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Phosphatidylserine Allows Observation of the Calcium−Myristoyl Switch of Recoverin and Its Preferential Binding

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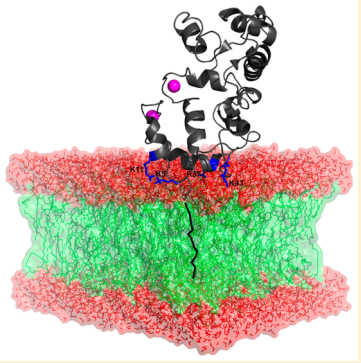
Supporting Information

ABSTRACT: Recoverin undergoes a calcium−myristoyl switch during visual phototransduction. Indeed, calcium binding by recoverin results in the extrusion of its myristoyl group, which allows its membrane binding. However, the contribution of particular lipids and of specific amino acids of recoverin in its membrane binding has not yet been demonstrated. In the present work, the affinity of recoverin for the negatively charged phosphatidylserine has been clearly shown to be governed by a cluster of positively charged residues located in its N-terminal segment. Moreover, the calcium−myristoyl switch of recoverin was only observed upon binding onto monolayers of phosphatidylserine and not in the case of other anionic phospholipids. Fluorescence microscopy experiments with mixed lipid monolayers allowed confirmation of the specific binding of myristoylated recoverin to phosphatidylserine, whereas the extent of penetration of recoverin in phosphatidylserine monolayers was estimated by ellipsometry. A model has thus been proposed for the membrane binding of myristoylated recoverin in the presence of calcium.



INTRODUCTION

Mammalian cells selectively maintain a high concentration of anionic phospholipids in the inner leaflet of the plasma membrane.[1] Numerous proteins are targeted to the cytosolic plasma membrane by means of electrostatic interactions between their clusters of basic residues and anionic phospholipids.[2,3] The inner leaflet of the plasma membrane is rich in anionic phosphatidylinositol (∼1 mol %) and phosphatidylserine (∼20 mol %).[3,4] Data have been reported on the involvement of phosphatidylinositol in the recruitement of cytosolic proteins,[3,5−8] and evidence is recently emerging to demonstrate the role of phosphatidylserine in the plasma membrane localization of such proteins.[2,9]

N-Myristoylation results from the acylation of the N-terminal glycine of numerous proteins.[10] The combination of myristoylation with the presence of a cluster of basic residues in the N-terminal segment has been proposed to highly potentiate protein binding to membranes.[11−16] Moreover, it has been predicted and partly shown experimentally that a large number of myristoylated proteins contain a cluster of positive charges in their N-terminal segment.[17] These proteins could thus bind membranes through the combined action of their N-terminal acylation and positively charged residues. Some of these proteins undergo a calcium−myristoyl switch. In the present study, recoverin has been used as an example to elucidate the mechanism of membrane binding of this type of proteins.

Recoverin is a 23 kDa N-acylated protein.[18] It is involved in rod visual phototransduction through its reversible binding to rhodopsin kinase and rod outer segment disk membranes, which is modulated by variations of the intracellular concentration of calcium.[19−23] Indeed, membrane binding of recoverin takes place at a high concentration of calcium in the dark, which results in the inhibition of rhodopsin kinase.[23−25] However, light absorption by rhodopsin results in a large decrease in intracellular calcium concentration.[26] As a result, recoverin undergoes a large conformational change leading to the sequestration of its myristoyl group in a hydrophobic cavity inside the protein (Figure S1A).[27] Consequently, recoverin dissociates from the membrane, which results in the activation of rhodopsin kinase.[19−23]

The membrane binding of recoverin is thus regulated by a calcium−myristoyl switch. This mechanism has been described in detail[28] on the basis of the structure of myristoylated recoverin in the absence[29] and presence[30] of calcium. Indeed, in the absence of calcium, the myristoyl group of recoverin is buried inside a hydrophobic cleft (Figure S1A).[29] The binding of calcium ions by the two functional EF-hand motifs of recoverin results in the extrusion of its myristoyl group (Figure S1B).[30] However, N-myristoylation was postulated to be barely sufficient for membrane binding of proteins.[31] Nevertheless, we have previously demonstrated that the myristoyl group of recoverin strongly accelerates its binding to lipid monolayers and allows binding to lipid bilayers.[32,33] Recoverin includes a cluster of positively charged amino acids (K5, K11, K37, and R43) in the vicinity of its N-terminal close to its myristoyl group. It has thus been postulated that these residues which become solvent-exposed during the calcium−myristoyl switch (Figure S1B) could allow binding of recoverin to negatively

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charged phospholipids thus complementing the hydrophobic anchoring provided by the myristoyl group.[34] Moreover, on the basis of surface plasmon resonance experiments, it has been suggested that the positively charged C-terminal domain of recoverin could also be involved in electrostatic interactions with negatively charged phospholipids.[35] Therefore, the parameters modulating the membrane binding of recoverin involving electrostatic interactions and their possible synergistic action with the myristoyl group are still not well-understood.

The particular and unique phospholipid composition of photoreceptor outer segment (POS) disk membranes could also modulate the membrane binding of recoverin. Indeed, more than 50% of the acyl chains of disk membranes are polyunsaturated,[36] which provides a large fluidity to those membranes.[37−39] The implication of an individual N-myristoylation in targeting peripheral proteins to the membrane is still a matter of debate.[40] Therefore, the dependence of the membrane binding of recoverin on lipid composition and on its myristoyl moiety is still unclear, which is also true for all myristoylated proteins and, in particular, for the EF-hand family of proteins.

