Measuring Ca$_{2+}$-Induced Structural Changes in Lipid Monayers: Implications for Synaptic Vesicle Exocytosis

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ABSTRACT Synaptic vesicles (SVs) are small, membrane-bound organelles that are found in the synaptic terminal of neurons. Although tremendous progress has been made in understanding the protein machinery that drives fusion of SVs with the presynaptic membrane, little progress has been made in understanding changes in the membrane structure that accompany this process. We used lipid monolayers of defined composition to mimic biological membranes, which were probed by x-ray reflectivity and grazing incidence x-ray diffraction. These techniques allowed us to successfully monitor structural changes in the membranes at molecular level, both in response to injection of SVs in the subphase below the monolayer, as well as to physiological cues involved in neurotransmitter release, such as increases in the concentration of the membro lipid PIP$_2$, or addition of physiological levels of Ca$_{2+}$. Such structural changes may well modulate vesicle fusion in vivo.

INTRODUCTION

Synaptic vesicles (SVs) are secretory organelles that store neurotransmitters in presynaptic nerve endings. Following action potential invasion of the nerve terminal, the plasma membrane is depolarized, leading to the opening of voltage-gated Ca$_{2+}$ channels in the plasma membrane. The accompanying rise in intracellular Ca$_{2+}$ leads to the fusion (exocytosis) of synaptic vesicles with the plasma membrane, resulting in the release of neurotransmitter (1–3).

The last two decades have seen tremendous progress in our understanding of the exocytic process at the molecular level. A whole network of proteins has been identified that are thought to play key roles in the process. In particular, attention has been focused on the so-called SNARE proteins, which are thought to drive membrane fusion, and a detailed, mechanistic molecular model of how these proteins function in membrane fusion has been proposed (which is discussed in the review by Jahn et al. (4)).

Perhaps unsurprisingly, our protein-centric view of the fusion process has meant that the role of lipids in the process (and by extension membranes and membrane structure) has been largely ignored. A case in point is the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Although a minor component of the total membrane lipid, PIP$_2$ is known to be essential for exocytosis and is found specifically enriched at sites of vesicle fusion (5,6).

To date, the role of PIP$_2$ in exocytosis has been attributed to its function as an effector lipid for the vesicular protein synaptotagmin. Synaptotagmin is thought to act as a molecular bridge between the vesicle and plasma membranes, facilitating SNARE protein interactions that drive the fusion reaction (4). However, multivalent cations such as Ca$_{2+}$ are also known to directly interact with membranes, altering the electrostatic environment with potential consequences for the biochemical and physiological activities of the membrane, including protein function (7–13). For instance, PIP$_2$ is a strongly anionic lipid, with a headgroup possessing 3–5 net negative charges (14), which, depending on orientation and flexibility, may well interact with Ca$_{2+}$ to produce highly localized membrane effects. Therefore, it is conceivable that structural changes in the plasma membrane induced by Ca$_{2+}$ binding directly to PIP$_2$ at the release site may play an important role in modifying the local membrane environment, thus modulating synaptic vesicle exocytosis. However, the structural basis of how Ca$_{2+}$ and PIP$_2$ function at the fusion site has been largely ignored, at least in part because of the overall complexity of biological systems and a lack of suitable experimental methods. We have recently taken a reductionist approach to such problems, using a Langmuir trough-based system to produce membrane monolayers of defined lipid composition, which can be investigated under precisely controlled conditions by x-ray reflectivity and grazing incidence x-ray diffraction (15). In this work, we use this approach to make a quantitative study of membrane structure in response to Ca$_{2+}$ and PIP$_2$ (16).

As in any biophysical model system, the advantage to probe structural changes at the molecular level comes at a price. Reducing the complexity of the synaptic terminal to a two-component monolayer system facing an aqueous suspension of SVs limits the conclusions to be drawn. However, the rationale of this study is as follows: the structure of the fatty acids, even if different from the real system, is used as a structural reporter for the changes in the headgroup region (lateral pressure, electrostatics), because the chain reorganizes after interaction of the headgroup
moieties with either small ions or SV proteins to lower the free energy. If these changes are significant in the monolayer model system, they are most likely also important in the biological membrane, even if the starting configuration is different in terms of composition and fluidity.

