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Interaction of Rabbit C-Reactive Protein with Phospholipid Monolayers Studied by Microfluorescence Film Balance with an Externally Applied Electric Field

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ABSTRACT C-reactive protein (CRP) is one of the most characteristic acute-phase proteins in humans and many other animals. It binds to phosphorylcholine in a calcium-dependent manner. In addition, CRP activates the complement systems via the classical pathway. The interaction between rabbit CRP (rCRP) and model biological membrane is studied using dimyristoylphosphatidylethanolamine and dipalmitoylphosphatidylcholine monolayers. Observations with fluorescence microscopy indicate that rCRP is more likely to be incorporated in the liquid phase of monolayers. Such incorporation does not depend on the presence of calcium and is not inhibited by phosphocholine. The area occupied by the protein when incorporated into the monolayer was estimated. The dipole moment density of the protein crossing the air/water interface was measured by appling an external electric field. Our results indicate that calcium binding leads to a conformational change in CPR, which might modify the orientation of CRP in the monolayer. In addition, a negative charge or negative difference in dipole moment density facilitates the incorporation of CPR into the monolayer.

INTRODUCTION

C-reactive protein (CRP) is one of the most characteristic acute-phase proteins, and it has been found in most vertebrates (Liu et al., 1987). The level of CRP in serum rapidly increases thousands of times in response to inflammation or injury. It has been reported that CRP is a cyclic pentamer composed of five identical nonglycosylated subunits (Osmond et al., 1977; Shrivel et al., 1996; Sui et al., 1996). Each subunit has 206 amino acids and a molecular mass of ~21 kDa. CRP was originally identified by its capacity to bind phosphorylcholine on C-polysaccharide, which leads to the precipitation of C-polysaccharide on the pneumococcal cell wall in a calcium-dependent manner. In addition, CRP binds specifically to other organic phosphate esters, which include monophosphate and phosphorylethanolamine. Various binding specificities of CRP were characterized. It has been discovered that CRP binds to chromatin histones, small nuclear ribonucleoproteins, and fibronectin (Robey et al., 1984; Du Clos et al., 1987; Du Clos, 1988; Potempa et al., 1987). In each case, the binding of CRP is inhibited by free phosphocholine (PC) and is calcium dependent. There was evidence that CRP binds to polycationic molecules (Potempa et al., 1981). This reaction does not require calcium.

The physiological role of CRP is not yet well defined. Some studies have suggested that CRP activates the complement system via the classical pathway. It has also been demonstrated that CRP promotes phagocytosis, as well as the activation of platelets. Although extensive studies had been carried out on the interactions between CRP and liposomes (Richards et al., 1979; Mold et al., 1981), the mechanism by which CRP binds to the model membrane is still unclear. It is essential to understand this mechanism because of its high relevance to the activation of the complement systems.

Compared with liposomes, a lipid monolayer on an air/water interface is easier to use for mimicking biological membranes. The composition, temperature, and ionic conditions of the monolayer can be easily controlled. In the present paper, we compared the distribution of rabbit CRP (rCRP) in the lipid monolayer, which contains PC and PE as headgroups at various conditions. Applying the presented method, we also quantitatively measured the area in the lipid monolayer occupied by rCRP penetration and the dipole moment density at which CRP protruded from the air/water interface. A possible mechanism is proposed to address the interaction of CRP with the model membrane on the basis of these comparisons and measurements.

MATERIALS AND METHODS

Materials

DPPC (dipalmitoylphosphatidylcholine) and DMPE (dimyristoylphosphatidylethanolamine) were obtained from Sigma Chemical Co. (St. Louis, MO). The lipid dye probe (rhodamine-DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). The monolayers were spread from a lipid solution in chloroform/methanol (3:1, v/v) containing 0.2 mol% of the dye probe. The water used was deionized, and the contamination by divalent ions was prevented by adding 10^{-5} M EDTA to the subphases. The electrolyte concentration in the subphases was 2 mM KCl. The pH was adjusted with KOH and HCl. Sepharose-PE, phosphocholine, and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co.