In this paper, we have used phospholipid monolayers to investigate whether a specific interaction takes place between recoverin and negatively charged phospholipids, as a result of electrostatic interactions. Moreover, measurements in the presence and absence of calcium allowed evaluation of whether a synergy takes place between the myristoyl group and the positive charges of recoverin in its membrane binding. Therefore, maximum insertion pressure, fluorescence microscopy, and ellipsometric measurements were performed to find out the importance of electrostatic interactions and of the calcium−myristoyl switch of recoverin in its membrane binding.

RESULTS

Preferential Binding of Recoverin to Negatively Charged Phosphatidylserine Monolayers. The plot of the increase in surface pressure ($\Delta\Pi$) measured after the injection of myristoylated recoverin (MRec) into the calcium-containing subphase of different phospholipid monolayers at several initial surface presures (Π_i) is presented in Figure 1A. This plot allowed calculation of the maximum insertion pressure (MIP) of MRec (intercept with the *x*-axis) in the presence of calcium (MRec-Ca) and of phospholipids with different polar head groups. It can be seen that very similar curves are obtained with monolayers of zwitterionic 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), which allowed calculation of very similar MIP values of MRec-Ca of 20.7 ± 0.7 and 21.7 ± 1.7 mN/m, respectively (inset of Figure 1A). In contrast, a much larger value of MIP of 36.7 ± 2.4 mN/m has been obtained for MRec-Ca in the presence of the negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS, inset of Figure 1A). This value is in the range of the estimated membrane lateral pressure of 30−35 mN/m.[41−47] These data suggest that the negative charge of DMPS favors membrane binding of MRec-Ca. The involvement of electrostatic interactions in this binding can be confirmed by shielding the charges of the phosphatidylserine monolayer using increasing concentrations of NaCl. Figure 1B shows that the larger the concentration of NaCl the smaller the value of MIP in the presence of a DMPS monolayer. Indeed, MIP values of 36.7 ± 2.4, 29.6 ± 2.3, and 26.3 ± 2.7 mN/m,

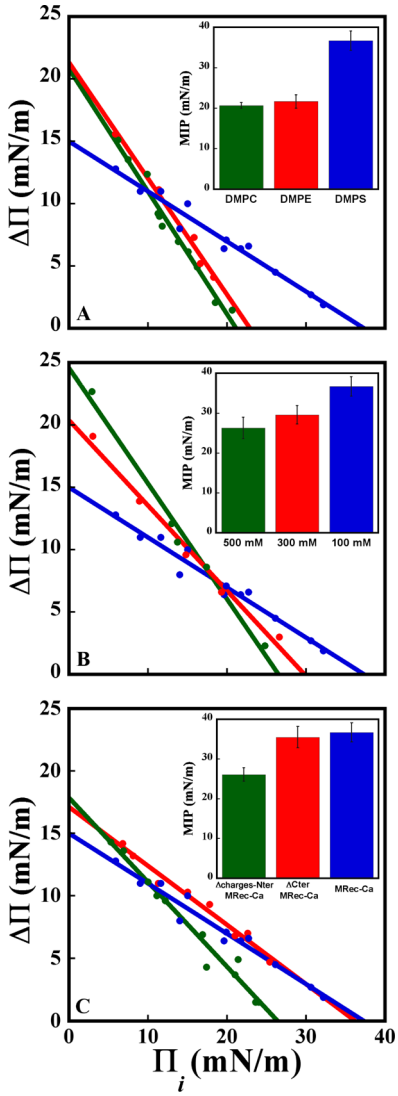

Figure 1. (A) MIP of MRec-Ca in the presence of calcium upon binding onto DMPC, DMPE, and DMPS monolayers. The histograms of the MIP are shown in the inset. The colors of the curves in the plot of $\Delta\Pi$ as a function of Π_i are the same as those used for each individual corresponding lipid in the histograms. (B) MIP of MRec-Ca upon binding onto DMPS monolayers at different concentrations of NaCl (100, 300, and 500 mM). The histograms of the MIP are shown in the inset. (C) MIP of MRec-Ca, ΔCter-MRec-Ca, and Δcharges-Nter-MRec-Ca upon binding onto a DMPS monolayer in the presence of calcium.

respectively, have been obtained for MRec-Ca in the presence of 100, 300, and 500 mM NaCl (inset of Figure 1B). Altogether, these data thus strongly suggest that electrostatic interactions are involved in the binding of MRec-Ca with DMPS.

