 mol % mellitin, virtually all the substrate molecules present in the outer monolayer of all the vesicles are hydrolyzed by the same amount of enzyme which in the absence of mellitin hydrolyzed only ∼5% of the total substrate. Furthermore, these results also rule out solubilization of the vesicles by mellitin, which would have made all the lipid accessible for the hydrolysis.

POPC Vesicles become Permeable in the Presence of Mellitin. The addition of dithionite to sonicated dispersions of POPC containing 0.6% NBD-PE results in a partial decrease in the fluorescence from NBD corresponding to the reaction of the probe present in the outer monolayer of the vesicles, that is ∼65% of the total. The same amount of probe reacts if mellitin is added to the vesicles at concentrations below 0.5 mol %, but above this concentration the amount of probe accessible to dithionite increases (Figure

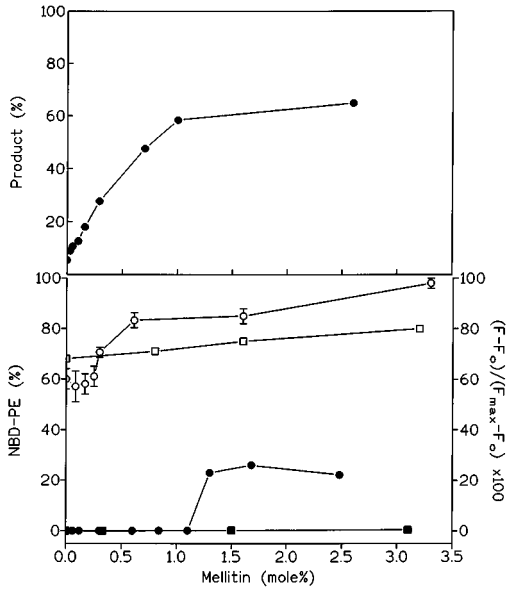

FIGURE 5: (Top) Extent of hydrolysis of POPC (520 nmol) vesicles as a function of mellitin. Mellitin was added to the reaction mixture containing vesicles and the reaction was initiated by bee PLA2 (21.4 pmol). (Bottom, left axis) Reaction of small sonicated vesicles containing 0.6 mol % NBD-PE (110 μM) with dithionite (10 mM): percentage of lipid reduced by dithionite as a function of mole percent of mellitin for POPC/DMPM vesicles (□); POPC vesicles (○), POPC and bee PLA2 (2.14 pmol) in the presence of 70 μM $CaCl_2$ (not shown but overlaps ○). (Bottom, right axis) RET fluorescence change of a 1:1 mixture of vesicles with NBD-PE labeled only in the inner monolayer and vesicles with Rh-PE (110 μM total lipid) as a function of mellitin, (●) vesicles of POPC; (■) vesicles of POPC/DMPM. Excitation 460 nm, emission 592 nm.

5, bottom). These results show that the probe in the inner monolayer of SUV vesicles of POPC is also modified. Since under these conditions, the inner monolayer lipids are not accessible to hydrolysis by PLA2 (as described above), the possibility of transbilayer movement of phospholipids is ruled out. In the case of POPC/DMPM covesicles, 68% of the probe reacted with dithionite in the absence of mellitin, and this percentage increased only slightly in the presence of the peptide (Figure 5). Also in this assay, bee or pig PLA2 did not have any effect on the permeability of the vesicles with or without mellitin (<0.4%), because the same amount of probe was modified by dithionite both in the presence or absence of PLA2.

Mellitin Does Not Induce Inner Monolayer Mixing. POPC or POPC/DMPM vesicles containing 0.6% NBD-PE were incubated with dithionite so that the NBD fluorescence from the outer leaflet of the vesicles was selectively quenched. In the absence of mellitin, dithionite does not significantly permeate the membrane of the vesicles over the time course of this experiment. Asymmetrically labeled NBD vesicles were mixed with an equal amount (in moles) of Rh-PE-containing vesicles, and then mellitin was added. As shown in Figure 5 (bottom), for POPC vesicles, below 1.3 mol % mellitin there is no noticeable increase in the RET signal, indicating that there is no mixing of inner monolayer lipids. Higher mole percent of mellitin results in a significant increase in the RET intensity that reaches ∼25% of the maximum [F_{max} was calculated with vesicles containing both probes, 0.3% each, and treated with dithionite (see Experimental Procedures section)]. In this experiment, NBD and Rh vesicles are mixed in a 1:1 ratio; therefore, lipid mixing