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Setup

The film balance, which contains a glass window at the bottom, is made of Teflon. Its dimensions are 20 × 8 × 0.8 cm. Monolayers were observed with a Nikon DIAPHOT-TMD inverted epifluorescence microscope equipped with extremely long working distance objectives. The objectives used for the observation of DPPC and DMPE monolayers are 40× and 10×, respectively. When the labeled protein was added, the monolayer was observed alternately through two interchangeable cutoff filters, the permissible wavelengths of which correspond to signals from the rhodamine-labeled lipid and FITC-labeled rCRP, respectively. The pictures were received with a low-light-level SIT camera (Hamamatsu c2400) and recorded with a VHS video recorder (Panasonic HD-100). The electrode consists of a metal syringe tip ~10 μm in radius (Miller and Möhwald, 1986). A micromanipulator (Nikon Narashige) was used to move the electrode in three orthogonal directions with an accuracy of 1 μm. A cover was designed to eliminate disturbance from air circulation and slow down the drift of domains.

Purification and fluorescent labeling of C-reactive protein

rCRP was purified by affinity chromatography on the basis of its specific binding to PE or PC (Bach et al., 1977). The purity of rCRP was judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The concentration of purified rCRP was measured spectrophotometrically at 280 nm using an $E^{1\%}_{1cm}$ of 19.5 for rCRP. After purification, rCRP was conjugated with FITC. We followed the method described by Nairn (1976), with some modifications. The labeled protein was further purified with G-25 gel filtration chromatography. The ratio of fluorochrome to rCRP was determined to be 1.5.

Monolayer formation

To properly reflect the interaction of rCRP with the monolayer, two different protein-adding procedures were compared. The first procedure was designed according to a recently reported method by Subirade et al. (1995), in which the monolayer was spread on the subphase first, and rCRP was injected into the subphase later. In this course, protein diffusion and the corresponding effect on the monolayer were monitored by fluorescence microscopy. Although the domain shape and size changed significantly after 4 h of protein injection, the distribution of rCRP in the monolayer was still inhomogeneous. Because the ordinary diffusion rate of protein in water is just on the order of 10^{-7} cm^2/s, the diffusion process will be too slow to be neglected. In just a few hours for ordinary experiments, the uniform distribution of protein cannot be ensured. Therefore, this protein-adding method is not suitable for the fluorescence microscopy study of the interaction between rCRP and the monolayer.

We adopted another method to add protein, because of the failure of the above method. In this method, the protein and the solution of subphase are thoroughly mixed first. The lipid monolayer was spread on the subphase immediately after adding the solution to the trough. Then the monolayer was compressed to the preferred area and incubated for 2 h. In this process, the protein completely interacts with the monolayer without introducing such uncertainty of distribution.

Measurement of the dipole moment density of the monolayer

At a certain temperature, lipid monolayers at the air/water interface undergo a phase transition between liquid and a more condensed gel phase, when the surface pressure is increased. In the two-phase region, the gel phase exists as discrete domains within the continuous liquid phase. The difference in the dipole moment density between the two phases is measured by examining domain redistribution under the electric field (Mi and Sui, 1996).

Under the action of an inhomogeneous electric field, lipid domains can be attracted to or repelled from the electrode and finally form a certain concentration distribution. In this process, the free energy of the system changes. The free energy change that results from the externally applied electric field could be counterbalanced by the one arising from domain redistribution. When the equilibrium state of the system was reached at the end, the total free energy is minimal. The equation for calculating the difference of the dipole moment density between the phases is as follows:

$$\mu = 4\pi\varepsilon_0 E(R_0)\cdot R_0 \cdot \left[-\phi \ln\frac{e^2\delta}{4R_0} + \phi + \frac{2R_0}{R_c^2}\frac{F_{\text{domain}}}{2\pi\mu^2}\right]^{-1} \tag{1}$$

where μ is the difference in the dipole moment density between the two phases. This equation is applicable to the situation in which the monolayer domains are repelled from the electrode.

As mentioned above, the monolayer domains are repelled from the electrode under certain electric fields; thus a radius R_0 can be used to indicate the magnitude of domain dispersion in the equilibrium state. This magnitude equals the radius of the area in which the monolayer domains are totally repelled from the electrode. ϕ represents the area fraction occupied by the dark lipid domains on the whole monolayer. δ is the distance from the closest neighboring dipoles. F_{domain} is the electrostatic energy of a single domain. For a circular domain with a radius of R_c, this energy can be expressed as

$$F_{\text{domain}} = -2\pi R_c\mu^2 \ln\frac{4R_c}{e^2\delta} \tag{2}$$

In Eq. 1, $E(r)$ represents the electric field at the air/water interface. Correspondingly, $E(R_0)$ is the magnitude of the electric field at the boundary of the domain dispersion. In our experiment, the electric field generated from a syringe tip electrode was idealized as follows. A conducting sphere of radius a, which holds a potential V_0 against the grounded subphase, is placed above the water surface at height h. Because of the high ionic concentration, the subphase can be treated as a good conducting medium. Correspondingly, any charge distributed under the interface is screened. In this model, the electric field $E(r)$ at the air/water interface is perpendicular to the surface of the subphase and is given by