This particular affinity of recoverin for the negatively charged phosphatidylserine could be favored by the presence of positively charged residues located in its N- and/or C-terminal segment(s), which have been proposed to be involved in its membrane binding.[34,35] Indeed, there is a cluster of 4 positive charges (Lys 5, 11, and 43 and Arg 37, Figure S1B) in the N-terminal segment and a net positive charge of +4 in the C-terminal segment (amino acids 190−202) of recoverin. Two mutants were therefore prepared to determine the involvement of these segments in the binding of recoverin to phosphati-

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dylserine monolayers in the presence of calcium: ΔCter-MRec-Ca and Δcharges-Nter-MRec-Ca. The amino acid sequence 190−202 has been removed in ΔCter-MRec-Ca, as previously performed by Senin et al.,[35] whereas Lys 5, 11, and 43, and Arg 37 have been mutated to Ser in Δcharges-Nter-MRec-Ca. Although Lys 83 was also suspected to participate in the N-terminal cluster of MRec-Ca,[34] it has not been mutated to prevent any perturbation of calcium binding by the EF-hand 2 motif of recoverin.[30] Figure 1C shows that the value of MIP obtained with ΔCter-MRec-Ca (35.5 ± 2.7 mN/m) is very similar to that measured with the wild type protein (36.7 ± 2.4 mN/m). These data thus suggest that the C-terminal domain of recoverin is not involved in its membrane binding in the presence of a negatively charged phospholipid. In contrast, the MIP observed with Δcharges-Nter-MRec-Ca (26.1 ± 1.7 mN/m, Figure 1C) is much smaller than that observed with the wild-type protein (36.7 ± 2.4 mN/m, Figure 1A,C) in the presence of the DMPS monolayer and calcium. Moreover, the MIP obtained with this mutant is almost identical to that observed when shielding the charges of the DMPS monolayer in the presence of 500 mM NaCl (26.3 ± 2.7 mN/m, Figure 1B). It therefore appears that the N-terminal cluster of positive charges of recoverin is involved in electrostatic interactions with the negatively charged phosphatidylserine in the presence of calcium.

Experiments were also conducted to determine whether the effect of large concentrations of NaCl on monolayers of DMPS could account for the observed decrease of the MIP of MRec-Ca at 300 and 500 mM NaCl (Figure 1B). Figure S2 shows that the isotherms of DMPS are shifted to larger molecular areas with increasing NaCl concentration. Moreover, an increase in the onset of the LE/LC phase transition can also be seen when increasing NaCl concentration. These changes are significant and cannot be readily disregarded. However, the MIP values are determined from recoverin adsorption isotherms at equilibrium surface pressures (Π_e), which are larger than 20 mN/m (inferred from Figure 1B, $\Delta\Pi + \Pi_i$). Small differences in molecular area can be observed between the isotherms at 100, 300, and 500 mM NaCl at surface pressures larger than 20 mN/m (Figure S2), which suggests a possible minor effect of large NaCl concentrations on the observed decrease of the MIP (Figure 1B). In addition, the isotherm DMPG undergoes a shift to larger molecular areas as a function of NaCl concentration,[48] in a manner similar to DMPS (Figure S2). DMPG has thus been used to find out whether isotherm shifts to larger molecular areas with increased NaCl concentrations (Figure S2) could account for the differences in MIPs observed with DMPS (Figure 1B). Figure S3 shows that there is no significant difference between the MIP of recoverin in the presence of a DMPG monolayer and 100 or 500 mM NaCl, which contrasts with the large decrease of the MIP of MRec-Ca in the presence of the negatively charged DMPS. These data thus suggest that the shift of the isotherms of DMPS and DMPG to large molecular areas at large concentrations of NaCl likely plays a minor role in the observed decrease of the MIP of MRec-Ca in the presence of DMPS at 300 and 500 mM calcium (Figure 1B). Nevertheless, this conclusion has to be taken with caution as these additional measurements provide indirect evidence that large concentrations of NaCl do not account for the observed differences in MIPs of MRec-Ca in the presence of DMPS (Figure 1B). However, additional evidence for the involvement of electrostatic interactions in the binding of recoverin to phosphatidylserine are provided by other approaches (see below).

Involvement of the Calcium−Myristoyl Switch of Recoverin on Its Binding to Phosphatidylserine Monolayers. Figure 2A,B respectively present measurements of the MIP and synergy of MRec with phospholipids bearing different polar head groups in the presence (MRec-Ca) and absence (MRec-EGTA) of calcium. It can be seen that very similar values of MIP are obtained with MRec-EGTA and MRec-Ca in the presence of zwitterionic phospholipids DMPC and DMPE.

