The Structure and Dynamics of Patch-clamped Membranes: A Study Using Differential Interference Contrast Light Microscopy

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Abstract. We have developed techniques for micromanipulation under high power video microscopy. We have used these to study the structure and motion of patch-clamped membranes when driven by pressure steps. Patch-clamped membranes do not consist of just a membrane, but rather a plug of membrane-covered cytoplasm. There are organelles and vesicles within the cytoplasm in the pipette tip of both cell-attached and excised patches. The cytoplasm is capable of active contraction normal to the plane of the membrane. With suction applied before seal formation, vesicles may be swept from the cell surface by shear stress generated from the flow of saline over the cell surface. In this case, patch recordings are made from membrane that was not originally present under the tip. The vesicles may break, or fuse and break, to form the gigasealed patch. Patch membranes adhere strongly to the wall of the pipette so that at zero transmural pressure the membranes tend to be normal to the wall. With transmural pressure gradients, the membranes generally become spherical; the radius of curvature decreasing with increasing pressure. Some patches have nonuniform curvature demonstrating that forces normal to the membrane may be significant. Membranes often do not respond quickly to changes in pipette pressure, probably because viscoelastic cytoplasm reduces the rate of flow through the tip of the pipette. Inside-out patches may be peeled from the walls of the pipette, and even everted (with positive pressure), without losing the seal. This suggests that the gigaseal is a distributed property of the membrane–glass interface.

THE patch clamp (13) has revolutionized electrophysiology. By providing a way to control the potential and chemical environment of cells and isolated membranes, the patch clamp has changed our view of cell excitability from something that was characteristic of nerve and muscle to a property of all cells. Despite the flood of papers that apply the technique in its various forms, there is almost no data available on the structure of the patch. In many cases, a detailed histology of the patch may be unnecessary in order to make sense of the data, but in others, an understanding of the patch structure is a necessity for interpreting the data. As part of our continuing studies of mechanosensitive ion channels, we needed to understand the structure of the patch in order to calculate stresses and strains that are involved in the control of channel activity. This paper constitutes a qualitative overview of patch structure visible at the light microscopic level. A paper in progress (Ruknudin, A., M. J. Song, and F. Sachs, manuscript submitted for publication) deals with the electron microscopic view of patch structure. Quantitative results from the light microscopic study are being prepared in another paper (Sokabe, M., F. Sachs, and Z. Jing, manuscript submitted for publication).

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Portions of this work have appeared in abstract form (1988. International Congress of Comparative Physiology and Biochemistry. 815.).

Materials and Methods

Imaging

The patches were observed in a modified Olympus IMT-II using differential interference optics. The Olympus Planapo 100×, 1.4 NA oil immersion objective was used for all high resolution studies. The Olympus condenser was replaced with a Leitz 50×, 1.0 NA, water immersion objective. This condenser was screwed into an Olympus Wollaston prism attachment which, in turn, was attached to a homemade support that rested on the condenser support. A film polarizer (Newport Corp., Fountain Valley, CA) was placed above the condenser support. To increase the available light, the halogen illuminator was replaced by the Olympus mercury arc lamp (75 W) normally used for fluorescence. A green filter centered at 546 nm, a heat reflecting mirror, and a diffuser were placed in the illumination path. In order to increase clearance of the patch pipette from the condenser, the front element (Fig. 1). To prevent metal contamination of the cell chamber by the brass lens barrel, the exposed surfaces were coated with a thin layer of Sylgard 184 (Dow Corning Corp., Midland, MI). Such treatment tends to make the lens surface hydrophobic due to creeping of low molecular weight components of the silicone polymer; and after treatment the lens surface needs to be cleaned to preserve wetting.

To image the patch clearly, the pipette had to be nearly parallel to the microscope stage, and we compromised at 7° (Fig. 1). Because of the small working distance of the condenser (500 μm) and the wide lens (4 mm), the cell chamber was made with very shallow walls. The cell chamber held a number 1 1/2 coverslip and was made of brass, Lexan, and Teflon, with only Lexan and Teflon coming in contact with the cells (Fig. 2).