What we observe here is a significant Ca\(^{2+}\)-dependent reorganization of the acyl chains as well as corresponding changes in the electron density profile, in particular in the presence of PIP\(_2\). Based on these results, we speculate that changes in membrane structure produced by directcation binding to lipids may regulate Ca\(^{2+}\)-dependent synaptic vesicle fusion with the plasma membrane. We believe these results call for a revision of the current protein-centered view of neurotransmitter release, to incorporate an active role for the lipid microenvironment during release.

**MATERIALS AND METHODS**

Lipid monolayers were prepared from the lipids dipalmitoyl-\(a\)-glycerol-3-phosphatidylcholine (DPPC) and phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)). To prepare lipid monolayers at an air-water interface, we used a custom-made shallow Langmuir trough, equipped with a single movable Teflon (DuPont, Wilmington, DE) barrier, available at ID10B of the European Synchrotron Radiation Facility (Grenoble, France). Each monolayer was exposed multiple times to collect the data at various salt concentrations in the subphase (see below). The special choice of the majority lipid DPPC in the monolayer follows from the fact that monolayer formation and chain ordering in this lipid is particularly easy, well described, and robust. The ordering in the saturated fatty acids corresponds more to a bilayer’s gel phase than to a bilayer’s fluid phase. Measurements on both the DPPC and DPPC/PIP\(_2\) monolayers were carried out at a temperature of 18°C, which is below the chain-melting temperature of DPPC. CaCl\(_2\) and/or synaptic vesicles were subsequently injected into the subphase using a microloader. The 1 M CaCl\(_2\) stock was added sequentially into the subphase to achieve the desired final Ca\(^{2+}\) concentration; in a first step the Ca\(^{2+}\) concentration was raised to 1 \(\mu\)M, and for subsequent experiments further Ca\(^{2+}\) was added to reach effective concentrations of 10 \(\mu\)M and 30 \(\mu\)M, respectively. Synaptic vesicles were purified from rat brain, as described in Takemori et al. (1), through differential centrifugation, sucrose density chromatography, and size-exclusion chromatography. Synaptic vesicles were routinely used at a final concentration of ~1.5 g/L.

FIGURE 1 Schematics of the experimental systems used. (A) Lipid monolayers were formed at an air-water interface. X-ray reflectivity was used to measure the electron density profile of the lipid layer \(\rho(z)\) in this system. GIXD was used to measure the tilt angle \(\tau\) of the lipid acyl chains. Where necessary, defined amounts of Ca\(^{2+}\), as well as synaptic vesicles, were introduced into the subphase. (B) The equipment used for x-ray scattering experiments. For reflectivity, the incident angle \(\alpha_i\) = reflected angle \(\alpha_r\) with \(2\theta = 0\). For GIXD, \(\alpha_i < \alpha_r\) and \(2\theta \neq 0\), where \(\alpha_i\) is the critical angle at the air-water interface. Note that the \(z\) axis is parallel to the sample normal.

**RESULTS**

**X-ray reflectivity measurements from lipid monolayers**

To begin, we used the technique of x-ray reflectivity to obtain the laterally averaged electron density profile for membranes of defined composition, as a function of the interface normal (\(z\) axis in Fig. 1). This profile can be used to deduce structural parameters of the membrane under investigation. We analyzed reflectivity data using kinematical approximation, with the electron density profile modeled using two boxes. One box was used to describe the hydrophilic headgroup, while the other described the hydrocarbon chain of the lipid. The electron densities of the subphase and the air were taken to be 0.334 e/\(\AA^3\) and zero, respectively. The capillary wave roughness of the water surface was fixed at 3.2 Å.

Baseline measurements of basic membrane parameters were obtained using a pure DPPC monolayer as a reference. Fig. 2 shows reflectivity data obtained from such a monolayer before and after injection of increasing amounts of
Ca$^{2+}$. In the electron density profiles, the peaks correspond to the headgroup regions of the measured monolayers. In the pure DPPC monolayer, the box model fit gave a headgroup size of 8.75 Å (box model), and an overall lipid thickness of 25 Å (headgroup plus chains). The box-model density value of the headgroup was 0.43 e$^{-}$/Å$^3$, visible as a shoulder in the profile between density maximum and air. These values are in agreement with previous measurements (15,17). Injection of CaCl$_2$ into the subphase led to a reduction in amplitude of the modulation in the respective reflectivity curves. Subsequent analysis showed that the amplitude of this reflectivity modulation depends critically on the headgroup contrast. Best fits to the data showed the electron density of the headgroup decreased monotonically, being 0.34 e$^{-}$/Å$^3$ at a final concentration of 30 μM CaCl$_2$ in the subphase ($\Delta \rho \sim 20\%$), with an overall headgroup size of 8.05 Å. In contrast, we found that the electron density of the lipid chain increased slightly to 0.32 e$^{-}$/Å$^3$.