$$E(r) = -V_0\frac{4SW}{(W-2S)(r^2+W^2)} \tag{3}$$

in which

$$W = \sqrt{h^2+2ah}$$
$$S = \frac{1}{2}\left(\frac{W^2}{W+h} - \frac{W^2}{W+h+2a}\right) \tag{4}$$

The coordination origin is set at the intersection of the interface and the line crossing the sphere center and perpendicular to the interface.

RESULTS

Incorporation of rCRP into the monolayer

rCRP binds to PC or PE in a calcium-dependent manner. Examination with fluorescence microscopy suggests that the incorporation of rCRP into the model membrane of PC or PE lipids cannot be characterized as specific binding. Fig. 1 shows the binding pattern of rCRP to DPPC monolayer under different conditions. First we examined the binding of

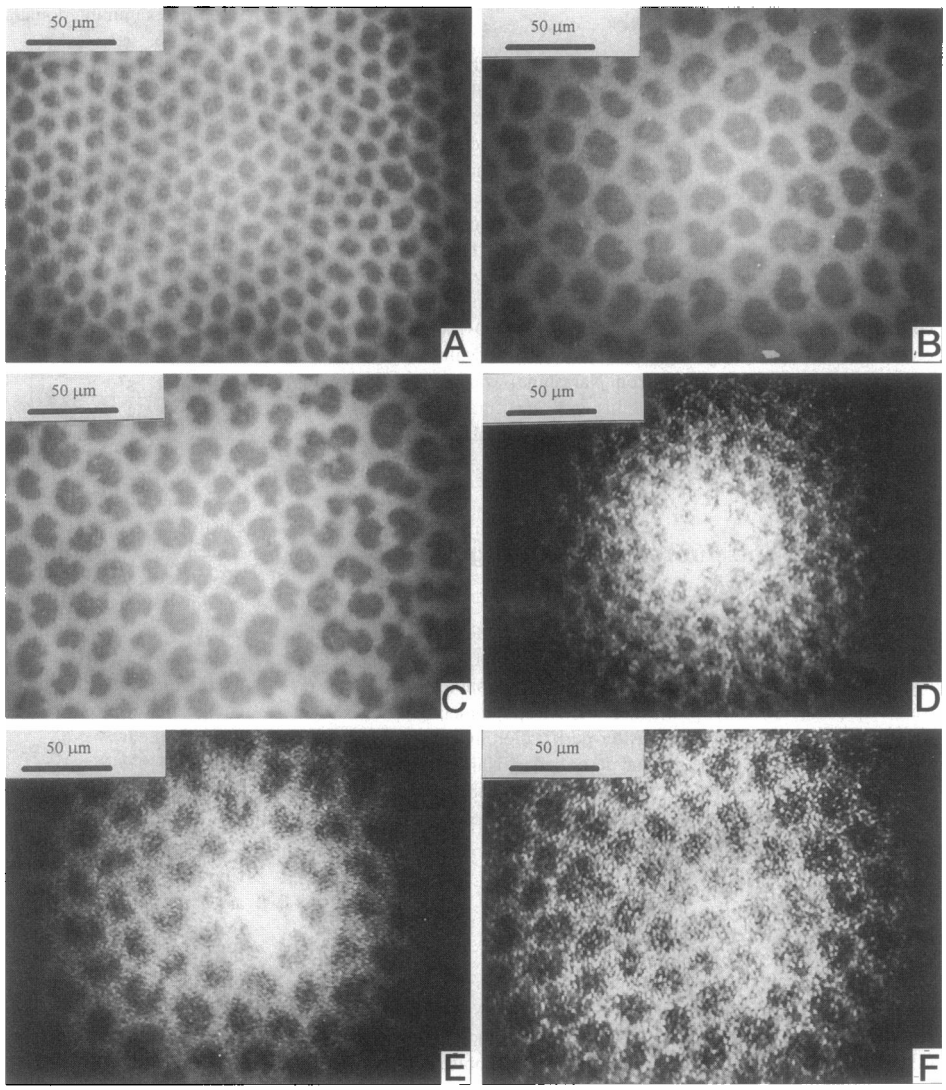

FIGURE 1 Fluorescence microscope observations of rCRP being incorporated into the DPPC monolayer under three different conditions. (*A* and *D*) Images of rCRPs binding to the DPPC monolayer in an EDTA environment. (*B* and *E*) Images taken when certain excess of calcium was added to the subphase. *C* and *F* were taken under conditions in which an excess of free PC and calcium was added to the subphase in advance to saturate the binding ability of rCRP. *A*, *B*, and *C* were imaged from the optical emission of the fluorescence lipid probe, and *D*, *E*, and *F* are corresponding images emitted from the FITC-labeled rCRP.