Patch pipettes had to be constructed with a long shank, a nearly parallel final taper, and a bend in order to clear the condenser and the edge of the...
Figure 1. Detailed view of patch pipette geometry for microscopy. The water immersion objective (Leitz 50X, 1.0 NA) has a working distance of 500 μm. The lens barrel was turned to the maximum possible taper in order to gain clearance for the pipette. The pipette had a long (~1 cm) shank in order to clear the edge of the lens and the dish. The final taper had to be shallow so that the tip of the pipette could make contact with the cells without the lower side touching the cover slip.

Figure 2. Diagram of the low clearance cell chamber. The chamber is round and shown in cross section, in scale. The inner chamber liner was made of Lexan, and, to permit tightening, we drilled a pair of holes which matched a custom spanner wrench.

Figure 3. Equipment layout.

screen and on one high fidelity audio track for computer synchronization (below). A multichannel oscilloscope display of the electrophysiological traces (RC Electronics, Santa Barbara, CA) was viewed with a surveillance TV camera and cut-in to the corner of the image with a screen splitter (Thalner Electric Laboratories) to provide correspondence between the image of the patch and the electrophysiological data. In later experiments, the whole system was controlled with a computer (HP Vectra RS/20) using PCLAMP software (Axon Instruments, Inc., Burlingame, CA). For videoanalog synchronization, PCLAMP was modified by Mr. David Borkowski to write the SIMPTE time code into the comment field of the disk file that contained the analog data. To translate the SIMPTE code into RS-232 format which could be read by the computer's serial port, Mr. Borkowski built a hardware translator. This enabled us to digitize particular frames from the video tape without using optical disk recording.

To analyze the data on video tape, we used a Data Translation DT-2851 frame grabber with a DT-2858 coprocessor, both located in the HP Vectra. The frame grabber was programmed to take an image, or series of images, upon receiving the proper time code. Images could be stored on disk at three full frames per second, although we commonly used composite subimages of the patch in order to save time and space. These could be accumulated at eight frames/s. In later work, we used a DT-2861 frame grabber which could acquire 16 full frames at video rates.

Tissue Culture

Tissue-cultured chick skeletal muscle cells were prepared according to the procedure of Guarnieri and Sachs (8). Pectoral muscles from 11-13-d-old White Leghorn chickens were sterilely dissected from decapitated embryos. The muscles were minced in divalent ion-free Hanks' solution and dissociated using 1 mg/ml Type I collagenase (Sigma Chemical Co., St. Louis, MO). The cells were rinsed, filtered free of large debris, and plated on polylsine-treated cover slips in DME containing 10% heat-inactivated horse serum, 2% embryo extract, and penicillin and streptomycin. After 24-36 h in culture, the cells were treated with 10 μg/ml cytosine arabinoside (Sigma Chemical Co.) for 1 d to reduce the fibroblast population.

Results

The basic process of patch formation is shown in Fig. 4, a time series taken at 4-s intervals. We found that, in many
cases, the process of patch formation looked very much like the idealization presented in the literature (9). The process of seal formation may be quite gradual. This is not due to a delay of the membrane entering the tip, but due to some "annealing" process by which invaginated membrane sticks to the glass. In Fig. 4, over a period of several minutes with constant suction applied, the seal resistance increased by a factor of ≈10 during the passage of the patch 10 μm up the tip. There are several distinctive features which differ from the classical picture of patch structure.

The patch tends to form a dome only when suction is applied (there are exceptions due to normal forces on the membrane, see below). When a seal is formed, the membrane sticks tightly to the glass. This tends to draw the membrane tight across the aperture of the pipette. In the absence of applied pressure, the pipette spanning region tends to be flat (Fig. 5). (It is important to note that zero pressure applied to the back of the pipette does not generally produce zero pressure at the tip because of capillary forces on the filling solution. We set the zero pressure before seal formation by adjusting the background pressure so that there is no visible flow through the tip. Flow was made visible by the movement of small particles in the media.)

