Lipid-Glass Adhesion in Giga-sealed Patch-clamped Membranes

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ABSTRACT Adhesion between patch-clamped lipid membranes and glass micropipettes is measured by high contrast video imaging of the mechanical response to the application of suction pressure across the patch. The free patch of membrane reversibly alters both its contact angle and radius of curvature on pressure changes. The assumption that an adhesive force between the membrane and the pipette can sustain normal tension up to a maximum T_a at the edge of the free patch accounts for the observed mechanical responses. When the normal component of the pressure-induced membrane tension exceeds T_a membrane at the contact point between the free patch and the lipid-glass interface is pulled away from the pipette wall, resulting in a decreased radius of curvature for the patch and an increased contact angle. Measurements of the membrane radius of curvature as a function of the suction pressure and pipette radius determine line adhesion tensions T_a which range from 0.5 to 4.0 dyn/cm. Similar behavior of patch-clamped cell membranes implies similar adhesion mechanics.

INTRODUCTION

High resistance, "gigaohm" seals ($R \geq 20$ GΩ) between glass pipettes and cell membranes have made possible single channel recording from patch-clamped membranes, a technique which has revolutionized the study of signal transduction through ion channel proteins (Hamill et al., 1981). High resistance seals are formed by applying gentle suction pressure to the patch pipette and drawing an Ω shaped bleb of membrane into the pipette. The tight seal is believed to be formed all along the interface between the membrane and the pipette walls, resulting in typical seal resistances of 10–100 GΩ in physiological saline (Hamill et al., 1981; Sackmann and Trube, 1984). Macroscopic resistance calculations predict that the separation between the lipid and glass must be on the order of angstroms across the area of the lipid-glass interface to account for the 20-GΩ resistance of the electrical seal. The atomic dimensions of the separation between the membrane and the glass has been attributed to the formation of hydrogen bonds, salt bridges, or van der Waals' forces between the membrane and the pipette glass (Corey and Stevens, 1983). Study of the membrane-glass adhesion responsible for this enormously successful technique has been limited primarily to pragmatic studies of experimental variables which affect the quality or consistency of high-resistance seals.

The physical mechanisms involved in seal formation are not well understood, particularly because it is not clear which components of cell membranes are involved in adhesion between the cell membrane and the glass pipette. There exists phenomenological evidence for seal formation through lipid-glass interactions as pointed out by Corey and Stevens (1983). Tight seals are readily obtained in a broad range of cells, among which phospolipid content is more highly conserved than is protein or extracellular matrix composition. In addition, enzymatic treatment which breaks down matrix proteins often facilitates patch formation (Corey and Stevens, 1983). The most convincing implication of lipid-glass interaction is the fact that pure lipid vesicles can be patch-clamped using the same procedures followed for cells, resulting in 10–200-GΩ seal resistances (Tank and Miller, 1983). Each of these observations implicates lipid-glass adhesion in the formation of tight seals to cell membranes. Arguments have also been made for seal formation in cells through interaction of either extracellular matrix constituents or of transmembrane proteins with the pipette glass. Recently the importance of the mechanical properties of patch-clamped membranes to the electrophysiological study of mechanically activated ion channels has prompted further investigation into the mechanical response of the membrane-glass seal (Sokabe et al., 1991; Ruknudin et al., 1991; Morris and Horn, 1991), the results of which have challenged some conventional views on the mechanics of patch formation. Membrane mechanics are extensively studied by application of pressure across an asperated liposome in a glass pipette. However those experiments are designed to exclude lipid glass adhesion and seal formation (Evans and Needham, 1987). Therefore the adhesion phenomena in gigaseal formation that we are reporting is eliminated in those studies.