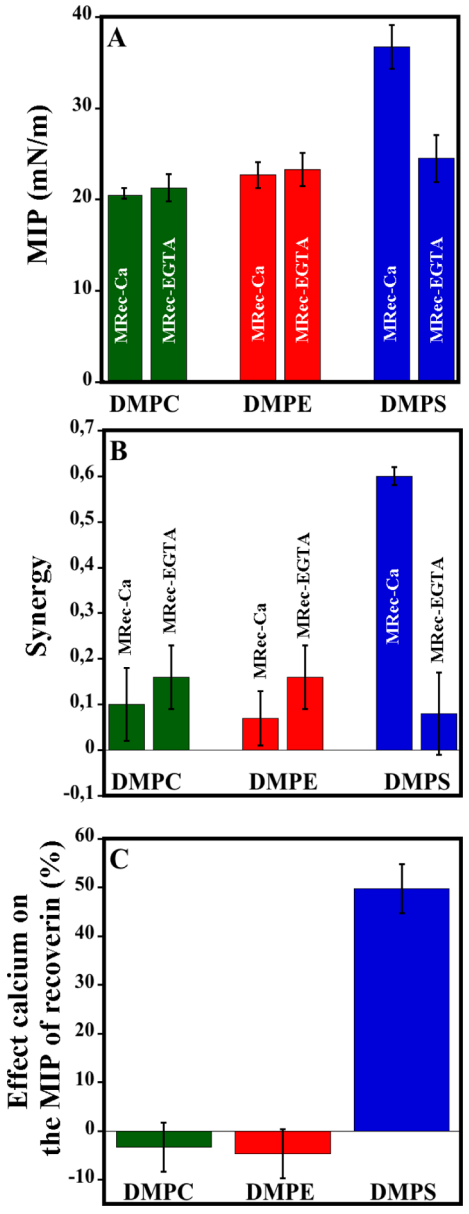

Figure 2. (A) MIP of myristoylated recoverin in the presence (MRec-Ca) and in the absence (MRec-EGTA) of calcium upon binding onto DMPC, DMPE, and DMPS monolayers. (B) Synergy of MRec-Ca and MRec-EGTA upon binding onto the same lipid monolayers. (C) Effect of calcium on the MIP of MRec-Ca, relative to that of MRec-EGTA, upon binding to lipid monolayers. The values have been calculated as follows: $(MIP_{MRec\text{-}Ca} - MIP_{MRec\text{-}EGTA}/MIP_{MRec\text{-}EGTA} \times 100)$. A significantly positive value in the presence of a particular lipid suggests that this lipid favors the observation of a calcium−myristoyl switch.

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In contrast, the MIP of MRec-Ca in the presence of DMPS (36.7 ± 2.4 mN/m, Figure 2A) is much larger than that obtained in the absence of calcium (MRec-EGTA; 24.5 ± 3.5 mN/m, Figure 2A). These observations strongly suggest that the calcium−myristoyl switch of recoverin resulting in both the extrusion of its myristoyl moiety and of positively charged residues in the presence of calcium is solely observed upon binding to the negatively charged phosphatidylserine monolayer.

The observation of the calcium−myristoyl switch of MRec-Ca upon binding only to phosphatidylserine is further supported by the data illustrated in Figure 2B which presents the values of synergy, such as previously reported.[49] The synergy provides additional information supporting the favorable and specific binding of MRec to the DMPS monolayer in the presence of calcium. Indeed, a much larger value of synergy of 0.6 ± 0.02 has been obtained for MRec-Ca than for MRec-EGTA (0.08 ± 0.09) (Figure 2B). When the synergy is close to 0, such as in the case of MRec-EGTA, the corresponding MIP value is called "stationary surface pressure" because protein monolayer binding is neither favored nor unfavored.[49] The much larger value of synergy in the presence of calcium suggests a preferential binding of MRec-Ca to the DMPS monolayer, whereas no significant difference can be seen between the synergy of MRec-Ca and MRec-EGTA in the presence of zwitterionic DMPC and DMPE (Figure 2B). The results shown in Figure 2C are an alternative representation of the same data shown in Figure 2A. It demonstrates more directly the effect of calcium to illustrate the selective observation of the calcium−myristoyl switch of recoverin in the presence of DMPS in comparison with other types of lipids. Altogether, the present data strongly suggest a major role of phosphatidylserine in the mechanism of calcium−myristoyl switch of MRec-Ca.

Additional experiments have been performed to determine whether other anionic phospholipids, such as phosphatidylinositol and phosphatidylglycerol, would also allow a specific preferential binding of recoverin through electrostatic interactions. However, as shown in Figure S4, no significant effect of calcium on the MIP of recoverin can be observed in the presence of either 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG, −10.7 ± 9.6%) or 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphoinositol (POPI, −12.7 ± 10.4%), whereas a strong effect of calcium can be seen with DMPS (49.8 ± 5.4%). These results suggest that the calcium−myristoyl switch of recoverin is modulated by a phosphatidylserine-specific phenomenon, which likely does not involve other negatively charged phospholipids.

Microscopic Observations of the Preferential Binding of MRec-Ca to Negatively Charged Phosphatidylserine Monolayers. Fluorescence microscopy measurements have been performed to further demonstrate that MRec-Ca specifically binds to negatively charged phosphatidylserine monolayers. Measurements were thus performed using pure DMPS monolayers or mixtures of phosphatidylserine with a zwitterionic phospholipid. The surface pressure isotherm of DMPS shows a phase transition from the fluid liquid-expanded (LE) to the solid/liquid-condensed (LC) states in the presence of calcium at 23 °C (see Figure S2). As shown in Figure 3A, dark LC domains of DMPS can be seen by fluorescence microscopy because the fluorescent lipid dye (Texas Red DHPE) is excluded from these condensed domains. However, a homogeneous fluorescence can be seen in Figure 3B when