The structural effect of adding PIP$_2$ into the monolayer is shown in Fig. 3. Interestingly, the electron density profile deduced from the best fit of the DPPC/PIP$_2$ data showed a slight decrease in the average electron density to 0.42 e$^{-}$/Å$^3$ of the lipid headgroups compared to pure DPPC (15,18). Again, addition of CaCl$_2$ to the subphase caused a monotonic decrease in the headgroup electron density. At 30 μM CaCl$_2$, the density was 0.37 e$^{-}$/Å$^3$ ($\Delta \rho \sim 12\%$). Note that this effect is less than seen with the pure DPPC monolayer. However, the effect on lipid headgroup size was much more dramatic; the overall size decreased from 7.18 Å to 4.3 Å. Compared to the expected size for the headgroup density maximum, which is in the range of 5–9 Å (17,19), this is an extremely low and unrealistic value. Although we cannot rule out an artifact in the fit, we offer a more speculative explanation. If the lateral area in the membrane per headgroup decreases along with the chain tilt angle (see subsection below), this is likely concomitant with a change in headgroup orientation to a more vertical direction. Correspondingly, if the headgroup volume is more elongated along the z axis (Fig. 1), it will contribute less to the overall electron density ($\rho(z)$) (averaged in xy), effectively being invisible against the buffer background. Thus, we propose that the small remaining peak is related to a highly localized positioning of Ca$^{2+}$ interconnecting the phosphate moieties of the headgroups. Note that the data analysis is based on a box model, with both the size and the electron density of the box as free fit parameters. The result for the PIP$_2$/Ca$^{2+}$ samples thus yield a smaller box width and a smaller electron density with respect to DPPC/zero Ca$^{2+}$. Hence, the box is reduced, and stands out as a small maximum with respect to the semiinfinite slab of the buffer. To avoid misunderstandings, we point out that the box names (i.e., headgroup box) are based on the interpretation of the control measurement without Ca$^{2+}$. As the structural features evolve, the fitted model parameters change and the box designators are only nominal, but still serve as useful fiducials when discussing the profiles. To summarize, our results from x-ray reflectivity measurements show that PIP$_2$ and Ca$^{2+}$ both alone and in combination are sufficient to induce profound structural changes in membranes.

**GIXD measurements from lipid monolayers**

To try and further understand the effects of PIP$_2$ and Ca$^{2+}$ on membrane structure we turned to GIXD, which is an experimental technique that provides information on the lateral ordering of the acyl chains in lipid molecules at an air-water interface (which, for the purpose of analysis, effectively represent a two-dimensional crystalline structure).

**FIGURE 2 X-ray reflectivity measurements made from DPPC monolayers.** (A) Measurements were made from pure DPPC monolayers (a, c) and DPPC monolayers with increasing concentrations of Ca$^{2+}$ in the subphase: 1 μM (b, d), 10 μM (e, f), and 30 μM (d, +). For clarity, the curves have been shifted vertically. (B) The corresponding electron density profiles obtained from the best fits (given by the solid lines in panel A).
scattering vectors are resolved. For all our measured samples, the data projected on the $q_{xy}$ axis (vertical integration of diffracted intensity) showed two Bragg peaks, which can be indexed as (11) and (02) of a centered rectangular unit cell (see Fig. 5). The presence of one out-of-plane Bragg rod at $q_z > 0$ indicates that the chains are tilted toward their nearest neighbors. However, it should be noted that such a centered rectangular lattice could also be interpreted as a distorted hexagonal lattice (20).