Using video microscopy and capacitance measurements Sokabe et al. (1991) demonstrated that the area of a cell membrane patch increases following the application of suction pressure by an amount which is considerably larger than the elastic limit for lipid membranes. They attributed this excess area increase to free flow of lipid through the mechanical seal into the patch, from which they concluded that lipid involvement was precluded from seal formation. Because the seals of patch-clamped lipid membranes have similar electrical properties to cellular membranes and the patches are obtained using the same techniques, it is reasonable to expect similar mechanical response in both systems. Since lipid membranes present a system in which physical models are more easily tested than in complex cell

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membranes, we have measured the response of patch-clamped pure lipid membranes to applied pressure gradients.

We have found that pure lipid membranes gigaohm sealed within patch clamp pipettes exhibit a reversible free area increase and curvature decrease in response to the application of suction pressure, a result which agrees with reported observations in patch-clamped cell membranes (Sokabe et al., 1991). In general we find that the free patch does not remain tangent to the pipette. Therefore equilibration of the normal mechanical tension component at the junction of the free patch with the glass implies the existence of an adhesion pressure between the membrane and the pipette that can sustain normal tension at the junction. A model is developed for the free area increase of the patch in response to the application of suction pressure which is based upon the assumption of a maximum tension T_a. Thus membrane at the edge of the lipid-glass interface is pulled away from the pipette wall when the normal component of the pressure-induced membrane tension exceeds T_a. Using this model to fit measurements of the membrane radius of curvature as a function of the suction pressure and pipette radius we are able to measure the adhesion force T_a. Close agreement between theoretical predictions and the experimental values of the membrane curvature as a function of the applied pressure gradient supports our proposed model for seal formation in lipid membranes.

MATERIALS AND METHODS

Standard patch-clamping procedures are used to obtain GΩ resistance seals to pure phospolipid membranes at the tip of patch pipettes (TW150-6; World Precision Instruments, Inc, Sarasota, FL). Pipettes are double-pulled using a tungsten wire filament and were not fire-polished.

Bilayer membranes are formed at the tip of the patch pipettes using the tip-dip technique of Coronado and Latorre (1983). The lipid monolayer is formed by adding 5 μl of a 10 mg/ml pentane solution of lipids to the bath surface in a 2-ml petri dish. After allowing 5–10 min for pentane evaporation, a patch pipette is twice dipped through the surface to form a bilayer across the tip. A solvent free method is also used in which 25 μl of a 40 mg/ml suspension of lipid vesicles in a 1 M NaCl aqueous solution is added to the 2-ml petri dish (Hanke et al., 1984). Similar results are obtained with both methods. Lipids used are dioleoyl phosphatidylethanolamine:dioleoyl phosphatidylserine:cholesterol at a concentration ratio of 3:1:1 (Avanti Polar Lipid, Inc., Pelham, AL). All experiments are conducted at room temperature.

The hydrostatic pressure difference Δp across the membrane is controlled by applying suction to the patch pipette. Suction pressure $0 \leq \Delta p \leq 2.5$ kPa (1.33 kPa = 1 cm HG) is stepped at intervals of 20–120 s by a computer-controlled piezoelectric valve (MaxTech, Inc., Torrance, CA) with a 5-ms rise time (Denk and Webb, 1992). The maximum magnitudes for Δp are approximately half those used in experiments involving cellular mechanically activated ion channels because the cytoskeleton-free lipid membranes rupture at lower applied pressures. Microscopic measurements of the membrane radii of curvature R_m and the pipette radius R_p are obtained from image analysis of high contrast images of the membrane profile. Membrane images are obtained by using buffers with different indices of refraction on either side of the membrane and imaging with Hoffman Modulation Contrast optics which create contrast based upon index of refraction gradients (Hoffman and Gross, 1975). The index of refraction gradient is produced by using a pipette buffer of 1 M NaCl, 5 mM $CaCl_2$, 10 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.0, and an equiosmotic bath buffer of 0.4 M sucrose, 0.8 M NaCl, 5 mM $CaCl_2$, 10 mM MOPS, pH 7.0. Reversal of the bath and pipette buffers has no observable effect on patch response. Images are recorded with a CCD video camera (CCD-72, Dage; MTI, Michigan City, IN), processed as digitized images in an RCI Trapix, and edge-enhanced, and the inner 80% of the membrane arc is fit using a nonlinear least squared fitting routine.