rCRP to the DPPC monolayer in an EDTA environment. Fig. 1, A and B, indicates that rCRP is incorporated into the liquid phase of the monolayer. (In Fig. 1, *A*, *C*, and *E* were imaged from the optical emission of the fluorescent lipid probe, and *B*, *D*, and *F* are corresponding images of FITC-labeled rCRP.) Because the binding of rCRP to PC is dependent on the presence of calcium, an excess of Ca^{2+} was added to the subphase in a separate experiment (see Fig. 1, *C* and *D*). It was also noticed that in the presence of calcium, rCRP was incorporated into the liquid phase of the monolayer. As the third control experiment, an excess of free PC and calcium was added to the protein solution in advance to saturate the binding capacity of rCRP. This subphase solution was then added to the trough, on which DPPC monolayer was spread later. Fig. 1, *E* and *F*, suggests that rCRP tends to be incorporated into the liquid phase. The effect of PC on the incorporation of rCRP into the monolayer is not obvious (compare Fig. 1, *E* and *F*, with Fig. 1, *C* and *D*). This phenomenon will be further discussed below.

Evaluation of the area in the lipid monolayer occupied by protein penetration and the dipole moment density of which protein extrudes from the interface

Fluorescence microscopy indicates that the incorporation of protein into the monolayer film not only raises the surface pressure, but also increases the size of the dark domains. Furthermore, rCRP is more likely to be incorporated into the liquid phase region of the monolayer. A plausible explanation for this phenomenon is that the size increase of the dark domain is caused by the compression of rCRP incorporation into the film. This compression converts some lipids from the liquid to the gel phase. This phase transition decreases the area occupied by the lipids. Assuming that all of the rCRP protein was incorporated into the liquid phase, the above area change could be attributed to the area occupied by the protein penetrating the film.

It is assumed that the molecular area of the gel phase lipids is A_s, and that of the liquid phase lipids is A_f. The area

fraction occupied by the gel phase domains in the absence and presence of protein is P_1 and P_2, respectively. The total area of the monolayer (S) is fixed.

On the basis of the above analysis and assumption, the number of lipid molecules experiencing phase transition equals

$$S \times (P_2 - P_1)/A_s \tag{5}$$

Therefore, the related area change (S_p) is

$$S_p = (A_f - A_s) \times S \times (P_2 - P_1)/A_s \tag{6}$$

This area is just the area occupied by the protein molecules penetrating the film.

When the penetration area of the protein molecules is calculated, the dipole moment density of the part of the protein that extrudes from the film can also be obtained. In the following discussion, parameters μ_s and μ_l represent the dipole moment densities of gel phase lipids and liquid phase lipids, respectively. Correspondingly, μ and μ' represent the difference in the dipole moment density between the two phases in the absence and presence of protein, respectively. The dipole moment density of the part of the protein that extrudes from the film is assumed to be μ_p.

When the protein is incorporated into the liquid phase of lipids, the dipole moment density of the "liquid phase" will be replaced by the mean dipole moment density of liquid phase lipids and the incorporated proteins. The definition of μ and μ' is described by the following equations:

$$\begin{aligned} \mu &= \mu_s - \mu_l \\ \mu' &= \mu_s - \frac{\mu_p \times S_p + \mu_l \times S_l}{S_p + S_l} \end{aligned} \tag{7}$$

where S_p is the area of penetration by protein into the film, and S_l is the area of liquid phase lipids. Hence,

$$\mu_p - \mu_l = \frac{S_p + S_l}{S_p}(\mu - \mu') = \frac{S(1 - P_2)}{S_p}(\mu - \mu') \tag{8}$$

This equation is used to evaluate the difference in dipole moment density between the protein and the lipids in liquid phase.