The striking adhesion of the membrane to the glass stands in clear contrast to the behavior of red cells and lipid vesicles in traditional pipette aspiration experiments (1, 4, 6) in which the membrane does not stick to the glass. In the traditional aspiration experiments designed to measure the mechanical properties of cell membranes, it is important that the membrane not stick so that the membrane stress is constant. In those experiments BSA is usually added to the bathing solutions to reduce adhesion. We have found, however, that we can make seals to cells which are bathed in tissue culture media that contains 10% serum and 2% embryo extract. Since the pipette contained only saline, nonadherent serum components might have been washed from the cell surface before seal formation. Regardless of what solutions we use in the pipette, we have found it nearly impossible to make tight seals to red cells, so that there may be some unique feature of the red cell surface that tends to prevent seal formation. Seal formation, incidentally, is not the same as adhesion. We have had cells which stuck strongly to the pipette but have refused to form a gigaseal. Yet we have covered cells with 40 nm gold particles conjugated to lectins and had the cells form satisfactory seals (12; Ruknudin, A., M. J. Song, and F. Sachs, manuscript submitted for publication). We think that seal formation involves denaturation of membrane proteins against the high energy surface of the
Figure 6. A time series showing extrusion of an excised inside out patch. The upper six frames were taken at 0.47 s/frame and the lower two frames are enlargements of the first and last frames of the series. The upper six images are at half the magnification of the lower two. There is no change in seal resistance during the extrusion, which in fact was carried out to completely evert the patch, and the seal was not lost until almost complete eversion. The image also shows vesicles that often form from the surface of the cell before seal formation. In this case, the vesicles settled back down against the patch. It is interesting that some of the vesicles are dark and some are light. This is not due to the direction of shear which lies along the axis of the pipette, but must represent some difference in the content of the vesicles. Between frames three and four one of the dark vesicles broke and may have fused with the patch. There are two peculiar "bubbles" indicated by the arrows. These extracellular objects, which are uncommon, prevent the membrane from sticking to the glass. During extrusion, the patch is pushed over these bubbles as though they were glass droplets, but after extrusion there was no visible structure in their place.

Figure 7. A time series (0.4 s/frame) showing a cytoplasmic barrier (arrows) to diffusion of small particles in the cytoplasm. Breakage of the cytoskeletal barrier is shown in Fig. 8.

glass much like a fried egg sticking to a frying pan. The space between the membrane and the glass may be filled with a concentrated solution of the sugar residues of the surface glycoproteins and these residues make an effective sucrose gap to prevent leakage of ions through the space (Sachs, F. 1984. Biophys. J. 42(2):57a). This distributed model would account for the ability to make seals with large spacers, such as colloidal gold beads, stuck between the membrane and the glass. In the presence of continued suction, the patch may be drawn quite far up the pipette before breaking. We have found patches as far as 30 μm from the tip. The mechanism of creep is not entirely clear, but quantitative analysis of the rate of movement, area change, and capacitance suggests that there are two components: a fairly rapid, ~1 s, lipid flow, during which the contact points of the membrane with the pipette do not change, and a continuing bulk flow, presumably lipid and protein, which moves the contact points of the membrane with the pipette (Sokabe, M., F. Sachs, and Z. Jing, manuscript submitted for publication). The same type of creep is observed in cell-attached and excised patches implying that the source of material to make the patch does not have to be drawn from the cell, but can represent a redistribution of material already stuck to the pipette. Electron microscopic data shows that during the formation of a patch, the inner walls of the pipette are coated with a continuous layer of membrane (12; Ruknudin, A., M. J. Song, and F. Sachs, manuscript submitted for publication).