ADHESION MODEL

Based upon macroscopic resistance analysis it is assumed that the high electrical resistance across patch seals results from the formation of bonds along the area of the membrane-glass interface (Corey and Stevens, 1983). The increase of patch area in response to applied suction pressure can be derived from the assumption that there is a definite lipid-glass adhesion force T_a which supports tension acting perpendicular to the interface. When the perpendicular tension $T_\perp$ exceeds T_a membrane will be pulled away from the edge of the membrane-glass interface until an equilibrium contact angle is established. This model can be used to predict the dependence of the membrane radius of curvature R_m on the pressure gradient Δp across the membrane.

The perpendicular component of the membrane tension acting at the membrane-glass interface is a function of the contact angle θ between the membrane and the pipette wall given by $T_\perp = T \sin(\theta)$, as illustrated in Fig. 1. Increasing

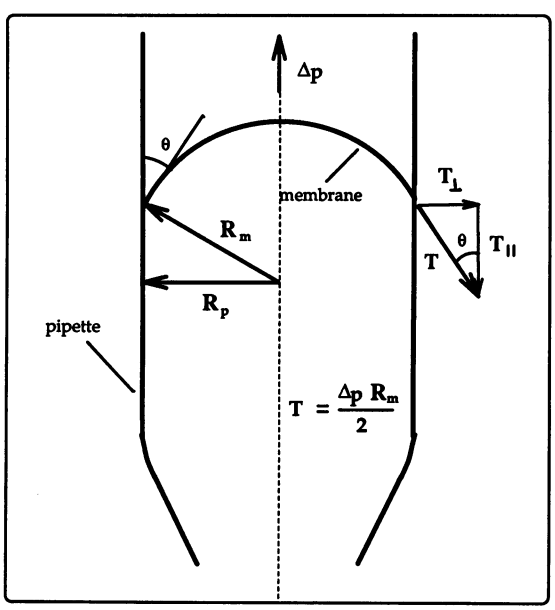

FIGURE 1 Schematic force diagram of a patch-clamped membrane with a pressure gradient Δp across the membrane. The pressure gradient creates a tension T in the membrane given by $T = \Delta p R_m/2$, where R_m is the radius of curvature of the membrane. The component of membrane tension acting perpendicular to the pipette at the glass-membrane interface is $T_\perp$ and that acting parallel to the pipette is $T_\parallel$. At equilibrium the line adhesion tension T_a at the edge of the interface must be equal and opposite to $T_\perp$. This model assumes an ideal membrane such that the radius of curvature R_m is much greater than the membrane thickness. In addition we assume that the walls of the pipette are parallel, an approximation that holds until the contact angle θ becomes very small.

the negative pressure so that $T_\perp > T_a$ will cause membrane to be pulled away from the pipette along the edge of the membrane-glass interface, reducing θ until at equilibrium the perpendicular tension will be equal to the adhesion force so that

$$T_\perp = T \sin(\theta) = T_a \quad (1)$$

The experimentally measured variables are the radius of curvature R_m of the patch and the applied transmembrane pressure Δp rather than θ and T. Assuming parallel pipette walls as in Fig. 1 (an approximation that holds in our system for $\theta \gg 0$) elementary geometry relates θ to R_m by $R_p/R_m = \cos(\theta)$, where R_p is the pipette radius, so that $\sin(\theta)$ as a function of R_m is given by

$$\sin(\theta) = (1 - R_p^2/R_m^2)^{1/2} \quad (2)$$

Assuming an ideal membrane the membrane tension T can be related to Δp by the equation

$$T = \Delta p R_m/2 \quad (3)$$

From Eqs. 1 and 2 the radius of curvature R_m can be expressed as a function of the variables T_a, Δp, and R_p as

$$R_m^2 = R_p^2 + 4T_a^2/\Delta p^2 \quad (4)$$

The force of adhesion T_a can be found experimentally by measuring the dependence of the radius of curvature R_m on the applied pressure difference Δp and fitting the data to Eq. 3.