For pure DPPC monolayers, the lattice parameters of the unit cell were found to be 5.42 Å and 8.54 Å, equating to an area of 23.11 Å² for each chain. The calculated tilt angle of the lipid chains (27.15°) was lower than the value previously reported (~30°) when using an aqueous subphase of ultrapure water (21). In contrast, the buffer used as the subphase in our study contained 150 mM KCl, 25 mM HEPES (pH 7.40 KOH), and 1 mM DTT, which we assume to account for this slight difference. Addition of CaCl₂ to the subphase caused significant structural changes in the lipid monolayer. Although two Bragg peaks could still be observed (indicating a similar unit cell structure), the lattice parameters were decreased slightly, resulting in a concomitant decrease in the area occupied by each acyl chain (see Fig. 5 and Table 1). Furthermore, we observed a monotonic

**FIGURE 5** Diffraction peaks in the horizontal plane derived from GIXD measurements on monolayers. The data correspond to a scan along $q_{xy}$, with vertically integrated intensity (sum over all position-sensitive detector channels), so that all peaks show up. Diffraction peaks were obtained for (A) pure DPPC and (B) DPPC/PIP₂ monolayers. Measurements were made in the absence of Ca²⁺ (a, ○) and with increasing concentrations of Ca²⁺ in the subphase: 1 µM (b, △), 10 µM (c, *), and 30 µM (d, +). For clarity, the curves have been shifted vertically. (B) The corresponding electron density profiles obtained from the best fits (given by the solid lines in panel A).

**FIGURE 3** X-ray reflectivity measurements made from DPPC/PIP₂ monolayers. (A) X-ray reflectivity measurements from DPPC/PIP₂ monolayers (a, ○) and DPPC/PIP₂ monolayers with increasing concentrations of Ca²⁺ in the subphase: 1 µM (b, △), 10 µM (c, *), and 30 µM (d, +). For clarity, the curves have been shifted vertically. (B) The corresponding electron density profiles obtained from the best fits (given by the solid lines in panel A).

**FIGURE 4** GIXD measurements made from DPPC and DPPC/PIP₂ monolayers. Contour plots of GIXD data from (A) a DPPC monolayer, (B) a DPPC monolayer with 1 µM Ca²⁺ in the subphase, (C) a DPPC/PIP₂ monolayer, and (D) a DPPC/PIP₂ monolayer with 1 µM Ca²⁺ in the subphase. (Crosses) Position of the [11] peak. (Inset) Averaged tilt angle of the lipid acyl chains under the corresponding conditions.
TABLE 1 Structural parameters derived from the in-plane Bragg peaks and out-of-plane Bragg rods obtained from GIXD experiments on lipid monolayers of various compositions

<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_{11}$ (Å)</th>
<th>$d_{02}$ (Å)</th>
<th>a (Å)</th>
<th>b (Å)</th>
<th>$A_1$ (Å$^2$)</th>
<th>$A_{02}$ (Å$^2$)</th>
<th>$L_{11}$ (Å)</th>
<th>$L_{02}$ (Å)</th>
<th>$\tau$ (deg)</th>
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<td>DPPC</td>
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<td>4.27</td>
<td>5.42</td>
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The $d$-spacings are given by $d_{11}$ and $d_{02}$. The dimensions of the rectangular unit cell are given by $a$ and $b$. The area per chain and the projected area per chain are given by $A_1$ and $A_{02}$, respectively. $L_{11}$, $L_{02}$ are the domain sizes in the direction of $q_{11}$ and $q_{02}$, and $\tau$ the tilt angle of the acyl chains.

decrease in the domain sizes $L_{11}$ and $L_{02}$, in the directions of $q_{11}$ and $q_{02}$, respectively, suggesting that Ca$^{2+}$ reorganizes the packing of the lipid molecules in the monolayer, which is consistent with other structural parameters obtained from the data. Injection of the Ca$^{2+}$ chelator EGTA into the subphase at the end of the experiment did not produce significant effects on the lattice structure of the acyl chains, although the domain sizes $L_{11}$ and $L_{02}$ were found to decrease further after EGTA addition.