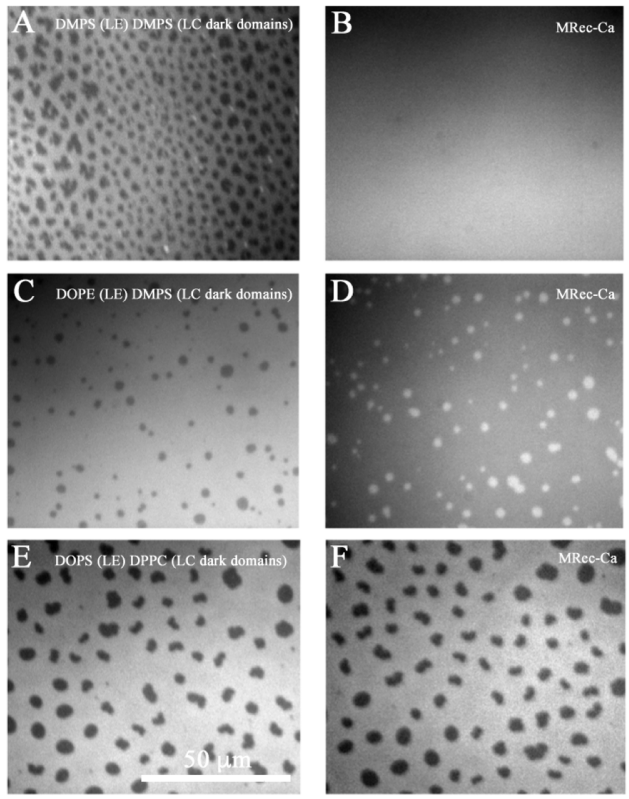

Figure 3. Fluorescently labeled myristoylated recoverin in the presence of calcium has been injected into the subphase of a pure DMPS monolayer (A, B), a DMPS/DOPE (50:50) monolayer (C, D), and a DOPS/DPPC (50:50) monolayer (E, F). Micrographs (A, C, E and B, D, F) allow us to respectively observe the fluorescence of Texas Red DHPE and of myristoylated recoverin labeled with Alexa Fluor 488.

observing the fluorescence of MRec-Ca labeled with Alexa Fluor 488, thus suggesting that MRec-Ca does not discriminate the fluid LE physical state from the LC, gel-like domains of DMPS.

Two different phospholipid mixtures have then been used to confirm that a specific binding of MRec-Ca to phosphatidylserine takes place in monolayers. The first mixture was used to allow observation of LC domains of DMPS in the fluid environment of an unsaturated zwitterionic phospholipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE). As suggested from its surface pressure isotherm (Figure S2), it can be expected that DMPS will form LC domains when mixed with DOPE given that DOPE shows a single fluid LE state at room temperature,[50] whereas a phase transition from 5 mN/m can be seen in the isotherm of DMPS in the presence of 1 mM calcium and 100 mM NaCl (Figure S2). This led to the observation of dark domains of DMPS by fluorescence microscopy when mixed with DOPE (Figure 3C). Then, the injection of fluorescently labeled MRec-Ca underneath this lipid mixture allowed the observation of protein fluorescence, which is preferentially associated with the LC domains of DMPS (Figure 3D). This preferential binding of MRec-Ca to the LC domains of DMPS strongly suggests that this negatively charged phospholipid favors binding of this protein. However, one can wonder whether MRec-Ca prefers binding negatively charged lipids in the LC state. Measurements have thus been performed with a phospholipid mixture using a phosphatidylserine in the fluid LE state (1,2-dioleoyl-*sn*-glycero-3-

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phosphoserine, DOPS). On the basis of the surface pressure isotherms of pure 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DOPS,[50] it can be concluded that the LC domains observed in Figure 3E consist of DPPC. The injection of MRec-Ca underneath this mixed monolayer allowed the observation of protein fluorescence except in the areas corresponding to the DPPC domains (Figure 3F). Altogether, these data demonstrate that MRec-Ca preferentially binds negatively charged phosphatidylserine whatever its physical state.

MRec-Ca Is Slightly Protuding into Phosphatidylserine Monolayers as Determined by Ellipsometry. The ellipsometric angle, $\Delta\delta$, provides information on the refractive index and thickness of the monolayer.[51] These two parameters evolve until the monolayer has reached its maximum density at equilibrium. Although the refractive index contributes to the ellipsometric signal at this film density, one can assume that this signal depends mainly on thickness to facilitate the analysis of the data. Indeed, for a thin and transparent optically isotropic monolayer at the air−water interface, the absolute change in ellipsometric angle is proportional to the thickness, within the Drude approximation,[52] at high monolayer coverage.[53,54] The ellipsometric data can then provide information on the extent of protein penetration in a lipid monolayer, as previously described,[55,56] on the basis of independent measurements with the lipid, the protein, and the lipid−protein monolayers. In fact, we have previously shown that the ellipsometric angle depends mainly on monolayer thickness except when a lipid phase transition takes place,[51] which is not the case in our experiments at equilibrium. The evolution of the ellipsometric angle upon MRec-Ca monolayer binding as a function of time is presented in Figure 4A. It can be seen that in the absence of a lipid monolayer an ellipsometric angle of ∼8.8° is obtained for pure MRec-Ca at equilibrium. The same experiment was carried out in the presence of a DMPS monolayer. The ellipsometric signal obtained at equilibrium upon binding of MRec-Ca to the DMPS monolayer is ∼14.5° (MRec-Ca/DMPS, Figure 4A). Pure DMPS contributes to this ellipsometric signal ($\Delta\delta_{DMPS}$ = 7.5°; dashed line in Figure 4A, $\Delta\delta$ DMPS). The binding of MRec-Ca to the DMPS monolayer thus starts at an ellipsometric angle of $\Delta\delta$ = 7.5°. However, the value of 14.5° for the MRec-Ca/DMPS monolayer does not correspond to the mere addition of the individual DMPS and MRec-Ca ellipsometric angles. Indeed, the sum of the ellipsometric angle of pure MRec-Ca in the absence of a lipid monolayer (8.8°, Figure 4A) and that of the pure DMPS monolayer (7.5°), i.e., 8.8° + 7.5° = 16.3°, is larger than the experimentally obtained $\Delta\delta$ for the MRec-Ca/DMPS monolayer, as described by $\Delta\delta_{Mrec\text{-}Ca} + \Delta\delta_{DMPS} > \Delta\delta_{MRec\text{-}Ca/DMPS}$ (16.3° > 14.5°). Consequently, MRec-Ca is not juxtaposed underneath the DMPS monolayer but rather slightly penetrates this lipid monolayer, as proposed in the model shown in Figure 4B.