In the process of forming a seal, when the pipette is close to the cell and suction is applied, vesicles may form from the surface of the plasmalemma (Fig. 6). These vesicles form in response to shear stresses produced by fluid flow over the surface of the cell in the manner of tether formation in red cells and lipid vesicles (2, 5). In this case, the membrane which forms the eventual patch is not the same as the membrane which existed in the same area before approach of the
patch pipette and may have altered properties. For example, in Fig. 6, some of the membrane vesicles appear dark and others light. Regardless of the reason for the difference in index of refraction, the structures are different. The vesicles may break and form an instantaneous seal, or fuse together before forming a seal, or remain intact. When the vesicles break to form the seal, the membrane may become inverted from the configuration expected in a cell-attached patch. This may cause anomalous voltage or agonist dependent responses. Although vesicle formation seems to come from the plasmalemma, it is possible that some of the material comes from intracellular membranes which are induced to fuse with the surface. Specific labeling experiments will be necessary to verify the origin of the vesicle membranes. It should be noted that there is no clear electrophysiological evidence for vesicle formation, so that without direct observation, an experimenter will not know that the patch has formed directly from plasmalemma beneath the pipette, plasmalemma that has flowed into the pipette after vesicle formation, or from the fusion of vesicles.

The patch does not consist of membrane alone. The tip is often filled with cytoplasm that appears in contact with the membrane. In cell-attached patches the cytoplasm may be in continuity with the cell's cytoplasm, but more commonly breaks free as a plug (Fig. 5). This cytoplasmic bleb may consist of cytoplasm that is "normal" in the sense that there is little Brownian motion of organelles. Sometimes, however, the cytoplasm is quite disrupted and organelles are seen to undergo Brownian motion as vigorous as that observed in water (Fig. 7).

We have not been able to unambiguously identify the nature of the organelles. There are round particles which are on the order of the resolution limit 0.2 μm (Figs. 7 and 8). These may be lysosomes, broken mitochondria, or other cellular organelles. We have also seen the formation of large vesicles which will change in size, sometimes becoming larger and sometimes smaller (Fig. 5). We do not know the properties of the vesicle interface; is it lipid or aqueous? To understand the origin and properties of these organelles, we are beginning studies with specific fluorescent probes.

**Figure 8.** The patch of Fig. 7 showing that particles have diffused beyond the cytoskeletal barrier. These particles are freely diffusing to the region of the plasmalemma. The patch structure is again seen as a plug of cytoplasm bounded by the plasmalemma at the right and some other interface (cytoplasm vs. saltwater?) at the right.

**Figure 9.** Separation of the cytoskeleton from the plasmalemma (0.17 s/frame). The patch was subjected to positive and negative pressure steps. Frames one through five correspond to positive pressure and show the plasmalemma pushed against the cytoplasm. In frame six the pressure has reversed and the plasmalemma (arrow) is pulling away from the cytoplasm. In frames seven through nine the plasmalemma (right arrow) separates from the cytoplasm (left arrow) while the space in between is filled, presumably, with saline. The dynamics of patch movement must be controlled in part by the viscous losses of flow of water through the cytoplasm. Fig. 11 shows the patch before separation of the plasmalemma from the cytoplasm.
We have seen structures within the tip, beside the plasmalemma, that are capable of retaining these organelles. Fig. 7 shows a tip with such a structure. The plasmalemma is at the left and this amorphous retaining network is to the right (arrow). The clear space between the retaining structure and the plasmalemma is maintained free of particles for several minutes. With time, the organelles which are actively diffusing about in the cytoplasm to the right, break through this net and reach the plasmalemma (Fig. 8). We presume that the network represents some kind of submembrane cytoskeletal network which has become separated from the lipid bilayer, but retains its netlike integrity for some time before rupturing. Another view of this kind of separation is shown in Fig. 9. Reading left to right, top to bottom, this patch has been subjected to a positive pressure (the tip is toward the upper right) which reverses in frame six. In this frame, the plasmalemma (arrow) begins to separate from the dark structure representing the cytoplasm–membrane interface. As time progresses, the flow up the pipette proceeds by pushing the plasmalemma up the tip (right facing arrow) while the cytoplasmic interface (left arrow) remains more or less fixed. Presumably, there is filtered salt water between the two interfaces.