Addition of PIP$_2$ to the monolayer caused the Bragg peaks to shift toward higher $q_{02}$ (see Figs. 4 and 5) (15). Interestingly, addition of Ca$^{2+}$ to monolayers containing PIP$_2$ produced more profound structural changes when compared to DPPC alone. A full comparison of the various effects of PIP$_2$ and Ca$^{2+}$ on monolayers of varying lipid compositions can be found in Table 1 and Fig. 6. Increasing the Ca$^{2+}$ in the subphase resulted in a gradual decrease of both the lattice parameters describing the unit cell and the tilt angle of the acyl chains. At 30 µM Ca$^{2+}$, the tilt angle of the acyl chains was reduced by ~25% in the case of the DPPC/PIP$_2$ monolayer, compared to <10% in the case of pure DPPC. Interestingly, incorporation of PIP$_2$ into the membrane also caused a differential effect with regard to the effects of the Ca$^{2+}$ chelator EGTA. In this case, injection of 10 mM EGTA into the subphase at the end of the experiment partially reversed the effects of the added Ca$^{2+}$; not only did the lattice parameters (and hence the area per lipid chain) increase, but we also observed a subsequent increase in the chain tilt angle from 18.47° to 20.20°.

**Synaptic vesicle interaction and its influence on membrane structure**

As both Ca$^{2+}$ and PIP$_2$ are essential for synaptic vesicle exocytosis, an obvious question is the possible role of structural changes in the fusion process. Fortunately, standard protocols exist for the preparation of ultrapure synaptic vesicles from rat brain, in amounts sufficient to perform our Langmuir trough-based measurements.

Thus, we have injected isolated synaptic vesicles directly into the subphase of a monolayer prepared from DPPC and PIP$_2$. The interaction of SVs with the monolayer was evidenced by a bimodal increase in the interfacial pressure within a few minutes, followed by a much more gradual increase, as reported in Ghosh et al. (15). The Ca$^{2+}$ concentration in the subphase was then progressively increased in the same fashion as in the experiments without SVs. Fig. 7 shows reflectivity data that was used to derive the laterally averaged electron density profiles for the measured

![FIGURE 6 Summary of diffraction data obtained from monolayers with varying amounts of Ca$^{2+}$ in the subphase. Effects of CaCl$_2$ injected in the subphase of monolayers consisting of DPPC (●) or DPPC/PIP$_2$ (▲). (A) Variation in tilt angle of the acyl chains from the surface normal, and (B) the area occupied by each chain. The effect of EGTA on the DPPC/PIP$_2$ monolayer is represented by a single data point (*) and indicated (arrow). In this figure, the error bars are estimated from the confidence intervals of the optimized parameters that provided the best fits to the data shown in Fig. 5.](image-url)
membranes. Addition of Ca\textsuperscript{2+} to the system resulted in structural changes to the monolayer, as shown by the distinct alterations in the electron density profiles we obtained. The structural changes followed a similar trend to that observed when synaptic vesicles were absent from the subphase: in the fit, both the width of the box (along \( z \)) attributed to the headgroup and its electron density decreased monotonically. At the highest concentration of Ca\textsuperscript{2+} in the subphase (30 \( \mu \)M), the box width was reduced to \( \sim 3 \) \( \AA \). As argued above, we attribute this to a change in headgroup tilt angle that in turn leads to an alteration in headgroup orientation. The small remaining peak would then be related to a highly localized positioning of Ca\textsuperscript{2+} interconnecting the phosphate moieties of the headgroups.

The structural basis of this rearrangement was investigated using GIXD to once again investigate the lateral ordering of the acyl chains in the lipid molecules. Results are shown in Figs. 8 and 9, and data are summarized in the lower panel of Table 1. The Bragg peaks obtained from the DPPC/P\( \text{IP}_2 \) monolayer, when synaptic vesicles were included in the subphase, showed a rectangular lattice structure, with an increased tilt angle compared to the DPPC/P\( \text{IP}_2 \) monolayer alone. Addition of increasing amounts of Ca\textsuperscript{2+} to the system resulted in structural changes to the monolayer, similar to the trend seen in both the DPPC and DPPC/P\( \text{IP}_2 \) systems. The tilt angle was found to decrease by \( \sim 12.5\% \) at the highest Ca\textsuperscript{2+} concentration used (30 \( \mu \)M), which is less than the effect seen on the DPPC/P\( \text{IP}_2 \) system in the absence of synaptic vesicles. The domain sizes \( L_{11} \) and \( L_{22} \) were smaller than those without SVs for all Ca\textsuperscript{2+} concentrations. The addition of 10 mM EGTA to the subphase at the end of the experiment partly reversed the effects on chain tilt angle. The observed increase was \( \sim 2\% \), similar to that seen in the DPPC/P\( \text{IP}_2 \) system.