In our experiments, two different monolayer systems (DPPC and DMPE) were used to study the interaction of rCRP with model membranes. The effects of Ca^{2+} and pH on the incorporation of rCRP into the film were also investigated (see Fig. 2 and Table 1).

In all experiments, the monolayer films were compressed into the two-phase coexistence region, and the total area of the films was kept constant in the process of the experiment. The molecular area of the DMPE monolayer was fixed at 62 Å^2 per molecule, and that of the DPPC monolayer was 72 Å^2 per molecule. The protein concentration in the subphase was 1.5×10^{-4} mg/ml, and the calcium concentration was 8×10^{-5} M. In the experiments we not only observed the growth of the dark domain in the presence of rCRP, but also found that the presence of calcium can also change the size of the domain and the character of the monolayer. These effects were taken into account in the measurements. The results obtained are shown in Table 1 and will be discussed below.

DISCUSSION

1. It was reported that the specific binding of CRP to PC is dependent on the presence of calcium (Shrivel, 1996). However, our observation by fluorescence microscopy showed that rCRP was incorporated into the film in an EDTA environment. The presence of an excess of competitive inhibitor of phosphocholine did not affect the incorporation of rCRP into the film (see Fig. 1, *E* and *F*). Furthermore, the pattern of rCRP distribution on the monolayer did not change in all three cases (in an EDTA environment, in the presence of calcium, and in the presence of both phosphocholine and calcium). Our observations suggest that the incorporation of rCRP into the DPPC monolayer is unsuitable for classification as specific binding. On the other hand, this incorporation could not result from electrostatic interaction. It is a well-established fact that the charge or dipole moment density of the gel phase domains is greater than that of the liquid phase surroundings. It would be difficult to understand the fact that rCRP is only incorporated into the liquid phase region, if electrostatic interaction is the major force. In light of our observations, we propose that the force incorporating CRP into the film comes from hydrophobic interactions. Steric hindrance might play an important role in supressing the binding of rCRP to the headgroups of the DPPC monolayer.

2. When rCRP binds to calcium ions, the sign of the dipole moment density of the part of the protein that extrudes from the film will change. This is the most significant result shown in Table 1. Because the sign of the dipole moment density is associated with the spatial charge distribution of the molecule, this sign change might be contributed to the change in the orientation of CRP incorporated into the film or to the significant change in the local charge distribution of the protein. Moreover, both changes reflect the fact that calcium binding could lead to a conformational change in CRP.

3. Comparison of the penetration area and the related dipole moment density difference in Table 1 leads to the following conclusion. For a specific type of lipid molecule at a specific pH, the protein penetration area is larger when the related dipole moment density difference is negative. For pure lipid systems, it had been determined that the difference in dipole moment density between the two phases is positive (Klingler and McConnell, 1993; Mi and Sui, 1996). The negative dipole moment density difference of the protein minimizes the system energy and facilitates its incorporation into the monolayer.

4. The effect of pH on CRP incorporation into the DPPC monolayer had been studied. With an isoelectric point of pH 5.5, rCRP is negatively charged at pH above 5.5 and posi-