The cytoplasm in the tip may play a significant role in determining stresses in the cell membrane. We have sometimes seen patches in which the membrane, at zero pressure, has nonuniform curvature (Fig. 10). The nonuniformity may be maintained even under moderate pressures, a behavior inconsistent with a membrane which has a low resistance to bending (3). Fig. 11 shows a time series (233 ms/frame) of a patch subjected to a change in pressure from positive to negative.

The first frame shows the membrane just after the step to positive pressure (the change in pressure is visible on the oscilloscope trace). Notice the marked nonuniformity in the curvature. In frame five (lower left) the pressure has just been made negative (see drop in oscilloscope trace). The membrane then bends inward, but still is nonuniform even in steady state. Approximately 0.5 s is required to reach a steady-state shape. Thus, in general, forces normal to the membrane cannot be ignored. We have found that the rate of reaching steady state is not symmetric in the pressure gradient. Some patches will respond slowly to positive pressure steps and rapidly to negative pressures, while other patches have the opposite behavior. We presume that this reflects the amount of cytoplasm that must be moved through the tip. If the plugging cytoplasm is external to the tip, then it will respond slowly to suction. If the plugging cytoplasm is inside the tip, then it will respond slowly to positive pressures. Clearly, without direct observation, it is not possible to estimate stresses in the patch.

**Nature of the Membrane–Glass Seal**

As shown above, the seal may form gradually suggesting that the seal has a distributed resistance rather than a local "spot weld." However, we found that once the seal has formed, by applying positive pressure we could peel the membrane back off the glass without observing a significant change in seal resistance. In fact we could exert an inside out patch without loss of the seal (Fig. 6). Since the resistance of the seal seems to be independent of length, either the resistance per unit length of the seal is extremely high, or the seal is located at the pipette tip. We favor the former explanation, because with a pipette containing an excised patch, it is possible to grossly maltreat the tip by reversibly pushing it into the bottom of a plastic tissue culture dish, or against a ball of Sylgard, without breaking the seal.

**Figure 10.** Nonuniform curvature of the patch. The left frame shows the (cell-attached) patch with positive pressure applied. The right patch shows the patch with a small negative pressure. The nonuniform curvature illustrates the existence of significant forces normal to the membrane. In the left image some diffuse material is seen on the extracellular surface of the membrane. We have seen such material in electron microscopic images, but have not yet been able to identify it. It may represent some kind of "dirt" adsorbed from the serum and embryo extract in the media, or it may represent the basement membrane separated from the plasmalemma.

**Figure 11.** Dynamic nonuniformity of curvature (0.23 s/frame). Alternating positive and negative pressures were applied to the patch. In frame one, the pressure (lowest trace on the oscilloscope screen) has just become positive and the membrane has begun to flow toward the tip. Notice the sharp bends in the membrane. In frame five the pressure has just become negative and the membrane flows back, but even in steady state, i.e., frame eight, the membrane curvature is nonuniform. These images are of the same patch shown in Fig. 9, but made before the plasmalemma separated from the cytoplasm.
Active Contractions

We have observed, on a number of occasions, active contractions of the patch. These contractions appeared in both excised and cell-attached patches. Fig. 12 shows a time series (4 s/frame) of a cell-attached patch with a slight suction applied. The patch was in steady state as shown in frame one. It then began to twitch spontaneously and it was pulled toward the tip of the pipette for more than a micron. The active movements appeared to occur as discrete twitches. After several seconds the contractions ceased and the patch relaxed back, close to its original location (frame nine). We don't know the nature of the contractile structures, but the existence of these forces has important consequences for mechanically sensitive enzymes, such as channels, which may be bound to the membrane.

Excised patches also showed contractile responses. Fig. 13 shows movement of an inside out patch that was everted by positive pressure. In this case there was no seal to the pipette, the everted vesicle (cytoplasmic side out) had merely stuck to the tip. Spontaneous dimpling appeared, lasted for several seconds, and stopped. In this case, the contractions are more mysterious because of the apparent lack of energy to drive them. The everted patch configuration should allow all diffusible substrates to leave. It is possible that mitochondria remain in the adherent cytoplasm and are capable of supplying a small amount of residual energy. Alternatively, the contractions may represent denaturation of cytoskeletal proteins which are using preexisting conformational energy as the energy source.