between vesicles containing the same probe is also possible, and such contacts will not result in any change in RET; consequently, the maximum mixing will be 50% of the ideal mixing (F_{max} in Figure 5) calculated with covesicles of 0.3% NBD and 0.3% Rh. As described above, and also shown in this figure, ≥0.5 mol % mellitin makes the POPC vesicles more permeable to dithionite, thus quenching the NBD-PE in the inner monolayer of the vesicles. For this reason, the RET intensity was monitored immediately (10 s) after addition of mellitin to the mixture of NBD-PE and Rh-PE vesicles; at this time, the intensity of the NBD-PE in the inner monolayer of the vesicles is still almost intact. These results suggest that above 1.3 mol %, mellitin somehow allows the mixing of lipids in the inner monolayer of POPC vesicles with either inner and/or outer monolayer on another vesicle. On the other hand, for covesicles of POPC/DMPM, no noticeable mixing of inner monolayer lipids was seen even at more than 3 mol % mellitin (Figure 5). The differences seen between the two lipids will be rationalized later, but in short they are due to the fact that, in POPC vesicles below 1.5 mol % mellitin, there is lipid exchange only between the outer monolayers of vesicles through contacts established by mellitin; on the other hand, above ~1.5 mol %, mellitin adopts a different form that probably spans the bilayer, causing leakage of aqueous content and the lipid mixing. Note that this high-R form (see Discussion and Figure 12) seen at a high mellitin/zwitterionic phospholipid ratio is not seen in POPC/DMPM vesicles even at >3 mol % mellitin. Control experiment: when the vesicles of POPC/DMPM containing NBD were not modified with dithionite, mellitin induced an increase in RET to Rh-PE vesicles (not shown). This is expected because lipid exchange between the outer monolayers of the vesicles in contact is induced by mellitin, as shown later.

Bee PLA2 Is Trapped in Product-Containing Vesicles. As shown in Figure 1, after the initial burst of hydrolysis of POPC vesicles by bee PLA2 the reaction progress essentially ceases. On the basis of the fact that bee PLA2 has a high affinity for an anionic interface, a possible reason for the cessation of the reaction would be that the enzyme is trapped on the product containing vesicles. This possibility is supported by results shown in Figure 6. In the set A, the bee enzyme added to a small amount of POPC vesicles hydrolyzes the substrate. Additional hydrolysis is not seen even when excess substrate is added; however, additional hydrolysis ensues immediately after addition of mellitin and the extent of hydrolysis increases with the amount of mellitin. As shown in set B, bee PLA2 added to vesicles of the products of hydrolysis of DMPC (a 1:1 mixture of lysoPC and fatty acid) is not available for the hydrolysis of POPC vesicles added afterward. However, the hydrolysis begins immediately after the addition of mellitin. These results are useful for resolving two possible mechanisms that may be at work here. The cessation of hydrolysis after the initial addition of the enzyme to POPC vesicles, could be due to either trapping of the enzyme on few vesicles which are essentially completely hydrolyzed, or the enzyme is trapped in the phase-separated domains of products on the whole population of the POPC vesicles [e.g., see Reichert et al. (1992), Maloney and Grainger (1993)]. The fact that mellitin initiates the hydrolysis of POPC vesicles added to bee PLA2 on the product vesicles (set B) essentially rules out the second possibility, i.e., segregation of PLA2 in the product domains

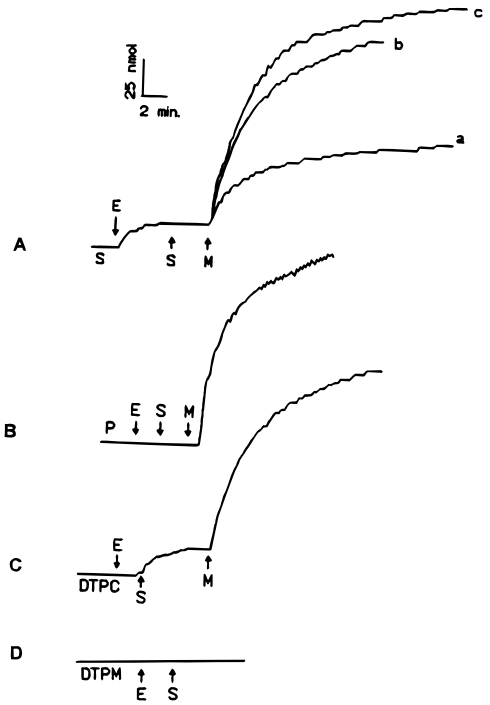

FIGURE 6: Mellitin-mediated lipid transfer between vesicles monitored by the reaction progress curves for the hydrolysis by PLA2 with different sequences of addition. (A) POPC vesicles (65 nmol) were hydrolyzed with PLA2 (18 pmol), and then more POPC vesicles (455 nmol) were added, followed by mellitin: 1.52 nmol (a), 6.11 nmol (b), or 15.2 nmol (c). (B) PLA2 (21.6 pmol) was added to vesicles of products (P) of hydrolysis (104 nmol), and POPC vesicles (520 nmol) were added afterward, followed by mellitin (6.1 nmol). (C) To a population of DTPC vesicles (260 nmol) containing PLA2 (21.6 pmol), POPC vesicles (520 nmol) were added, and after cessation of the hydrolysis, mellitin (6.1 nmol) was added. (D) Vesicles of POPC (520 nmol) were added to a population of vesicles of DTPM (260 nmol) containing PLA2 (21.6 pmol). S, POPC; E, bee PLA2; M, mellitin; P, lysoPC + fatty acid mixture.

present on most of the vesicles is not the basis for cessation of hydrolysis. As shown in set C, enzyme added to nonhydrolyzable zwitterionic DTPC vesicles is readily available to POPC vesicles added afterward, supporting the idea that negative charges in the interface are necessary for enzyme binding. A control (set D) shows that PLA2 bound to DTPM vesicles is not available to POPC vesicles added next.