RESULTS

High contrast video imaging of patch-clamped lipid membranes is used to measure the radius R_m of membrane profiles as a function of the applied transmembrane pressure difference Δp. Patch-clamped membranes are imaged for up to 10 h during which the only resolvable motion of the membrane in response to application of Δp is a reversible rounding of the patch. Movement of the membrane along the axis of the pipette occurs very slowly, at a rate of 1–2 μm/h. Because there is a large index of refraction difference between glass and water the images of the membrane profiles are obscured near their edges and near the pipette tips. Therefore R_m measurements are taken from long-lived patches which have crept for several hours and are located 5–10 μm up the pipette axis where the pipette radius R_p is typically 5–6.5 μm. The taper of our pipettes is shallow in this region making the pipette walls nearly parallel, justifying the approximation made in Eq. 4.

The response of the membrane patch to negative pressure gradients Δp is measured using Hoffman optics as illustrated in Fig. 2. Increasing Δp causes the membrane to round up, decreasing its radius of curvature R_m and increasing the total area of the patch. At zero applied pressure the membrane lies flat across the pipette, as predicted by Eq. 5. Rounding of the patch in response to pressure is reversible in that the patch returns to a flat plane when suction is released. The edge of the lipid-glass interface at which the patch contacts the pipette appears free to move reversibly to satisfy the new tension equilibrium after a change in the pressure gradient. The membrane response to positive pressure is also tested. Under positive pressure the patch is pushed back toward the tip of the pipette at a rate at least an order of magnitude greater than the creep rate observed under negative pressure. At low positive pressures some sections of the membrane remain hysteretically pinned to the pipette making the patch an irregular shape. Increasing positive pressure doubles the membrane

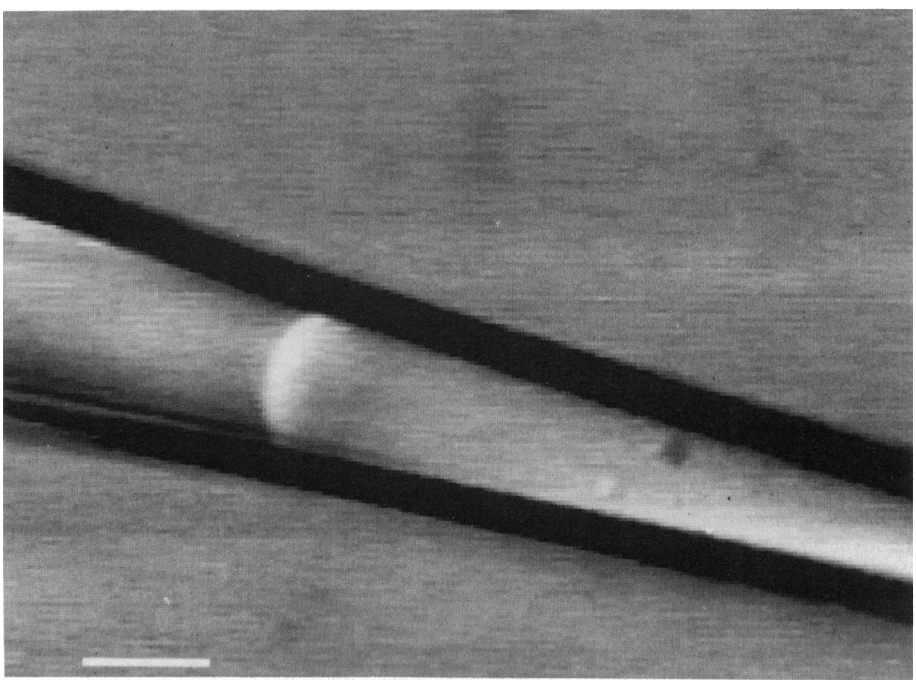

FIGURE 2 Video image of a patch-clamped lipid membrane. Application of a negative pressure gradient across the membrane has caused the membrane to round up and decrease its radius of curvature. High contrast images of the membrane profile are obtained using Hoffman illumination optics which create contrast from index of refraction gradients. An index of refraction gradient is created across the membrane using a high sucrose buffer in the bath and an equal osmolar solution of NaCl in the pipette. Scale bar = 10 μm.

back along the glass surface and eventually pushes the patch of membrane out of the pipette, destroying the seal.