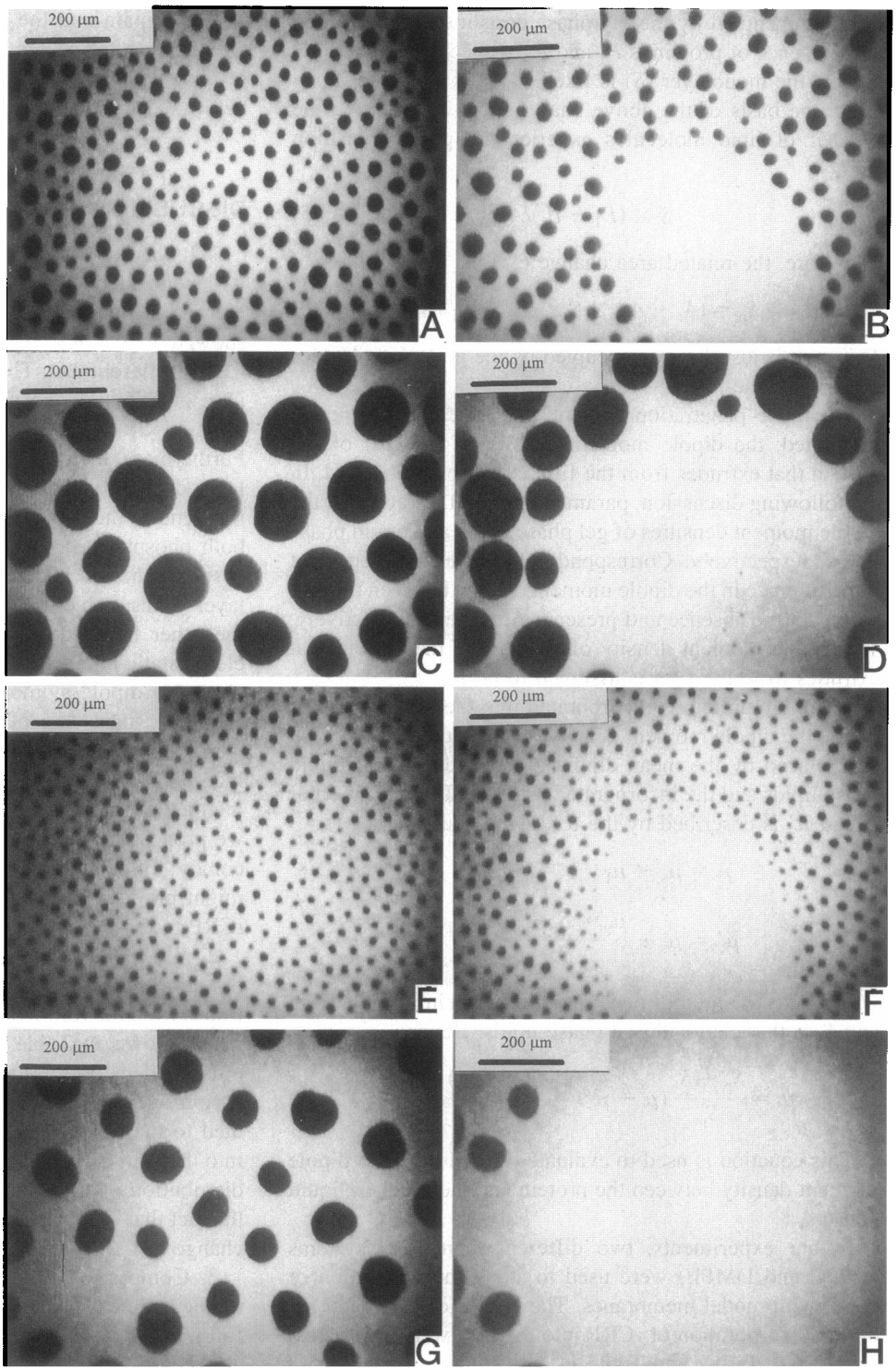

FIGURE 2 Comparison of the DMPE monolayer domain size and related domain redistribution under the externally applied electric field in the absence (*A*, *B*, *E*, *F*) and presence (*C*, *D*, *G*, *H*) of rCRP. *A*, *B*, *C*, and *D* were taken in an EDTA environment; *E*, *F*, *G*, and *H* were taken in a calcium environment. (*B*, *D*, *F*, *H*) Effects of the externally applied electric field on the lipid monolayer. The applied voltage was +30 V.

tively charged at lower pH. As shown in Table 1, when the pH of the subphase was increased, the related penetration area increased dramatically. This tendency indicates that negatively charged protein is more likely to be incorporated into the film.

5. The effect of calcium ions on the incorporation of rCRP into the DPPC monolayer is different from their effect on the incorporation of rCRP into the DMPE monolayer. The binding of CRP to calcium ions promoted its incorporation into the DPPC monolayer, but inhibited its incorporation into the DMPE monolayer. This phenomenon may be related to the different performances of DMPE and DPPC liposomes in activating the complement system (Mold et al., 1981). But the molecular mechanism leading to the different performances of DPPC and DMPE must be further addressed.

TABLE 1 Comparison of the characteristics of rCRP incorporation into phospholipid monolayers

Phospholipid	pH	Presence or absence of calcium	Area occupied by rCRP penatration (cm^2)	Difference in dipole moment density ($\mu_p - \mu_1$) (Debye/nm^2)
DMPE	4.0	A	22 ± 2	−0.10 ± 0.02
DMPE	4.0	P	5.0 ± 1	0.05 ± 0.01
DPPC	4.0	A	2.8 ± 1	1.4 ± 0.3
DPPC	4.0	P	7.5 ± 1	−0.47 ± 0.09
DPPC	6.0	A	11.6 ± 1	0.21 ± 0.04
DPPC	6.0	P	19 ± 2	−0.17 ± 0.03

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