Collectively, results described so far show that the hydrolysis seen after the addition of mellitin is due to the substrate replenishment in the enzyme-containing vesicles. The possibility of intervesicle exchange of the enzyme promoted by mellitin is ruled out on the basis of the fact that such a mechanism will show a continuous reaction progress in the presence of mellitin until all the available substrate is hydrolyzed; only a limited extent of hydrolysis is seen, and it depends on the amount of mellitin (Figures 3 and 4). On the basis of the results described thus far, we suggest that a possible mechanism for the mellitin-induced intervesicle exchange of phospholipids is through vesicle−vesicle contacts. Indeed, as shown by the biophysical studies described next, mellitin promotes formation of such contacts under the kinetically relevant conditions.

Mellitin Does Not Enhance the Binding of PLA2 to the Interface. A possible interpretation of results in Figure 1 is that mellitin promotes binding of bee PLA2 to zwitterionic

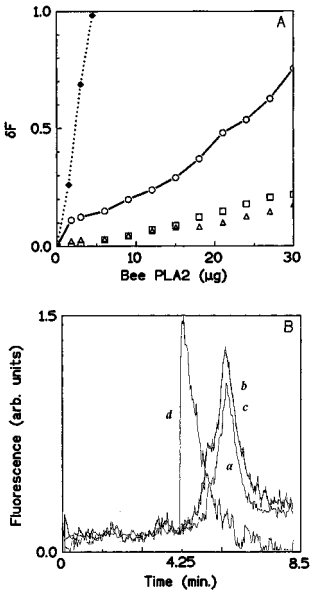

FIGURE 7: (Panel A) Effect of mellitin on the binding of bee PLA2 to SUV vesicles of different lipid compositions containing 2% DTPE-DNS. PLA2 was added from a stock solution to vesicles of DTPM (◆); POPC in the presence of 100 mM $CaCl_2$ (○); POPC-ether (△); POPC-ether containing mellitin (□). RET intensity from Trp of PLA2 to the DNS groups at the interface was measured at 500 nm (excitation at 290 nm). Lipid, 37 μM; Mellitin 0.5 μM. (Panel B) Size exclusion chromatography of (a) bee PLA2 (375 pmol) in solution; (b) PLA2 mixed with POPC-ether vesicles (256 nmol); (c) PLA2 incubated with POPC-ether (256 nmol) and mellitin (1.35 nmol); (d) PLA2 incubated for 10 min with vesicles of POPC (130 nmol) in the presence of 0.5 mM $CaCl_2$ to allow hydrolysis. In all the chromatogram the contribution of the lipid was subtracted. Detection by fluorescence (see Experimental Procedures).

vesicles, which is ruled out by results summarized in Figure 7. Binding of bee PLA2 to DTPM vesicles containing 2% DTPE-DNS is accompanied by an increase in the RET intensity at 500 nm (Figure 7A). Such an increase is not seen on the addition of bee PLA2 to zwitterionic vesicles alone or in the presence of mellitin. As also shown in this figure, a somewhat enhanced RET is seen with vesicles of hydrolyzable POPC. In this case, there is some uncertainty in the magnitude of the RET signal because the reading is taken while the hydrolysis is still in progress. In fact, addition of the products of hydrolysis to POPC-ether vesicles increases the binding of bee PLA2, as well as other secreted PLA2. Quantitative methods to determine high-affinity binding of bee PLA2 are not available due a lack of suitable tryptophan residue (Yu et al., 1997); however, on the basis of the results described, next it is estimated that the apparent dissociation constant < 10 μM for bee PLA2 bound to codispersions of POPC-ether with 10 mol % products of hydrolysis, compared to an estimated K_d of >2 mM with vesicles POPC-ether alone.

The RET results are consistent with the gel filtration results shown in Figure 7B. The retention time for bee PLA2 does not change in the presence of zwitterionic vesicles of POPC-ether with or without mellitin. Nevertheless, when bee PLA2 was preincubated for 10 min with vesicles of hydrolyzable POPC in the presence of Ca^{2+} prior to gel filtration, the enzyme binds with high affinity to the lipid and is eluted together with the lipid fraction near the void volume (Figure 7B). This indicates that the presence of negatively charged lipids or products of hydrolysis is essential for the irreversible

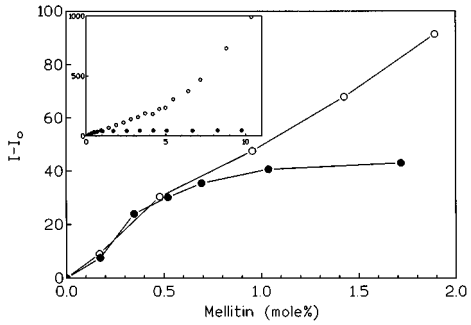

FIGURE 8: Mellitin induced changes in the intensity of 90 °C scattered light at 360 nm as a function of the mole % of mellitin for vesicles (106 μM) of (○) POPC/DMPM; (●) POPC. Inset: Scattering at higher mellitin concentrations.

binding of bee PLA2 to the interface. Moreover, the high-affinity binding of the enzyme in the presence of the products of hydrolysis is not influenced by mellitin, i.e., the bound enzyme is not desorbed by mellitin; if this was the case, the hydrolysis of POPC would not stop after only a small amount of additional substrate is hydrolyzed (Figures 1 and 4). Collectively, these results show that the binding of bee PLA2 to zwitterionic vesicles is not affected by mellitin.