Capacitance and Area Changes

When the patch membrane is stressed, its area increases and its capacitance increases. The change is reversible if the stress is not maintained for too long. With long lasting stimuli (>10 s) or with high pressures, the patch tends to creep so that there is a drift toward higher capacitances. The change in capacitance is proportional to the change in area by approximately $0.6 \mu F/cm^2$, suggesting that the new area...
is obtained from an influx of new lipid (Sachs, F., and M. Sokabe. 1989. International Conference on Video Microscopy. 37). These observations are treated quantitatively elsewhere (Sokabe, M., F. Sachs, and Z. Jing, manuscript submitted for publication).

Discussion

The patch is more like a small piece of cell than a piece of isolated membrane (10, 12; Ruknudin, A., M. J. Song, and F. Sachs, manuscript submitted for publication). It contains cytoskeleton and organelles as well as membrane. If mitochondria are present, they may provide a temporary energy source even in isolated patches. Such an energy source may affect the interpretation of data obtained with isolated patches which are generally assumed to be free of stored energy.

The complex cytoskeletal structure can provide significant passive and active forces normal to the plane of the membrane. These forces complicate the interpretation of data on mechanosensitive ion channels (11), but probably account for much of the scatter observed in the dose-response relationship for these channels. The patch imaging has provided a number of important insights into the interpretation of data from mechanosensitive ion channels.

To stimulate stretch-activated (or inactivated) ion channels the pressure in the pipette is varied. The results of this paper affect the quantitative interpretation of that data. It has been assumed that the membrane tension ($T$) is proportional to the pressure by Laplace's law, $T = \Delta P r / 2$, where $\Delta P$ is the pressure drop across the membrane and $r$ is the radius of curvature. The law is only true for a thin membrane which has no resistance to bending, a good approximation for a lipid layer (3). However, patch membranes can exhibit nonuniform curvature showing that there may be significant forces normal to the membrane.

Another possible error in SA channel experiments is that the viscosity (and elasticity) of cytoplasm will limit the rate at which the transmembrane pressure, $\Delta P$, is created. Even though the pressure on the pipette side of the membrane may be known with high precision, the pressure on the bath side of the membrane may not be atmospheric and may change in time as well. The membrane tension therefore is not certain, independent of any influence of cytoplasm on patch shape and stiffness (Fig. 11).

We have been able to understand some of the factors influencing the shape of the dose-response curve for stretch-activated channels. In earlier work it had been assumed that the membrane tension was proportional to the applied pressure. Aside from the considerations mentioned above, the assumption of constant radius of curvature is not valid. Even for an ideal membrane, if it sticks to the walls of the pipette, the radius of curvature is a function of the pressure (Fig. 5). At zero pressure the membrane tends to be planar. As the pressure (or suction) is increased the radius decreases. Thus, the increment in tension per unit pressure is larger at low pressures than at high pressures. The radius of curvature for the ideal membrane is constrained to lie between infinity and the radius of the pipette, a wide range indeed. This wide range of radii means that the membrane tension is not a linear function of pressure and may explain much of the scatter observed in the sensitivity of SA channels. If the dose-response data for SA channels are replotted as a function of membrane tension (having measured the radius of curvature, rather than pressure), the scatter between different patches is much reduced (Sokabe, M., F. Sachs, and Z. Jing, manuscript submitted for publication).

The future of patch clamp mechanics is promising. The method permits us to measure not only the capacitance and the channel activity in real time, but also the membrane strain. With appropriate fluorescent dyes and immunogold probes it should be possible to monitor movement of specific components of the patch. Finally, it is possible to examine the ultrastructure of the same patch observed by these other techniques to correlate structure and function (Ruknudin, A., M. J. Song, and F. Sachs, manuscript submitted for publication).

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