Binding of MRec-Ca to Phospholipids with Fatty Acyl Chains Typical of POS Membranes. MIP values of MRec-Ca have also been measured in the presence of monolayers of phosphatidylserines bearing either saturated (18:0) or polyunsaturated (22:6) fatty acyl chains as well as in the presence of total POS lipids and a lipid mixture mimicking the POS lipid content. These results are shown in Figure 5. The MIP values obtained with these phosphatidylserine monolayers are larger than the estimated membrane lateral pressure of 30−35 mN/m.[41−47] However, significant differences were observed depending on the type of fatty acyl chain. Indeed, the MIP of MRec-Ca in the presence of the saturated 1,2-distearoyl-*sn*-glycero-3-phosphoserine (DSPS, 41.8 ± 4.8 mN/m) is much larger than that in the presence of the polyunsaturated 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoserine (DDPS, 31 ± 1.2 mN/m, inset of Figure 5). The MIP obtained in the presence of DMPS (36.7 ± 2.4 mN/m, Figure 1A) is in between the values obtained with DSPS and DDPS. Nevertheless, whatever the fatty acyl chain, a functional calcium−myristoyl switch can be

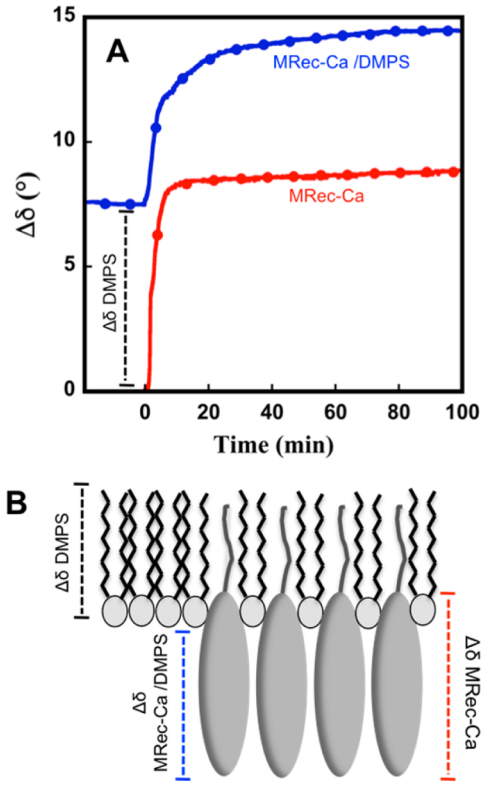

Figure 4. (A) Ellipsometric angle of MRec-Ca in the presence (MrecCa/DMPS) and in the absence (MrecCa) of a DMPS monolayer. (B) Model of the binding of MRecCa to the lipid monolayer on the basis of the different values of ellipsometric angles.

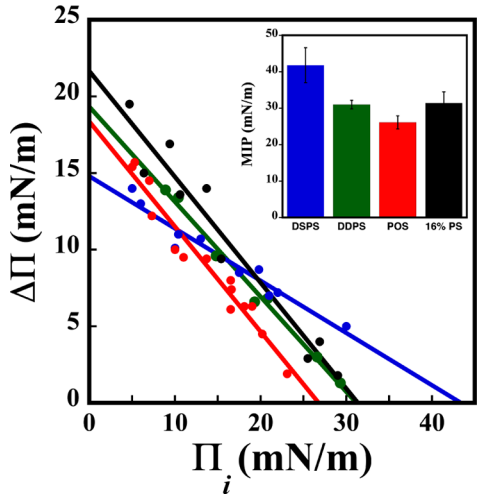

Figure 5. MIP of MRec-Ca upon binding onto DSPS, DDPS, lipids extracted from photoreceptor outer segment (POS) membranes, and a lipid mixture (DSPC, DSPE, and DSPS) containing 16% phosphatidylserine. The histograms of the MIPs are shown in the inset.

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observed in the presence of these phosphatidylserines as discussed below.

Figure 6 illustrates the effect of calcium on the MIP of MRec-Ca relative to that of MRec-EGTA and thus provides

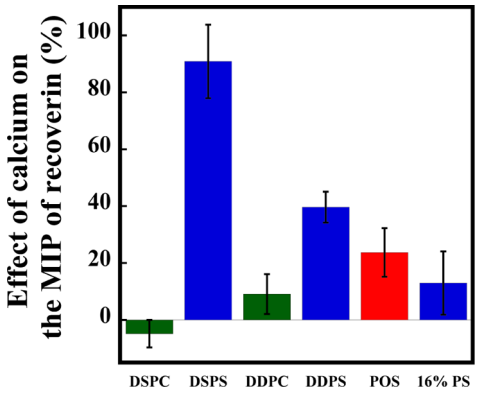

Figure 6. Effect of calcium on the MIP of MRec-Ca upon binding onto monolayers of DSPC, DSPS, DDPC, DDPS POS, or a lipid mixture (DSPC, DSPE, and DSPS) containing 16% phosphatidylserine. The values of the effect of calcium have been calculated as described in the legend of Figure 2C. The values of MIP of MRec-EGTA used for these calculations are presented in Table S1.