**DISCUSSION**

In this article, we have used x-ray reflectivity and grazing incidence x-ray diffraction to study the membrane structure at the initial stages of the fusion process, which we regard as equivalent to docking, the recruitment of SVs to the plasma membrane.

**Lipid composition at the release site has a profound influence on membrane structure**

A basic requirement for exocytosis is the membrane lipid P\( \text{IP}_2 \) (5,6). Interestingly, the addition of a small amount of P\( \text{IP}_2 \) was sufficient to induce a measurable structural effect in our system (Figs. 3–6). Hence, P\( \text{IP}_2 \) most likely plays an

![FIGURE 7 X-ray reflectivity measurements of DPPC/P\( \text{IP}_2 \) monolayers and the effect of synaptic vesicle addition. (A) X-ray reflectivity measurements from DPPC/P\( \text{IP}_2 \) monolayers with synaptic vesicles in the subphase both in the absence of Ca\textsuperscript{2+} (a, o), and with increasing Ca\textsuperscript{2+} concentrations in the subphase: 1 \( \mu \)M (b, △), 10 \( \mu \)M (c, *), and 30 \( \mu \)M (d, +). For clarity, the curves have been shifted vertically. (B) Electron density profiles (obtained from the solid lines shown in panel A).](https://example.com/figure7)

![FIGURE 8 Diffraction peaks in the horizontal plane derived from GIXD measurements on monolayers in the presence of synaptic vesicles. Diffraction peaks were obtained for DPPC/P\( \text{IP}_2 \) monolayers with synaptic vesicles in the subphase both in the absence of Ca\textsuperscript{2+} (a, o), and with increasing Ca\textsuperscript{2+} concentrations in the subphase: 1 \( \mu \)M (b, △), 10 \( \mu \)M (c, *), and 30 \( \mu \)M (d, +). Reversibility of Ca\textsuperscript{2+}-induced changes was tested by adding 10 mM of the Ca\textsuperscript{2+} chelator EGTA to the subphase at the end of the experiment (e, □). The Bragg peaks were fitted to two Lorentzians (solid lines). (Inset) Hydrocarbon chains of the lipid molecules in the monolayers could be described by a centered rectangular unit cell.](https://example.com/figure8)
addition of PIP 2 enhances and shifts the dependence to the membrane lipids, these studies have all used high concentrations of Ca\(^2+\) could interact directly with Ca\(^2+\). The electrostatic effects of Ca\(^2+\) in their organization within membranes due to the presence of strongly charged lipids are reported to undergo structural changes at release sites. Even zwitterionic lipids are reported to undergo structural changes in their organization within membranes due to the presence of this ion (7,9,10). Although previous studies have used a variety of advanced physical techniques to investigate the electrostatic effects of Ca\(^2+\) on the arrangement of various membrane lipids, these studies have all used high concentrations of Ca\(^2+\) (12,13).

In this study, we have measured the changes in monolayer structure that may occur at release sites under quasiphysiological conditions (22). Interestingly, we found profound effects on membrane structure induced by the electrostatic association of Ca\(^2+\) with phospholipid headgroups, both in x-ray reflectivity and grazing incidence x-ray diffraction measurements. PIP 2 is an anionic lipid with three-to-five net negative charges and is more likely to adsorb multivalent cations in the headgroup regions of the lipid film, with the effects scaling in proportion to the concentration of Ca\(^2+\) used. For example, in pure DPPC, the tilt angle \(\tau\) decreases with Ca\(^2+\) concentration from 27.2° to 25.9° at a maximum concentration of 30 \(\mu\)M, while addition of PIP 2 enhances and shifts the dependence to the range of 24.5° to 18.5°.

In this work, we measured two main structural parameters; the tilt of the acyl chains and the area per lipid were measured as averages across an entire monolayer. These values are tightly interrelated. The interface properties (for example, electrostatic interactions, binding of macromolecules to the membrane surface, and the pressure of the film balance) contribute to fixing the area per lipid, while reorganization of the lipids through chain tilting serves to maximize van der Waals interactions. This effectively means the smaller the area per headgroup, the smaller the tilt angle. In other words, compensatory tilting of the acyl chains acts to maintain the hydrophobic core of the membrane. As discussed above, Ca\(^2+\) induces a significant reduction in both the area and tilt angle (Fig. 6), indicative of Ca\(^2+\)-induced compaction of the film; for example, by bridging the anionic phosphate groups of the lipid. This trend is partially counteracted by incubation of synaptic vesicles with the monolayer (Fig. 9), but is intensified with addition of PIP 2. A corresponding intensified effect with PIP 2 was manifested as a progressive reduction in the size of the headgroup box in the presence of PIP 2 and Ca\(^2+\), suggesting that Ca\(^2+\) ions were effectively bridging-up the lipid molecules and compacting the lipids. Intuitively, this packing of lipids into a smaller unit cell resulted in a reduced area per chain and a reduction in tilt angle of the acyl chains as revealed by grazing incidence experiments.