Experimental measurements of R_m as a function of Δp at negative pressures are plotted as solid circles in Fig. 3. A nonlinear least squares fitting routine is used to find the value of the adhesion force T_a which best fits the data to the functional form of Eq. 6. The solid curve in Fig. 3 represents the best fit to the data and and agrees well with the data. For this particular patch the adhesive force $T_a = 2.6 \pm 0.1$ dyn/cm. Analysis has been done independently on five patch-clamped lipid membranes, with the functional dependence of R_m on Δp fitting the theory in each case, but in each case with somewhat differing values of T_a. The best fit values for the adhesion force T_a vary between patches, falling within a range of 0.5–4 dyn/cm. Variations in T_a reflect the all too familiar real and uncontrollable differences in seal stability and resistance between the patches.

DISCUSSION

The response of membrane patches to applied suction pressure is particularly relevant to research on mechanically activated channels, because these ubiquitous ion channels are identified by their response to the application of suction pressure to a patch-clamped membrane just as voltage-sensitive channels are identified by their response to transmembrane voltage (Sachs, 1988; Morris, 1990). The mechanics of membrane-glass adhesion determines the manner in which the pressure-induced tension in cell membranes is distributed between the lipid membrane and the underlying cytoskeletal network and therefore affects the interpretation of studies of

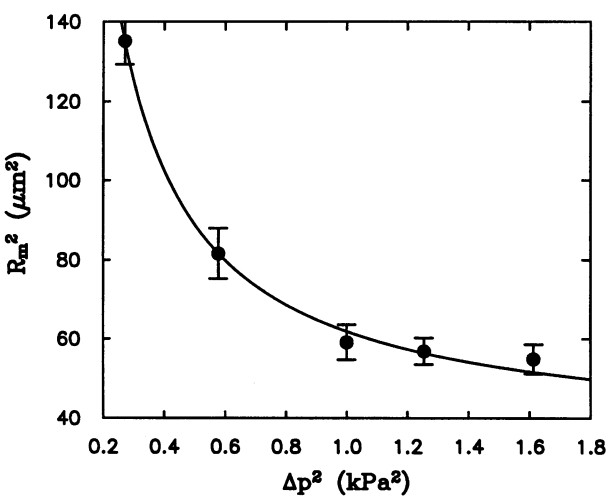

FIGURE 3 Membrane radius of curvature R_m plotted as a function of the applied pressure gradient Δp. The circles represent the experimental values for R_m obtained from image analysis of membrane profiles as a function of Δp. The solid line represents the theoretical fit to the data using the functional form derived in Eq. 7, $R_p^2 = R_p^2 + 4T_a^2/\Delta p^2$ with a measured pipette radius $R_p = 5.9$ μm at the point of membrane contact. A best fit value for the adhesion line force $T_a = 2.6 \pm 0.1$ dyne/cm is obtained from a nonlinear least squares fit to the data. Pressure is plotted in units of kPa^2, where 100 kPa $\approx$ 1 atm.

mechanical activation of ion channels in patch-clamped membranes. The increased probability of cellular mechanically activated channel openings in response to the application of a pressure gradient Δp across the patch of membrane is believed to be due to the resulting tensile stress in the membrane-cytoskeletal system. In many cases it remains an open question whether tension is coupled to mechanically activated channel proteins via cytoskeletal linkages, lipid interactions, or a combination of both. Cell adaptation experiments provide convincing evidence for the influence of cytoskeleton on mechanically activated channels in frog oocytes (Hamill and McBride, 1992) and also in the tension-sensitive channels of the inner hair cell (Assad et al., 1989; Howard et al., 1988), as frequently advocated Sachs (1988). However we have shown that cytoskeletal coupling is not always necessary for mechanical channel activation, as proven by the tension dependence of alamethicin channels reconstituted into pure phospolipid membranes (Opsahl et al., 1990).