Mellitin Induces Aggregation of Vesicles. Addition of mellitin to POPC/DMPM vesicles results in a significant increase in light scattering with the mole percent of mellitin (Figure 8). The increase in scattering, even at low mole percent, is rapid (complete in 5 s) and concentration dependent. These results show that mellitin induces an increase in the size of the particles, which is consistent with the idea of vesicle−vesicle contact formation by mellitin. On the other hand, in POPC or POPC-ether vesicles <1 mol % mellitin induces an increase in the light scattering, but the scattering reaches a plateau above 1.5 mol %. A second key difference between the zwitterionic and anionic vesicles may be noted. The increase in scattering after the addition of mellitin to anionic vesicles is virtually instantaneous; with zwitterionic vesicles, there is a second time-dependent component, suggesting a fusion-related process (not shown). These results are consistent with the formation of vesicle−vesicle contacts by mellitin, resulting in clusters of vesicles; in POPC vesicles, clustering saturates at ∼1.5 mol % mellitin, whereas in POPC/DMPM vesicles, clustering continues even above 4 mol % mellitin. However, at these high mellitin concentrations, the slope of the increase in scattering is significantly steeper (Figure 8, inset), probably due to fusion of vesicles.

Mellitin Induces Lipid Exchange. Transfer of phospholipids between vesicles across mellitin-contacts was directly monitored as the change in the fluorescence intensity of pyrene-labeled phospholipids on dilution with unlabeled phospholipids. Emission from vesicles of pyrene-phospholipid is dominated by the excimer band (480 nm), and the intensity of the monomer band (395 nm) increases as the probe is diluted due to exchange with phospholipids from vesicles in contact. As shown in Figure 9A, lipid exchange was observed on the addition of mellitin to a mixture of covesicles of POPC/pyPM (3:7) with 125-fold excess of vesicles of POPC/DMPM (3:7); here the mellitin induced exchange of pyrene lipid is comparable to that induced by PxB [not shown, however, see Cajal et al. (1996a)]. The exchange is complete in less than 5 s after addition of mellitin (for example, see Figure 9B), comparable to the rapid change

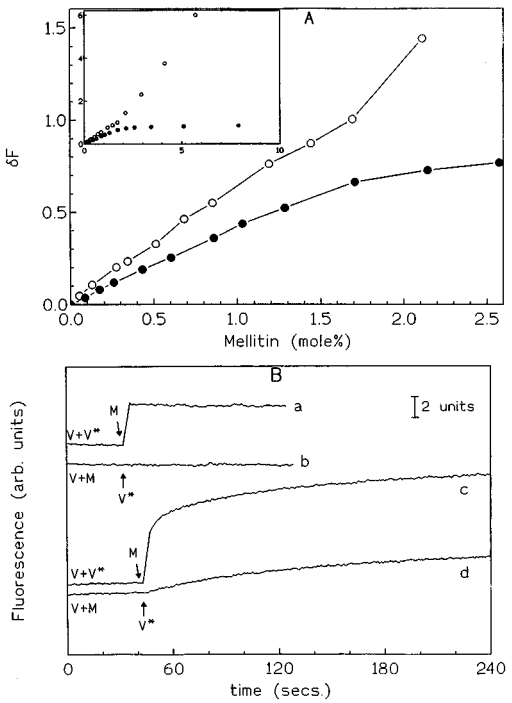

FIGURE 9: Mellitin-mediated lipid exchange between vesicles monitored by fluorescence. (A) Fluorescence intensity of pyrene monomer as a function of mellitin mole percent in a mixture of vesicles of POPC/pyPM (3:7) with POPC/DMPM (3:7) (○); pyPC with POPC-ether (●). Bulk lipid concentration was 1.7 μM for py-vesicles and 211 μM for unlabeled acceptor vesicles. Inset: Lipid exchange at higher mellitin concentrations. (B) Time course of the fluorescence change under different conditions: (a) Mellitin (0.9 mol %) was added (arrow) to a mixture of POPC/pyPM (V^*) and POPC/DMPM (V) vesicles; (b) mellitin was premixed with POPC/DMPM vesicles and then the labeled vesicles were added (arrow); (c,d) same as (a,b) but for vesicles of pyPC and POPC-ether. Experiments are in 10 mM Tris, pH 8.0, excitation 346 nm, emission 395 nm.

in the light scattering. After the rapid increase in fluorescence, there is no time-dependent additional change, which argues against a slower process such as fusion. The lipid exchange is detectable even below 0.05 mol % mellitin, and the increase in the monomer pyrene fluorescence is proportional to the concentration of mellitin added. These results imply that mellitin bound to POPC/DMPM vesicles forms vesicle−vesicle contact, and thus it remains trapped in those contacts and is unable to exchange with vesicles that are not in contact.