Our results are in line both with numerical and experimental studies of the effects of cations on membrane structure. For instance, a molecular dynamics study has previously predicted a tight packing of zwitterionic lipids around Ca\(^2+\) ions, which leads to an increase in the ordering of the lipid chains (7). High-resolution neutron diffraction studies directly showed these Ca\(^2+\) ions to localize within the headgroup region of the DPPC bilayer in a lamellar phase (23), while NMR and IR spectroscopy studies suggested a conformational change in the polar region of the DPPC bilayer (10) and rearrangement in the carbonyl region of the lipid palmitoyloleoylphosphatidylcholine as a result of Ca\(^2+\) bridging (11).

Membrane structure and synaptic vesicle association

Unfortunately, it has proved difficult to unambiguously disentangle the structural changes in the lipid monolayer brought about by our various manipulations. The strong effects of Ca\(^2+\) and PIP 2 are likely brought about by simple electrostatic effects, resulting in “charge bridging” that compacts the membrane. The situation becomes more complex, however, when considering the effects seen on synaptic vesicle incubation—although two possibilities are readily apparent.

First, monolayer isotherm and interfacial pressure measurements have previously shown that isolated synaptic vesicles interact with lipid membranes in a Ca\(^2+\)-dependent manner (15), leading us to speculate on the possible insertion of synaptic vesicle-associated proteins into the monolayer. Our GIXD measurements of PIP 2-containing monolayers, incubated with synaptic vesicles, add considerable weight to this hypothesis. Although Ca\(^2+\) addition still leads to a reduction in the tilt of the acyl chains in this system, the effect is significantly less than seen in the absence of synaptic vesicles (Table 1).

Although we can make no direct judgment, it is tempting to speculate that synaptotagmin is most likely responsible...
for mediating the interaction between vesicles and the membrane. Synaptotagmin is a major vesicle protein that contains tandem C2 domains—C2A and C2B (M. Koch and M. Holt, unpublished). Interestingly, synaptotagmin interacts with PIP2 at concentrations of calcium required for transmitter release (24). It is thought that Ca2+ binding may serve merely to stiffen the membrane in areas denuded of proteins, facilitating buckling by synaptotagmin insertion (31). Such a collective, interdependent reorganization of the plasma membrane may then explain the nonlinear Ca2+ dependence of vesicle fusion at release sites across the synaptic terminal (22), providing part of the 40–200 kBT of free energy thought necessary for synaptic vesicle fusion (32,33), and we argue that it should be considered in future schemes of vesicle release.

Further experiments will be needed to fully understand the structural changes that occur in membranes during the fusion reaction. These experiments will need to introduce further complexity in a defined, reproducible manner. First, membranes of a more complex lipid composition (reflecting more accurately the plasma membrane will need to be used. Second, future experiments will also have to consider that proteins participate at multiple stages of the fusion pathway in neurons. For example, synaptotagmin is known to interact with the plasma membrane SNARE proteins syntaxin 1 and SNAP-25, which, together with the vesicular protein synaptobrevin, are thought to constitute the minimal fusion machinery (6,34).

Our choice of a lipid only monolayer was influenced by recent work that suggests that synaptotagmin is responsible for Ca2+-independent docking of the vesicle to PIP2 at exocytic sites via a polybasic patch on the side of C2B domain, arguably the first stage in the release process (35–37). This is thought to occur separately from its membrane penetration activities, which require reorientation of the C2B domain on the membrane surface.

Unfortunately, the incorporation of SNAREs into artificial membranes usually leads directly to synaptic vesicle fusion, irrespective of Ca2+ addition (38). Hence, to fully understand the fusion pathway, syntaxin and SNAP-25 will eventually have to be reconstituted into membranes in a way in which vesicles remain stably associated until Ca2+ addition.

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