Similarity between the mechanical response of our patch-clamped pure lipid membranes and that of patch-clamped frog oocyte membranes indicates similar adhesion properties. Using video microscopy as well as capacitance measurements Sokabe et al. (1991) demonstrated that the area of a membrane patch in a pipette increases up to 10% following the application of suction pressures of up to 3.0 kPa. Because this area increase is considerably larger than the 2% elastic limit of lipid membranes, they ruled out membrane stretching as the source of the area change. Capacitance measurements confirmed that the area increase in their video images was the result of a net increase in the amount of material comprising the patch, an increase which they interpreted as evidence for pressure-induced free flow of lipid from the cell into the patch of membrane (Sokabe et al., 1991). They concluded that the lipid bilayers of patch-clamped cellular membranes are unable to sustain tension and therefore cannot be involved in the coupling of stress to mechanically activated ion channels.

In contrast our experiments on the response of pure lipid membrane patches to applied pressure jumps clearly show that the patch-clamped membranes can sustain tensions even in the presence of slow creep of the gigaohm membrane seal. Unlike previous measurements of mechanical response in patch-clamped cell membranes, our studies of the response of patch-clamped membranes to applied pressure gradients are done on pure lipid membranes in which there is no doubt that the high resistance ($R \geq 20$ gigaohm) seal and the mechanical bond between the membrane and the patch pipette involves lipid-glass adhesion. In our model of membrane-glass adhesion lipid flow along the glass surface is not necessary for the increase in patch area and corresponding change in shape which we observe in response to incremental changes in the pressure gradient. A patch-clamped membrane in a pipette of radius R_p can change from a flat disc with area R_p^2 to a hemisphere with area $2R_p^2$ by pulling membrane away from the pipette walls to account for a change in membrane area as large as 100% without invoking lipid flow across the mechanical seal. Although lipid flow adjacent to

the glass surface is necessary for initial patch formation the membrane appears to be move less than 2 μm/h along the pipette during our mechanical measurements. The close similarity between the mechanical response of cell membranes and pure lipid bilayers suggests that our model also applies to patch-clamped cell membranes. The 10% area changes observed in patch-clamped oocyte membranes can be easily explained within the context of our model without requiring lipid flow through the mechanical seal along the surface of the pipette.

Study of the response of patch-clamped pure lipid membranes to applied pressure gradients has added some insight into the mechanics of attachment of lipid membranes to glass pipette surfaces. Our results measure the mechanical adhesion tension at the intersection of the patch with the glass-membrane interface. We show that morphological change in patch-clamped lipid membranes in response to applied suction is the result of membrane pulling away from the interface in order to establish force equilibrium with the line adhesion force T_a. From microscopic measurement of the membrane curvature as a function of Δp we have measured line adhesion tension of T_a = 0.5–4.0 dyn/cm between the lipid and the glass pipettes in our system. We suppose that an adhesion pressure extends along the area of the membrane-pipette interface based upon: 1) earlier resistance calculations suggesting close spacing in the van der Waals' force regime as well as 2) our observation of the reversibility of the contact point between the patch and the membrane-glass interface. We have not investigated the physical mechanism for this adhesion pressure. The similarity in the mechanical response of patch-clamped cell membranes suggests a similar model for patch-clamped cells.

We conclude that lipid bilayers are able to support tension in cellular patch-clamped membranes and that coupling of membrane to the adjacent glass of the pipette supports a significant perpendicular tension applied through the lipid membrane. These results confirm assumptions of previous patch-clamp experiments identifying membrane tension as a driving force for mechanically activated channel gating. Further work is needed in order to understand the molecular level mechanisms responsible for the adhesion tension T_a of a few dyn/cm which we have measured.

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