When mellitin was added to a mixture of pyPC and excess POPC-ether vesicles (Figure 9A), the increase in monomer fluorescence was similar to that of the POPC/DMPM mixture at low mellitin concentrations ($\leq$1.5 mol %), but at higher mole fractions (inset) the behavior of mellitin was different in the two lipids. The exchange of PC lipids shows a time-dependent component after the initial rapid increase in fluorescence (Figure 9B) and reaches a plateau at $>$1.6 mol % mellitin, indicating that no new intervesicle contacts are formed with additional amounts of mellitin. On the other hand, exchange of lipids between anionic vesicles is instantaneous and increases linearly with mol % of mellitin. This effect also parallels the scattering results (Figure 8).

Mellitin in Contacts Does Not Exchange between Anionic Vesicles. As shown in Figure 9B, addition of mellitin to a mixture of POPC/pyPM and POPC/DMPM vesicles induces formation of contacts and exchange of pyPM between the vesicles, as deduced from the increase of monomer fluorescence immediately after mellitin addition (curve a). On the other hand, no transfer of pyPM was observed when pyPM/POPC vesicles were added to a mixture of POPC/DMPM and 0.9 mol % mellitin (curve b). These results indicate that mellitin does not exchange freely after it is bound to the covesicles and becomes a part of vesicle−vesicle contact. As also shown in Figure 9B, for mixtures of pyPC and POPC-ether vesicles (curve c), mellitin not only induces rapid lipid exchange, as seen for anionic vesicles, but there is also a slow time-dependent increase in fluorescence. When mellitin was premixed with unlabeled vesicles, and then pyPC vesicles were added, the overall increase in fluorescence was significantly smaller, because the rapid initial component is absent (curve d), indicating that mellitin does not exchange freely. The slow phase is probably due to fusion of vesicles or slow exchange of bound mellitin. Direct evidence in support of the nonexchangeability of bound mellitin was obtained by gel filtration chromatography. Mellitin (0.5 mol %) mixed with POPC-ether vesicles coeluted with the lipid fraction, as deducted from the ~4-fold increase in fluorescence at 340 nm (excitation = 285 nm). These results still leave open the possibility that slow exchange with POPC/pyPC mixtures (Figure 9B, curves c and d) could be due to a slow exchange of vesicles in contact.

Specificity for the Exchange of Phospholipids. The pyPM dequenching assay (Figure 9) shows that mellitin induces lipid exchange between vesicles. This raises the question whether such exchange occurs through intervesicle molecular contacts where mellitin acts as a "filter" to sort the lipids that exchange, or it promotes lipid mixing due to (hemi)-fusion. In the first case, there should be an indication of the selectivity for the (probe) molecules that exchange. On the other hand, during (hemi)fusion, lipid mixing in one or both monolayers will be expected. Of course, such a sorting criterion for a functional contact may be overly stringent if the exchange does not show selectivity. Several probes were used to determine if mellitin-contacts display selectivity for the exchange.

Covesicles containing 0.3% each NBD-PE and Rh-PE in a matrix of POPC/DMPM or of POPC were mixed with a 50-fold excess unlabeled vesicles and then titrated with increasing amounts of mellitin. As shown in Figure 10A, there is a decrease in RET intensity, indicating that surface dilution of the probes due to lipid exchange with the unlabeled vesicles takes place rapidly. At low concentrations of mellitin ($\leq$2 mol %), dilution of the probes is similar in both POPC and POPC/DMPM vesicles. Above this concentration, dilution of the probes continues to increase in POPC/DMPM vesicles, but it reaches a plateau in POPC vesicles. The overall behavior is comparable to that seen with the increase in scattering (Figure 8) and pyrene exchange (Figure 9).

Finally, lipid mixing was monitored as the release of self-quenching of R18 due to surface dilution of the probe with excess unlabeled vesicles. The relative change in fluorescence induced by mellitin is shown in Figure 10B. In POPC vesicles, the exchange of this cationic probe is seen up to 1.5−2 mol % mellitin, and beyond this concentration no further dilution of the probe was seen, in agreement with the results in Figures 8−10A. Remarkably, at low mole fractions of mellitin, the extent of dilution of R18 by exchange was significantly higher with POPC vesicles,

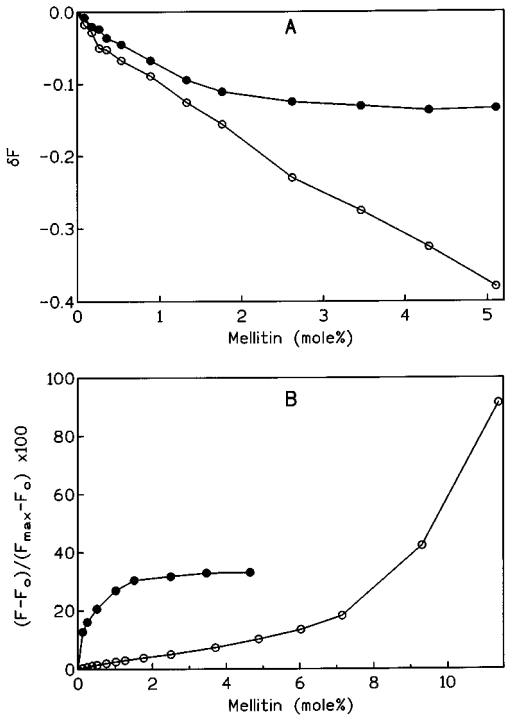

FIGURE 10: Specificity of intervesicle lipid exchange by mellitin. (A) Change in the RET intensity as a function of the mole fraction of mellitin added to a mixture of covesicles containing 0.3% of NBD-PE and 0.3% of Rh-PE (21.8 μM) added to excess unlabeled vesicles (1.06 mM) of POPC/DMPM (○), or POPC (●). Excitation 460 nm, emission 592 nm. (B) Increase in the emission due to dequenching of 6% R18 in a mixture of labeled covesicles (1.7 μM) and unlabeled vesicles (211 μM) induced by mellitin: POPC/DMPM (○); POPC-ether (●). Excitation 556 nm, emission 577 nm.