information on the observation of a calcium−myristoyl switch, such as depicted in Figure 2C. Figure 6 shows that large significant differences can be observed between the effect of calcium on MRec-Ca in the presence of DSPS (90 ± 12.3%) and DDPS (39.6 ± 5.4%). However, a slight effect can be observed in the presence of 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine (DDPC, 9.1 ± 7.0%), and no significant effect can be seen in the presence of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, −4.8 ± 4.9%) (Figure 6). The MIP of MRec-Ca in the presence of POS lipids (26.1 ± 1.8 mN/m, Figure 5) is smaller than the estimated lateral pressure of membranes.[41−47] A MIP value of 31.4 ± 3.1 mN/m has been obtained using a mixture of lipids bearing steroyl fatty acyl chains (DSPC/DSPE/DSPS, 42:42:16 mol %) which have been selected because of their abundance in photoreceptors.[57,58] This MIP value is similar to that measured with DDPS but larger than that obtained with POS lipids. Nonetheless, the calcium−myristoyl switch of recoverin can also be observed in the presence of both POS lipids and 16% PS (23.7 ± 8.5% and 13% ± 11%, respectively, Figure 6) although much less extensively than in the case of pure phosphatidylserine monolayers (DSPS and DDPS, Figure 6; DMPS, Figure S4). Given that POS membranes consist of ∼16% phosphatidylserine,[57,58] the observation of a smaller effect of calcium in the presence of the 16% phosphatidylserine lipid mixture is consistent with the small content of this negatively charged phospholipid in these membranes. It also suggests that the richer fatty acyl chain content of phosphatidylserine in POS (see Discussion) is more favorable to MRec-Ca binding.

Comprehensive Model Integrating All Data Gathered through the Conducted Experiments. A model has been built using the NMR structure of MRec-Ca (Protein Database ID: 1JSA-12), which allows us to illustrate the knowledge gained through the conducted experiments. As shown in Figure 7, the N-terminal group of MRec-Ca slightly penetrates into the DMPS monolayer, which nevertheless leads to a rather extensive penetration of the myristoyl group. The positive

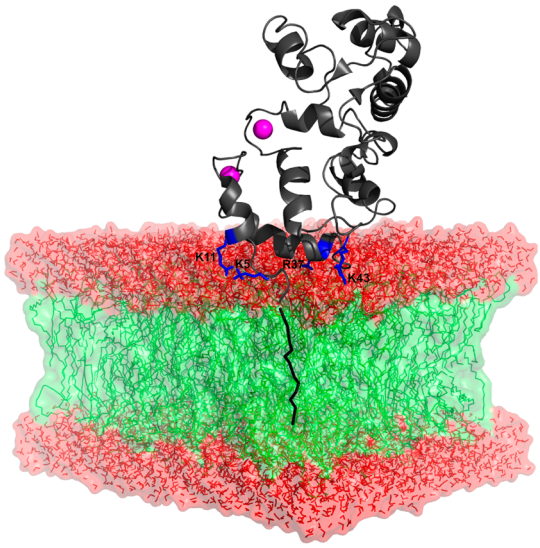

Figure 7. Schematic model of the binding of recoverin to a DMPS monolayer in the presence of calcium.

charges formed by Lys 5, 11, and 43 and Arg 37 of MRec-Ca have been positioned in close proximity to the negatively charged carboxylic group of the phosphatidylserine headgroup but away from the location of its negatively charged phosphate group located in between the serine and the glycerol backbone, in agreement with the data presented in Figures 1−6. Moreover, the lack of a mechanism of calcium−myristoyl switch of MRec-Ca in the presence of DMPG and POPI monolayers (Figure S4) strongly suggests that the negatively charged phosphate group of these phospholipids is not accessible to the positively charged amino acids located at the N-terminal of recoverin in the presence of calcium. One can thus postulate that MRec-Ca is not closely located to the phosphate group of phosphatidylserine. The likely binding of the positive charges of MRec-Ca to the negatively charged carboxylic group of phosphatidylserine has allowed us to appropriately locate both the myristoyl group and the extended N-terminal segment of recoverin into the DMPS bilayer.

DISCUSSION

It has been postulated but never shown experimentally that recoverin could show affinity for negatively charged phospholipids because it includes a cluster of positively charged amino acids in the vicinity of its N-terminal segment close to its myristoyl group.[34] Measurements were thus performed to clarify this issue and to provide more general information for myristoylated proteins which contain a cluster of positive charges in their N-terminal segment.[17] The present data strongly suggest that MRec-Ca preferentially binds negatively charged phosphatidylserine. Several observations argue in favor of the existence of a calcium−myristoyl switch of MRec-Ca solely in the presence of this type of phospholipid: (1) a larger MIP of MRec-Ca in the presence of DMPS than the estimated lateral pressure of membranes, (2) a much larger MIP in the presence than in the absence of calcium, (3) a decrease of the MIP when screening the charges of DMPS with increasing concentrations of NaCl or by using Δcharges-Nter-MRec-Ca (Lys 5, 11, 43 and Arg 37), (4) the absence of a calcium−myristoyl switch of recoverin in the presence of other anionic phospholipids, and (5) on the basis of the fluorescence micrographs showing specific binding of MRec-Ca to the