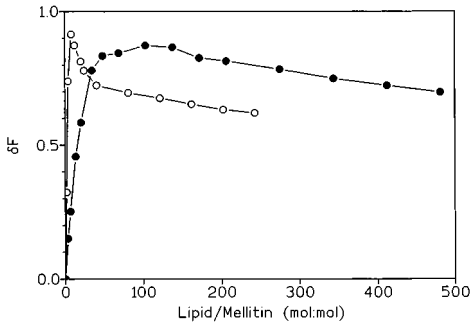

FIGURE 11: Binding of mellitin (5.22 μM) to vesicles of POPC/DMPM (○) or POPC-ether (●). Excitation 285 nm, emission 330 nm.

compared to POPC/DMPM vesicles. This indicates that in fact, mellitin contacts between POPC/DMPM vesicles show selectivity during the exchange. That cationic R18 is excluded (sorted out) from cationic mellitin in the contact is the most likely explanation; that is, mellitin will preferentially interact with and exchange anionic lipids, if available.

Mellitin Binds to Anionic and Zwitterionic Interfaces. The effect of the negatively charged lipids on the binding of mellitin to the interface is dramatic. As shown in Figure 11, the change in the tryptophan-19 fluorescence emission on the binding of mellitin to POPC/DMPM and POPC-ether vesicles as a function of lipid concentration is complex. These results indicate the existence of at least two forms of bound mellitin with different spectroscopic properties. Although we have not investigated the molecular basis of this phenomenon in detail, based on the published results [reviewed in Cornut et al. 1993)], it is clear that mellitin does form a series of monomer and tetramer species in the aqueous phase as well as at the lipid interface. Nevertheless, we show that the two bound forms are functionally different.

Table 1: Fluorescence Characteristics of Mellitin and Accessibility to Aqueous Quencher Acrylamide in Different Environments

lipid	lipid/mellitin	$\Delta\lambda_{max}$ (nm)[a]	δF(330 nm)	K_{SV}[b]
none	0:1	0	0	26.0
POPC	48:1	17	0.83	
POPC	66:1		0.84	5.24
POPC	300:1		0.76	3.88
POPC	480:1	19	0.70	
POPC/DMPM	8:1		0.91	4.72
POPC/DMPM	28:1	17	0.78	
POPC/DMPM	162:1	19	0.65	
POPC/DMPM	280:1		0.61	3.82

[a] Blue shift in wavelength of emission maximum of mellitin upon binding (λ_{max} = 350 nm in aqueous solution). [b] K_{SV}, Stern−Volmer quenching constant (M^{-1}) from 0 to 150 mM acrylamide. Excitation 285 nm, 10 mM Tris, pH 8.0.

Binding of mellitin to the anionic vesicles of POPC/DMPM results in an increase of the tryptophan-19 emission intensity at high mole fractions of peptide that reaches a maximum at lipid to mellitin = 5:1, and then there is a sharp decrease in emission intensity at higher lipid to mellitin ratios. The early part of the binding isotherms extrapolated to the maximum change in the signal suggests that the POPC/DMPM:mellitin stoichiometry is ~5 for the maximum signal and apparent $K_D \ll 1$ μM. Besides the change in emission intensity at 330 nm, there is a shift in the emission maximum from 350 nm in aqueous phase to 333 nm (POPC/DMPM to mellitin 28:1) or to 321 nm (162:1) as summarized in Table 1. These results suggest that there are at least two different forms of bound mellitin in POPC/DMPM vesicles, depending on the concentration of the peptide in the interface. Binding of mellitin to zwiterionic vesicles of POPC-ether shows essentially the same behavior, but the intensity maximum is reached at a higher lipid:mellitin ratio (~100:1); there is a decrease in intensity at higher lipid concentrations (Figure 11). The shape of the binding curve with POPC-ether suggests that the apparent K_D is ~50 μM. The emission maximum of mellitin also shows a blue-shift upon binding to POPC-ether vesicles. The shift is more prominent at low mellitin concentration, as also seen with POPC/DMPM vesicles (Table 1). In conjunction with other results, this also rules out the possibility of decrease in binding of mellitin at high lipid:mellitin ratios, as an explanation for the decrease in emission intensity.

Quenching of Mellitin by Acrylamide. As summarized in Table 1, the tryptophan-19 fluorescence emission from mellitin in buffer is readily quenched by acrylamide, with $K_{SV} = 26$ M^{-1}. On the other hand, mellitin bound to vesicles is ~7-fold less accessible to acrylamide, indicating a higher degree of shielding (Table 1). The form of mellitin at high lipid/mellitin ratios, or contact-forming mellitin, is less exposed to acrylamide quenching, with a similar K_{SV} for both POPC and POPC/DMPM vesicles. On the other hand, at low lipid/mellitin ratios, the degree of quenching is somewhat higher. Control experiments show that acrylamide has no effect on the shape or on the wavelength of emission maximum of mellitin.

DISCUSSION

Modulation of PLA2 activity by a variety of agents has a rather confusing past. Most of the commonly used assays do not distinguish between the various kinetic possibilities in the context of the interface, where special considerations and precautions are necessary to satisfy the microscopic steady state condition for the interpretation of the reaction progress (Jain et al., 1995). For example, the fraction of the enzyme at the interface is reduced by nonspecific inhibitors; similarly, anionic additives increase the fraction of the enzyme at the interface and thus act as activators (Jain & Berg, 1989). In addition, apparent activation of PLA2 by polymyxin B due to a rapid substrate replenishment on the enzyme-containing anionic vesicles (Jain et al., 1991b) is now established to be due to a novel phenomenon, i.e., selective and rapid exchange of anionic phospholipids through the peptide-mediated intervesicle contacts (Cajal et al., 1996a,b). Salt-triggered intervesicle exchange of phospholipids mediated by myelin basic protein (Cajal et al., 1997) provides yet another dimension in the regulation of direct intervesicle exchange of phospholipids. Now, our studies show that apparent activation of PLA2-catalyzed hydrolysis of phospholipid vesicles by mellitin is due to substrate replenishment through mellitin-mediated vesicle−vesicle contacts.

Results reported here show that mellitin, an amphipathic α-helical peptide, makes stable vesicle−vesicle contacts, which mediate intervesicle exchange of zwitterionic and anionic phospholipids without (hemi)fusion or solubilization of vesicles. Mellitin-contacts between zwitterionic POPC or anionic POPC/DMPM vesicles promote similar level of exchange of anionic and zwitterionic phospholipids, but the exchange of cationic octadecylrhodamine (R18) is significantly lower in anionic vesicles (Figure 10B), indicating selectivity or sorting through the contact, rather than hemifusion.

Exchange takes place between the outer monolayers of vesicles in contact. Although the exchange process accounts for the apparent activation of PLA2 by substrate replenishment, it is also clear that the origin of the apparent specificity for certain PLA2 but not others (Figure 1) is due to the requirement of anionic charge for the binding of PLA2 to a zwitterionic interface. This requirement differs significantly for the enzymes from different sources (Jain et al., 1982). As is the case with most other PLA2, the bee enzyme does not bind to zwitterionic interfaces; however, formation of the products of hydrolysis promote subsequent binding of the enzyme to the interface. A dramatic difference in the effect of mellitin on bee and pig PLA2 (Figure 1)-catalyzed hydrolysis is apparently due to a difference in the abilities of these enzymes to exchange between vesicles (Jain & Berg, 1989). If the bee PLA2 binds rather tightly to the product containing vesicles, initial hydrolysis would promote binding of more enzyme which would sequester the enzyme as is the case with vesicles of anionic phospholipids (Figure 6). Under these conditions excess substrate is hydrolyzed as it becomes accessible to the bound enzyme by intervesicle exchange mediated by mellitin. The pig PLA2 has poor affinity for the zwitterionic vesicles as well as to those containing a small mole fraction of the products. Therefore, if not enough product is formed, the enzyme is not sequestered. In fact, it is possible to establish conditions

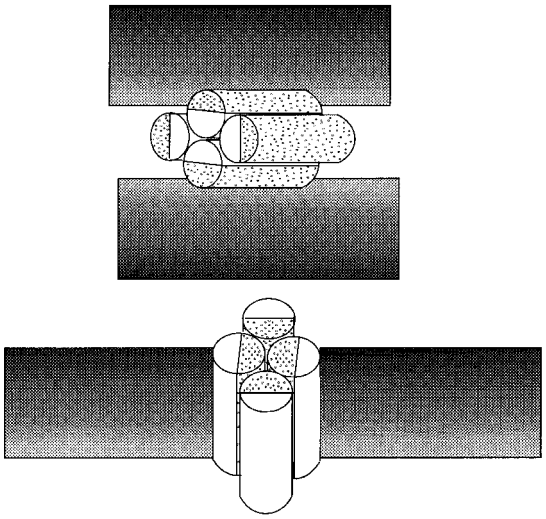

FIGURE 12: A heuristic model for the interaction of mellitin with membranes. Mellitin is shown as an amphipathic α-helix in which the polar side is dotted. In the high-R form, at high lipid/mellitin ratios (top), mellitin forms aggregates where the monomers lie horizontally in the membrane surface and establish contacts between vesicles; in the aggregates, the polar face of the monomers is oriented toward the lipid headgroups or the aqueous phase, and the hydrophobic sides face each other, forming and apolar interior that facilitates lipid exchange between the outer monolayers of the vesicles in contact. In the low-R form, at low lipid/mellitin ratios (bottom), mellitin adopts a transmembrane conformation, where the hydrophobic side of the helices are in contact with the membrane interior, thus forming a channel with polar interior. The transition from one form to the other depends on the lipid composition as well as the mellitin concentration in the interface.