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negatively charged phosphatidylserine when mixed with zwitterionic lipid monolayers. Altogether, these data demonstrate that electrostatic interactions are involved in the preferential membrane binding of recoverin in the presence of calcium. In addition, the ellipsometric data have allowed us to postulate that the N-terminal segment of recoverin slightly penetrates in the DMPS monolayer. The model proposed in Figure 7 integrates this information and the possible location of the positively charged amino acids with respect to that of the negatively charged carboxylic group in the headgroup of phosphatidylserine. The membrane binding of recoverin is thus most likely regulated by an electrostatic switch, such as that postulated for other proteins,[31,59] given that a calcium−myristoyl switch has exclusively been observed in the presence of phosphatidylserine. Moreover, a small calcium−myristoyl switch signal has been observed in the presence of POS membranes, which contain ∼16% phosphatidylserine, as well as a lipid mixture containing the same percentage of phosphatidylserine.

We have previously shown that polyunsaturated zwitterionic phospholipids favor monolayer binding of recoverin.[49] However, the present data show that the calcium−myristoyl switch of recoverin can barely be observed in the presence of such monolayers because only slightly larger values of MIP have been obtained with MRec-Ca compared to MRec-EGTA in the presence of the polyunsaturated DDPC monolayer (Figure 6). In contrast, the MIP value of recoverin of 31 ± 1.2 mN/m (Figure 5), which is larger than the lateral pressure of membranes, as well as the observation of a calcium−myristoyl switch in the presence of the polyunsaturated DDPS (Figure 6) are consistent with the involvement of electrostatic interactions in the membrane binding of this protein. Moreover, much larger MIP values of MRec-Ca have been observed in the presence of phosphatidylserines in a condensed rather than in a fluid state. Indeed, the MIP of MRec-Ca in the presence of DSPS (41.8 ± 4.8 mN/m) in a gel-like, condensed state is much larger than that observed with the DMPS monolayer (36.7 ± 2.4 mN/m), which shows a phase transition between fluid LE to LC states (Figure S3) and that of DDPS (31 ± 1.2 mN/m), which is in the fluid LE state. As stated above, POS membranes contain a rather small amount of phosphatidylserine.[57,58] However, phosphatidylserine is the most unsaturated POS phospholipid as it contains ∼20% saturated (16:0 and 18:0) and ∼74% polyunsaturated (22:4, 22:5, 22:6, 24:4, and 24:5) fatty acyl chains.[57,58] In addition, photoreceptor membranes were shown to contain 8% detergent-resistant membranes (DRM),[60] which are enriched in saturated phospholipids and cholesterol as well as in 16:0 and 18:0 phosphatidylserine.[60] One could thus postulate that the larger concentration of saturated phosphatidylserine in POS DRMs might favor the location of recoverin in these membrane heterogeneities. However, binding of acylated proteins to DRMs has been solely observed for proteins bearing two acyl groups, either a N-acylation and a S-acylation or two S-acylations,[40] except for the c-SRC protein found in neuronal cells, which is N-acylated.[61] Recoverin behaves in a manner similar to that of c-SRC as it was also shown to be partly associated with DRMs.[62] Moreover, the location of recoverin in DRMs was shown to increase in the presence of calcium, which is consistent with the present data. Indeed, our fluorescence micrographs allowed to demonstrate that MRec-Ca binds phosphatidylserine in the LC state as efficiently as in the LE state, thereby suggesting that the myristoyl group of recoverin is essential for the binding of anionic phospholipids in the LC state (Figure 4) although the largest share of recoverin is not associated with DRMs.[62] Interestingly, recoverin is heteroacylated in vivo.[63] Indeed, ∼90% of bovine recoverin is N-linked with unsaturated fatty acyl chains (14:1 and 14:2).[63] This particular N-acylation could influence the binding parameters of recoverin. One could indeed postulate that binding of N-unsaturated recoverin would be energetically more favorable to unsaturated or polyunsaturated phospholipids such as DDPS than to saturated phospholipids in the gel-like state such as DSPS. It would thus be interesting to determine the extent of membrane binding of hetroacylated recoverins in the presence of phosphatidylserine with fatty acyl chains typical of POS membranes.

The present data allowed us to propose a model where both the charges in the N-terminal of recoverin and its myristoyl group provide a proper binding of recoverin to the negatively charged phosphatidylserine.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information provides The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04218.

Details on the experimental methods used in this manuscript, structure of recoverin in the presence and absence of calcium, Π-A isotherms of DMPS in the presence and absence of calcium and increasing concentrations of NaCl, the comparison between the MIP of myristoylated recoverin in the presence and absence of calcium as well as in the presence of calcium and 500 mM NaCl upon binding onto DMPG and DMPS monolayers, comparison between the effect of calcium on the MIP of MRec-Ca relative to that of MRec-EGTA upon binding onto POPI, DMPG, and DMPS monolayers, and a table listing the MIP of MRec in the presence and absence of calcium upon binding onto monolayers of lipids typical of photoreceptor outer segment disk membranes (PDF)

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Notes

The authors declare no competing financial interest.

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