(results not shown) with vesicles of other phospholipids, such as near the phase transition temperature of dimyristoylphosphatidylcholine vesicles, where apparent activation of the pig enzyme is also seen due to substrate replenishment by mellitin.

Molecular Features of Mellitin Contacts. Structural constraints for intervesicle contact formed by mellitin or any other peptide are not known. As a model for lipid−protein interactions, mellitin has provided structural motifs for ordered structure (Lavaialle et al., 1982; Cornut et al., 1993), aggregation (Hermetter & Lackowicz, 1986; Weaver et al., 1992; Georghiou et al., 1982; Chattopadhyay & Rukmini, 1993), and salt effects (Schoch & Sargent, 1980; Bello et al., 1982; Hider et al., 1983). Such a wealth of structural information makes mellitin an attractive target for speculations about the molecular features of vesicle−vesicle contacts.

A model shown in Figure 12 emphasizes two membrane-bound forms of mellitin: the high-R and low-R forms, which may coexist under certain conditions. Thus, depending on the conditions, mellitin makes vesicle−vesicle contact, or promotes leakage. For example, leakage is seen at >1.5 mol % in zwitterionic vesicles but not in anionic vesicles (Figure 5). Note that a bundle of amphiphilic helices invoked in this model is only an operational structural device that accounts for two functional states. As is the case with numerous bioactive peptides (Lehtonen et al., 1996; Mancheño et al., 1996; Saberwal & Nagaraj, 1994; Cornut, 1993), mellitin spans the bilayer at high mole fractions presumably by breaching the hydrophobic barrier to form pores or channels. Two related factors may be at work here: the amphipathic helical structure and the existence of multiple

aggregated forms. The primary sequence of mellitin, GIGAVLKVLT$_{10}$TGLPALISWI$_{20}$KRKRQQ, has a charged region (21−26) and a relatively nonpolar region (1−20) with a hydrophilic and a hydrophobic face. Mellitin is almost unstructured in diluted aqueous solution, but it acquires α-helical structure when the polarity of environment decreases, e.g., at high ionic strength (Talbot et al., 1979). The molecular structure in crystalline form, membrane bound form, and in methanolic solution is remarkably similar: the sequence folds into a bent helix rod with a 120−160^o kink in 11−14 hinge region (Terwilliger & Eisenberg, 1982; Vogel & Jahnig, 1986; Inagaki et al., 1989). The hydrophobic residues 1, 5, 6, 8, 9, 13, 16, 17, 19, and 20 form an apolar face. The emerging consensus is that mellitin can exist as a monomer as well as an aggregate, possibly a tetramer. There is evidence that at least in the aqueous phase, the equilibrium between such forms is readily shifted by salt, peptide concentration, pH, and surface pressure at the air−water interface, probably because generation of an apolar face in the amphipathic α-helix favors self-association (Faucon et al., 1979; Talbot et al., 1979; Wackerbauer et al., 1996). Existence of an equilibrium between two bound forms of a peptide in the interface has been proposed for other peptides (Stanislawski & Ruterjeans, 1987; Pouny et al., 1992).

On the basis of the structural features and balance of forces that act on mellitin at the bilayer interface, our working hypothesis is that at a low lipid:mellitin ratio, the α-helices are perpendicular to the plane of the membrane and form a bundle that spans the bilayer, separated by chains of phospholipid molecules (Figure 12, bottom). In such a complex the acyl chains interact with the hydrophobic face of each of the helices, and their polar faces form a hydrophilic channel. This transbilayer structure is similar to that conceptualized by Vogel and Jahnig (1986). Such an aggregated structure will have a strong propensity to form highly curved surfaces which could act as fusion intermediates. In short, the low-R structure is suited for lysis, as also demonstrated by others (Talbot et al., 1987; Benachir & Lefleur, 1996).

The high-R form of mellitin at the interface (Figure 12, top) is also represented as an aggregated structure. This arrangement derives inspiration from the unit cell of mellitin crystal (Terwilliger & Eisenberg, 1982): a tetrameric bundle, with apolar residues buried inside, is made up of two antiparallel dimers to decrease the electrostatic repulsion between the C-termini. For phospholipid exchange the acyl chains will come in contact with the hydrophobic space at the center of the bundle. The interfacial equilibrium between the high-R and low-R forms of mellitin depends on lipid composition. Charge interactions involving the cationic surface of the bundle are indicated (Figure 5 and 8), which provides an explanation for several observations: (a) The high-R form is less favored in zwitterionic POPC vesicles, where mellitin in excess of about 1.5 mol % forms leakage pathway (Figure 5); (b) contacts with mellitin in the proximity of anionic phospholipids provide a basis for selectivity against cationic R18 (Figure 10B), whereas such a selectivity is not seen for contacts between POPC vesicles; (c) anionic phospholipids in vesicles inhibit the lytic activity of mellitin (Benachir & Lefleur, 1995, 1996; Hincha and Crow, 1996). In short, while raising a cautionary flag for the interpretation of membrane-bound peptides as a single structure, results reported in this paper suggest that the two functional forms of mellitin may have a structural basis in terms of the arrangement of molecules in or at the surface of bilayers.

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